Molecular Mechanisms of Differential Activation of Naive T cells by Weak and Strong Antigen-presenting Cells

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Publications during thesis

Professional articles

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Transiently reduced PI3K/Akt activity drives the development of regulatory function in antigen-stimulated naïve T cells. PLoS ONE 8(7): e68378. doi:10.1371/journal.pone.0068378

Peter Reichardt*, Irene Patzak*, Kristian Jones, **Eloho Etemire**, Matthias Gunzer[#] and Nancy Hogg[#]. A role for LFA-1 in delaying T lymphocyte egress from lymph nodes. The EMBO Journal (2013) 32, 829–843.

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Oral talks

- **Eloho Etemire**, Marco Krull, Peter Reichardt and Matthias Gunzer. Early Signaling dynamics driving the generation of induced Tregs from naïve T cell pools. *43rd Annual Meeting of German Society for Immunology (DGfI). Mainz. Sept 2013*

- **Eloho Etemire**, Marco Krull, Peter Reichardt and Matthias Gunzer. Functional Roles for Defective PI3K Signaling and differing Co-stimulatory activity of APCs in T-Cell Plasticity. *MACSQuant users' day. Bergisch Gladbach. Nov 2012*

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Poster presentations

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Abbreviations

APCs	antigen presenting cells
BD	Becton Dickinson
BMDC	Bone Marrow Dendritic Cell
Bp	Base pair
BSA	Bovine Serum Albumin
CCD	Charge Coupled Device
CCL21	Chemokine (C-C motif) ligand 21
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxy Fluorescein Succinimidyl Ester
CpG	Dinucleotide with the base sequence CG in 5'-3' orientation
Ct	Cycle-threshold
CTL	cytotoxic T-cell
CXCR4	C-X-C chemokine receptor type 4
CXCR5	C-X-C chemokine receptor type 5
DC	Dendritic Cells
DMRs	Differentially methylated regions
DMSO	Dimethyl Sufoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotidetriphosphate
DTT	Dithiothreitol
e.g.	*Latin "exempli gratia"* = for example
EDTA	Ethylenediamine Tetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FasL	Fas ligand
FasR	Fas receptor
FCS	Fetal Calf Serum
Fig	Figure
FITC	Fluorescein Isothiocyanate

Foxp3	forkhead box protein 3
FSC	forward scatter
GC	Germinal centre
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
h	Hour
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HEV	High endothelial venules
i.e.	*Latin: "id est"*
ICAM1	inter-cellular-adhesion molecule
ICOS	Inducible T-cell COStimulator
IFN-γ	Interferon gamma
IgG	immunoglobulin G
IL-	Interleukin
ITAM	Immunoreceptor Tyrosine based Activation Motif
KCl	Potassium chloride
kD	Kilodalton
ko	Knock out
LAT	linker for Activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
miRNA	microRNA
mRNA	messenger RNA
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium chloride
NEAA	Non-Essential Amino Acids
NFkB	Nuclear factor kappa B
NK	Natural killer
nM	Nanomolar

ns	non significant
OvGU	Otto-von-Guericke University
p value	probability value
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PI3K	phosphatidylinositol-3-kinase
PLCγ1	phospholipase C gamma 1
PMT	photo-multiplier
PRR	Pattern Recognition Receptors
qPCR	quantitative PCR
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLOs	Secondary lymphoid organs
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
SPF	specific pathogen free
SSC	side scatter
STAT	Signal Transducer and Activator of Transcription
TCR	T-cell receptor
TGF-β	Transforming Growth Factor-beta
Th-cell	T helper cell
TLR	Toll-like Receptors
TNF-α	Tumour Necrosis Factor-alpha
Tr1	Type 1 regulatory cell
Treg	regulatory T cell
Tween 20	Polyoxyethylene sorbitan monolaurate

VLA	very-late antigen
ZAP70	zeta-associated protein of 70kDa
STAT	Signal transducers and activator of transcription
TSA	Trichostatin A
°C	Degree celcius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
%	Percent

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ABSTRACT

Regulatory T cells (Tregs) are a special branch of T cells which function primarily to keep immune responses at a non harmful level and are principally of two types: naturally occurring Tregs (nTreg) which develop in the thymus and induced Tregs (iTregs) which develop from naïve T cell pools in the periphery. Tregs are widely depicted by their expression of the Foxp3 transcription factor and there is an increasing awareness of the existence and activity of non-Foxp3 Tregs which also play a role in modulating immune responses. iTregs have been extensively studied in recent years and while much is now known about their biology, there however exists a knowledge gap about initial signaling events which underlie the process of naïve T cell conversion into iTregs in the periphery. With the aid of transgenic T cells specific for the pOVA peptide, this study investigated the processes involved in the conversion of naïve T cells into CD4+ CD25+ Foxp3- iTregs following activation of the T cell receptors (TCRs) on these T cells by weak antigen presenting cells (APC).

Results of this study showed that after activation by weak APC, iTregs initially shed the lymph node homing molecule CD62-L and after 24h begin to be re-express this molecule again and by 72h, had returned to values as high as in naïve T cells. This was in contrast to T cells triggered by mature dendritic cells (DCs) which shed CD62-L just as iTregs but maintained consistently low amounts of CD62-L after the initial shedding phase. Based on pharmacological dissection, a differential role for the PI3K/mTOR signaling pathway was implicated in the dynamics of CD62-L regulation between iTreg and effector T cells. After TCR triggering by weak APC, iTregs exhibited an attenuated phopsho-status at the hydrophobic motif of Akt; Ser473 and this signaling profile was found to be associated with upregulated levels of the novel molecule PHLPP1 which has been reported to specifically target the hydrophobic motif of Akt. Lack of co-stimulatory molecules was also identified as playing a critical role in acquisition of regulatory function in T cells triggered by weak APC and augmented CD28 stimulation was able to abrogate regulatory function but interestingly had no effect on CD62-L expression, thus emphasizing the importance of suboptimal CD28 signaling for conferment of regulatory behaviour in iTregs but not in modulating its migratory potential as assessed by its CD62-L expression level. This work also highlighted the differential requirements of PI3K and mTOR signaling for acquisition of regulatory function as pharmacological inhibition of PI3K but not mTOR drove T cells from effector lineage into regulatory cell lineage.

In the final part of the thesis, we began investigating the role of adhesion molecules of the microenvironment stroma in T cell activation. Adaptively transferred transgenic OT 2 cells were equally activated by transferred antigen laden wildtype dendritic cells with regards to CD25 expression but the percentage of proliferating cells was significantly lower in Intercellular adhesion molecule (ICAM) deficient mice which lack ICAM expression on the stroma of the lymph node compared to wildtype thus suggesting that in situations of ICAM deficiency, T cell immune response might be delayed or insufficient compared to wild type conditions.

In conclusion, this present work apart from giving insight into early signaling events in iTreg generation also proposes that there exists a temporal window of sensitivity after TCR triggering within which the signaling threshold of the PI3K/Akt axis determines cells fate lineage into effector or regulatory T cell fate lineage and provides hint that *in vivo,* adhesion molecules on the stroma of lymph nodes play a role in efficiency of T cell responses.

1.0 INTRODUCTION

1.1 The Mammalian Immune System

The immune system is a pathogen fighting system found in invertebrates (at a basic rudimentary level) down through to vertebrates which have evolved a more complex system. The main function of this system is to eliminate foreign antigens, promote formation of immunological memory and drive the establishment of tolerance to self-antigen (Luckheeram et al. 2012). These functions are very important as mammals live in the environment shared with several organisms ranging from single cellular to multicellular organisms and most times, there will be contact with these organisms co-habiting the ecosystem which may trigger disease conditions. To control these incidences, the immune system has evolved and developed over several thousand years to produce a tightly controlled mechanism to clear off and keep down adverse cellular incidents due to encounters with other organisms or matter on the planet. The immune system is made up of various cell types and mediating agents both physical and chemical that interlink with one another and several other non-immune cells in complex regulatory circuits to effect clearance and protection against foreign harmful antigens as well as ensuring tolerance towards self-antigens. On the basis of antigen specificity and response timing, the immune system can be divided into 2 somewhat distinct compartments which have evolved to tackle foreign body invasion into the body using distinct mechanism, viz: the innate and adaptive immune systems (Kindt et al. 2007; Murphy, 2012).

1.1.1 The Innate Immune System

The innate immune system is non-specific in action (Kindt et al. 2007). Innate immune cells recognize tissue insult and pathogen-associated molecular patterns (PAMP) by agency of pattern recognition receptors (PRR). One of the key classes of PRR is the Toll-like receptor (TLR) family which are type 1 transmembrane proteins with divergent leucine rich extracellular domains as well as a highly conserved Toll-IL-IR (TIR) cytoplasmic domain (Tabeta et al. 2004). TLR signaling links with intracellular signaling pathways using adaptor molecules and generally drives transcription of NF-kB target genes as well as other signaling pathways some of which drive initiation of the adaptive immune response in addition to shaping terminal differentiation/cell lineage fate decision (Schnare et al. 2001). If foreign organisms/matter/antigens manage to get over physical barriers such as the skin and mucosal membranes, then they are confronted by the innate immune system which forms the first line of defence. The innate response is mediated by phagocytic cells namely neutrophils, dendritic

cells (DCs) and macrophages in addition to basophils, eosinophils, mast cells and natural killer (NK) cells which act in a non-phagocytic manner. DCs and other phagocytic cells primarily reside in tissues and act as sentinels monitoring the microenvironment for signs of unwanted immune events**.** In situations that lead to the perturbation of cellular or tissue homeostasis, sentinel cells such as DCs and macrophages are the first responders releasing chemical mediators such reactive oxygen species (ROS), matrix proteases, chemokines and cytokines in addition to biologically active substances such as histamine that boost the accumulation of leucocytes at such inflamed (damaged) regions. Concurrently, macrophages and mast cells activate vascular and fibroblast responses that facilitate elimination of invading organisms and drive local tissue repair. While this response is in play, DCs scavenge up these foreign antigens and migrate to lymphoid organs to present these antigens to cells of the adaptive immune compartment thus sub-serving their functions as links between the innate and adaptive immune systems (de Visser et al. 2006). NK cells also bridge the innate and adaptive immune divide by interacting bi-directionally with DCs: some NK cell subsets dispose of immature DCs while others drive DC maturation which can then in a feedback manner regulate NK cell activation. They act against bacteria and viruses by secreting cytotoxic granules which contain cytotoxic granzymes and perforins which attach onto target cells thereby inducing programmed cell death. Distinctive features of NK cells that make them generally regarded as innate immune cells are their possession of invariant receptors and the fact that NK cell killing is dependent on germ-line encoded receptors with preference for molecules on the cell surface of infected or damaged cells (Degli-Esposti and Smyth 2005;Hamerman et al. 2005). A unique feature of all innate immune cells is the ability to react quickly to tissue injury without previous memory of such insults (de Visser et al. 2006).

1.1.2 The Adaptive Immune System

The acute spontaneous reactions of the innate immune system to cellular homeostatic perturbation sets the stage for the activation of the more specific and direct acting adaptive immune system. Induction of this system is dependent on interaction between mature sentinel cells acting as antigen presenting cells (APCs) and lymphocytes in an inflamed tissue environment. Cells of the adaptive immune system i.e. T cells and B cells act in a very specific manner due to their expression of somatically generated, diverse antigen specific receptors which are formed based on the random gene rearrangements that allow for a more broad antigen specific repertoire than innate immune cells which as aforementioned possess germline-encoded receptors. Given this restricted receptor repertoire, clonal expansion of

cells bearing receptors specific for antigens of invading pathogens is vital before an adaptive immune response can be effectively mounted. This need for clonal expansion is the reason for the relatively slow pace of the adaptive immune response when compared to the rapid response associated with the innate immune system (Kindt et al. 2007; de Visser et al. 2006). Another distinct feature of the adaptive immune system is the fact that following primary response, a subset of cells involved in the response go on to differentiate into long lived "memory cells" which can drive a more aggressive response were the tissue insult to occur again (Murphy, 2012). As aforementioned, T cells and B cells are the 2 main cell types involved in mediating the adaptive immune response.

1.1.2.1 B Cells

B lineage cells play a key role in humoral adaptive immune response and terminally differentiate into plasma cells (PCs). B cells act to terminate pathogen activity by secreting pathogen specific targeted antibodies which upon binding to these pathogens by the process of opsonisation marks these pathogens for phagocytosis by phagocytic cells of the innate immune system (Kurosaki et al. 2010).

B cells develop from lymphoid progenitors in the microenvironment of the bone marrow and early steps in their development are dependent on contact between precursor B cells and the stromal structures of the bone marrow in addition to occurring in the absence of antigen. This phase of development leads to rearrangement of immunoglobulin genes leading to production of immature B cells which bear an antigen receptor in the form of cell surface expressed immunoglobulin M (IgM) which is capable of interacting with antigens in the environment. The production of immature B cells centres around functional rearrangement of the B cell receptor (BCR) and is subject to rigid positive and negative selection steps that eliminate overtly reactive immature B cells and end with the licensing of these immature cells to traffic to the peripheral lymphoid tissues where they following maturation status begin to express immunoglobulin D (IgD) and IgM (Kato et al. 2013).

Mature naïve B cells circulate in the periphery through secondary lymphoid organs (SLOs) until they are activated following an encounter with their cognate antigen and express high levels of the chemokine receptor CXCR5 which enables them to respond to CXCL13 produced by DCs and stromal cells of secondary lymphoid organs in addition to enabling them home to the B cell rich follicles of SLOs from where an adequate antibody response can be mounted (Kato, Hulse, Tan, & Schleimer 2013;Reif et al. 2002) (Kato et al. 2013; Reif et

al. 2002). In order to mount an optimal antibody response, B cells must be activated by helper T (Th) cells which inhabit a distinct T cell rich region of the SLO. To bridge this divide, follicular B cells via BCR signaling upregulate CCR7 and following its interaction with the chemokine CCL21 produced by the high endothelial venules (HEVs) and fibroblastic reticular cells of the T cell zone, traffic to the border of the T and B cell zones of the SLO and can then interact with cognate Th cells (Cyster 2010;Gunzer et al. 2004). Following on from instruction by cognate Th cells, based on strength of BCR antigen recognition and differential expression of chemoattractant receptors, primed B cells differentiate into one of three possible lineages as either extrafollicular plasma cells, Germinal Centre (GC) B cells or early memory B cells that possess the ability to recirculate in the periphery. Cells with strongly activated BCRs mostly become extrafollicular cells with concomitant express of the EBV-induced protein 2 (EBI2) and increased expression of CXCR4, while those destined to become GC B cells do not express EBI2 or upregulate CxCR5 (Goodnow et al. 2010;Paus et al. 2006). This interaction of B and T cells is the final step required in the production of terminally differentiated plasma cells which can thereafter begin to secrete Immunoglobulins (Ruprecht and Lanzavecchia 2006).

1.1.2.2 T Cells

T cells constitute the other arm of the adaptive immune system next to B cells and are made up of a plethora of cells distinguishable based on cell surface expression of different key molecules and functionality. T cell precursors originate from a common lymphoid haematopoietic stem cell that upon leaving the bone marrow localize to the thymus for maturation. The microenvironment of the thymus based on the combination of stromal cells, chemokines and cytokines factors make it conducive for the generation of functional T cells from the precursors (thymocytes). In maturation from thymocytes to T cells, T cell receptor (TCR) gene rearrangement and thymic selection are vital steps undertaken to ensure that the T cell pool contains a vast repertoire of cells capable of recognizing several different antigens and also setting the threshold for TCR reactivity/affinity for antigen. During this process, thymocytes migrate thorough the microenvironment of the thymus where they make contact with peptide major histocompatibility complex (pMHC) on distinct thymic APCs including medullary thymic epithelial cells (mTECs), cortical thymic epithelial cells (cTECs) and DCs which serve vital functions in shaping the T cell repertoire for antigen recognition, selection process and surface expression of such molecules as CD8 and CD4 (Gill et al. 2003;Klein et al. 2009).

T cell selection, commonly depicted by the affinity model proposes that thymocytes possessing TCRs with too weak or too strong affinity for the pMHC are destroyed leaving only those with intermediate binding affinity for the pMHC to thrive going on to differentiate into mature CD4+ or CD8+ T cells after undergoing the process of positive selection (Daniels et al. 2006). TCRs which are vital and indispensible components in the mediation of T cell responses are multicomplexed molecular structures consisting of either αβ or γδ chains which are bonded with 4 CD3 subunits (γ,δ,ε and ζ). The TCR α chain consists of V (variable) and J (joining) gene segments while the β chain consists of a V, J and D (diversity) gene segment. The vast repertoire of antigen specific TCRs is built based on random gene rearrangement between exons of the V-D-J segments of the β chain and the V-J segements of the α chain (Starr et al. 2003). In addition to the major populations of CD4+ and CD8+ cells produced via this process, some T cell precursors by interacting with pMHCs differentiate into non-conventional T cells such as the CD1d-reactive NKT cells (Luckheeram et al. 2012). While the TCR binds with the major histocompatibility complex (MHC) molecule on the APC bearing cognate antigen, the CD3 chain mediates T cell activation dynamics (Rudolph et al. 2006).

Following completion of the thymic maturation phase, former thymocytes exit the thymus as naïve T cells expressing L-selectin (CD62-L), CCR7 and leucocyte function antigen-l (LFA-1) into the periphery. These molecules confer on the naïve T cell the ability to roll, adhere and extravasate through the HEVs in peripheral and mucosal lymphoid structures where these naïve cells scan APCs for cognate antigen recognition. Naïve T cell survive for months in the periphery due to the influence of pro-survival signals from interleukin 17 (IL 17) as well as the low affinity TCR/ self-antigen interaction of the naive T cells (Kindt et al. 2007). As aforementioned, there are 2 main classes of T cells produced following thymic maturation, viz: CD8+ T cells and CD4+ T cells.

1.1.2.2.1 CD8+ T Cells

CD8+ T cells otherwise known as cytotoxic T lymphocytes (CTLs) bear the CD8 molecule, which is a glycoprotein of about 32-35 kDa (Littman 1987), and is a disulfide-linked dimer consisting of an α and β chain each containing an immunoglobulin-like domain linked to membrane by a polypeptide. CD8 binds weakly to an invariant site on the α3 domain of the MHC class I molecule. MHC class I molecules are restrictedly expressed by non-classic APCs including almost all cells of the mammalian system and present peptides from mostly

viruses and injured cells to CTLs which have evolved to recognize and kill such cells. This is very important as viruses can infect almost all nucleated cells with the exception of a few cases e.g. erythrocytes which are non-nucleated and are thus not known to be subject to infection by viruses (Murphy, 2012). CTLs proliferate under the influence of IL-2 with the capacity to expand clonally over several multiple hundred folds during a primary immune response. This greatly increased ability to expand on a clonal basis compared to CD4+ is likely due to the relatively easy activation of the Ag-MHC class 1 complex and generally higher survival rate of CD8+ T cells in the general circulation. Rapid expansion and the highly specific and selective killing ability of CTLs make them very efficient antigen specific effector cells as they are able to kill several target cells while leaving uninfected bystander cells unharmed (Broere et al. 2011).

CTL killing is a contact dependent process requiring the establishment of contact between the CTL and its target cell following antigen recognition leading to release of cytolytic granules from CTLs into the immunological synapse formed between both cells. There are 2 main pathways via which CTLs act on target cells (Berke 1995;Harty et al. 2000); the Ca^{2+} dependent perforin/granzyme mediated apoptosis and the Ca^{2+} independent Fas ligand/ Fas-mediated apoptosis. Both of these pathways are initiated following TCR signaling and ultimately involve induction of apoptotic mechanisms in the target cell (Shresta et al. 1998). In the perforin/granzyme killing pathway, lytic granules containing granzymes, perforin and the proteoglycan serglycin are transported into target cells as one complex. Following secretion, granzymes which are capable of inducing apoptosis via either caspase dependent or independent pathways enter into target cells using pores bored into the cell by perforins or by receptor induced endocytosis. When mediated by endocytosis, perforin drives the translocation of granyzmes from endocytic vesicles into the cytosol of the target cells with the proteoglycan serglycin acting as a chaperone to perforin until the complex reaches the plasma membrane of the target cells after travelling across the immunological synapse formed between the CTL and target cells. This way, granzymes induce apoptosis in target cells without inflammation, leaving behind fragments of cell debris that are taken up by macrophages (Broere et al. 2011; Kindt et al. 2007). The second pathway for CTL killing activity is via the Fas ligand dependent pathway. In this pathway, the Fas ligand (FasL) expressed on the CTL binds onto the Fas receptor (FasR) on the target cell. The Fas molecule belongs to the TNF receptor family known to possess an intracellular death domain which when triggered initiates caspase dependent apoptosis and is known to be upregulated on the cell surface of CTLs following TCR crosslinking. Thus, the binding of FasL and FasR in CTL

and target cell interaction consequently leads to triggering of caspase 1 activation which ultimately leads to DNA cleavage and cell death in the target cell in a non-inflammatory manner (Broere et al. 2011; Kindt et al. 2007).

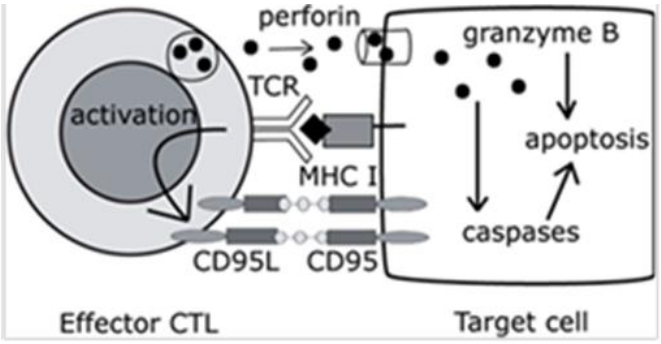


Figure 1.1 Mechanisms of action for CTL cytotoxicity. Both the perforin/granzyme pathway and the FAS-L dependent pathway lead to apoptosis of infected or damaged target cell (Broere et al. 2011)

1.1.2.2.2 CD4+ T Cells

CD4 is a 55 kDa glycoprotein expressed on the cell surface of many haematopoietic cells (Maddon et al. 1987) and is a member of the immunoglobulin superfamily and has 4 Immunoglobulin domains expressed on the cell surface, the D_1-D_4 domains. D_1 and D_3 domain have semblance to the Immunoglobulin variable (IgV) domains while the D_2 and D_4 domains resemble the immunoglobulin constant (IgC) domains. CD4 T cells are restricted to recognition and triggering by MHC class II molecules as the CD4 protein only interacts via its D_1 domain with the β_2 domain of MHC class II molecules which are expressed only on antigen presenting cells unlike CD8+ T cells which recognize the MHC I molecule. CD4+ T cells otherwise called helper T cells are so called because one of their main functions is to send signals to others cells of the immune system including B cells and CD8+ cells. (Kindt et al. 2007; Murphy, 2012). Apart from its function in MHC class recognition, CD4 via its cytoplasmic tail acts as a co-receptor during TCR interaction with APC. In this role, the cytoplasmic/intracellular tail of CD4 serves to amplify TCR signaling by recruiting the Lck tyrosine kinase which is central to activation of naïve CD4 T cells (Brady et al. 1993;Foti et al. 2002).

Naïve CD4 T cells ordinarily lack the ability to mount an immune response as they are non-cytokine secreting cells and only acquire effector functions when activated and differentiated into cytokine producing cells. The step of differentiation places a range of possibilities before the naïve T cell as based on environmental milieu, cytokine signaling and transcription factor expression along with epigenetic tuning, naïve cells can differentiate into different subsets/terminal lineages namely T helper 1 (Th1), T helper 2 (Th2), T helper 9 (Th9), T helper 17 (Th17) among several other lineages (Boothby et al. 2001; Luckheeram et al. 2012).

Apart from the classic CD4 T cell subsets, there is also increasing awareness of the existence of cytotoxic CD4 T cells which act like CD8 cells but express the CD4 marker (Appay 2004;Fang et al. 2012;Marshall and Swain 2011;Quezada et al. 2010) The concept of T cell subsets was first introduced by Mossman and Coffman (Mosmann et al. 1986) who found that T cells could be divided into 2 groups; an IFNγ producing group and an IL-4, IL-5 and IL-13 producing group. The importance of this concept was further put in context by the work of Locksley and coworkers who found that mice which mounted a predominantly Th1 response could clear off *Leishmania major* while mice with a predominant Th2 cell response could not clear off such infection (Heinzel et al. 1989). The array of so far discovered and researched CD4+ T cells is enumerated below:

T helper 1 (Th1)

Th1 cells drive pro-inflammatory cell mediated immunity and have been demonstrated to induce delayed-type hypersensitivity (DTH), drive B cell production of opsonizing antibodies of the IgG isotype while also mediating the response of the mammalian system against protozoa such as *Leishmania* and *Trypanosoma* (Broere et al. 2011). They are generally involved in fighting immune events involving viral, bacterial and parasitic infections (Stassen et al. 2012). In addition, Th1 cells also work closely with CD8+ T cells in mounting antitumour responses and are considered the T cell subset with the most potent antitumour activity (Dunn et al. 2006;Nishimura et al. 2000). Th1 cells secrete Interferon γ (IFN γ) and IL-12 which are also the key cytokines that mediate the signaling cascade needed to drive differentiation of naïve T cells into Th1 cells (Trinchieri et al. 2003). During the process of T cell activation by APCs, IL-12 is produced in copious amounts by the APC following activation of the pattern recognition receptors on the APC and this IL-12 leads to production of IFNγ by NK cells which further helps drive Th1 differentiation (Iwasaki and Medzhitov 2004;Steinman et al. 2003).

The master regulator for the induction of Th1 cells is the T-box transcription factor (T-bet) (Szabo et al. 2000). Additionally, other transcription factors such as STAT1, Runx 3, Eomes and Hlx have been implicated in Th1 cell induction (Luckheeram et al. 2012). The T-bet gene apart from promoting the differentiation of naïve T cells into Th1 cells also suppresses the development of other cell lineages such as the Th2 and Th17 subsets (Afkarian et al. 2002;Lazarevic et al. 2011;Lugo-Villarino et al. 2003). T-bet expression is strongly driven by the signal transducer and activator of transcription 1 (STAT 1) rather than on IL-12 dependent

STAT4 which is in turn driven by IFNγ. T-bet has also been demonstrated to drive IFNγ production by the differentiating naïve T cell which by a positive feedback mechanism amplifies the production of T-bet thereby further perpetuating the differentiation of naïve T cells into the Th1 lineage (Afkarian et al. 2002; Luckheeram et al. 2012).

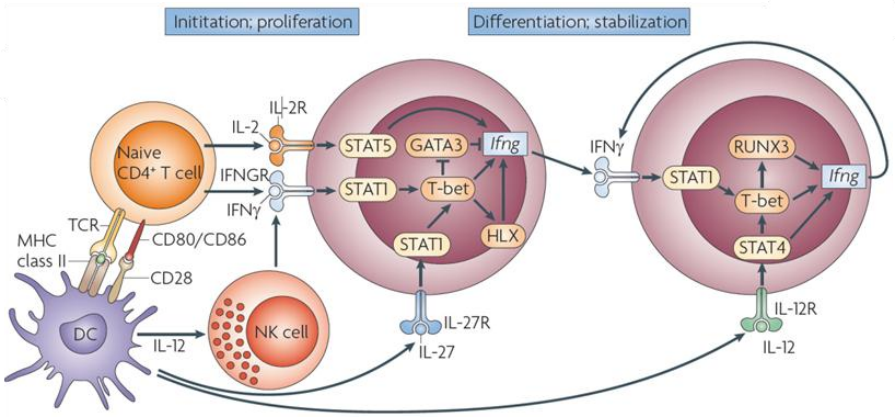


Figure 1.2 Differentiation of Th1 cells from naïve T cells. Modified from Wilson et al. 2009.

T helper 2 (Th2)

Th2 cells mediate the non-inflammatory adaptive immune response. They have been extensively demonstrated to be intrinsically vital for B cell production of IgA, IgE and IgG immunoglobulins and have also been shown to secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Luckheeram et al. 2012). In addition to providing support to humoral immune responses, Th2 cells marshal host defence against helminthic infections and allergic diseases, and generally act against extracellular parasites (Zeng 2013). They also mount antitumour activity via the recruitment of tumouricidal macrophages and eosinophils into the tumour microenvironment which secrete superoxides to breakdown the tumour (Hung et al. 1998).

IL-4 and IL-2 are important for differentiation of Th2 cells from the naïve T cell pool. In terms of transcriptional control, IL-4 induced STAT6 has been shown to drive GATA3 (GATA-binding protein) which has been identified as the master regulator for Th2 cells (Kaplan et al. 1996;Zeng 2013). The transcription factor GATA3 has been demonstrated to induce Th2 differentiation through such mechanisms as increased Th2 cytokine production, driving selective proliferation of Th2 cells via Gfi-1 recruitment and also by inhibition of Th1 differentiation by repressing the expression of T-bet possibly by downregulation of STAT4 (Usui et al. 2003;Zhu et al. 2006). In addition to GATA3, other transcription factors such as

STAT5, STAT3, c-Maf and IRF4 have been shown to play roles in Th2 differentiation (Luckheeram et al. 2012).

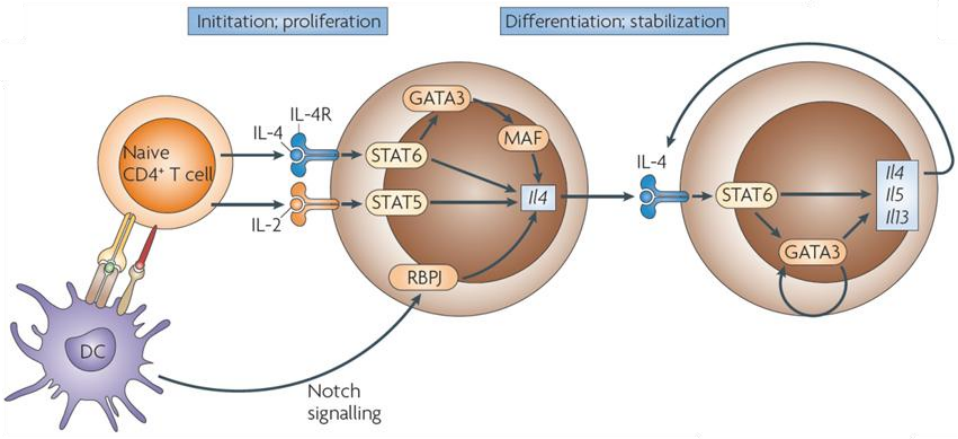


Figure 1.3 Differentiation of Th2 cells from naïve T cells. Modified from Wilson et al. 2009.

T helper 9 (Th9)

The Th9 subset is a newly characterized subset which secretes IL-9 and 10, and is currently under intense investigation to further clarify its role in the immune response. They are known to be pro-inflammatory with a wide spectrum of activity in autoimmune and allergic inflammation (Akdis et al. 2012;Shimbara et al. 2000). Specifically, presence of Th9 cells has been demonstrated to be an important causative factor in the development and progression of allergic airway disease such as asthma. Transcriptionally, 2 master regulators have been identified for the Th9 cell subset; IRF4 and and PU.1 which both bind directly on the IL-9 promoter (Chang et al. 2010;Goswami and Kaplan 2012;Ramming et al. 2012;Staudt et al. 2010). TGF-β has also been found capable of skewing the differentiation of Th2 cells towards Th9 generation. Additionally, TGF-β acting simultaneously with IL-4 has been demonstrated to the induce differentiation of Th9 cells from Th2 cells (Veldhoen et al. 2008).

T helper 17 (Th17)

Th17 cells are IL-17 and IL-22 secreting effector T cell produced following naive T cell triggering under the influence of IL-23 and are reported to be widely associated with inflammation induced and associated conditions such as rheumatoid arthritis, psoriasis and Crohn's disease (Broere et al. 2011) as well as systemic lupus erythematosus, multiple sclerosis and asthma (Dong 2006;Kolls and Linden 2004). Additionally, Th17 cells have been

found to play an important role in the pathogenesis of autoimmune encephalomyelitis (Aranami and Yamamura 2008). The presence of IL-17 induces expression of pro-inflammatory cytokines such as IL-6 and granulocyte colony-stimulating factor (G-CSF), matrix metalloproteases (MMP) and chemokines (CXCL8, CXCL2). These factors lead to macrophage and neutrophil recruitment to sites of infection. Also, IL-22 secreted by Th17 cells works in a synergistic manner with IL-17 to induce antimicrobial peptides such as β-defensins in keratinocytes which help to boost the innate immune response against infections (Schwandner et al. 2000).

The major cytokines involved in Th17 cell differentiation are Il-6, IL-21, IL-23 and TGFβ with the retinoic acid receptor acid receptor-related orphan receptor gamma-T (RORγt) as master transcription factor (Luckheeram et al. 2012). RORα has also been shown to act synergistically with RORγt in Th17 lineage committal and its absence has been demonstrated to totally abort the development of Th17 cells (Yang et al. 2008). TGFβ signaling is also vital to Th17 induction but only at low concentrations and in the presence of IL-6. However, when present in high concentrations, TGFβ drives iTreg production via FoxP3 induction (Chen et al. 2003;Zhou et al. 2008a). In Th17 differentiation, STAT 3 activated downstream of IL-6, IL-21 and IL-23 is important for the induction of the master transcription factor RORγt and STAT3 deficiency has been found to drive enhanced expression of T-bet and Foxp3 which are master regulators for the differentiation of other cell lineages (Yang et al. 2007). Additionally, other transcription factors such as Runx1, Batf, IRF4 and the Aryl hydrocarbon receptor (AhR) are known to drive Th17 induction via mechanisms such as inhibition of STAT1 and STAT5 signaling which are known to inhibit Th17 differentiation (Kimura et al. 2008).

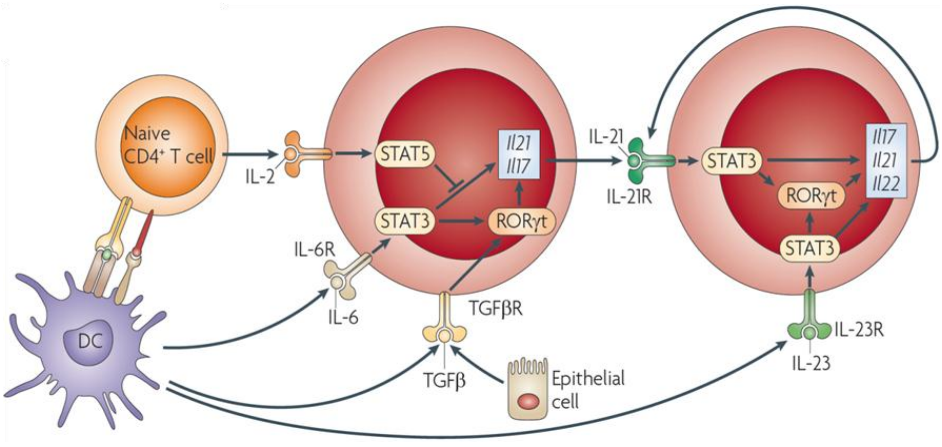


Figure 1.4 Differentiation of Th17 cells from naïve T cells. Modified from Wilson et al. 2009.

T helper 22 (Th22)

Th22 cells are characterized by the production of IL-22 and contribute to epidermal hyperplasia in patients with cases of inflammatory skin disease. While Th17 cells and NK cells also produce IL-22 in addition to their main cytokines, cells identified as "Th22" produce very high amounts of IL-22 independent of IL-17 dependent mechanisms (Eyerich et al. 2009;Nograles et al. 2009). Although initially described as been an offshoot of Th1 cells (Gurney 2004), they are now known to be a distinct lineage subset. IL-22 which is highly expressed by Th22 cells exhibits antimicrobial defence of keratinocytes by enhancing the expression of Calgranulin A, calgranulin B, psoriasin, β-defensin 2 and β-defensin 3, working in synergism with IL-17 (Liang et al. 2006;Wolk et al. 2006). Th22 cells are also important for the clearance of enteropathogenic bacteria (Basu et al. 2012). In terms of transcriptional control, little is known about how Th22 cells are induced but AhR and T-bet have been reported to be vitally involved in Th22 differentiation and function along with signaling along the IL-6 cascade (Basu et al. 2012).

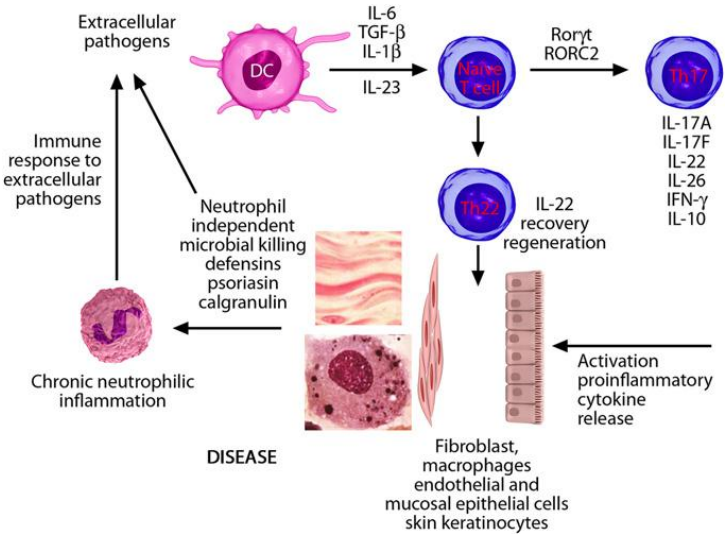


Figure 1.5 Role of Th17 and Th22 cells in inflammation and immunity. Schematic depicting overview of cytokine released from Th17 and Th22 during an immune response (Akdis et al. 2012).

Follicular Helper (Tfh) T cells

Tfh cells otherwise known as follicular B helper T cells are a specialized cell lineage which is primarily concerned with helping B cells produce antibodies and by their expression of the CXCR5 are able to localize to the B cell regions of SLO (Breitfeld et al. 2000). IL-6 and IL-21 are the key cytokines needed for Tfh differentiation acting via the STAT3 signaling pathway in a TGFβ and RORγt independent manner. Inducible co-stimulator (ICOS) is also

important for Tfh induction as it has been demonstrated that in ICOSL deficient mice, Tfh cells are significantly reduced (Nurieva et al. 2008;Vogelzang et al. 2008). Recently, Bcl6 has been identified as a candidate for master regulator status in Tfh induction as its expression has been demonstrated to inhibit the production of other cell lineages (Nurieva et al. 2009).

1.1.2.2.3 Regulatory T cells (Tregs)

Treg cells as a subset include more than one cell type and are important for maintenance of peripheral resistance, down modulation of the amplitude of immune responses and prevention of autoimmune diseases (Murphy, 2012). The concept of Treg existence and activity was first proposed by R. K. Gershon in 1975 (Jonuleit and Schmitt 2003), and finally proven by the seminal work of Shimon Sakaguchi and co-workers in 1995 (Sakaguchi et al. 1995). It is now known that Tregs play a major role in immunologically relevant situations such as cancer, inflammatory conditions and transplant acceptance or rejection and are distinguishable based on cytokine profile and cellular markers and differentiation under the influence of antigen stimuli (Roncarolo et al. 2006).

Tregs are mostly differentiated based on the expression of the Foxp3 transcription factor (Roncarolo and Gregori 2008). Foxp3 is a member of the FOX protein family and is widely projected as the master transcription factor in Treg development and function (Marson et al. 2007;Zhang and Zhao 2007). Lack of the Foxp3 gene product scurfin leads to acutely lethal lymphoproliferative disease (Khattri et al. 2003). Tregs are generally designated as Foxp3+ or Foxp3-. For the Foxp3+ subset, there are 2 major cell types; the induced Tregs (iTreg) and the naturally occurring Tregs (nTreg). The Foxp3 subset encompasses both the iTreg and nTregs. iTregs develop from naïve T cells in the periphery after antigen triggering as against nTregs which are derived from the thymus as a terminally differentiated lineage with the Foxp3 transcription factor already expressed (Chen et al. 2003). For iTregs, TGF-β is the major cytokine required for their induction from naïve T cells following triggering through the TCR (Chen et al. 2003; Luckheeram et al. 2012). Smad2 and Smad3 activation via TGF-β pathways also plays a role in Foxp3 induction during iTreg development (Takimoto et al. 2010). Also important for iTreg induction is the presence of IL-2. IL-2 acting via STAT5 signaling enhances Foxp3 expression skewing naïve T cells towards the iTreg lineage. Conversely, defective IL-2/STAT5 signaling inhibits iTreg development instead favouring Th17 induction (Burchill et al. 2007;Davidson et al. 2007). Absence or mutation of Foxp3 is

associated with severe systemic autoimmune diseases in mammals (Passerini et al. 2011). Aside the classic Foxp3+ iTregs, there are other iTreg populations which are non-Foxp3+.

The Foxp3- Treg subset of which the T regulatory type 1(Tr1) is the most studied are IL-10 producing cells which have been shown to be important in suppressing autoimmune processes and inflammation. IL-27 and IL-10 have been described as being key cytokines in Tr1 induction (Awasthi et al. 2007;Gregori et al. 2010). While the mechanism via which IL-10 acts to induce Tr1 cells is yet to be elucidated, it is known that IL-27 triggers 4 key transcription factors necessary for Tr1 induction; c-Maf, IL-21, AhR and ICOS (Apetoh et al. 2010;Pot et al. 2009).

The inhibitory activity of Treg require TCR stimulation and are mostly mediated via a cell contact dependent mechanism and secretion of such factors as Il-10 and TGF-β (Broere et al. 2011) and TNF-α (Kindt et al. 2007) among several other factors. These factors function by suppressing differentiation of other T cell subsets in addition to prohibiting MHC and co-stimulatory molecule upregulation on APCs thereby leading to reduced and inefficient activation of T cells (Murphy et al. 2012). Other mechanisms such as IL-2 deprivation leading to apoptosis (Pandiyan et al. 2007) and blockade of cyclic adenosine monophosphate activity (Bopp et al. 2007) have been implicated as means via which Tregs mediate their activity. Tr1 cells exert their effect via IL-10 secretion as evidenced via experiments with IL-10 blocking antibodies (Levings et al. 2002). The effect of IL-10 secretion leads to suppression of Th1 and Th2 immune responses to pathogens, cancer cells and allo-antigens while supernatants from cultures of activated Tr1 cells decrease the potency of DCs in allogeneic T cell activation and proliferation (Jonuleit & Schmitt 2003).

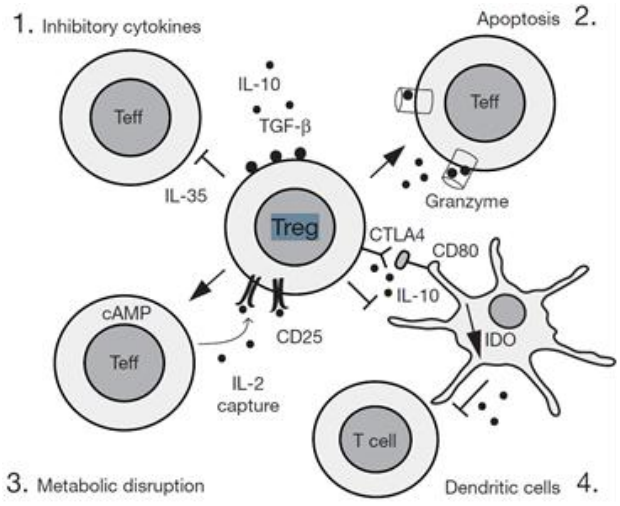


Figure 1.6 Numerous mechanisms of action of Tregs. Tregs demonstrate different mechanisms for mediating suppressory activity some of which are enumerated above (Broere et al. 2011).

1.1.3 Antigen Presenting Cells (APCs)

For T cells to differentiate and become functional there needs to be TCR triggering/activation. This triggering/activation step is performed by a group of specialized cells generally referred to as APCs (Reichardt et al. 2010). APCs are extremely important in marshaling an immune response as T cells lacks the ability to respond to free unbound antigens and only respond to antigen presented specifically on the MHC molecule (Murphy, 2012; Kindt et al. 2007).

Of all APCs, DCs are the most potent and efficient at T cell activation. They are relatively short-lived bone-marrow derived cells which are located in the skin (Langerhans cells), airways (within epithelial cells) and in the lamina propria of the gut as well as SLOs. At these locations, DCs are immature as denoted by their low expression levels of co-stimulatory markers and highly phagocytic behaviour (MacDonald et al. 2009). Immature DCs are highly endocytic and after encountering vaccine adjuvants or infectious agents using their multitude of pattern recognition receptors they take up microbes or other immunogenic matter, begin to upregulate co-stimulatory molecules following which they process and present peptides on their MHC. The process of change from immature to mature DCs is essential to DC antigen presenting capacity as demonstrated by the fact that freshly isolated sentinel Langerhans cells are known to be very poor APCs and only after maturation when they begin to express very high levels of MHC II molecules of the magnitude of 10^6 per cell do they become efficient APCs (Schuler and Steinman 1985). Following on from this step, matured DC exit the tissue and migrate via afferent lymph nodes to the T cell zones of the nearest draining lymph node where they initiate immune responses by activating naïve T cells (Pulendran et al. 2001;Reis e Sousa 2006). Macrophages in addition to their well-documented ability to phagocytose large particulate bodies are also able to break down these bodies and present peptides from these foreign matters on their MHC molecules (Murphy, 2012; Kindt et al. 2007). However, macrophages due to their lower expression rates of MHC and co-stimulatory molecules are less efficient APCs than DCs (Santin et al. 1999). B cells while researched more for their antibody producing roles are also known to be capable of presenting antigens to T cells. It has been argued that the ability of B cells to take up and present antigens as peptide fragments is an important step in mounting an adaptive immune response (Mitchison 2004). B cells function as APC firstly by recognition and capture of foreign antigen by the BCR, then degrading antigen within the internal compartments of the cell and

then transporting peptides out of the cell where they are loaded on the MHC II molecules following which these peptides are presented to T cells (Yuseff et al. 2013). B cells encounter both soluble and large particulate antigens from such cells as macrophages and DCs as well as those in the circulation (Qi et al. 2006;Suzuki et al. 2009). B cells have limitations taking up large particulate antigens instead favouring soluble antigens. This limitation, could contribute to reasons why B cells are regarded as the least efficient APC of all three APCs addressed so far (Unanue 1984), in addition to the fact that B cells express extremely low levels of co-stimulatory molecules (Lenschow et al. 1996).

There are 2 factors that make for qualifying a cell as an APC. Firstly, such a cell must be able to present antigen on their MHC to the TCR in order to trigger what is considered "signal 1" which is antigen specific and secondly, to be considered an APC, such a cell should be able to provide "signal 2" which is the co-stimulatory input and non-antigen specific (Jenkins 1992;Schwartz 1990). While DCs, macrophages and B cells meet these requirements to different extents, there are other cells such as keratinocytes and epithelial cells which are thought to be able to present antigen but lack co-stimulatory capacity and could under conditions of inflammation upregulate the co-stimulatory B7 proteins. These cells are regarded as non-professional APCs while the former comprising of the macrophages, B cells and DCs are professional APCs (Mellman et al. 1998;Nickoloff and Turka 1994;Sprent 1995).

1.1.4 Dynamics of CD4+ T cell Activation

In order to mount an immune response, T cells need to be activated from their naïve state. Naïve cells expressing the molecules CCR7 and CD62-L journey through the general circulation and enter into the SLO (mostly lymph nodes) using these homing molecules under the influence of a CCR7 gradient. Naïve T cells expressing CCR7 and CD62-L enter into SLO structures by trans-endothelial migration through the high endothelial venules of the micro-circulation based on their expression of these molecules which enable them to adhere and roll on the endothelium of the HEV. After this rolling step on the endothelium, naive T cells are "arrested" by the interaction with integrins present on the endothelium following which they extravasate through the endothelial layer and get into the lymph node (Arbones et al. 1994;Galkina et al. 2007;Weninger et al. 2001). In the lymph node, T cells scan against antigen bearing APCs until they find APC bearing cognate antigen (Goodnow and Cyster 1997;Reichardt et al. 2010). Interactions between APCs and T cells have been shown to be dynamic in nature (Gunzer et al. 2000). This interaction leads to binding of the TCR and CD4 co-receptor onto the MHC II complex and co-stimulatory B7 proteins on the APC to create an

immunological synapse. The immune synapse is a dynamic structure consisting of clusters of signaling molecules from both T cell and APC with these cells being bound to each other by the adhesion molecules LFA-1 and intercellular adhesion molecule-1 (ICAM-1) respectively with an inner signaling hub called the central supramolecular activation complexes (c-SMAC) which contains the signaling molecules and the peripheral supramolecular activation complexes (p-SMAC) made up of adhesion molecule which protect the integrity of the structure and maintains the contact for effective T cell activation (Monks et al. 1998;Philipsen et al. 2013).

During T cell-APC interaction, the αβ chains of the TCR which are non-covalently bound with invariant chains of the CD3 molecule play a major role in driving T cell activation as its multiple immunoreceptor tyrosine-based activation motifs (ITAMs) provide sites for the activity of protein tyrosine kinases (PTK) which serve to perpetuate the intracellular signaling events necessary for optimal T cell activation, proliferation and differentiation. TCR signaling along with CD3 activation sets off cellular and molecular processes involving recruitment of downstream kinases such as Lck and ZAP-70 that ultimately lead to proliferation and differentiation of these naïve T cells into effector lineages dependent on APC type, environmental cue and amounts of co-stimulatory molecules present (Brownlie and Zamoyska 2013; Tao et al. 1997). Src family kinase members such as Fyn and Lck phosphorylate ITAMs leading to recruitment of ZAP-70 via their Src-Homology (SH2) 2 domains. This recruitment leads to phosphorylation and activation of ZAP-70 by Lck. pZAP-70 in turn phosphorylates SLP-76 and LAT. SLP-70 has been implicated in actin cytoskeleton rearrangement which plays a role in immune synapse development dynamics while pLAT as a cell membrane adaptor protein provides binding sites for such proteins as Grb2, Ras and PLC-γ. PLC-γ then plays a critical role in Ca^{2+} flux as it cleaves 4,5-biphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) following activation by phosphatidylinositide 3-kinase (PI3K). DAG in turn stimulates Protein Kinase C (PKC) and the accumulation of IP3, as the activation stimuli persists, it triggers the first wave of intracellular Ca^{2+} release that leads to opening of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Ultimately, these tightly co-ordinated signaling events lead to activation of transcription factors such as NF-AT, ELK-1, Jun in T cells and other genes needed to induce T cell activation (Brownlie and Zamoyska 2013;Weiss 2010).

Co-stimulatory signals augment TCR signals to promote proliferation and differentiation. The main co-stimulatory molecule is CD28 on the T cell which has the B7 proteins CD80 (B7-1) and CD86 (B7-2) on APC as ligands. There are other less potent co-stimulatory molecules

involved in T cell activation dynamics such as ICOS, OX-40 and CD27 which are also expressed on APCs (Croft 2009;Greenwald et al. 2005). At the inception of T cell activation, cytokines initially secreted from the APCs and also from innate immune cells help to drive the process and then as the process progresses, the differentiating T cell begin to produce cytokines which then by a positive feedback loop perpetuate the differentiation process (Luckheeram et al. 2012). After the process of triggering, activated T cells exit the SLO to the exterior where they mount an immune response by secreting cytokines (Reichardt et al. 2013;Seder and Ahmed 2003). This egress is possible based on the downregulation of the homing molecules CD62-L and CCR7 as well as the concomitant upregulation of receptors which drive homing of T cells to sites of inflammation such as very late antigen 4 (VLA-4), P- and E-selectin ligands, and inflammatory chemokine receptors such as CXCR3 and CCR5 (Forster et al. 2008;Mora and von Andrian 2006). Of extreme importance in T cell egress is the upregulation of the sphingosine-1-phospahate receptor on T cells which leads to T cell chemotaxis towards high levels of sphingosine-1-phosphate in deep regions of the lymph node from where T cell migrates through the LN medulla across medullary cords and sinuses before reaching efferent lymphatic vessels which takes the T cells into the general circulation (Reichardt et al. 2010). While most of the activated T cells egress as effectors, a percentage of them differentiate into memory T cells that retain "immunological memory" and thus retain the capacity to respond with even more efficiency and strength against the antigens they recognize in a secondary infection (Seder & Ahmed 2003).

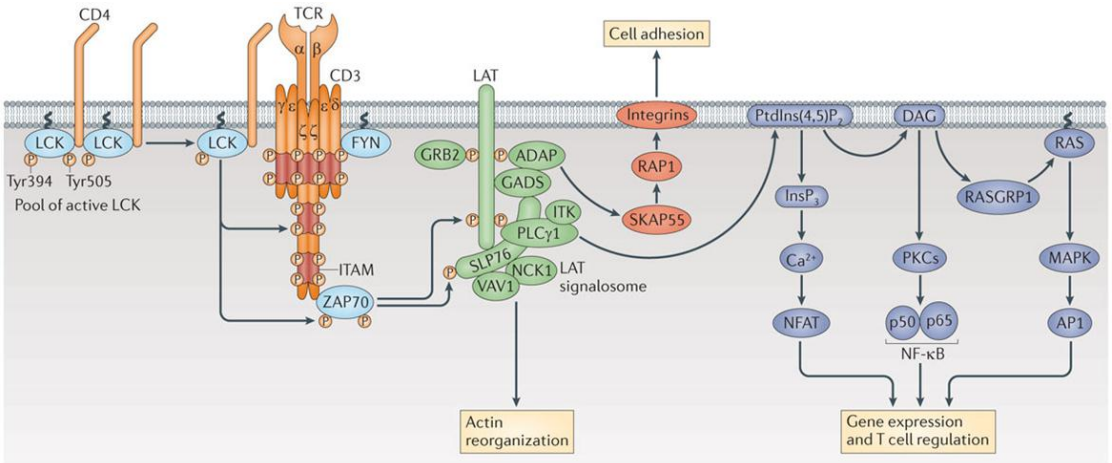


Figure 1.7 Schematic of signaling circuit in T cells. APCs with cognate antigen binds onto the TCR of T cells while B7 proteins from APCs bind on the CD4 protein in T cell setting off signaling cascades leading to T cell activation and the acquisition of functionality based on cytokine profile (Brownlie and Zamoyska 2013).

1.1.5 The Role of the PI3K/mTOR Pathway in T cell Activation and Function

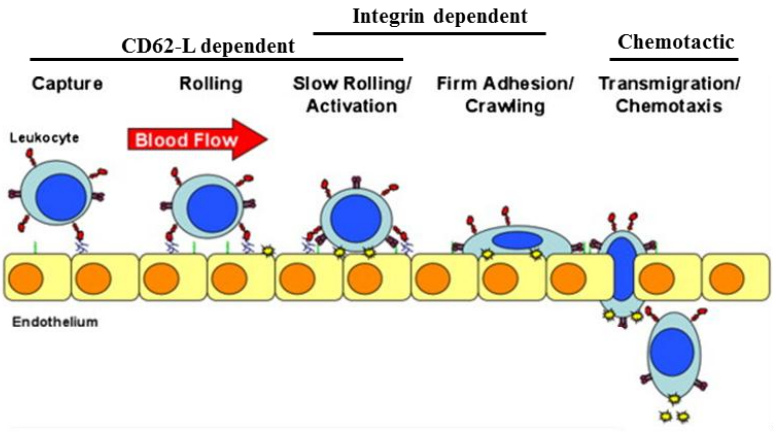
The PI3K pathway is an ancient conserved signaling pathway in metazoans (Wang et al. 2008a). In T cells this pathway regulates a wide range of functions such as cell growth, proliferation, survival, differentiation and metabolism and can be activated by several means including TCR activation, co-stimulatory molecule activation, as well as cytokine and chemokine receptor triggering (Okkenhaug et al. 2004;Wang et al. 2008a). Triggering of the TCR leads to phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) which is recruited to the cell membrane to produce phosphatidylinositol-3,4,5-triphosphate (PIP3) (Han et al. 2012). The secondary messenger PIP3 plays a crucial role in initiation of PI3K signaling activity. Although present in very low amounts in naïve T cells, this lipid secondary messenger is rapidly upregulated following TCR triggering by MHC bearing cognate antigen. As the activated T cell remains in contact with APC, PIP3 increasingly accumulates at the cell plasma membrane (Costello et al. 2002;Finlay and Cantrell 2010;Harriague and Bismuth 2002). Recruitment of PIP3 in turn leads to recruitment of Phosphoinositide-dependent kinase 1 (PDK1) as well as Protein Kinase B (PKB) otherwise called Akt. PDK1 then phosphorylates Akt at Thr308 and in order to achieve full Akt activation, the mammalian target of Rapamycin complex 2 (mTORC2) phosphorylates Akt on Ser473 (Bhaskar and Hay 2007;Fayard et al. 2010). mTOR activation leads to protein synthesis and regulation of metabolic processes needed for growth and cell differentiation as well as survival (Laplante and Sabatini 2009;Laplante and Sabatini 2012). Akt activation leads to exclusion of members of the Foxo transcription factors such as Foxo1, Foxo3a, Foxo4 and Foxo6 from the cell nucleus (Fabre et al. 2005;Hay 2011). Foxo1 has been implicated as a major transcription factor driving the production of KLF-2 which in turn drives the promoter regions of the genes of the T cell homing molecules CD62-L and CCR7 (Fabre et al. 2008;Sinclair et al. 2008). Thus, in addition to being implicated in regulating T cell metabolism after activation, the PI3K pathway also regulates T cell migration. This function is important because for T cells to function optimally, they have to be at the right site of the body to deliver their cytokines by which they influence the immune response (Finlay and Cantrell 2010).

1.1.6 Function of CD62-L in T cells

CD62-L (L-selectin or Mel-14 antigen) is a cell adhesion molecule which is made up of a large highly glycosylated extracellular Ca^{2+}- dependent lectin domain, a single spanning transmembrane domain resembling an epidermal growth factor-like region as well as a small cytoplasmic tail which are similar to complement regulatory proteins (Phong et al. 2003).

CD62-L is expressed on most leukocytes such as neutrophils, T cells and B cells where it is involved in the rolling of leukocytes on inflamed endothelial of the vasculature, preceding the firm arrest of T cells by interaction with integrins on the vascular endothelium and subsequent transmigration/extravasation of these cells through the endothelium into SLOs (Kindt et al. 2007; Murphy 2012). Naïve T cells circulate continually in the general circulation via the lymphatic system and blood through SLOs such as lymph nodes and the protein L-selectin plays a key role in driving the migration of these cells into the lymph nodes where they encounter antigen (Finlay and Cantrell 2010). This trafficking of T cells to the SLO begins with the attachment of circulating T cells to the high endothelial venules (HEVs) of lymph nodes via agency of the interaction between the lectin like glycoprotein L-selectin, expressed on T cells and carbohydrates on the endothelium such as the mucin-like ligands, GLYCAM-1 and CD34. The density of L-Selectin expression per cell is directly proportional to its ability to bind onto the endothelium as *in vivo* studies have shown that cells (both normal and malignant) with high level expression of L-Selectin traffic more efficiently to lymph nodes than cells which lack this receptor (Kaldjian and Stoolman 1995). Thus L-selectin plays a role in localization of cells *in vivo* as it serves as a homing molecule.

Following T cell activation, the extracellular domain of L-Selectin is cut by the enzymatic activity of the Tumour Necrosis Factor converting enzyme (TNF-Converting Enzyme or TACE, also called disintegrin and metalloprotease (ADAM) 17) (Smalley and Ley 2005) using an ERK dependent mechanism (Sinclair et al. 2008). Shedding activity apart from its importance in T cell localization is also used as a measure of assessing T cell activation status as naïve T cells are denoted by their high expression of CD62-L (Moreau et al. 2012). There are however T cell subsets that constitutively express high CD62-L levels, examples of which include memory T cells (Sinclair et al. 2008) as well as regulatory T cells (Grailer et al. 2009), as high CD62-L enable these cells localize to the lymphoid organs where they subserve their functions.



Capture	Rolling	Slow Rolling/ Activation	Firm Adhesion/ Crawling	Transmigration/ Chemotaxis
Mediates capture through binding to endothelial- and leukocyte-expressed ligands	Supports rolling at higher velocities than E- or P-selectin	Functions synergistically with ICAM-1 to reduce leukocyte rolling velocities	Ligation stimulates β_1 and β_2 integrin-dependent adhesion	Ligation enhances chemotaxis to SLC, IL-8, SDF-1α, and PAF, and increases surface CXCR4 expression

Figure 1.8 Role of CD62-L in T cell migration to lymph nodes. Steps involved in T cell trafficking to the lymph node depicting the CD62-L dependent steps of T cell capture, rolling and slow rolling following which activation of integrins sets in to mediate firm adhesion and subsequent chemotaxis under the influence of chemoattractants to the lymph nodes. Modified from Grailer et al. 2009.

1.1.7 The Role of Epigenetics in T cell Differentiation and Function

Gene transcription is regulated by the complexing of transcription factors onto the regulatory regions of genes. In more intricate details, transcription is regulated based on the availability of transcription factors, their localization and higher order interactions with other transcription factors, co-activators or repressors (Wilson et al. 2005). Accessibility of regulatory regions of target genes which is greatly influenced by post-translational modifications (PTM) acting via conformational changes in chromatin represent a second level at which gene transcription is controlled. These changes which affect chromatin accessibility to transcription factors are referred to as epigenetic changes and include such changes as DNA methylation status and histone modifications (Berger 2007;Rowell et al. 2008;Wilson et al. 2009). Epigenetics is the study of the mechanisms inducing phenotypic changes in cell behaviour which are independent of actual DNA sequence changes but are rather driven by conformational changes in DNA structures such as the aforementioned modifications of chromatins which affect the timing and extent of specific gene expression in a particular cell. Therefore, by regulating the chances of transcription factor binding onto gene regulatory regions thus limiting access of RNA polymerase II containing complexes necessary for commencement of gene transcription, epigenetic factors are able to ablate, attenuate or drive efficient gene

transcription (Ansel et al. 2003;Li 2002;Smale and Fisher 2002). Since DNA sequence is unchanged, epigenetic modification and attendant phenotypical results they encode are heritable traits. This heritability is possible by agency of the DNA methytransferase 1 enzyme which faithfully copies methylation patterns from methylated cytosines from parental DNA strands onto daughter DNA strands (Wilson et al. 2005).

DNA methylation is the most well studied of all epigenetic modifications. In mammals, only the cytosine residue on CpG dinucleotides of DNA can be methylated via the activity of 2 types of DNA methyltranferase (DNA MTase or Dnmt) family enzymes which catalyze the process of transferring a methyl group to DNA with S-adenosyl methionine (SAM) acting as methyl donor. There are 2 types of Dnmts important for induction of DNA methylation patterns; Dnmt1 and Dnmt3 which has 2 isoforms; Dnmt3a and Dnmt3b (Bheemanaik et al. 2006;Malygin and Hattman 2012). DNA methylation is begun by Dnmt3a and DNMT3b which induce de novo methylation on unmodified CpG sites and then Dnmt1 is upregulated during the S-phase where it is recruited by proliferating cell nuclear antigen (PCNA) and preferentially binds onto hemi-methylated CpG sites on dividing cells where it serves to maintain methylation patterns similar to those of parent cells. Dnmt1 thus serve as maintenance transferases (Okano et al. 1998;Pradhan and Esteve 2003). In general, DNA methylation represses gene activity with the notion that gene silencing correlates with DNA methylation at promoter regions of such genes while hypomethylation at same region is linked to gene activation and expression (Li et al. 2012; Wilson et al. 2005; Wilson et al. 2009). Next to DNA methylation, histone modification of which acetylation is a major player has been implicated and extensively investigated for its role in gene regulation. Reversible acetylation of the amino group of lysine in histone tails by histone acetylases (HATSs)/histone deacetylases (HDACs) is one of the characterized histone modifications and is linked to transcriptional activation/repression respectively. Hyper-acetylated histones are transcriptionally active while hypoacetylated histones are transcriptionally inactive/repressed (Moreira et al. 2003).

In T cell biology, epigenetics plays a major role in the development of T cells from precursor stage up to full maturation status in the thymus (Carbone et al. 1988;Wu et al. 1990). There is a vast body of literature implicating epigenetic modifications as major players in the differentiation process of naïve T cells after activation (Kanno et al. 2012;Thomas et al. 2012). Naïve T cells when activated with antigen initially secrete low levels of both IFNγ (Th1) and IL-4 (Th2) cytokines thus indicating that naïve T cells are poised at the chromatin level to respond to signals that drive differentiation. These changes are common to both Th1

and Th2 skewing conditions and are known to be independent of STAT4 and STAT6 signaling along with the transcription factors needed ultimately for cell fate lineage determination (Grogan et al. 2001). However, by mechanisms not fully understood, during differentiation these cells decide on one cell fate over the other. During this differentiation process, cells with Th1 lineage fate begin to exclusively produce IFNγ while silencing the IL-4 gene transcription and for cells destined for Th2 cell fate, the IL-4 gene locus is activated while the IFNγ producing locus is transcriptionally silenced. These changes are underlined by changes on chromatin structure and heritably transmitted over multiple cycles proliferation and involves several factors such as co-ordination of DNA, histone modifications and marshaling of feedback processes that enhance the expression of some genes while suppressing expression of others based on cytokine feedback mechanisms (Avni et al. 2002;Fields et al. 2002;Murphy and Reiner 2002). Epigenetic modifications have also been demonstrated as setting the threshold for Th9 cells differentiation by histone modifications at the PU.1 gene locus that favour gene transcription (Ramming et al. 2012). In Tregs, the Foxp3 locus has been shown to be demethylated as against methylation of the same locus in naïve T cells (Floess et al. 2007) while on a global level, there have been demonstrated to be over 100 differentially methylated regions (DMRs) between Tregs and conventional naïve T cells including such genes as Foxp3 and IL-2 (Schmidl et al. 2009). Demethylation of the Foxp3 locus probably licenses the T cell to express the Foxp3 protein which confers regulatory ability.

Another level of epigenetic control of T cell differentiation involves small RNA (RNAi) molecules which typically inhibit gene expression. This inhibitory activity is driven by small 20-30 nucleotide non-coding RNA and was first discovered in *Caenorbabditis elegans*. There are several types of inhibitory RNA such as the endogenous micro RNA (miRNA or miR) and the synthetic/exogenous Short Interference RNA (siRNA) (Lee et al. 1993;Wilson and Doudna 2013). Of this group of small inhibitory proteins are the miRNAs which have been widely studied and are thought to regulate as much as 50% of all genes, with almost all mammal cells engaging miRNA processes for gene regulation (Bartel 2009;Friedman et al. 2009). In T cells, miRNAs are known to regulate such processes as proliferation, survival as well as controlling the differentiation process from naïve to effector lineages or regulating cytokine production. It is also known that miRNA deficient T cells still maintain their capacity to become effectors. It is therefore thought that miRNAs are more important in T cells for enforcing differentiation states by inhibiting transcription factors that drive other lineage fates. This line of thought is further supported by the fact that miRNA deficient T

cells tend to lose their effector status and acquire the characteristics of other T cell subsets (Jeker and Bluestone 2013;Muljo et al. 2005;Zhou et al. 2008b). Some known functions of specific miRNAs in T cell include and are not limited to the following: miR-10a restrains conversion of iTregs into Tfh cells (Takahashi et al. 2012); miR-29ab represses IFNγ (Ma et al. 2011;Steiner et al. 2011); miR146a promotes Treg function as well as inhibiting IFNγ production in CD4 T effector cells (Lu et al. 2010).

1.1.8 Aim of Study

Based on type of APCs, cytokines present and environmental factors, naïve T cells can differentiate into several types of effectors (Reichardt et al. 2010). Previous studies from our group have shown that the activation of antigen specific naïve T cells by antigen laden naive B cells (TofB) led to the induction of Tregs which could significantly inhibit heart organ transplant rejection and ear swelling in an allogeneic context when compared against T cells activated by DCs (TofDC) which had an T effector function and phenotype (Reichardt et al. 2007). TofB cells were found to be CD25+CD62-L+ in contrast to TofDCs which were CD25+CD62-L- and could therefore preferentially home to the lymph nodes. While DCs are the major APCs *in vivo*, it is pertinent to note that there are strong hints in the literature that B cells do come into contact with T cells with different functional outcomes (Garside et al. 1998;Zheng et al. 2009;Zheng et al. 2010).

The convenience of this co-culture system therefore offered the opportunity to kinetically study early signaling events governing Treg development in a model more physiological than obtainable with antibody stimulation. This was important as while a lot is known about function/signaling in established Tregs, very little is known about the processes governing their generation from naïve T cells. This work utilized APCs extensively for T cell triggering as against antibody stimulation as this was more physiologically and it is known that antibody stimulation dynamics differ from physiological conditions of stimulation by APCs (Arndt et al. 2013;Berg et al. 1998;Wang et al. 2008b). Armed with these facts, we set out to investigate the following:

1. What pathways drive the differential regulation of CD62-L following the activation of naïve T cells by weak and strong APCs?

2. What role does co-stimulatory input play in T cell differentiation with respect to acquisition of regulatory properties? Also, does re-expression of CD62-L link acquisition of regulatory function and homing behaviour?

3. What role does epigenetics play in the differential homing capacities of the TofB, TofDC and TofiDC?

4. What role does adhesion molecules on the stroma of the microenvironment play in T cell activation?

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Product	Manufacturer
Acetic acid	Roth
Agarose	Invitrogen
Ammonium persulfate (APS)	Bio-Rad
Aprotinin	Sigma
Bovine serum albumin (BSA)	Sigma
Calcium Chloride ($CaCl_2$)	Sigma
Commassie blue	Roth
Decitabine	Selleck Chemicals
Dimethyl sulfoxide (DMSO)	Fluka
1,4-Dithiothreitol (DTT)	Biocheringer
ECL Western Blotting Substrate	Thermo Scientific Pierce
Ethylene glycol tetraacetic acid (EGTA)	Fluka
Ethanol	Roth
Ethylendiaminetetraacetic acid (EDTA)	Fluka
Fetal calf serum (FCS)	bio west
Gentamycin	Sigma
GM-CSF	in house produced
Glycerol	Roth
Glycine	Roth
HEPES	BIOCHROM AG
IGEPAL CA-630	Sigma
Interleukin-4 (IL-4)	in house produced
Isoflurane	DeltaSelect
Isopropanol	Roth
Leupeptin	Sigma
L-Glutamine	BIOCHROM AG
Lipopolysaccharide (LPS)	Sigma
Ly294002	Promega
Magnesium chloride ($MgCl_2$)	Merck

β-Mercaptoethanol	Fluka
Methanol	Roth
Non-Essential Amino Acid (NEAA)	BIOCHROM AG
Ova Peptide (pOVA)	Peptide Core facility, HZI, Braunschweig
Paraformaldehyde	Roth
Poly Acrylamide (PAA)	Roth
Penicillin/Streptamycin	Gibco
Phenylmethanesulfonyl fluoride (PMSF)	Fluka
Ponceau S	Sigma
Potassium chloride (KCl)	Fluka
Potassium hydroxide (KOH)	Merck
Protein ladder for western blot	Fermentas
Protease Inhibitory Cocktail	Roche
Rapamycin	Calbiochem
Skim milk powder	Roth
Sodium azide (NaN_3)	Fluka
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Roth
Sodium orthovanadate (Na_3VO_4)	Sigma
Sodium pyruvate	BIOCHROM AG
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Bio-Rad
Trichostatin-A (TSA)	Sigma
Tris	Roth
Trypan Blue	Sigma
Tween 20	Roth

2.1.2 Buffers and Media

2.1.2.1 Complete Medium

500ml RPMI Media

500µL β-Mercaptoethanol (50 µM)

50mL FCS (10%)

5mL HEPES (10 mM)

5mL L-Glutamine (2 mM)

5mL Sodium-Pyruvate (1 mM)

1 x NEAA

5mL Penicillin / Streptomycin (100 U/ml)

2.1.2.2 BMDC Medium

500ml RPMI Media

500µL β-Mercaptoethanol (50 µM)

25mL FCS (5%)

500µL Gentamycin (50 µg/ml)

5mM L-Glutamine (2 mM)

250ng GM-CSF

25ng IL-4

1 x NEAA

***** Media for generating Immature DCs was exactly the same as above except that the IL-4 component was totally omitted.

2.1.2.3 Erythrocyte-Lysis buffer

4.15 g NH4Cl (0.15 mM)

0.5 g KHCO3 (1 mM)

1.85 mg Na-EDTA (0.1 mM)

Make up volume to 500ml with distilled H_2O and sterile filter.

2.1.2.4 PBS

80.0 g NaCl (136.9 mM)

2.0 g KCl (2.7 mM)

14.3 g Na_2HPO4 + 2 H_2O (8.1 mM)

2.0 g K_2HPO_4 (1.47 mM)

Make up volume to 1000ml with distilled H_2O and sterile filter. pH 7.4

2.1.2.5 PBS + 1% FCS

1000 ml PBS (1x), pH 7,4

10 ml FCS

2.1.2.6 MACS-Puffer

986.8 ml PBS

10ml FCS

3.2mL 0.5M EDTA (0.58 g)

2.1.2.7 Tris Buffered Saline (TBS) (10x)

24.22 g Tris-HCl (500mM)

80.0 g NaCl (1.5M)

Make up volume to 1000ml with distilled H_2O and sterile filter add conc. HCl to make up pH to 7.6

2.1.2.8 TBS-T

1x TBS + 0.1% Tween-20

2.1.2.9 10% SDS Solution

100g SDS

Add DD H_2O to 1L

2.1.2.10 Electrophoresis Buffer (10x)

30.28g Tris (0.25M)

144.12g Glycin (1.92M)

100ml 10% SDS (1%SDS)

Make up volume to 1L with DD H_20

To make 1x solution, dilute 1 part buffer with 9 part DD H_20

2.1.2.11 Transfer Buffer (10x)

144.12g Glycin (1.92M)

30.28g Tris (0.25M)

Make up volume to 1L with DD H_20

To make 1x working solution, mix

100ml 10x buffer

200ml MeOH

700ml DD H_2O

2.1.2.12 Ponceau S Working Solution

Ponceau S (0.5%)

Methanol (40%)

Acetic acid

2.1.2.13 Cytoplamic Lysis Buffer "Buffer A"

100µl 1M HEPES (10mM)

100µl IM KCl (10mM)

2µl 0.5M EDTA (0.1mM)

1ml IGEPAL (10% IGEPAL)

Add DD H_2O to 10ml

2.1.2.14 Nuclear Lysis Buffer "Buffer C"

100µl 1M HEPES (10mM)

20µl 0.5M EDTA (1mM)

896µl 4.6M NaCl (0.4M)

1ml Glycerol (10% Glycerol)

Add DD H_2O to 10ml

* Add the following protease inhibitors to both buffers just before use:

Phosphatase inhibitor 10 mM NaF

protease inhibitors 0.5 mM Sodium vanadate

1 mM Phenylmethylsulfonylfluoride (PMSF)

Protease Inhibitory Cocktail (1/25th volume to total lysis buffer volume)

2.1.2.15 SDS Gel

10 x SDS running buffer

250 mM Tris

2 M Glycine

1% SDS

pH 8.3

For an 8×10×0.1 cm gel, the following volumes were used:

Running gel (12%)

4.08 ml deionized H_2O

3.04 ml Tris-HCl [1,5 M], pH 8,8

4.8 ml PAA

120 µl SDS solution [10% (w/v)]

16 µl TEMED

16 µl APS (25%)

Stacking gel

1.8 ml deion. H2O

320 µl Tris-HCl [0,5 M], pH 6,8

330 µl PAA

25 µl SDS solution [10% (w/v)]

12.5 µl TEMED

12.5 µl APS [25%]

2.1.3 Antibodies and Cell staining

Western Blot

Specificity	Source	Clone	Final dilution
Foxo1	Cell Signalling	C29H4	1:1000
Lamin B	Santa Cruz	-	1:1000
Anti-rabbit Horseradish peroxidase	Cell Signalling	-	1:3000
Anti-mouse Horseradish peroxidase	Cell Signalling	-	1:3000

T cell co-stimulation

Specificity	Source	Isotype	Clone	Final Conc
CD28	BD	IgG2	37.51	10.0 µg/ml

FACS

Surface Antibodies

Specificity	Source	Isotype	Clone	Final Conc
CD4 PerCP	BD Biosciences	IgG2a	RM4-5	0.33 µg/ml
CD4 PE	BD Biosciences	IgG2b	L3T4/GK1.5	0.20 µg/ml
CD4 FITC	BD Biosciences	IgG2b	L3T4/GK1.5	0.33 µg/ml
CD4 VB 421	Biolegend	IgG2b	L3T4/GK1.5	0.33 µg/ml
CD25 PE	BD Biosciences	IgG1	PC61	0.20 µg/ml
CD25 APC	BD Biosciences	IgG1	PC61	0.33 µg/ml
CD62-L APC	BD Biosciences	IgG2a	MEL-14	0.33 µg/ml
CD62-L PE	BD Biosciences	IgG2a	MEL-14	0.33 µg/ml
CD80 PE	abcam	IgG1	2D10	0.1µg/ml
CD86 PE-Cy7	abcam	IgG2a	GL1	0.20 µg/ml
MHC II FITC	eBioscience	IgG2b	NIMR-4	0.83 µg/ml
CD11c APC	Caltag	IgG	N418	0.33 µg/ml

Intracellular Antibodies

Specificity	Source	Isotype	Clone	Final Conc
Total Akt FITC	Cell Signalling	IgG	C67E7	0.25 µg/ml
pAkt Thr308 FITC	Cell Signalling	IgG	C31E5E	0.25 µg/ml
pAkt Ser473 FITC	Cell Signalling	IgG	D9E	0.25 µg/ml
Total S6 FITC	Cell Signalling	IgG1	54D2	0.25 µg/ml
pS6 Ser235/6 FITC	Cell Signalling	IgG	D57.2.2E	0.25 µg/ml
pS6 Ser240/4 FITC	Cell Signalling	IgG	D68F8	0.25 µg/ml

* Cognate isotype antibodies were applied for proper gating and data interpretation.

Fluorescence microscopy

Specificity	Source	Isotype	Clone	Final dilution
Foxo1	Cell Signalling	IgG	C29H4	1:100
DAPI	Invitrogen	-	-	1:500
Rabbit anti-mouse 2^{o} antibody	BD Biosciences	IgG2a	11-26c.2a	1:500

2.1.4 Kits

2D QuantKit	GE healthcare Life Sciences
Carboxy Fluoroscein Succinimidyl Ester (CFSE)	Invitrogen
Cytoperm/fix kit	BD
DAPI	Invitrogen
DNeasy Blood and Tissue kit	QIAGEN
Epitect Bisulfite kit	QIAGEN
LS magnetic separation columns	Miltenyi
Mice B cell isolation kit	Miltenyi
Mice T cell isolation kit	Miltenyi
MISCRIPT reverse Transcription Kit	QIAGEN
MISCRIPT RNEasy Isolation kit	QIAGEN
ProLong® Antifade Kit	Invitrogen
QIAquick PCR Purification kit	QIAGEN
Reverse Transcription Kit	QIAGEN
RNEasy Isolation kit	QIAGEN
SYBR green kit	QIAGEN

2.1.5 Primers

The following primer kits were purchased as ready made from QIAGEN

Mice PHLPP1

Mice Actin

Mice let-7b

Mice RNUS6

Methylation specific PCR for the Klf-2 locus was designed using methprimer. Primer characteristics are as given below:

CpG Island 1 (Product size: 298kb)

Primer	Tm (^{o}C)	GC%	Sequence
Forward	54.99	75.00	GGAGTTTTAGGTGTTATTTTTTTT
Reverse	56.92	66.67	ACCCACCCAAACCTTATAAAC

CpG Island 2 (Product size: 142kb)

Primer	Tm (^{o}C)	GC%	Sequence
Forward	59.93	70.83	GGGGTGTAGGGTAGTAGGAGGTAT
Reverse	58.33	52.00	CCAATCCCATAAAAAAAATAAAATC

2.1.6 Cell Culture Materials and Laboratory ware

20°C freezer	Liebherr Premium
4 °C refrigerator	Liebherr Premium
80°C freezer	Thermo Fischer Scientific
Bottle top filters (22µM)	Millipore
Cell Culture Microscope	Leica
Cell culture plates (6, well, 24 well & 96 well round bottom)	Nunc
Cell strainers (40µm and 70 µm)	BD Falcon
Centrifuge (mini) 2ml, 1.5ml	eppendorf
Centrifuge for plastic tubes (15 und 50 ml)	eppendorf
CO_2 Incubator	Thermo Fischer Scientific
Culturewell coverslips (12mm)	neo Lab
Electrophoresis chamber	BioRad
Eppendorf tubes (1.5 ml, 2ml)	eppendorf
FACS Calibur	BD Biosciences
FACS LSR	BD Biosciences
FACS tubes	Corning Costar®

Falcon tubes (15, 50 ml)	Greiner bio-one
Heating block	eppendorf
Ice machine	Scotsman
Micropipettes	Gilson
Neubauer Counting chamber	Marienfeld
Objective slide	Thermo Fischer Scientific
T3 Kombi PCR Thermocycler	Biometra
PCR Tubes	Greiner bio-one
Petri dishes	Corning
pH Meter	Schott
Photometer	SmartSpec Plus
Pipette tips (20 µl & 1000 µl)	Greiner bio-one
Pipette tips (200 µl)	eppendorf
Precision weighing scale	Sartorius
SDS PAGE Apparatus	Bio-Rad
Sterile bench	Haraeus LaminAir
Syringes (1 ml, 5 ml, 20 ml)	Omnifix®
Syringes (1ml)	Omnican
Vortex	Scientific Industries
Water bath	Julabo
Western blot Apparatus	BIO-RAD

2.1.7 Software Used

The following software was used in this work from aspects ranging from word and document processing to data analysis and experiment design.

I. MS office: word processing and data presentation

II. Flowjo-Tristar: Analysis of FACS data

III. Graphpad prism: Data presentation

IV. Fiji-Image J: Image Analysis

V. Methprimer: Design of primers for Methylation Specific PCR (MSP)

VI. BiQ Analyser: Analysis of sequences after Bisulfite treatment

VII. ZEN pro 2011: Data acqusition on Zeiss Apotome microscope

2.2 Methods

2.2.1 Mice

Wildtype mice of the C57Bl/6 strain were sourced from Harlan Winkelmann, Charles Rivers or Jackson labs while Balb/c mice were obtained from Harlan and Jackson labs. Both wildtype strains were used as sources of B cells and dendritic cells (mature or immature). OT-II (based on a C57BL/6 genetic background with recognition for the MHC-II-IAb-molecule) (Barnden et al. 1998) and DO.11.10 (based on the genetic background of the Balb/C with recognition for the MHC-II IAd molecule) (Murphy et al. 1990) transgenic mice expressing TCR complexes engineered to recognize a peptide of chicken-ovalbumin (pOVA AA323-339) were used as sources of T cells. ICAM-1$^{-/-}$ knockout mice were obtained from Dr Nancy Hogg of the Leukocyte Adhesion Laboratory, Cancer Research UK London Research Institute, London, UK. Mice between the ages of 8-16 weeks were sacrificed while under deep anesthetic conditions by cervical dislocation following which lymph nodes, bone marrow or spleen cells were isolated.

All mice were housed and bred either in the animal facility of the medical faculty of the Otto-von Guericke University, Germany or the animal facility of the centre for Medical Biotechnology (ZMB) of the University of Duisburg Essen, Germany under SPF conditions and allowed free access to chow and water ad libitum.

2.2.2 Lymphocyte Isolation and purification

Mice were killed by cervical dislocation after been put into deep narcosis by use of Isoflurane and spleen was removed under antiseptic conditions. The spleen was meshed in a 70µM cell strainer/sieve using the piston of a syringe in a petri-dish containing PBS/1 % FCS. Following this homogenization process, the cell suspension was centrifuged for 5 min at 400 x g and supernatant was discarded. To rid off erythrocytes in the recovered pellet, erythrolysis was performed by addition of erythrolysis buffer to the cell pellet (3ml per spleen). The cell pellet was resuspended in this buffer for 1 min and left to stand for 4 min at room temperature. Lysis activity was stopped by filling up the 50ml tube within which the reaction is occurring with PBS/1 % FCS. This suspension was passed through a 70µM sieve to rid off debris and the eluent was centrifuged as described above. After centrifugation, the cell pellet was washed once with PBS/1% FCS and then after discarding supernatant, cell count was determined. For determination of cell count, a 10µl cell suspension aliquot was mixed 1:1 with 0.4% trypan

blue, pipetted into a Neubauer chamber for counting under a light microscope. All cells stained blue in the Neubauer chamber were excluded as dead cells.

After counting spleenocytes, cells were ready for isolation of either B cells or T cells using the Miltenyi negative immune-depletion technique. For every 1×10^8 of total spleenocytes, cells were incubated with 20µl MACS buffer and 5µl B or T cell antibody cocktail, incubated in a fridge for 10 min and then 15µl MACS buffer with 10µl antibiotin microbeads were added with further incubation for another 15 min in a fridge making a total incubation time of 25 min. After this incubation period, total volume was made up to 10mL with MACS buffer and centrifuged to pellet cells. At same time, 3ml of MACS buffer was passed through the MACS separation column to equilibrate it. After centrifugation, antibody cocktail and biotin microbead stained spleenocytes were suspended in 1ml MACS buffer and put into the separation column. 1ml MACS buffer was passed through the separation column 3 times and 3ml of MACS buffer was passed through 2 times making a total volume of 9ml MACS buffer flow-through excluding the original 1ml in which the spleenocytes suspension was initially re-suspended in. After this, the MACS column holding all unwanted fractions of cells was discarded while the eluent was centrifuged to recover the cells of interest in this case, either B cells or T cells. After decanting supernatant, cells were washed in PBS/ 1% FCS, centrifuged and the cell pellet was suspended in complete media for downstream applications. Cells were usually enriched to purities of about 90-95%, a sample of which is shown below by FACS staining.

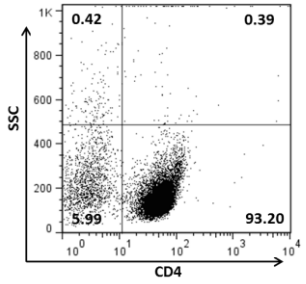


Fig 2.1 Purity of MACS isolated cells. A sample showing T cell purity after isolation using the MACS T cell isolation kit showing T cell purity of about 93% as observed following surface staining of T cell by CD antibody.

Following cell counting as previously described, cells were seeded in cell culture plates for downstream procedures. B cells were typically loaded with pOVA at a final concentration of 1µg/ml and in some cases, additionally with LPS titrated from 20ng/ml to 1µg/ml and incubated overnight before use. T cells were typically immediately after isolation put together with APCs.

2.2.3 Creation of Dendritic cells from mice bone marrow

Dendritic cells were created using from modified technique from previously published work (Inaba et al. 2001;Labeur et al. 1999) as described hereafter: Mice hind legs were recovered and bone marrow flushed out using a 21G needle with syringe filled with PBS/ 1%FCS in a Falcon tube. The bone marrow was disintegrated by vigorous pipetting and centrifuged at 400 x g for 5 min to pellet cells. After discarding supernatant, erythrocytes were lysed using 3ml erythrolysis buffer per mice and left to stand for 4 min following which lysis reaction was stopped by adding 40ml PBS/1% FCS. This suspension was passed through a 70µM cell sieve to rid off debris and the eluent was centrifuged to recover cells. After this centrifuging step, cell pellet was washed once more with PBS/1% FCS. After this, the recovered cells were placed in 10cm petri-dish with 20ml of either BMDC media or immature DC media dependent on cell type been generated. After standing for 2 hours in an incubator at 37^{o}C with 5% CO_2, non-adherent cells were collected in supernatant and after pelleting by centrifuging, plated in 6 well plates at a density of 3×10^6 cells per 5ml in each well. On day 3 after isolation, culture media was refreshed by taking off 2.5ml and replacing with fresh culture media. On day 7, non-adherent cells were recovered and after pelleting by centrifuging, platted in fresh media and stimulated with 20ng/ml LPS overnight to induce expression of co-stimulatory molecules. Peptide OVA (pOVA) was added to cell cultures at a final concentration of 1µg/ml 2-4 hours before DCs or immature dendritic cells (ImmDCs) were used to activate naive T cells.

2.2.4 *In vitro* T cell activation

Antigen specific naive T cells from the spleen(s) of DO11.10 or OT-2 mice were co-cultured with mature (or immature) peptide OVA loaded LPS stimulated DC or naive splenic B cells from wildtype mice of similar genetic background at a ratio of 1:10 or 1:1 respectively. The cell culture medium was RPMI-based and supplemented with 10% fetal calf serum. To obtain B-cell– and DC-primed T cells for further downstream applications such as immunoblots or realtime PCR in which cell purity is essential for the integrity of analysis, co-cultures underwent immunomagnetic depletion of non-CD4 cells using the MACS system at designated timepoints as indicated in "Results".

T cell activation was modulated pharmacologically using Ly294002 at 1µM, 10µM and 100 µM (but mostly at 10 µM (Sinclair et al. 2008)) and Rapamycin was used applied at concentrations of 10nM, 100nM and 1 µM (mostly at 100nM (Procaccini et al. 2010)).

Soluble CD28 was used for co-stimulation at a concentration of 10µg/mL. For investigation of epigenetic phenomena, Decitabine was added to co-cultures at 24h into the 72h timepoint at 5µM as described (Syrbe et al. 2004) while Trichostatin-A was used added to co-cultures at 56h during a 72h kinetic i.e. for 16 hours at 100nM (Moreira et al. 2003).

2.2.5 Proliferation and Inhibitory Assays

For inhibition assays, B cell (TofB), ImmDC (TofImmDC) or DC (TofDC)–primed T cells (with or without pharmacological modulators as indicated in "Results") were created as described in **2.2.4.** Differentially activated T cells were recovered by the MACS system. These "recovered" T cells (TofB, TofDC and TofiDC) were then tested for their inhibitory capacity by addition to freshly isolated CFSE stained naive T cells primed with pOVA loaded DCs at a ratio of 1:1 (T primed: T naive). Readouts for proliferation or activation markers were taken at 72h.

2.2.6 Fluorescence Activated Cell Sorting (FACS)

FACS (otherwise called flow cytometry) exploits the principles of light scatter, excitation and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells. This cell biology technique is extremely useful to immunologists for the purpose of determining antigen/protein expression levels on cell membranes as well as to determining expression levels of intracellular antigens/proteins. In flow cytometry, cells are hydro-dynamically focused in a sheath of fluid before incidenting on an optimally focused light source which are in most cases lasers. As cells or particles of interest incident the light source, they scatter this light and fluorochromes are excited to a higher energy state. As the molecules return to their original lower energy state, energy is released as a photon of light with specific spectral properties unique to different fluorochromes.

Photo multiplier tubes (PMTs) are detectors which collect the photon emissions from each cell or particle which is technically referred to as an "event" and converts them to analog voltages. The electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for strong or specific fluorescence signals. After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC) and recorded as data files. Optical filters are placed before the detectors so that only wavelengths

of light corresponding to specific fluorochrome emissions are collected by each detector (e.g. FITC emits in the green region therefore a 30 nm band pass filter centered at 525 nm could be used to collect light from this fluorochrome). Light scattered at the same wavelength and direction as the laser light, primarily from the surface of the cell, correlates with relative cell size (Forward Angle Light Scatter (FSC)) while light scattered 90 degrees to the laser (Side Scatter (SSC)) usually from internal structures, correlates with granularity. By correlating these two parameters, one can discriminate subpopulations of cells in peripheral blood samples, spleen cells, lymph nodes among several others. Signals/events emanating from cell debris or aggregates can also be detected and excluded from analysis on the basis of forward and side scatter. One unique feature of flow cytometry is that it measures fluorescence per cell or particle in contrast to spectrophotometry measures percentage absorption and transmission of specific wavelengths of light in a bulk volume of sample. Staining cells with multiple fluorochromes conjugated to antibodies or fluorochromes directed at other specific targets such as DNA, cytokines, or other proteins distinguishes cell subpopulations which can be quantified and also allows more thorough characterization of cell development and activity. Flow cytometry data is displayed and analyzed using histograms or two-dimensional dot plots.

One-Parameter Histograms: A one parameter histogram is a graph of cell count on the y-axis and the measurement parameter on x-axis. All one-parameter histograms have 1,024 channels. These channels correspond to the original voltage generated by a specific light event detected by the PMT detector. In other words, the ADC assigns a channel number based on the pulse height for individual events. Therefore, brighter specific fluorescence events will yield a higher pulse height and thus a higher channel number when displayed as a histogram.

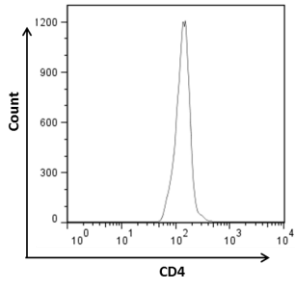


Fig 2.2 A one parameter FACS histogram. Depicts cell population as a histogram on the x-axis with cell count on the y-axis.

Two-Parameter Histograms: A graph representing two measurement parameters, on the x- and y-axes, and cell count height on a density gradient. This is similar to a topographical map. One can select 64 or 256 channels on each axis of two-parameter histograms. Particle counts are shown by dot density or contour plots.

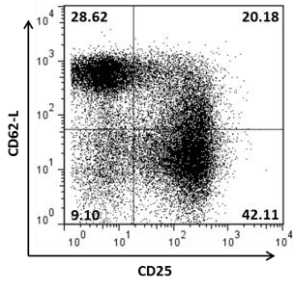


Fig 2.3 A two parameter FACS dot-plot. Depicts cell characteristics with respect to expression profile of 2 molecules**,** e.g., in this blot with CD62-L on the y-axis and CD25 on the x-axis.

For surface staining, cells were washed in cold PBS supplemented with 1% FCS to rid off media. After washing, cells were stained in PBS/FCS buffer with antibodies of interest at predetermined concentrations and left to stand on ice for 20 minutes after which cells were washed to rid of unbound antibodies and resuspended in PBS/FCS for data/event acquisition.

For performing intracellular staining, surface antigens of interest were first stained as indicated above following which cells were fixed with the cytofix component of the BD cytoperm/cytofix kit on ice for 30 minutes. After fixation, cells were centrifuged and after discarding fixation buffer washed with the cytoperm buffer which by virtue of its saponin component opens up pores in the cells making them accessible to intracellular antibodies. Intracellular antibodies dissolved in cytoperm buffer was applied to cells and then incubated for 1 hour on ice. After incubation in the presence of antibody, cells were washed in PBS/FCS and resuspended in PBS/FCS buffer ready for event acquisition and analysis.

With respect to event analysis, dead cells were gated out using side and forward scatter characteristics and then CD4 positive cells or other cells of interest were gated upon based on specific surface antigen staining.

2.2.7 Protein Detection via Immunoblot

Immunoblotting (alternatively called western blotting) is a method designed to detect a specific protein in a given sample of tissue lysate. It utilizes gel electrophoresis as a means to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are thereafter transferred onto a membrane (typically nitrocellulose or PVDF) where they are probed using cognate antibodies to the protein of interest whose expression profile is under investigation. The most common type of gel electrophoresis employs polyacrylamide gels and buffers containing the protein denaturing agent, SDS. SDS is an anionic detergent applied to

protein sample to linearize proteins and to impart a negative charge to linearized proteins. Therefore proteins can be separated according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge). Besides the addition of SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, which further denatures the proteins thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. S-S disulfide bonds to SH and SH) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilo Daltons, kD). The concentration of acrylamide determines the resolution of the gel i.e. the greater the acrylamide concentration of the gel, the better the resolution of smaller molecular weight proteins.

2.2.7.1 Protein extraction

Total cell extraction in NP-40 lysis buffer

After washing cells in ice-cold PBS, following T cell recovery from co-cultures by immune-magnetic depletion, cells were collected by centrifugation. 5×10^6 T cells were resuspended in 80-120µl of Buffer A (with freshly added phosphatase and protease inhibitors) in a 1.5ml eppendorf and incubated on ice for 10 min. Cell lysate was then centrifuged at 13,200 rpm for 10 min 4°C following which the cytoplasmic lysate was carefully transferred into a fresh eppendorf tube and immediately stored at -20°C. To recover nuclear proteins, cell pellet from cytoplasmic lysis was washed with 100µl of Buffer A (without IGEPAL) and then centrifuged after which the buffer was pipetted off. Ice cold Buffer C was added to washed cell pellet and incubated with vigorous shaking overnight in a cold room at 4°C. Following this incubation period, cell suspension was centrifuged as aforementioned and nuclear proteins carefully transferred by pipetting into a fresh eppendorf tube.

2.2.7.2 Measurement of protein concentration

Protein concentrations were determined using the 2D Quantkit (GE healthcare Life Sciences) following the manufacturers' protocol.

2.2.7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to separate proteins on the basis of their molecular size. The system normally involves usage of two gels both contained in one panel: a "stacking gel" with a low level of cross linkage and of low pH thus allowing proteins to enter the gel and compact without smearing; and a "separation gel" which is of higher pH in addition to having more cross linkage, where the proteins are separated according to molecular size.

Protein samples derived from cell extracts were denatured by heating at 95°C for 5 min in 1 x SDS sample buffer, and then cooled immediately on ice. The samples were collected by brief centrifugation and then equal amounts of protein were loaded on a SDS gel. The electrophoresis was performed with a vertical gel chamber in 1 x SDS running buffer. After electrophoresis, the gel containing the separated proteins is used for Western blotting analysis.

3.2.7.4 Western blotting

The separated proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore) by wet blotting (Bio-Rad), according to the manufacturer's instructions. After blocking in 1 x TBS containing 0.1% Tween 20 and 5% non-fat milk powder or BSA for 1-2 hour at room temperature, the membrane was incubated overnight with the appropriate primary antibodies at 4°C.

Following 2-3 washes in TBS-T and 2 washes in TBS each wash lasting for about 6 minutes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After another round of washes as aforementioned, the bound proteins were then detected using the Thermo Scientific Pierce enhanced chemiluminescence system (ECL).

2.2.8 RNA isolation and gene expression analysis

qRT-PCR is a molecular biology laboratory technique for amplifying a specific portion of a ribonucleic acid molecule. The RNA strand is first reverse transcribed into its DNA complement (cDNA), after which this cDNA is amplified using PCR over a predetermined number of cycles. This can be accomplished either by a 1 or 2 step process. During the first step of RT-PCR called "first strand reaction," cDNA is made from a messenger RNA template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase) through the process of reverse transcription. qRT-PCR takes advantage of a unique feature of mature mRNAs; the 3' polyadenylated region, commonly called the poly(A) tail as a binding site for

Oligo(dT) DNA primers. These primers will anneal to the 3' end of every mRNA in the solution, allowing 5'->3' synthesis of cDNA by the reverse transcriptase enzyme. cDNA can also be prepared from mRNA by using gene specific primer or random hexamer primers.

After the reverse transcriptase reaction is complete and cDNA has been generated from the original single-stranded mRNA, standard PCR termed the "second strand reaction," is initiated. If the initial mRNA templates were derived from the same tissue, subsequent PCR reactions can be used to probe the cDNA library that was created by reverse transcription. Primers can be designed to amplify target genes being expressed in the source tissue. Quantitative real-time PCR can then be used to compare levels of gene expression.

2.2.8.1 Isolation of total RNA

Total RNA was isolated using the Qiagen RNeasy Mini kit following the kit protocol.

2.2.8.2 Measurement of RNA concentration

RNA concentration and quality was determined using the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington). Isolated RNA purity was checked by ratio of sample absorbance at 260 and 280 nm (260/280), which should be approximately 2.0 for pure RNA. Sample concentration was read in ng/µl based on its absorbance at 260 nm.

2.2.8.3 cDNA Synthesis

mRNAs were converted by reverse transcription into cDNAs using Oligo (dT) primers and reverse transcriptase kit (QIAGEN). Briefly, 200 ng of total RNA from naïve and differentially activated T cells and from different timepoints was prepared using the Qiagen QuantiTect® Reverse Transcription Kit. For cleaning up the RNA, DNA elimination step was undertaken using the following prep.

Genomic DNA elimination Reaction set-up:

Component	Volume/Reaction (µl)
gDNA Wipeout Buffer, 7x	2
Template RNA, up to 1 µg*	10
RNase-free water	2

Total reaction volume	14

Following DNA clean up, RNA was converted to cDNA using the following prep of the aforementioned Qiagen kit:

Component	Volume/Reaction (µl)
Reverse-transcription master mix Quantiscript Reverse Transcriptase	1
Quantiscript RT Buffer, 5x	4
RT Primer Mix	1
Template RNA Entire genomic DNA elimination reaction	14
Total reaction volume	**20**

use 2µl 10x miScript Universal Primer when amplifying miRNA and reduce volume of RNase free water to keep total reaction volume at 20µl. This step was used in assaying miRNA let7-b levels.

2.2.8.4 Real-time PCR analysis

Real-time PCR was used to amplify a segment of known/target sequence from cDNA with gene specific primers following the protocol of the miScript SYBR® Green PCR Kit. The reaction volume is as shown below:

Component	Volume/Reaction (µl)
2x QuantiTect SYBR Green PCR Master mix	10
10x Quantitect Primer Assay	2
Template cDNA	1
RNase-free water	7
Total Volume	**20**

use 2µl 10x miScript Universal Primer when amplifying miRNA and reduce volume of RNase free water to keep total reaction volume at 20µl. This step was used in assaying miRNA let7-b levels.

One step Quantitative real-time PCR was performed using either the Qiagen Rotor-Gene Q or ABI prism 7000 under the following cycling conditions programme:

Steps	Temp	Time	Cycle no
Enzyme activation	95°C	15 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

The threshold value ct for each individual PCR product was determined manually and ct values obtained for the target gene were normalized by subtracting the ct values obtained from genes of interest with the reference (housekeeping) gene which was beta-actin for this study. Samples were assayed in duplicates and results were derived using the comparative Ct method ($\Delta\Delta C_t$).

2.2.9 Methylation specific PCR

Methylation specific PCR (MSP) is a multistep molecular biology PCR-based method of choice for investigating epigenetic modifications induced by gene methylation. Basically the technique utilizes Bisulfite treatment of genomic DNA which leads to conversion of unmethylated cytosine in the genome to uracil.

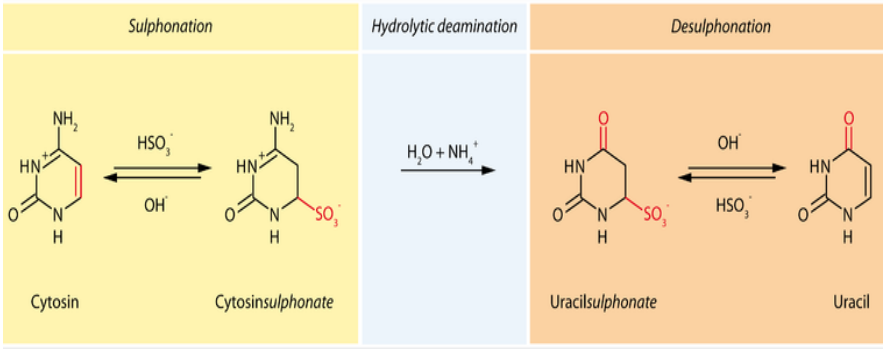


Fig 2.4 Chemical depiction of bisulfite treatment of DNA. Source: Tollefsbol T (ed): Handbook of Epigenetics: The new Molecular and Medical Genetics. 1st edition. London, San Diego: Academic Press, 2011.

Following this conversion, primers specific for the locus of interest are designed. n. After this step of PCR amplification, differences in the DNA of treated samples can be analysed using techniques such as sequencing. Overall, results are obtained based on differences in nucleotide sequences judging by the presence of uracil nucleotides in the gene sequence.

2.2.9.1 Genomic DNA Prep

DNA preps were prepared from MACS purified T cells following 72 hours of activation by either pOVA peptide laden B cells, Immature or mature DCs. Naïve T cells were used as control. DNA was extracted using the QIAGEN QIAamp DNA Mini Kit according to the manufacturers' instruction. DNA integrity was checked using the NanoDrop.

2.2.9.2 Bisulfite treatment

Bisulfite treatment of genomic DNA is an important step in the MSP procedure serving to provide a system for differentiating methylated and unmethylated cytosine which is converted to uracil following this treatment. For this phase of the work, the QIAGEN Epitect Bisulfite kit was used according to the manufacturers' instruction with thermal cycling conditions as shown below:

Step	Time	Temperature
Denaturation	5 min	95^{o}C
Incubation	25 min	60^{o}C
Denaturation	5 min	95^{o}C
Incubation	85 min	60^{o}C
Denaturation	5 min	95^{o}C
Incubation	175 min	60^{o}C
Hold	Indefinite(overnight)	20^{o}C

2.2.9.3 PCR

After Bisulfite treatment and subsequent DNA clean-up using the QIAGEN QIAquick PCR purification kit, cleaned DNA was subjected to PCR using the HotStarTaq DNA Polymerase from QIAGEN with cognate primers designed to amplify CpG islands previously identified on the Klf-2 locus in mouse T cells. Reaction set-up for the PCR/sample is as shown below:

Component	Volume/ Reaction (µl)	Final concentration
Reaction mix 10x PCR buffer	10	1x
5x Q-solution	20	1x
dNTP (10mM of each)	2	200µM
Forward Primer	1	0.5µM
Reverse Primer	1	0.5µM
HotStarTaq DNA Polymerase	0.5	2.5 units/reaction
Distilled water	61.5	-
Template DNA	4	1µg
Total volume	100	-

2.2.9.4 Sequencing

Amplified sequences were outsourced for sequencing at GATC Biotech AG, Konstanz and results were analysed using the BiQ Analyzer from the Max Planck Institute for Informatics, Saarbrücken.

2.2.10 Immunocytochemistry- Fluorescence Microscopy

Naïve T cells and differentially activated T cells were recovered working on ice after triggering and placed on acid treated 12mm glass slips contained in a 24 well plate. Acid treatment increases positive charge on glass slips in order to boost adhesion between slip and negatively charges cells. After allowing to stand for one hour to adhere, slips were washed with PBS to get rid off complete media. Following on from this, cells were fixed in 4% PFA for 30 min and then washed with PBS 3 times. After washing, cells were blocked for 2 hours in solution consisting of PBS/ 1% BSA/ 0.5% Tween 20. After blocking, cover slips were washed with PBS 2 times and then incubated overnight at 4^{o}C in unconjugated Foxo1 antibody diluted in PBS/ 1% BSA/ 0.5% Tween 20. After overnight incubation, cover slips were washed 2 times with PBS/ 1% BSA/ 0.5% Tween 20 and then incubated with secondary antibody for 1 hour in the dark at room temperature following which nuclear staining was

performed using DAPI. After nuclear staining, cover slips were washed 2 times with PBS/1% BSA/ 0.5% Tween 20 and then with PBS alone. For final wash, distilled water was used. For mounting, objective slides were coated with 15µl of the Prolong antifade Kit mounting media and then cover slips were placed over these and allowed to stand overnight in the dark at room temperature to cure. After this, slides were ready for imaging and data acquisition.

Mounted cells were imaged using a Zeiss AxioObserver.Z1 and ApoTome at the Imaging Centre Essen (IMCES). The Zeiss AxioObserver.Z1 is an inverted epifluorescent microscope with a metal halide HXP-120 lamp capable of acquiring DIC and bright field images using a Zeiss AxioCam MRm 1.2 Megapixel monochrome charge-coupled device (ccd) camera with a frame size of 1388x1040 pixels. It possesses 4 filters in addition to air, water and oil immersion lenses with the Zen 2011 software which affords the opportunity to perform multi-channel fluorescence image acquisition, z-stacks and time series data acquisition.

The Zeiss ApoTome uses a structured illumination grid giving wide field optical sectioning which slides into the optical path and projects a grid onto the image plane which is laterally shifted in 3 predefined steps with an image collected at each step. This set-up serves to rid of blurred out of focus fluorescence signals. Thus this system backed up with complex algorithms is able to yield enhanced images with better resolution and less blur and out of focus planes. For this part of the work, images from T cells were analysed in order to gauge the relative amounts of Foxo1 in the nucleus of these cells. Image processing was performed using Image J while Foxo1 quantification was done using the cell-pro programme.

2.2.11 Role of adhesion molecules on the stroma of SLOs in T cell activation *in vivo*

DC (0.5×10^6) were injected in 20µl PBS into the footpad of age matched wildtype or ICAM-$1^{-/-}$ mice. As control DC without pOVA were injected into the footpad of a wildtype host. After 16 h, 5×10^6 pOVA antigen specific CFSE stained OT-2 cells were injected retro-orbitally into each host. 72h after the introduction of OT-2 T cells, ipsilateral and contralateral popliteal nodes were prepared separately, passed through a 70µM nylon mesh to create a single cell suspension and labeled with fluorochrome conjugated antibodies against CD4 and CD25. After labeling and washing, events were acquired on a FACS and the CD4 population was gated upon and analyzed for proliferation based on CFSE dilution.

2.2.12 Statistics

Data in this body of work are presented as mean +/- SEM from a minimum of 2 independent experiments in order to ensure data reproducibility. Statistical significance was determined using student's t-test as provided on the GraphPad software package (Prism Software, GraphPad, CA). A p value < 0.05 was considered as statistically significant, $p < 0.01$ very significant and $p < 0.001$ extremely significant.

3.0 RESULTS

3.1 Critical Previous Work

This thesis builds upon critical previous work which began on the topic. In the previous part of the work, it was demonstrated that CD62-L is re-expressed following activation of naïve antigen specific T cells by naïve antigen laden B cells (TofB) showing an initial phase of CD62-L shedding followed by re-expression at later time points. This was in contrast to the dynamics of CD62-L regulation in the same naïve T cells triggered by matured DCs. Using the pharmacological agents PD184352 and TAPI-2 which inhibit MEK-Erk phosphorylation and TACE shedding respectively, the underlying mechanism for initial shedding was found to be Erk mediated TACE sheddase activity. In terms of long term regulation, the PI3K/mTOR signaling axis was found to mediate sustained downregulation of CD62-L in TofDC but not in TofB where addition of pharmacological agents could induce no upregulation of CD62-L as was observed in the case of the TofDC.

The next phase of the work involved investigating CD62-L regulation on the transcriptional level. In summary, the expression levels of 2 key transcription factors; KLF-2 and CD62-L which have been reported in the literature to regulate CD62-L transcriptional activity were investigated by real time PCR in a kinetic manner. It was observed that following activation of antigen specific T cells by either antigen laden DC or B cells, CD62-L and KLF-2 mRNA were equally downregulated 24h after activation but while levels remained low in the TofDC, both mRNAs were re-expressed as at the 72h timepoint. Targeting the PI3K/mTOR pathways with Rapamycin or LY294002 was shown to induce re-expression of both CD62-L in the TofDC and had only a short term effect on TofB failing to induce any upregulation of both mRNAs at 72h. These results are published together with other data generated in this thesis in the paper; Etemire et al. 2013.

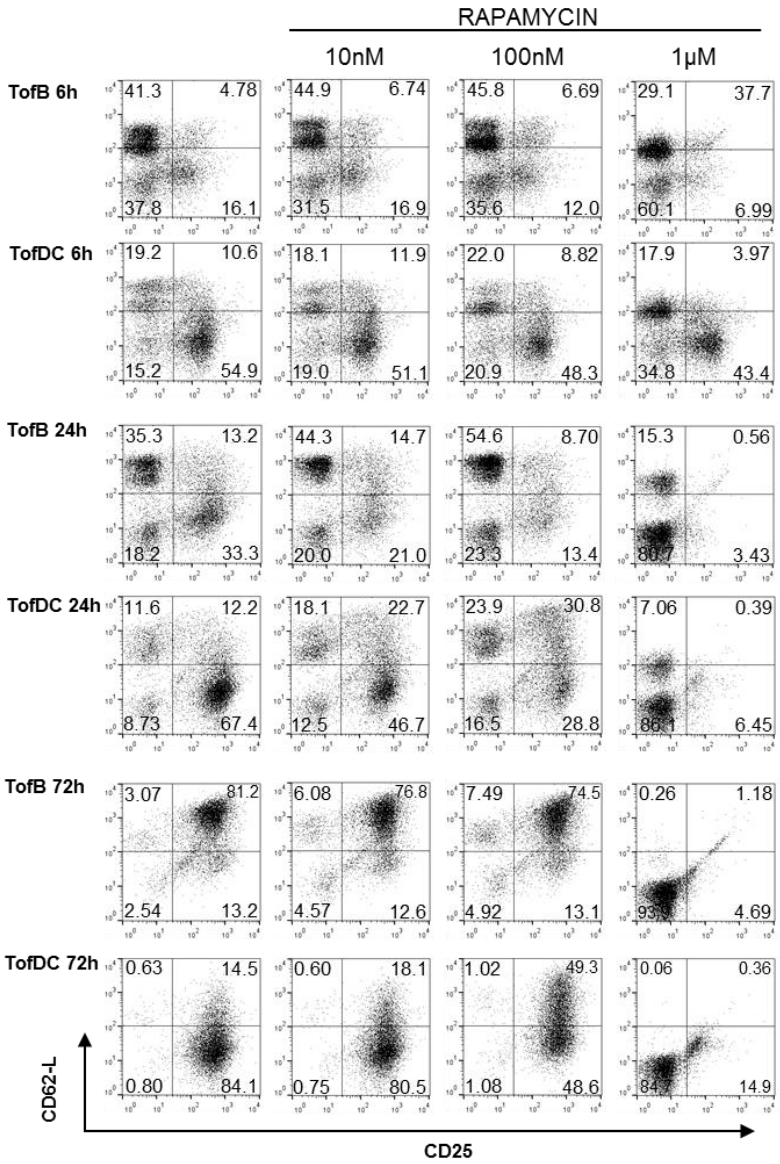
3.2 Rapamycin and Ly294002 regulate CD62-L in a dose dependent manner

To further gain insight into the regulation of CD62-L via the PI3K/mTOR signaling pathway, we titrated the concentration of Rapamycin and Ly294002. Having previously used Rapamycin at 100nM we titrated this inhibitor at 10nM and 1μM concentrations. mTOR signaling occurs via 2 complexes; TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2). TORC1 is Rapamycin sensitive contains Rheb, regulatory associated protein of mTOR (raptor), G protein β-subunit-like protein (GβL), protein kinase B/Akt substrate 40 kDa (PRAS40). This complex is linked with phosphorylation of such targets as the ribosomal S6

kinase (S6K1). TORC2 is considered Rapamycin insensitive and contains the Rapamycin-insensitive companion of mTOR (rictor), GβL, mammalian stress activated protein kinase interacting protein-1 (mSin1) as well as mTOR (Delgoffe et al. 2009). Concentrations up to about 150nM of Rapamycin inhibits TORC1 while usage at doses from 500nM inhibit TORC2 and induce cell death (Foster and Toschi 2009).

mTOR inhibition by Rapamycin had no influence on CD62-L expression profile of both TofB and TofDC at 6h. However, at 24h a divergent pattern of response to the inhibitor began to appear. In TofB, CD62-L expression level increased from 48% to 58% when treated with 10nM Rapamycin and 62% at 100nM while in TofDC, CD62-L levels increased from 24% to 40% when treated with 10nM Rapamycin and 53% at 100nM (Figure 3.1a).

a)



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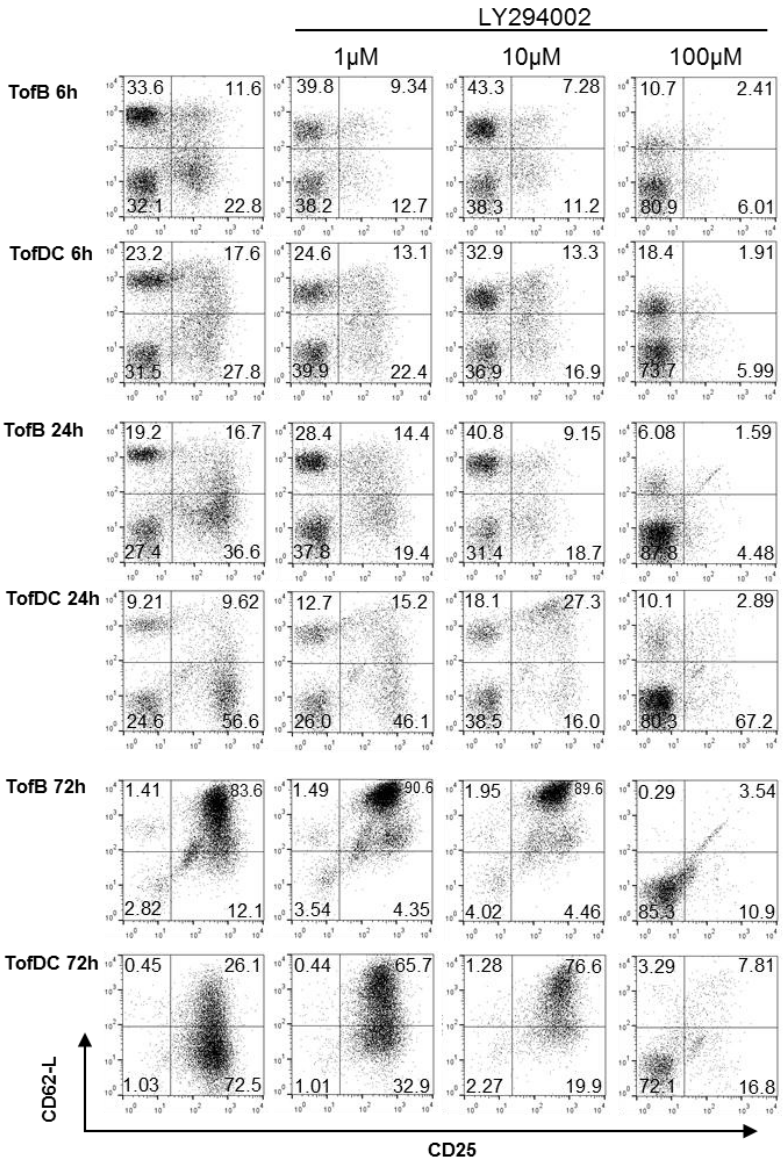


Fig 3.1 Effect of mTOR and PI3K inhibitor titration on CD62-L expression levels. Antigen specific naïve T cells were triggered with pOVA laden naïve B cells (TofB) or DC (TofDC) for specified timepoint in the presence or absence of inhibitors. Subsequently, CD62-L dynamics was analysed by gating on T cell population from events acquired by flow cytometry. (A) One representative FACS blot depicting dose dependent regulation of CD62-L in T cells by the mTOR inhibitor Rapamycin. (B) One representative FACS blot showing the dose dependent regulation of CD62-L in T cells by the PI3K inhibitor LY294002. Data are representative of 3 independent experiments.

At the supra-optimal concentration of 1μM, both cell models began to show signs of cell death. At 72h, the upregulating effect of Rapamycin on CD62-L was lost in the TofB as the level remains constant in both untreated TofB as well as the 10nM and 100nM Rapamycin treated samples. This was in contrast to the TofDC where Rapamycin at 10nM induced no upregulation while inducing a rise from 15% CD62-L expression in untreated TofDC to 50%

in TofDC treated with 100nM. In both cell models, treatment with Rapamycin at 1µM induced cell death.

Titration of the PI3K inhibitor LY294002 was performed at 3 different concentrations; 1µM, 10µM and 100 µM. A 6h in both TofB and TofDC, PI3K inhibition had no discernible effect on CD62-L expression levels at 1µM and 10µM while signs of cell death were already visible in cells treated with 100µM LY294002. In the TofB at 24h, CD62-L expression was increased from 33% to 43% and 51% in 1µM and 10µM treated samples respectively. This pattern of upregulation was also observed in TofDCs which showed increase from 19% to 28% and 45% CD62-L expression when treated with LY294002 at 1µM and 10µM. however, supra-effective levels of LY294002 at 100µM induced cell death. Data at the long-term timepoint of 72h showed that at this timepoint, in TofB PI3K inhibition had no effect on CD62-L expression while the upregulation of CD6-L in TofDC following LY294002 application was sustained and even potentiated with values rising from 27% in untreated cells to 66% and 78% in 1µM and 10µM treated cells respectively (Figure 3.1b).

These results are in agreement with data from Sinclair et al. 2008 which reported upregulation of CD62-L following PI3K and mTOR inhibition . This data also indicate that previous work reported under "Previous Work" had been conducted with these inhibitors at an optimal physiological concentration and also indicate very strongly that the PI3K/mTOR signaling pathway is involved in the regulation of CD62-L in both TofB and TofDC.

3.3 CD62-L dynamics is independent of lipopolysaccharide (LPS) induced Toll Like receptors (TLR) activation in B cells

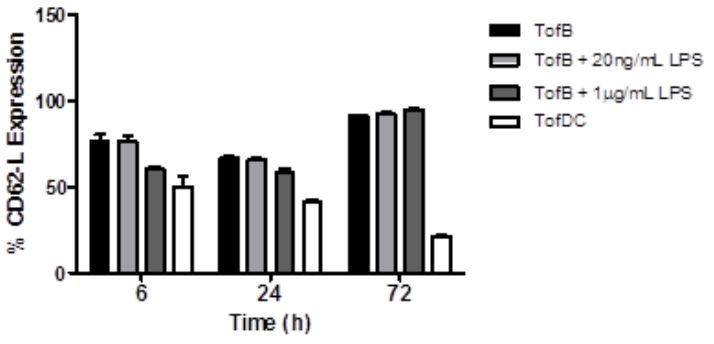
Having implicated the PI3K/mTOR axis in CD62-L regulation in both cell model systems, we moved on to identify new pathways that might perhaps drive this differential regulation of CD62-L. TLR triggering has been demonstrated to induce loss of CD62-L in B cells (Morrison et al. 2010) and naïve B cells express low amounts of TLRs (Ruprecht & Lanzavecchia 2006) as opposed to DCs which due to their innate immune functions abundantly express these receptors (Edwards et al. 2003;Schreibelt et al. 2010). To test our hypothesis that the increased presence and activity of TLRs on DC might be responsible for the differential regulation of CD62-L on TofDC vs TofB, we decided to trigger TLR on B cell using LPS as it has been previously demonstrated by LPS is a potent trigger of TLRs especially TLR4 which is highly expressed on DCs (Acosta-Rodriguez et al. 2007;Chow et al.

1999;Hoshino et al. 1999). LPS triggering of TLRs has been demonstrated mice and human T cells (Zanin-Zhorov et al. 2007) as well as in B cells (Ruprecht & Lanzavecchia 2006).

LPS was used at both the regular concentration used for maturation of DCs in this thesis (20ng/mL) as well at a high concentration of 1ug/mL. At 6h, T cells activated with B cells and 20ng/mL LPS added in culture showed similar CD62-L kinetic to TofB while there was a marked reduction in CD62-L expression of T cells triggered with 1µg/mL LPS in culture solution. The decrease in CD62-L expression was transient as T cells triggered with LPS treated B cells showed similar % expression of CD62-L as the regular TofB at 24 and 72h (Figure 3.2a). Also, LPS incubated TofB cells were able to induce faster upregulation of CD25 than non LPS treated TofB cells. However, at the terminal timepoint of 72h, TofB generated in the presence or absence of LPS expressed equal amounts of CD25 (Figure 3.2b). This experiment was repeated in a modified manner where B cells were treated overnight with LPS at 20ng/mL and 1µg/mL before being used in co-culture with T cells to generate TofB (data not shown). Under these conditions, CD62-L and CD25 kinetics were indistinguishable from the results reported above.

On the basis of these observations, we could exclude a role for the innate signaling mechanism of TLRs in the dynamics of CD62-L as well as CD25 regulation in the T cells in this study. It was also instructive that even with the direct addition of the potent TLR trigger LPS in culture, TofBs CD62-L dynamics was unchanged thus further suggesting that CD62-L regulation in this context is independent of TLR activity. This data thus strengthened the need to focus on signaling events driven directly by TCR stimulation as it did appear that receptors and mechanisms from the innate arm of the immune system were not vitally important for subserving the functions under investigation and might play no discernible role in iTreg induction in the system under investigation here.

a)



b)

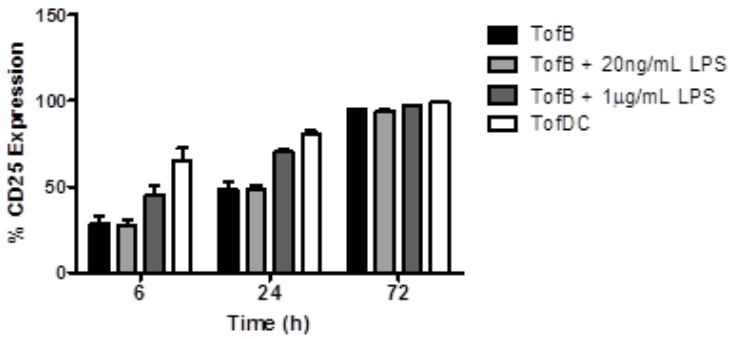


Fig 3.2 Role of TLRs in the regulation of CD62-L in TofB. TofB and TofDC was prepared as previously described with LPS added at indicated concentrations. CD62-L and CD25 dynamics were analysed by gating on the T cell population from events acquired by flow cytometry. (A) CD62-L levels in TofB with or without LPS in culture. (B) CD25 expression levels in TofB with or without LPS in culture. Data are means + SEM of 3 independent experiments.

3.4 Readout of mTOR induced S6 phosphorylation status as a surrogate marker for PI3K activity in TofB and TofDC

Based on the observation that the PI3K inhibitor LY294002 as well as the mTOR inhibitor Rapamycin both exerted the same influence on the dynamics of CD62-L regulation in the TofB vs the TofDC (with Rapamycin showing less potent effect), we hypothesized that differences in the underlying signaling pathway of the PI3K/mTOR signaling axis might be responsible for this disparity in CD62-L expression. This would offer explanation as to why silencing of these pathways which are activated following TCR triggering was able to upregulate CD62-L in the TofDC back to naïve T cell expression levels. To test this hypothesis, we investigated the phosphorylation status of the mTOR downstream effector molecule, S6. Phosphorylation status of the S6 protein has previously been used as a readout for PI3K activity by Levings and co-workers (Crellin et al. 2007). We investigated in a kinetic manner from 6, 24 and 72h the expression of pS6 looking at 2 different phospho-sites; Ser235/236 and Ser240/244 in addition to examining total S6 expression. Total S6 levels were more or less constant between TofB and TofDC at all timepoints examined and no significant differences were observed (Figure 3.3a). For both phosphorylation sites examined in Figure 3.1b-c, there was no significant difference found between TofB and TofDC as well across all timepoints investigated. It was however observed that as a trend, pS6 Ser235/6 expression levels were very even between both cell models as against the decreased albeit not in significant d levels in pS6 Ser240/4 status in the TofB at 72h which was lower than that of pS6 expression levels in TofDC (Figure 3.3b-c). These results indicated that there was some

regulation at the S6 level in both TofB and TofDC but did not fully clarify the signaling profile of the PI3K/mTOR axis with respect to CD62-L regulation in both cell models.

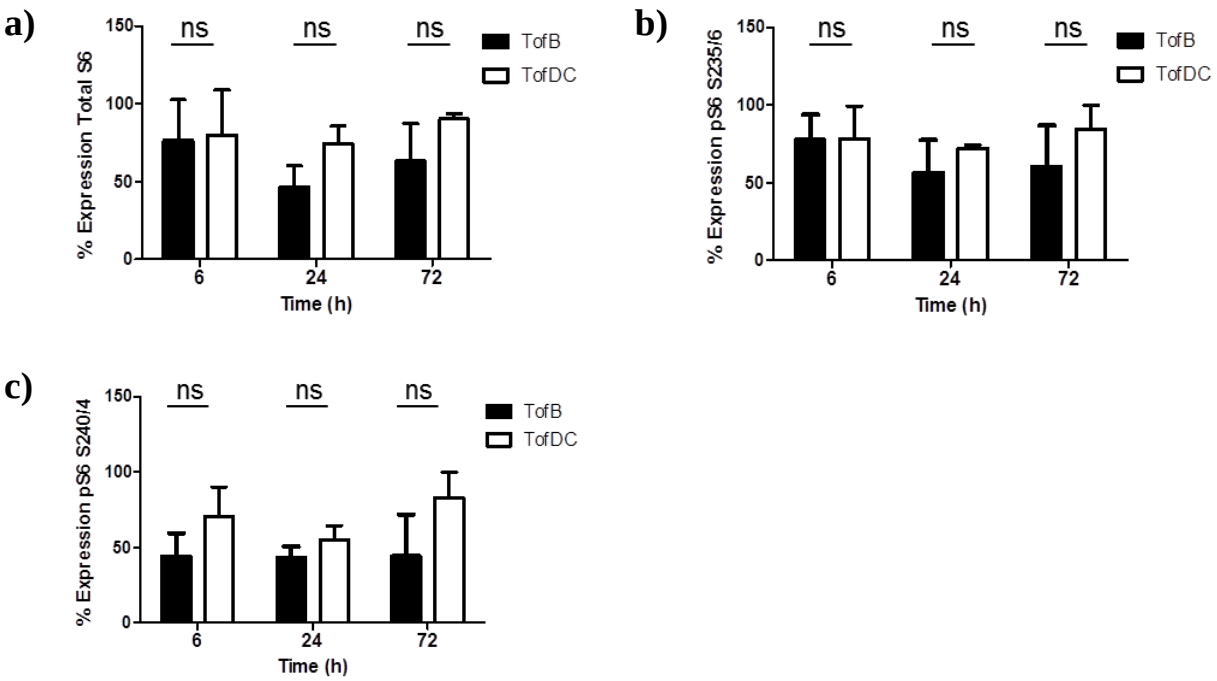


Fig 3.3 pS6 as surrogate readout for PI3K/mTOR signaling. TofB and TofDC were prepared and acquired as events by flow cytometry following which the T cell population was gated upon**.** (A) Time course of % expression for total S6 in T cells (B) Time course of % expression for pS6 Ser235/6 in T cells (C) Time course for % expression of pS6 Ser240/4 in T cells. Data are means + SEM of 5 independent experiments.

3.5 Induction of Tregs using immature DC (iDC) as weak APC

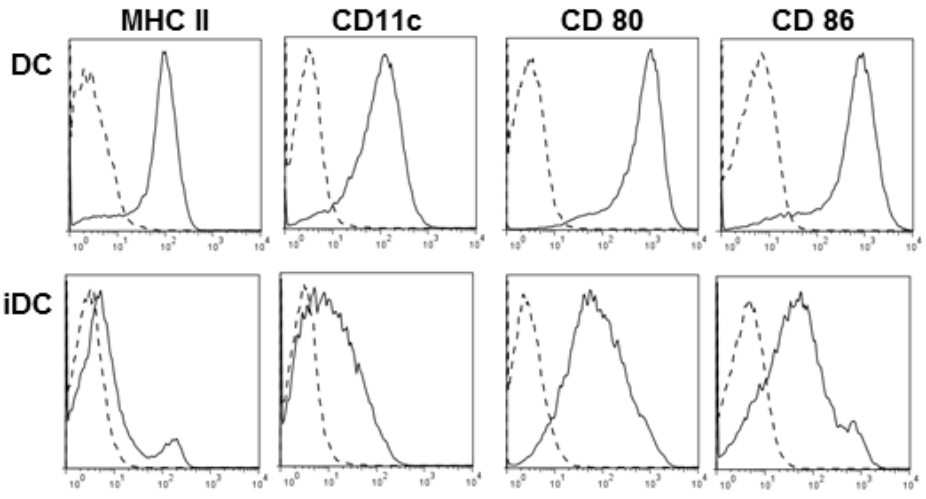
Having performed the study so far with B cell as weak APC, we decided to incorporate a more physiological weak APC into the study. The reason for this being that while it is known that B cells interact *in vivo* with T cells, it is still a matter of discussion as to the functional relevance of these interactions (Barr et al. 2007). In filling this gap, DCs were of choice because while they function as the most potent APC under conditions of inflammation, in non-inflammatory situations when they exist in an immature state and function to induce self-tolerance by inducing iTregs *in vivo* (Dudziak et al. 2007;Ohnmacht et al. 2009;Steinman and Nussenzweig 2002). Based on these facts, we generated immature DCs for use as additional APC for the remaining part of this work.

3.5.1 Generation and characterization of immature dendritic cells (iDCs)

iDC were generated using the same technique as BMDC generation but in the absence of IL-4 as culture media containing GM-CSF only has been demonstrated to induce an immature phenotype in bone marrow derived cells (Lutz et al. 2000). Cells were characterized with respect to 3 key markers; CD11c, MHC II and B7 (CD80 and CD86) protein expression levels. Only few cells of the iDC population generated expressed MHC II and those which did had similar expression levels as matured DCs. CD11c and B7 protein expression were markedly lower in iDC compared to DC (Figure 3.4a). On the basis of % expression, iDCs expressed about 3 times less MHC II than DCs while DCs expressed 50% more CD11c than iDCs. In terms of B7 protein expression, DCs were 95% and 90% positive for CD80 and CD86 expression respectively. This was in contrast to iDCs which had expression levels of about 80% for both B7 proteins (Figure 3.4b). While these percentages do not reveal very prominent differences in the phenotype of DCs vis a vis iDCs, analysis on per cell basis revealed in more details important differences between both cell types. While MHC II was more or less equally expressed on both cell types, CD11c expression was about 5 times higher in DC than iDCs (Figure 3.4c). With regards to co-stimulatory molecule expression, DCs expressed about 4-6 times more CD80 and 3-5 times more CD86 than iDCs (Figure 3.4d).

These results validated our system for the generation of iDCs in addition to hinting very strongly that iDCs will function as far less efficient APC than DC and fit very nicely into our study as we characterize the dynamics of differential T cell activation based on APC efficiency.

a)



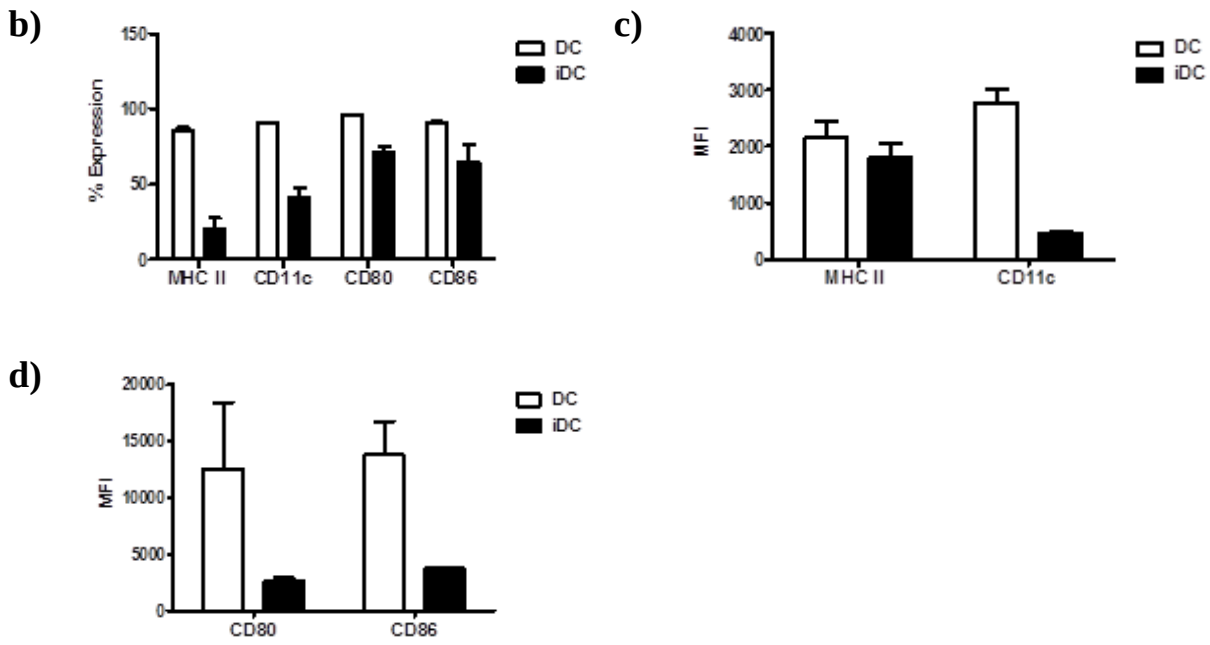


Fig 3.4 Characterization of iDCs against mature DCs. iDC was contrasted against matured DCs to measure the relative expression of lineage markers, stimulatory and co-stimulatory molecules. (A) One representative FACS blot showing expression of markers investigated. Solid lines depict expression profile of target molecules while dotted lines are isotype controls (B) Comparison of % surface expression of MHC II, CD11c and B7 proteins on DC and iDC. (C) Per cell analysis show that DC express more CD11c than iDC while both cells possess equal amounts of MHC II (D) cell cell analysis reveal that DCs express more co-stimulatory molecules than iDC. Data are means + SEM of 2 independent experiments.

3.5.2 Immature DC triggered T cells show the same Phenotypic Characteristics as TofB

Next to generating TofiDCs, it was important to characterize them in terms of key molecules whose expression patterns are presently known in the TofB. TofBs have been reported as being $CD25^{+}CD62\text{-}L^{+}$ as well as $Foxp3^{-}$ (Reichardt et al. 2007). After analysis of T cells activated by iDC (TofiDC), we observed that TofiDC were about 90% positive for CD25 thus expressing values similar to the TofB and TofDC (Figure 3.5a). Interestingly, TofiDCs just like both other differentially activated T cells in this study were found to be $Foxp3^{-}$ (Figure 3.5b). Next to characterizing CD25 and Foxp3 expression status, we turned to investigate the dynamics of CD62-L expression in TofiDC during the co-incubation phase of their generation. Here we found that TofiDC regulated CD62-L expression in a manner similar to TofB. Initially, T cells triggered by iDC shed CD62-L at the early timepoint of 6h but starting from 24h onwards began re-expressing CD62-L up to levels which were significantly higher than the expression rate in TofDC. However, despite the fact that there was no significant difference between TofB and TofiDC at 6h and 24h, CD62-L expression level were significantly higher in the TofB and TofiDC at 72h compared to TofDC (Figure 3.5c-d).

These results showed that the iDCs generated were still more potent APCs than B cells but for the fact that they could induce T cells in a manner similar to B cells acting as APCs, we considered the TofiDC a suitable cell type for incorporation into our study subject to functional testing which should show that they do possess regulatory function just as TofB.

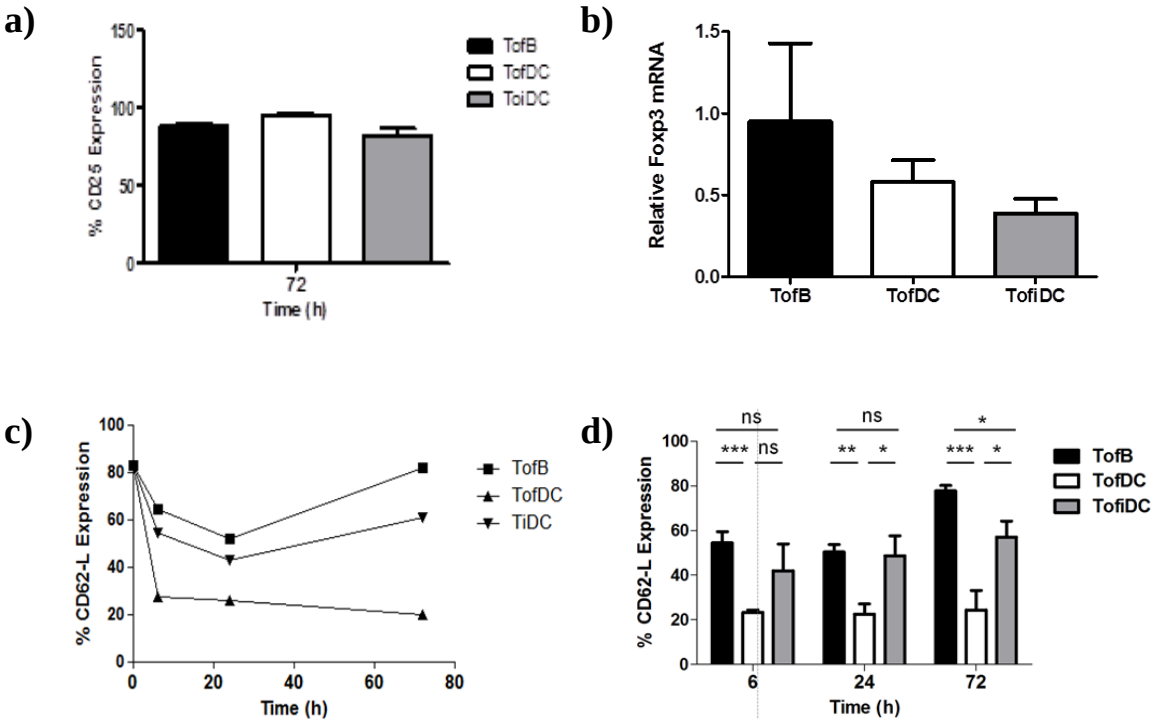


Fig 3.5 Phenotypic characterization of T cells triggered by iDC. Antigen specific naïve T cells were triggered with antigen laden iDC to produce TofiDCs in addition to co-cultures for TofB and TofDC. Expression profiles of specific markers were assessed by flow cytometry and analysis performed on the T cell gated population. (A) CD25 is expressed in TofiDC at the same levels as TofB and TofDC. (B) Foxp3 expression was not upregulated in TofiDC, as well as in TofB and TofDC. Naïve T cells were assigned a value of 1 for analysis. (C) One representative line graph of CD62-L regulation in TofiDC vis a vis TofB and TofDC. (D) Statistical analysis of CD62-L expression in all three cell types show that TofiDC CD62-L dynamics mirror that of TofB and is significantly higher than on the TofDC. Data for Figures a,b and d represent means + SEM of 3-4 independent experiments.

3.5.3 Functional characterisation of TofiDC and delineation of the involvement of the PI3K and mTOR pathways in acquisition of regulatory function

Having characterized TofiDCs phenotypically, we set out to characterize them functionally in order to gain information as to whether or not they exhibit regulatory behaviour while at same time looking to see if mTOR and PI3K inhibition-induced CD62-L upregulation in TofDC could lead to induction of regulatory function in the inhibitor treated TofDCs. This question was asked based on the hypothesis that incomplete signaling via the mTOR/PI3K pathway drove upregulation of CD62-L in TofB as well as in TofDC were subjected to incomplete signaling profile of these pathways via pharmacological interference using Rapamycin and

LY294002. In addition to this, we hypothesized that re-expression of CD62-L following activation of T cells perhaps links homing behaviour and acquisition of regulatory function.

To test our hypothesis, *in vitro* T cell activation assays were performed wherein we examined 2 parameters in order to judge the activation and proliferation status of naïve T cells under the influence of these differentially activated/primed T cells: firstly CD25 expression and secondly, CFSE dilution. Our data showed that after 72h of assay, as expected, naïve T cells left standing alone did not upregulate CD25. In contrast to this, naïve T cells incubated with antigen laden DC were 97% positive for CD25 and this was unchanged even with the addition of TofDC and TofDC+Rapamycin modulator samples to the co-culture. However, same T cells incubated with DC separately could only induce about 41-47% CD25 expression when co-cultures were performed in the presence of the modulators TofB, TofiDC as well as TofDC+LY294002. This result was of great interest as it gave strong hints to the fact that although both Rapamycin and LY294002 induced CD62-L upregulation in TofDC, there could very well be differences in terms of functional outcome based on the mechanism of action of these inhibitors. While PI3K inhibition conferred on TofDC the ability to cut CD25 expression in naïve T cells, mTOR inhibition by Rapamycin on these same cells in parallel could not confer this functionality. Also of interest was the observation that functionally, in terms of inhibition of CD25 expression, TofiDCs were as potent as TofB (Figure 3.6a).

We next investigated the regulatory capabilities of TofiDC as well as TofDCs treated with PI3K or Rapamycin inhibitors in terms of T cell proliferation as can be judged by CFSE dilution using an inhibitory assay run for 72h. Our results show that the TofiDCs were functionally equivalent to TofBs as both cell models equally inhibited about 70% of naïve CFSE stained T cells from proliferating when stimulated with antigen carrying DCs. In control conditions, i.e. in the absence of these cells, 96% of naïve T cells assayed did proliferate under the influence of DC stimulation. Interestingly, TofDC+LY294002 strongly reduced the proliferatory potential of stimulated naïve T cells as only 24% of such cells were able to divide in the presence of LY294002 treated TofDC. This was in contrast to TofDC+Rapamycin treated cells which could not inhibit proliferation to the same extent and potency as TofDC+LY294002. 82% of naïve T cells did proliferate under DC stimulation even when co cultured with TofDC+Rapamycin (Figure 3.6b). Statistical analysis of this data showed that TofB, TofiDC and TofDC+LY294002 very significantly inhibited naïve T cell proliferation while TofDC and TofDC+Rapamycin had no significant effect on the proliferation of the same T cells in separate pools (Figure 3.6c).

Taken together, our results showed the TofiDC to be a functional equivalent of TofBs and also strongly indicated that blockade of the PI3K but not mTOR signaling cascade is responsible for the induction of iTregs in our model system. These results further bring into focus the role and underlying functioning of the PI3K signaling pathway in the induction of iTregs so far studied in this body of work.

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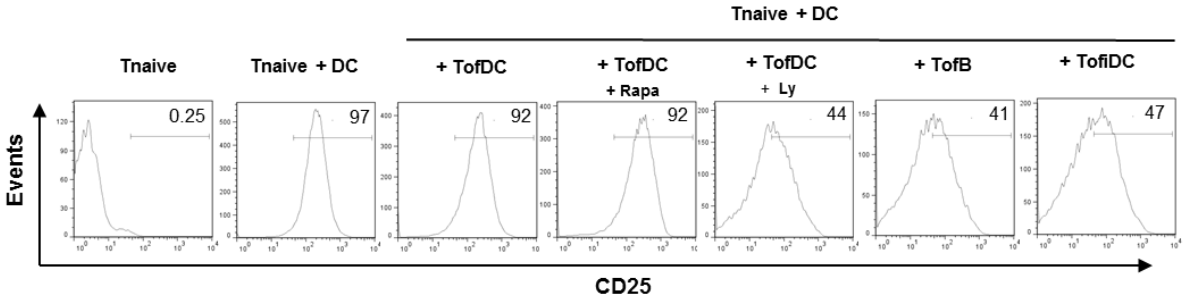

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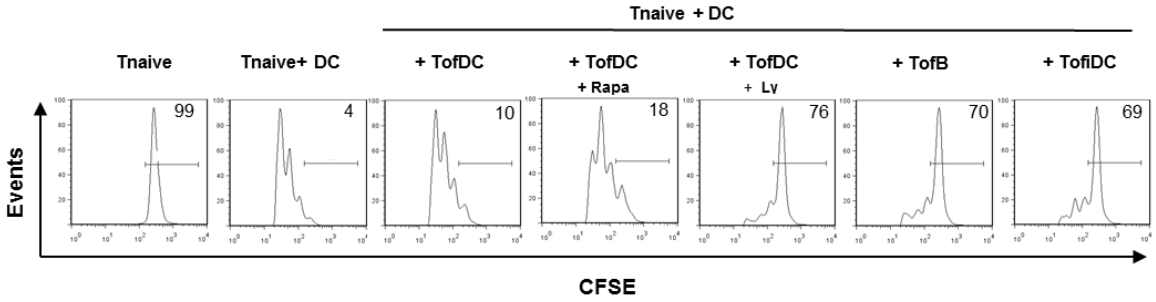

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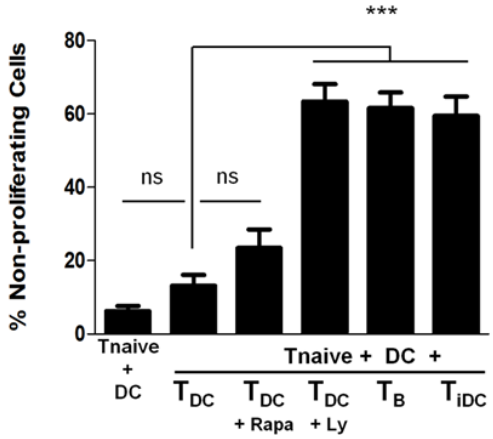
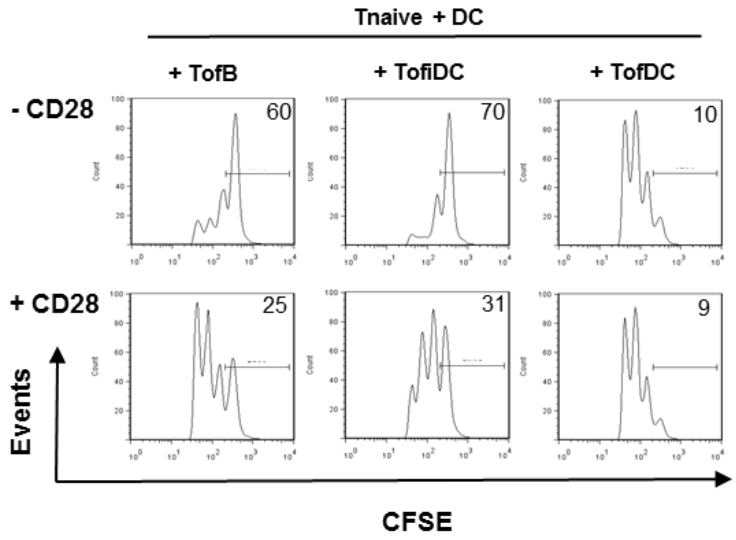


Fig 3.6 Functional characterisation of TofiDC and delineation of the involvement of the PI3K and mTOR pathways in the acquisition of regulatory function. Co-cultures of CFSE stained antigen specific naïve T cell stimulated by DC. Differentially activated T cells recovered by MACS separation were added to test their inhibitory/functional capacity on naïve T cells activated by DC over a 72h time course. (A) One representative FACS blot depicting the effect of differentially activated and or pharmacologically treated cells on CD25 expression. (B) One representative FACS blot depicting the effect of differentially activated and or pharmacologically treated cells on T cell proliferation. (C) Statistical analysis of the inhibitory effect of differentially activated T cells on naïve T cell proliferation show that TofB, TofiDC and TofDC+LY294002 but not TofDC and TofDC+Rapamycin exhibit regulatory function. Data for Figure 3.6a-b are representative of 4 independent experiments and Figure 3.6c represent means + SEM of 4 independent experiments.

3.6 Sub-optimal co-stimulation drives acquisition of regulatory function but exerts negligible influence on CD62-L dynamics

The study so far had identified iDCs which lacked high expression levels of co-stimulatory molecules (Figure 3.4) as sharing both phenotypic and functional similarities with B cells which also lack co-stimulatory capacity (Reichardt et al. 2007). While activation via co-stimulatory input does not on its own trigger PI3K signaling which our data indicate might play a prominent role in the induction of iTregs in our study model system, it is however known that co-stimulation plays a prominent role in fine tuning the activity of the PI3K signaling cascade (Garcon et al. 2008;Riha and Rudd 2010). It was therefore interesting to further investigate the role of the lack or absence of co-stimulatory input into the induction of iTregs vis a vis upregulation of CD62-L in our system.

a)



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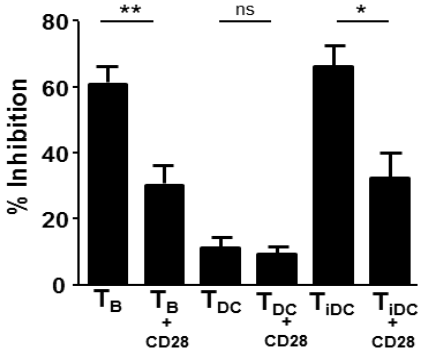

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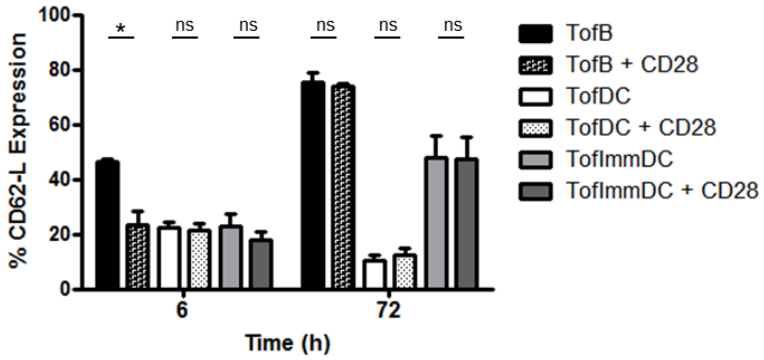


Fig 3.7 Role of co-stimulatory input in acquisition of regulatory behaviour and migratory potential of iTregs. Co-cultures of all 3 model cell types were created in the presence and absence of soluble CD28. Recovered differentially activated T cells were used in inhibition assays or analysed for CD62-L expression. (A) One representative FACS blot showing the effect of augmented CD28 stimulation on the acquisition of regulatory behaviour. (B) Statistical analysis of the impact of augmented co-stimulation on acquisition of regulatory function. Addition of soluble CD28 led to the significant reduction of regulatory properties of TofB and TofiDC but not TofDC. (C) Statistical analysis of the impact of augmented co-stimulation on CD62-L dynamics. Addition of soluble CD28 led to transiently significantly increased shedding of CD62-L only the TofB at 6h and had no effect on CD62-L dynamics in all cell models long term. Data for Figure 3.7a are representative of 4 independent experiments and Figure 3.7b-c represent means + SEM of 4 independent experiments.

To this end, we added soluble CD28 to cell cultures during the creation of all 3 cell models in addition to controls without CD28 i.e. regular TofB, TofDC and TofiDC. After this step, we tested the regulatory potency of recovered differentially activated T cells in an inhibition assay with naïve T cells triggered by DC. Our data showed that addition of CD28 to TofB and TofiDC during the generation phase of these cells led to reduction of the regulatory properties of both cell types. Naïve T cells stimulated with DC assayed with TofB and TofDC had 60% and 70% of non-proliferating cells as against CD28 augmented TofB and TofiDC which could only inhibit 25 and 31% of stimulated naïve T cells from proliferating (Figure 3.7a). Statistical analysis of this data showed that in samples co-incubated with TofB+CD28 and TofiDC+CD28 there was a significantly increased percentage of proliferating cells when compared to the non-CD28 treated TofB and TofiDC. Interestingly, there was no significant difference in the rate of naïve T cell proliferation in the presence of TofDC+CD28 and regular TofDC samples (Figure 3.7b) thus suggesting that DCs generated in this study had attained a "maximum proliferatory and co-stimulatory capacity" that could not be exceeded by the addition of soluble CD28 even at the high concentration of 10µg/mL as used in this present study.

Having clarified the role of co-stimulation in the context of regulatory function acquisition, we next focused on its role in the migration potential/capacity of the iTregs in our study i.e. TofB and TofiDC. We investigated this by again augmenting the co-stimulatory input during the generation phase using soluble CD28. At 6 hours after commencement of co-culture, we observed that CD28 augmentation induced a significant increase in CD62-L shedding in the TofB+CD28 sample in contrast to untreated TofB. At 24h, CD28 augmentation had no effect on CD62-L expression (data not shown) as well as at 72h. In both TofDC and TofiDC, there was no significant difference observed between CD28 augmented or non-augmented cells across all timepoints investigated (Figure 3.7c).

These results reveal a divergence in the function of co-stimulatory input with respect to iTreg generation from naïve T cells. While lack of CD28 activity seemed important for acquisition of regulatory function, our data also indicated that it played no discernible role in regulating the migratory/homing potential of these cell types as it exerts no influence on the dynamics of CD62-L regulation.

3.7 Phospho-site specific defective Akt signaling observed following T-cell triggering by weak APCs

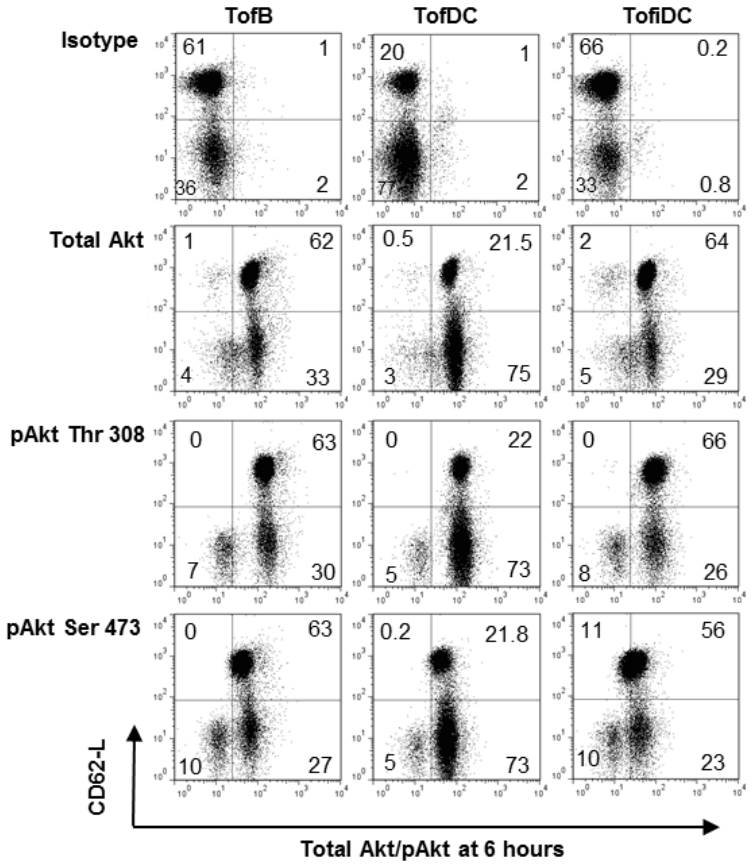
Having clarified the role of low co-stimulatory input for the generation of TofB and TofiDC, we next turned our attention to better understanding the underlying signaling mechanisms driving this induction. Previous data (Figure 3.1) had suggested suboptimal PI3K/mTOR triggering following T cell activation as a possible mechanism via which weak APC (naïve B cells) drive iTreg production. Examination of the S6 phosphorylation status as surrogate readout for the signaling cascade did not yield clear cut results as there was no significant difference between iTreg and control and TofB (Figure 3.3). To address in more detail the underlying signaling profile in all 3 cell models in focus, we decided to investigate a more upstream molecule in this cascade. We focused on Akt phosphorylation dynamics as previously studied by Levings and co-workers (Crellin et al. 2007). TofB and TofiDC just as TofDC were viable metabolically-active proliferating cells as observed by their expression of CD25 (Figure 3.5a). This suggested that they require a system for utilizing nutrients and driving metabolism. Such metabolic function is recognized as being dependent on functional PI3K/mTOR signaling which is mediated via the agency of the downstream effector Akt (Finlay & Cantrell 2010). In view of these observations and reports, it was important to understand how suspected sub-optimal PI3K/mTOR signaling could be compatible with viability and proliferation as observed in the iTregs of our study.

Akt kinetics was studied by testing phosphorylation levels of the Akt activation loop at Thr308 and the hydrophobic motif at Ser473 following differential activation of naïve T cells using all 3 APCs of our study in separate wells. pAkt Ser473 has previously been reported as possessing a defective signaling profile in Tregs (Ouyang et al. 2012). In one representative experiment, we observed that across all timepoints, levels of total Akt were virtually the same with identical expression levels in all 3 cell models. The phosphorylation status was also the same in the pAkt Thr308 activation loop. However, interestingly, examination of the hydrophobic motif pAkt Ser473 showed an interesting phenotype. At 6h, TofB, TofiDC and TofDC had about 90% pAkt Ser473 expressing T cells. This number dropped down to 30% and 26% in TofB and TofiDC at 24h while 70% of TofDC still maintained pAkt Ser473 expression. On a per cell (MFI) basis, TofB had an expression level of 64, TofiDC 43 while TofDC had the highest value of 79. This trend of pronounced pAkt Ser473 deficiency was reversed at 72h when pAkt Ser473 levels increased to the high levels of 80-82% in TofB and TofiDC while TofDC had about 94% pAkt Ser473 positive T cells (Figure 3.8a-c). Statistically, we found significantly reduced pAkt Ser473 levels in TofB and TofiDCs relative to TofDC at 24h. At other timepoints examined, when normalized against total Akt

expression, there was no significant difference between the profile of pAkt Ser473 of iTregs and TofDC (Figure 3.8d-f).

Put together, these results provided clear indications that there was some form of regulation on the PI3K/Akt signaling axis that could be important for Treg induction.

a)



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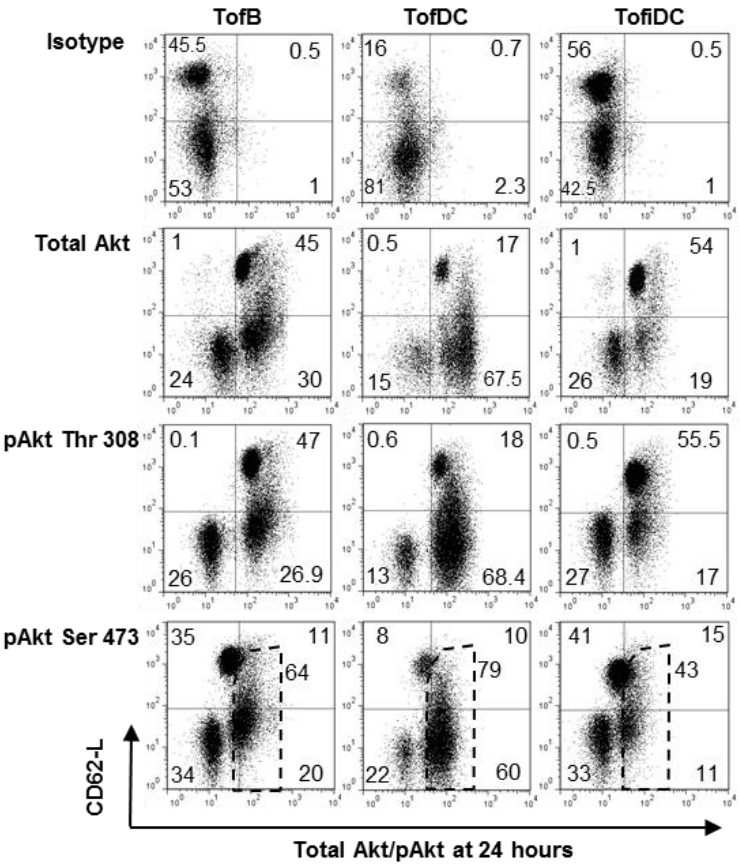
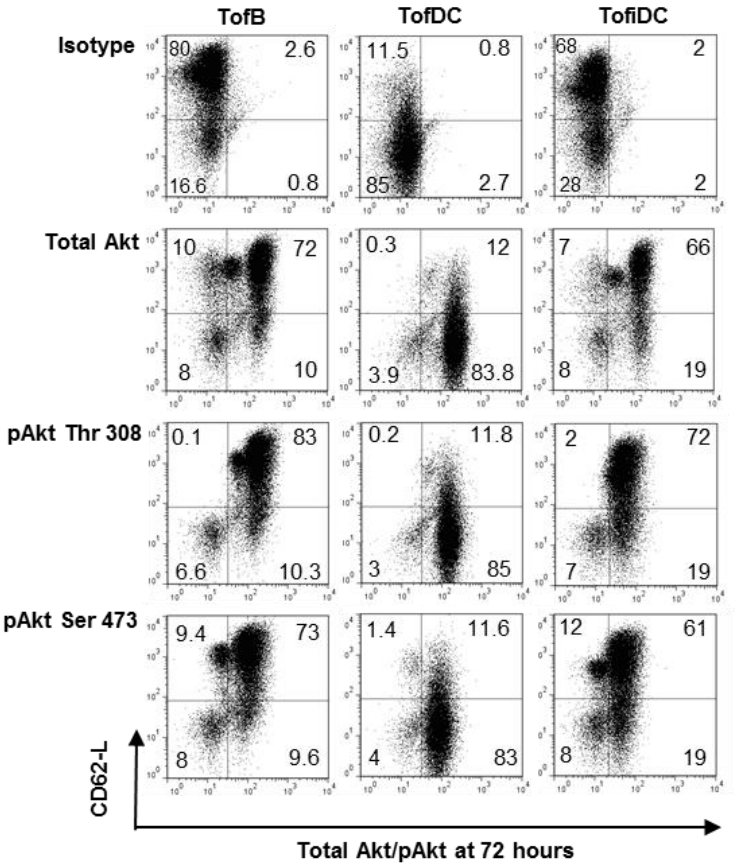

TofB
TofDC
TofiDC
Isotype
45.5
0.5
53
1
16
0.7
81
2.3
56
0.5
42.5
1
Total Akt
1
45
24
30
0.5
17
15
67.5
1
54
26
19
pAkt Thr 308
0.1
47
26
26.9
0.6
18
13
68.4
0.5
55.5
27
17
pAkt Ser 473
35
11
64
34
20
8
10
79
22
60
41
15
43
33
11
CD62-L
Total Akt/pAkt at 24 hours

c)


TofB
TofDC
TofiDC
Isotype
80
2.6
16.6
0.8
11.5
0.8
85
2.7
68
2
28
2
Total Akt
10
72
8
10
0.3
12
3.9
83.8
7
66
8
19
pAkt Thr 308
0.1
83
6.6
10.3
0.2
11.8
3
85
2
72
7
19
pAkt Ser 473
9.4
73
8
9.6
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4
83
12
61
8
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CD62-L
Total Akt/pAkt at 72 hours

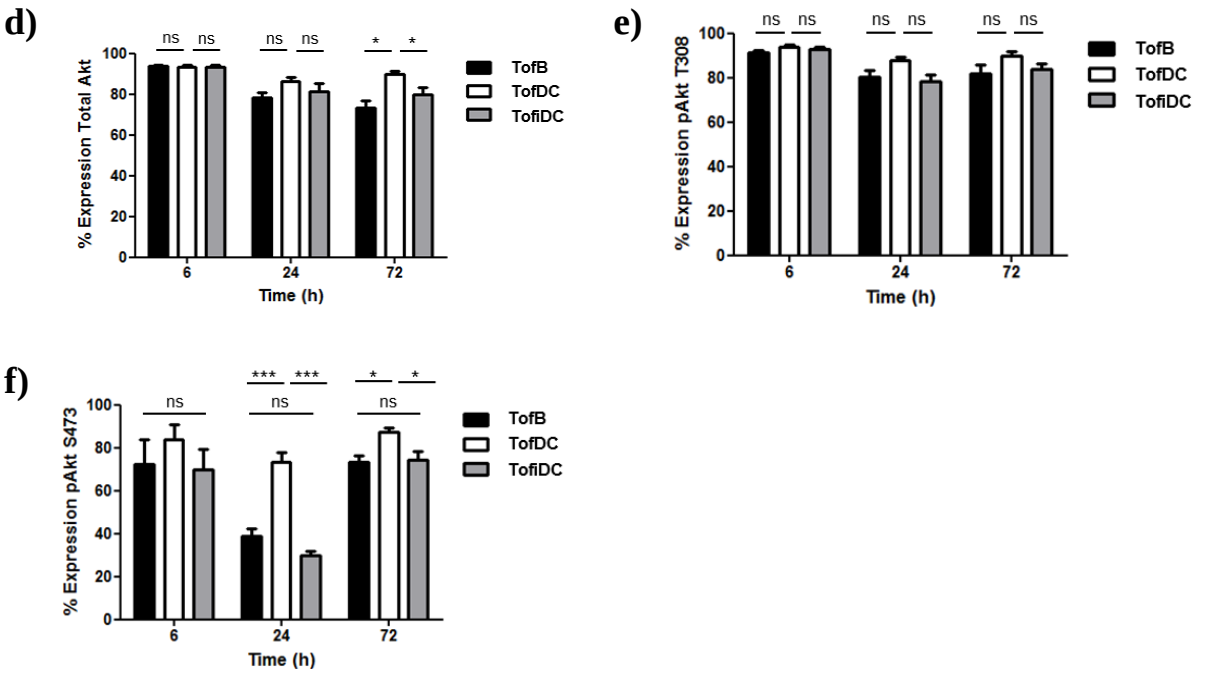


Fig 3.8 Analysis of Akt signaling activity as readout of PI3K/mTOR signaling. TofB, TofiDC and TofDC were prepared and acquired as events by flow cytometry following which the T cell population was gated upon. (A) Representative FACS blot showing Akt dynamics in T cells after triggering by APC at 6h. (B) Representative FACS blots showing Akt dynamics in T cells after triggering by APC at 24h. MFI values of pAKT Ser473 are indicated in dotted lines. (C) Representative FACS blots showing Akt dynamics in T cells after triggering by APC at 72h. (D) Statistical analysis of Total Akt expression in T cells over indicated the timepoints. (E) Statistical analysis of pAkt Thr308 expression in T cells over the indicated timepoints. (F) Statistical analysis of pAkt Ser473 expression in T cells over the indicated timepoints. Data for Figure 3.8a-c are representative of 4 independent experiments and Figure 3.8d-f represent means + SEM of 4 independent experiments.

3.8 Temporal upregulation of PHLPP1 drives induction of regulatory function

Regulation on the level of PI3K/mTOR signaling albeit via transiently defective signaling could provide the opportunity for upegulation or downregulation of genes that could be important for driving the production of Tregs from naïve T cells. This would also answer the question as to how defective PI3K/mTOR signaling could be compatible with sustained cell viability and proliferation. The pressing question therefore was by what mechanism(s) are the iTregs in this study able to regulate PI3K signaling specifically at one phosphosite? To answer this question, we probed the expression of PH domain and Leucine rich repeat Protein Phosphatases (PHLPP). PHLPP is a phosphatase which has been recently reported as mediating a specific defect in phosphorylation of Akt Ser473 (Patterson et al. 2011). We assayed mRNA expression levels as done in the aforementioned study and interestingly observed a temporal regulation in the dynamics of PHLPP1 expression in both TofB and TofiDC. PHLPP1 mRNA was significantly increased in both iTregs at 6h compared to TofDC and was maintained at high levels albeit not significantly over 24 and 72h (Figure 3.9). This

pattern of expression fitted in with defective pAKT Ser473 at 24h in addition to coinciding with the timing of commencement of CD62-L re-expression in both iTregs.

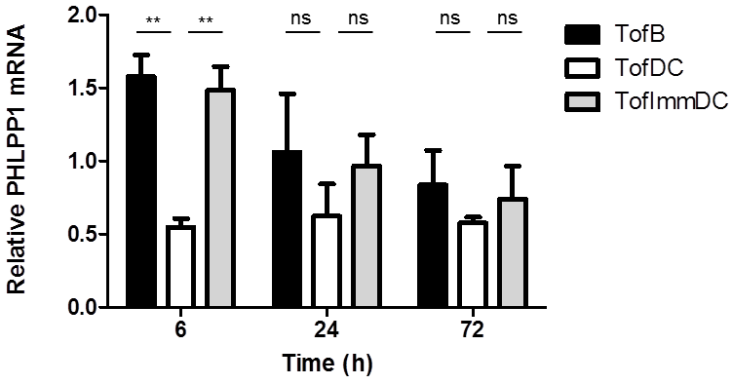


Fig 3.9 PHLPP1upregulation underlies defective PI3K/mTOR signaling. At 6h, PHLPP1 mRNA is selectively upregulated in iTregs but not TofDC and retains high but non-significant levels at 24 and 72h. Data represent means + SEM of 3 independent experiments.

Having observed selective upregulation of PHLPP1 in TofB and TofiDC, we wondered if this phosphatase might also be selectively upregulated in TofDC+LY294002 samples which we previously showed to exhibit regulatory function of same potency as both TofB and TofiDC (Figure 3.6b-c). To this end, we assayed TofDC+LY294002 against regular TofDC for expression levels of PHLPP1 mRNA. TofDC+Rapamycin treated samples were also assayed given that they induced nominal but non-significant inhibition of naïve T cell proliferation at a basal level higher than that obtainable with TofDC as moderating agent in inhibition assays (Figure 3.6b). Interestingly, we observed that LY294002 treated TofDC upregulated PHLPP1significantly when compared to levels in TofDC while Rapamycin treated TofDC expressed slightly higher but not significant levels of PHLPP1 also (Figure 3.10).

These results together with fig 3.8 show that induction of regulatory activity is linked to PHLPP1 induction. iTreg generation utilizes this transient phase of PI3K/mTOR signaling to perhaps tune genes to enhance Treg properties.

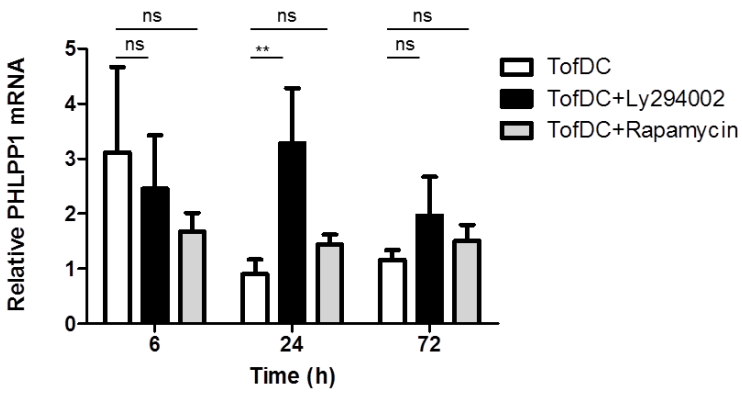


Fig 3.10 PHLPP1 is upregulated by PI3K but not Rapamycin inhibition. Addition of LY294002 but not Rapamycin leads to the significant induction of PHLPP1 in TofDC. Data represent means + SEM of 3 independent experiments.

3.9 Clarifying Foxo1 activity during Treg induction

3.9.1 Foxo1 is temporarily excluded at early timepoints from the T cell nucleus following activation but returns at later timepoints

Having examined generation of Tregs from naïve T cell pools with focus on regulation of CD62-L re-expression dynamics as well as mechanisms governing regulatory behaviour acquisition, we decided to examine nuclear related events that could be relevant for CD62-L regulation. To investigate this, we turned to the transcription factor Foxo1which apart from its known function in CD62-L regulation (Fabre et al. 2005) has recently been demonstrated to be relevant for Treg development (Ouyang et al. 2012). Following TCR triggering, Foxo1 is exported to the cytoplasm where it is phosphorylated and rendered transcriptionally inactive by 14-3-3 proteins (Tzivion et al. 2011). We therefore hypothesized that Foxo1 was excluded from T cell nucleus following TCR triggering by all APCs in our study and was re-expressed in the nucleus in T cells triggered by weak APC thus leading to CD62-L re-expression while being permanently excluded from the nucleus of TofDC which express consistently low levels of CD62-L after the initial shedding phase (Figure 3.1). To test our hypothesis, we performed nuclear extraction of Foxo1 protein from TofB and TofDC and assayed by western blot after establishment of a functional protocol for clean nuclear extraction (Figure 3.11a). Data obtained indicated that while as expected the nucleus was depleted of Foxo1 following TCR signaling just as shown by Fabre at al. 2005, there was an unexpected finding as we observed that Foxo1 was again present in the nucleus of both TofB and TofDC at 24 and 72h at levels (Figure 3.11b).

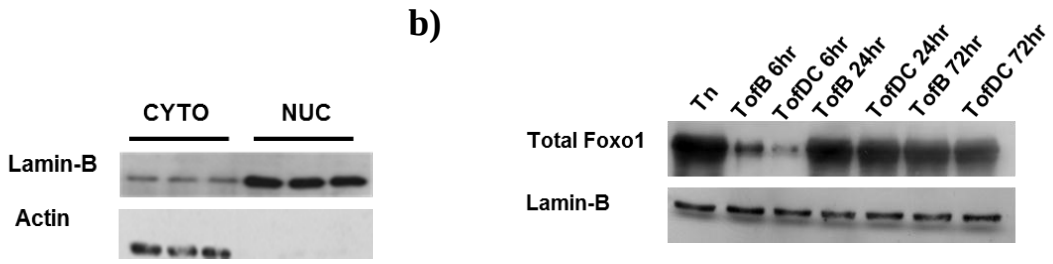
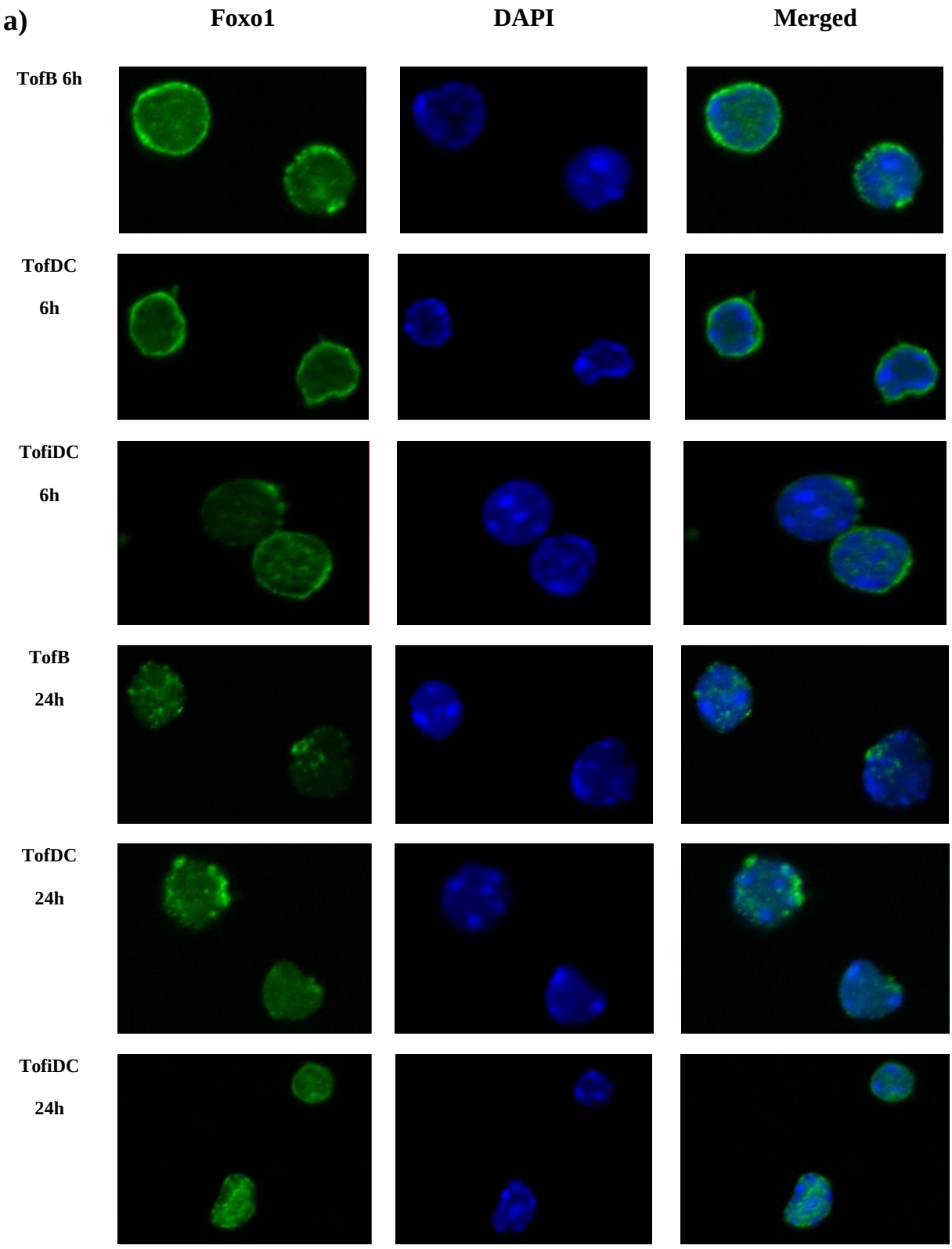


Fig 3.11 Foxo1 is re-expressed in the nucleus of activated T cells. (A) Control test confirming good technique for extraction of nuclear protein from T cells. (B) Foxo1 expression levels in TofB and TofDC showing exclusion at 6h and return at later timepoints. Data are representative of 3 independent experiments.

To confirm this rather unexpected finding of Foxo1 being present in the nucleus of TofDCs which we hypothesized had permanently excluded Foxo1thus its consistently low expression of CD62-L, we performed microscopic studies on differentially activated T cells at 6 and 24h. In all 3 cell models at 6 hours of co-culture, there was enriched expression of Foxo1 at the exterior of the nucleus as depicted by DAPI staining with less Foxo1 visible in the region co-localized with DAPI staining. At 24h, there was a marked loss of this concentric ring of enriched Foxo1 around the DAPI staining which was observable at 6 hours with more Foxo1 now been visible in the region of the cell co-localized with DAPI (Figure 3.12a). Quantification of Foxo1 levels in the nucleus indeed showed that there was more Foxo1 present in the nucleus of all 3 differentially activated T cells at 24h compared to 6h (Figure 3.12b).

These results fit in with the concept of initial nuclear exclusion of Foxo1after TCR triggering and then subsequent re-appearance at later timepoints even in the presence of sustained PI3K signaling.


a)
Foxo1
DAPI
Merged
TofB 6h
TofDC 6h
TofiDC 6h
TofB 24h
TofDC 24h
TofiDC 24h

b)

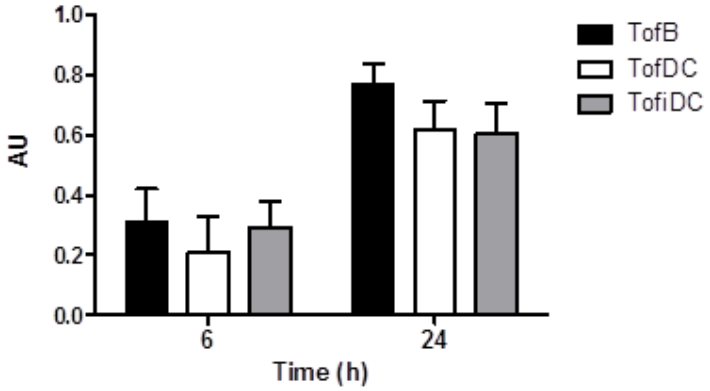


Figure 3.12 Confirmation of presence of Foxo1 in the nucleus of activated T cells. (A) Apotome immunofluorescence microscpy images of differentially activated T cells at indicated timepoints showing enrichment of Foxo1 outside the nucleus at 6h after TCR triggering and loss of Foxo1 cytoplasmic enrichment as well as increased expression of nuclear Foxo1 at 24h. (B) Quantification of Foxo1 expression level in differentially activated T cells. Naïve T cell nuclear Foxo1 expression level was assigned a nominal value of 1 and data is represented as arbitrary unit (AU) relative to expression level in naïve T cells. Data in Figure 3.12b is Means + SEM of 12-14 T cells quantified for nuclear Foxo1 expression.

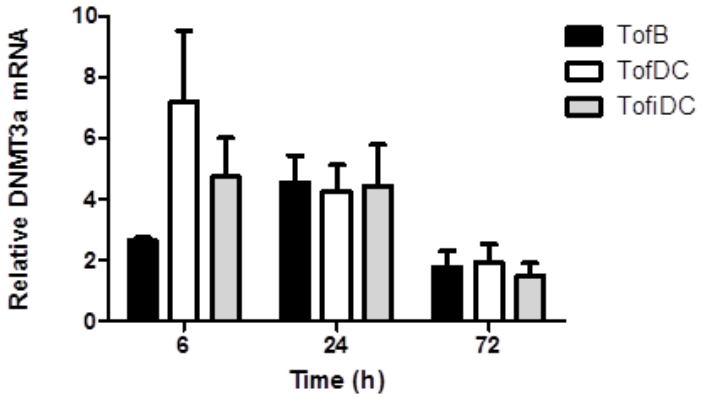
3.9.2 TCR triggering leads to global changes in DNMT expression

With the observation of Foxo1 been present in the nucleus of activated T cells, we sought to better understand this phenomenon. It has been described that the forkhead box O transcription factor family of which Foxo1is a member can be regulated epigenetically (Daitoku et al. 2011). We hypothesized that indeed Foxo1 could be re-expressed in the nucleus but epigenetically modulated such that while it was able to bind onto the promoter locus of KLF-2 in iTregs and thus drive CD62-L transcription, it was unable to bind onto the KLF-s locus in TofDC. To explore this possibility, we began with studying the dynamics of DNA methylation as this is the most common and widely studied epigenetic modification (Singal and Ginder 1999). In order to gain insight into DNA methylation dynamics, we assayed global mRNA levels of DNMTs in differentially activated cells against expression levels in naïve T cells.

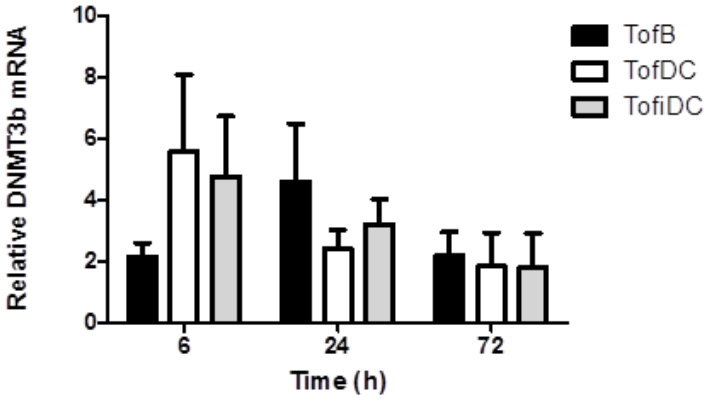
Measurement of DNMTs mRNA showed that 6h after T cell activation, both de novo DNMT levels were 3 to 8 times higher in all 3 cell models than in naïve T cells which were assigned a value of 1. At 24h, there were 4-5 times more de novo DNMTs expressed in TofB, TofDC and TofiDC but at 72h, de novo DNMT mRNA expression dropped down to same levels as in naïve T cells (Figure 3.13a-b). The converse was the case with the maintenance transferase DNMT1 which at 6h remained at same level as in naïve T cells but rose to levels 2 to 5 times more at 24h and remained high at 72h with values ranging from 2-4 times above naïve T cell DNMT1 mRNA level (Figure 3.13c).

These results while unable to give pinpoint details about the DNA methylation status at a specific gene locus did provide hints that following TCR priming there was a re-arrangement of the DNA methylation pattern to perhaps fit in with future cell fate requirements as observed with the initial surge and fall in expression levels of de novo transferases (DNMT 3a and DNMT 3b) which form hemi-methylated residues and then increased expression of the maintenance transferases (DNMT 1) at later timepoints which then make the methylation pattern permanent and by extension maintain differentiated cell lineages.

a)



b)



c)

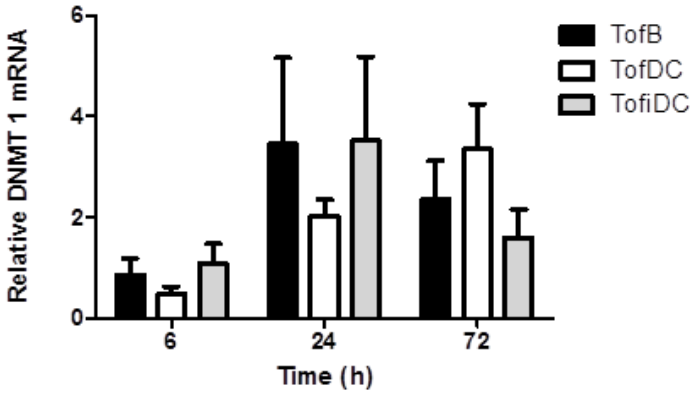


Fig 3.13 Global mRNA expression levels of DNMTs following differential T cell activation. (A) DNMT3a mRNA expression level in differentially activated T cells. (B) DNMT3b mRNA expression level in differentially activated T cells. (C) DNMT1 mRNA expression level in differentially activated T cells. Data represent means + SEM of 3 independent experiments.

3.9.3 Global DNA hypomethylation selectively upregulates CD62-L

Having established that Foxo1 is retained in the nucleus of activated T cells even in the presence of sustained PI3K signaling as evidenced by high expression of phosphorylated Akt along with the fact that there is some form of regulation on the level of DNMTs during the activation process, we decided to home in on the role of epigenetic mechanisms in CD62-L regulation. To accomplish this we induced hypomethylation of the genome using the pharmacological agent Decitabine. Decitabine (5-aza-2'-deoxycytidine, generic name: Dacogen) is a DNMT inhibitor commonly used to study re-expression of genes silenced by promoter methylation (Yang et al. 2001) and functions by sequestering DNMT1 to 5-aza-dC substituted DNA by irreversible binding of cysteine in the catalytic domain of the DNMT1 enzyme to the 6 position of the cytidine ring thus preventing the methylation of cytosine by DNMTs (Juttermann et al. 1994). Thus if indeed DNA methylation regulates CD62-L dynamics, then especially in the case of TofDC there should be upregulation of CD62-L following hypomethylation of the genome by Decitabine.

Following addition of Decitabine to cell cultures during the generation phase of our study cell models, CD62-L levels remained unchanged in TofB and TofiDC after 72h with 82% and 70% CD62-L expressing T cells respectively. In contrast, genomic hypomethylation led to increased CD62-L expression in the TofDC population with values rising from 16% in untreated conditions to 30% in Decitabine treated TofDC, representing a raise of about 95%. We also measured CD25 levels across all 3 cell models and it was largely unaltered in both treated and untreated conditions with values ranging between 83-95% CD25 expressing T cells thus indicating that Decitabine treated T cells were still viable.

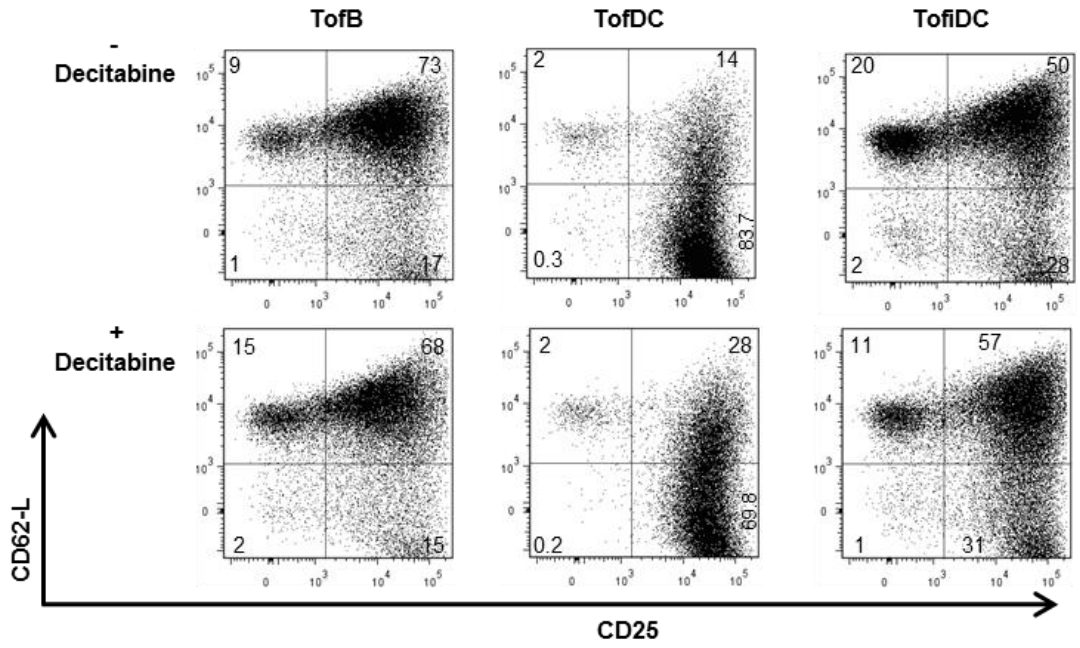


Fig 3.14 Genomic hypomethylation leads to upregulation of CD62-L levels in TofDC but not iTregs. All 3 cell models were treated with Decitabine and CD62-L expression measured at 72h. DNA hypomethylation led to doubling of CD62-L expression in TofDC but had no effect in iTregs. CD25 expression was unaffected. Data are representative of 3 independent experiments.

3.9.4 CD62-L is independent of HDAC inhibitor activity

Another common epigenetic modification we investigated for its involvement in CD62-L regulation was histone acetylation. Since we suspected gene repression as the possible mechanism via which histone acetylation might inhibit CD62-L expression in the in presence of total Foxo1 in the nucleus, we focused on HDACs which are generally associated with gene silencing (Moreira et al. 2003). HDACs function by removing acetyl groups from histone tails thereby making the chromatin more positively charged thus increasing the strength of association between DNA and the chromatin. Ultimately, this leads to very tight bonding that blocks access of transcription factors to promoter sites (Richon and O'Brien 2002). In view of the above facts, we utilized the HDAC inhibitor (HDACi) Trichostatin (TSA) to induce chromatin opening in order to understand the role of suspected acetylation-linked gene repression in CD62-L regulation.

We read out CD62-L expression at 72hours after incubation in the presence of TSA for the last 16 hours of incubation of T cells with different APC. Our data could exclude a role for HDAC activity in CD62-L regulation as addition of the inhibitor TSA had no influence on CD62-L expression levels in all 3 cell models; CD62-L levels remained at the same levels, both in plus or minus TSA samples. This was in contrast to the upregulation of CD62-L in

TofDC treated with Decitabine which was observable once again in this experimental run, confirming data from Figure 3.14. We then tested for synergism between HDACs and DNMTs as HDACs have been demonstrated to interact synergistically with DNMTs to promote gene silencing (Robertson et al. 2000;Rountree et al. 2000). Synergistic application of Decitabine and TSA yielded values which were not different from those obtained with Decitabine addition alone thus excluding a possible synergistic effect of both inhibitors in the regulation of CD62-L (Figure 3.15).

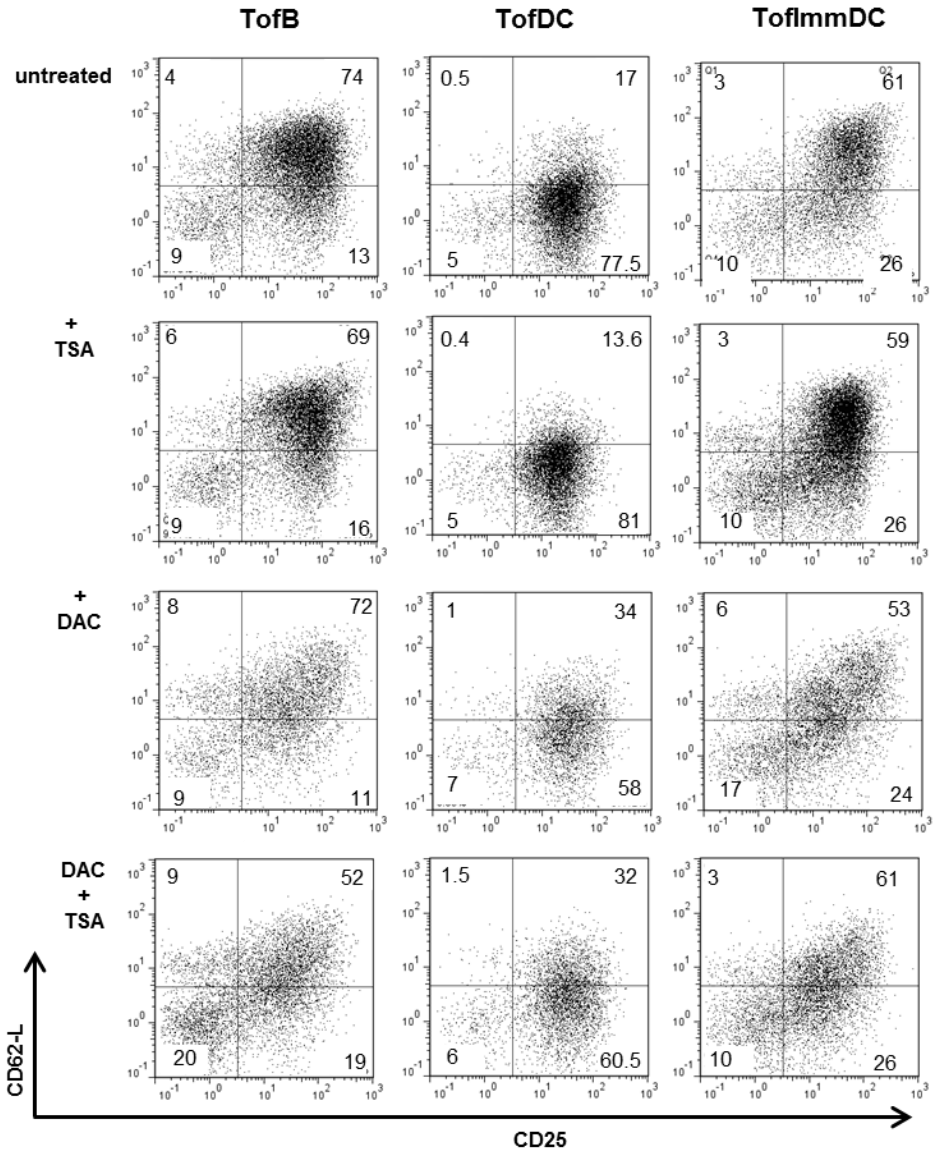


Fig 3.15 HDAC activity is dispensable for CD62-L regulation. T cells triggered by different APCs for 72h were treated with TSA at the 56h timepoint and CD62-L levels were measured by FACS. Addition of TSA had no effect on CD62-L expression level and when applied in synergy with Decitabine showed no synergistic effect. Data are representative of 2 independent experiments.

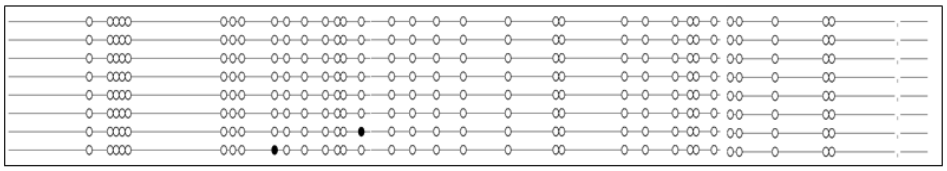
3.9.5 Methylation specific PCR of KLF-2 CpG islands in naïve and differentially activated T cells

With data from experiments with Decitabine indicating a possibility that DNA methylation could play a role in CD62-L transcription, we decided to examine the KLF-2 locus of the T cell genome to identify CpG islands that might be important in the context of this study. By gene mapping (supplementary figure 1-2), 2 CpG islands on the mice KLF-2 locus were identified, methylation primers designed and samples probed and analysed together with Dr Juliane Weski of the Institute for Experimental Immunology and Imaging, Uniklinikum Essen. Naïve T cells which, based on our hypothesis, should possess a methylation free KLF-2 locus served as control while the KLF-2 locus of TofDC with consistently low expression levels of CD62-L ought to be methylated thus inhibiting CD62-L transcription. We also hypothesized that both iTregs in our study would possess a hypomethylated KLF-2 locus as in naïve T cells which would be permissive for CD62-L transcription. Given that DNA methylation is a heritable and thus transferable trait, we focused on just the 72h timepoint as all cells at this point in the culture would have terminally differentiated and should therefore be equipped with all the necessary epigenetic signatures driving underlying their differentiation. Tested experimentally, our data albeit preliminary showed that in CpG island 1 of naïve T cells there was very low level methylation which would hypothetically allow for gene transcription but TofB, TofiDC and TofDC shared more or less the same amount of methylation status (Figure 3.16a). Examination of CpG island 2 showed a minimal amount of methylation in naïve T cells while both iTregs showed the same amount of methylation. Interestingly and contrary to projections, the KLF-2 locus in TofDC was totally unmethylated (Figure 3.16b).

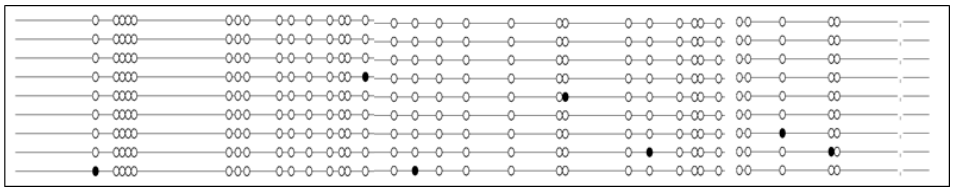
Overall, our results suggest that the KLF-2 locus shares the same status in both effector T cells and iTregs of this study, and may thus not be the optimal target for understanding the mechanism via which Decitabine was able to able to upregulate CD62-L in TofDC as demonstrated in Figures 3.14 and 3.15.

a) Methylation pattern of KLF-2 CpG Island 1

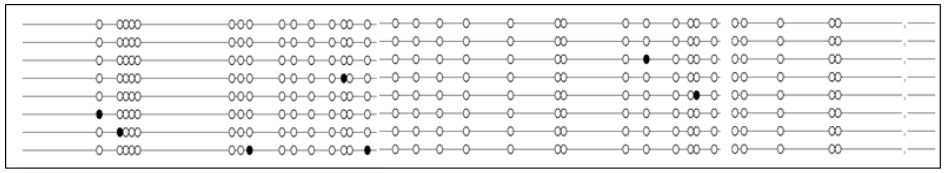
Tnaive



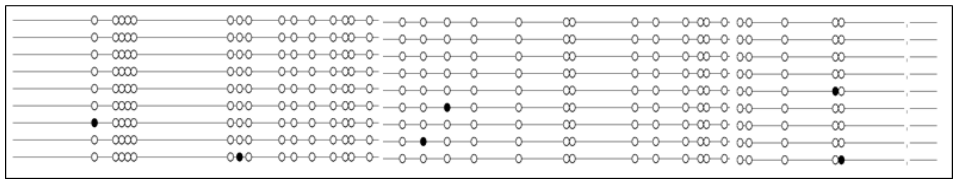
TofB



TofiDC

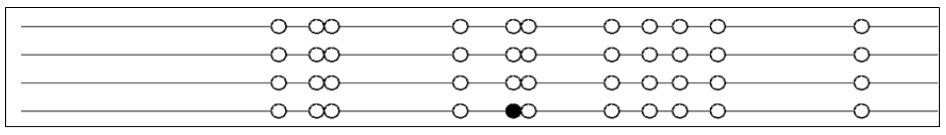


TofDC

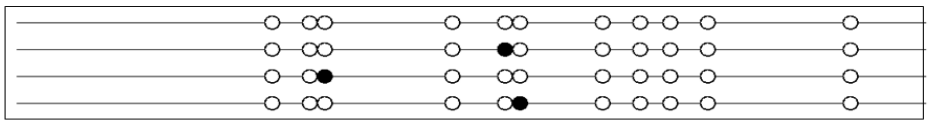


b) Methylation pattern of KLF-2 CpG Island 2

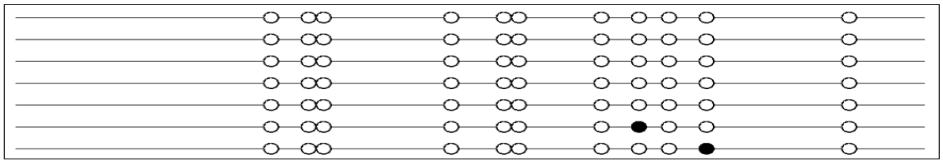
Tnaive



TofB



TofiDC



TofDC

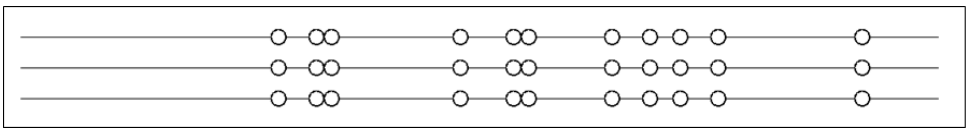


Fig 3.16 Methylation patterns of KLF-2 CpG Islands in naïve and 72h differentially activated T cells. (A) Methylation pattern from CpG island 1 (B) Methylation pattern from CpG island 2. Black dots represent methylated clones while white dots represent unmethylated clones. Data depicts 1 independent experiment.

3.9.6 Role of miRNA let-7b in CD62-L regulation

Epigenetic regulation of CD62-L has also been linked with miRNA expression. Under conditions of *Mycobacterium ulcerans* infection, mycolactone, a macrolide produced by this pathogen leads to T cell depletion as well as loss of CD62-L and downregulation of let-7b RNA levels. This downregulation of CD62-L was reversible when cells were treated with miRNA let-7b (Guenin-Mace et al. 2011). We therefore postulated that following T cell activation, let-7b is downregulated in iTregs as well as TofDC but while it remains consistently downregulated in TofDC, it is re-expressed in iTregs. To test our hypothesis, we quantified miRNA let-7b RNA levels in TofB and TofDC and found that it was equally expressed in both cell models and followed the same trend after T cell activation. While at 6h after commencement of co-cultures, let-7b expression remained at naïve T cell levels, at 24 and 72h let-7b levels were downregulated in both cell models at values of about 60% and 70% respectively (Figure 3.17).

These results indicated that under conditions of T cell activation, irrespective of the inducing APC, let-7b activity is dispensable for CD62-L regulation.

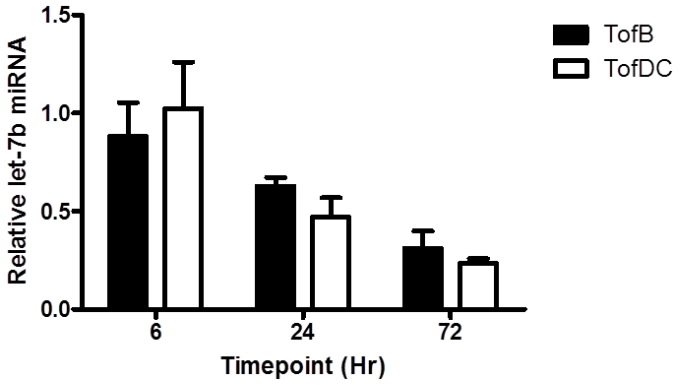


Fig 3.17 CD62-L regulation is independent of let-7b. Naive T cells activated by either B cells or DCs equally downregulate let-7b thus excluding a role of this miRNA in CD62-L regulation. Data represent means + SEM of 3 independent experiments.

3.10. Modulation of T cell activation dynamics by the microenvironment

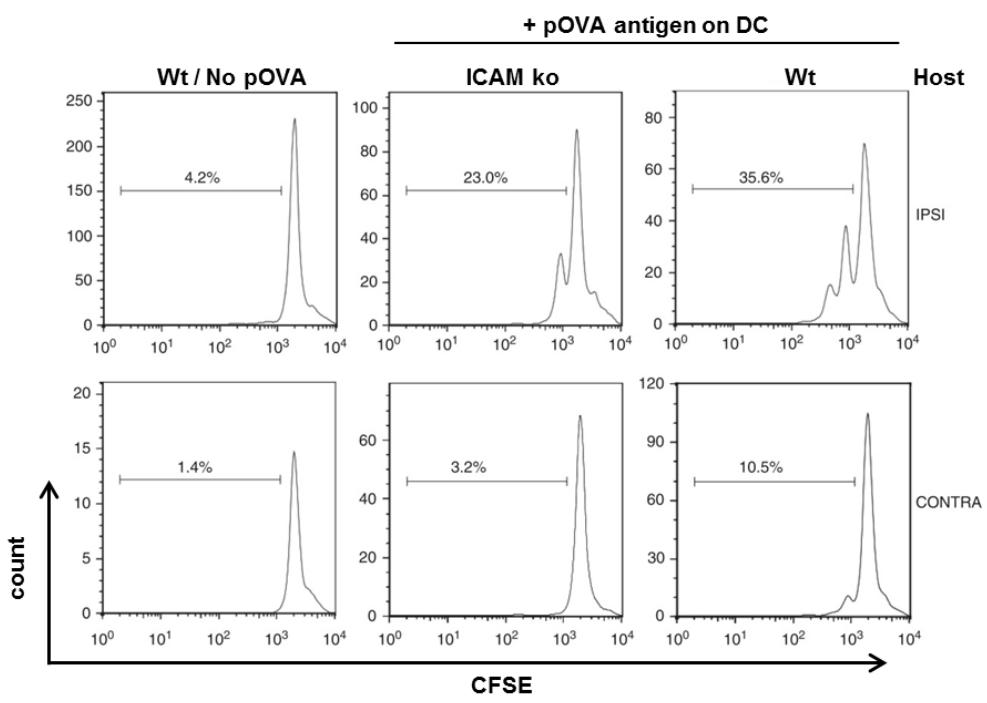
Work reported so far in this thesis had addressed T cell signaling mechanisms upon activation by APCs of different strengths and functional outcomes thereof in an *in vitro* setting. *In vivo,* T cell activation occurs in SLOs which are composed of stromal cells and adhesive molecules and integrins such as ICAM 1 and LFA-1 (Reichardt et al. 2010; Reichardt et al. 2013). In this part of the thesis we set out to begin understanding in more detail the dynamics of T cell activation under conditions where these adhesion molecules and or stromal cells are defective or missing. To begin with, we tested T cell activation dynamics in the context of ICAM 1 knockout using $ICAM^{-/-}$ knockout mice. Here we adoptively transferred a predetermined number of wildtype DCs with or without pOVA and at later timepoints wildtype naïve T cells into wildtype and ICAM knockout mice so the only difference in the experimental set up was the lack of ICAM on the stroma of the ICAM deficient mice. Thus the aim of this round of experiments was to observe if ICAM on the stroma of the SLO plays any role in T cell activation *in vivo*.

Following on from experimental protocol, popliteal lymph nodes (PLN) from all experimental animals were recovered and CFSE stained T cells were analysed from both ipsi and contra lateral nodes. In the no pOVA control sample, there was basal homeostatic proliferation of 4.2% T cells and 1.4% T cells in ipsi and contralateral nodes. When specific antigen was introduced, 23% of T cells in the ipsilateral lymph node of the ICAM ko mice proliferated while in wildtype C57Bl/6 mice 35.6% of T cells proliferated representing a difference of about 30% in proliferation between both mice types. Interestingly, in the contralateral node, there was an observed discrepancy as more proliferating T cells (10.5%) were found in the contralateral nodes of wildtype mice compared to 3.2% of proliferating T cells in the

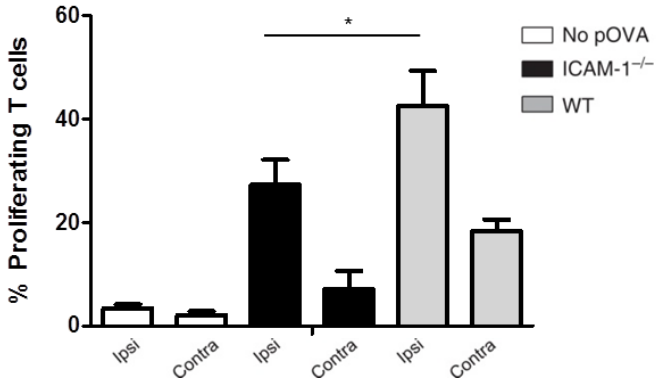
contralateral nodes (Figure 3.18a). In a wild type host, T cells proliferated one cycle more in 2 out of 3 experiments compared to ICAM deficient host further depicting the difference in efficiency of activation in wild type compared to ICAM deficient host. When subjected to statistical analysis, the number of proliferating T cells were found to be significantly more in wildtype mice compared to ICAM ko mice (Figure 3.18b). Examination of these CFSE stained T cells on the basis of per cell expression of CD25 showed no significant difference between activation status of T cell adaptively transferred and activated in wildtype or ICAM ko mice (Figure 3.18c).

These results gave strong hints that ICAM deficiency in the microenvironment of SLOs could play a role in tuning the immune response as evidenced by disparity in the difference in rate of T cell proliferation in the presence or absence of ICAM. This role might however be restricted to only the rate of proliferation and not the ability to bring cells to full activation status as evidenced by expression of CD25.

a)



b)



C)

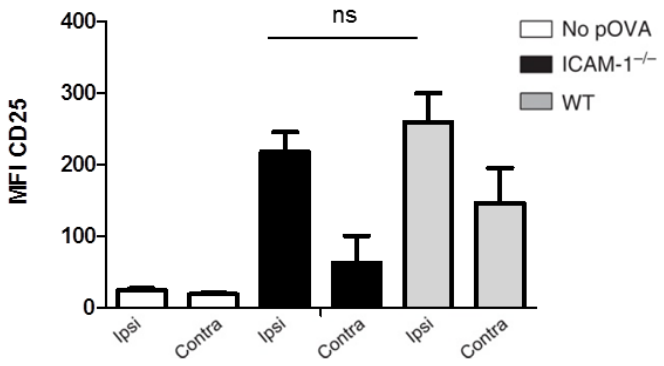


Fig 3.18 Modulation of T cell activation in ICAM deficient *in vivo* microenvironment. Adaptively transferred CFSE stained T cells were recovered and analysed by FACS after activation for 72h *in vivo.* (A) One representative FACS blot of T cell proliferation activated in the presence or absence of ICAM. (B) Statistical analysis of T cell proliferation rates in the presence or absence of ICAM. (C) Statistical analysis of MFI values of CD25 expression in T cells activated in the presence or absence of ICAM. Data for Figure 3.18a are representative of 3 independent experiments and Figure 3.18b-c represent means + SEM of 3 independent experiments.

4. DISCUSSION

This thesis was designed to elucidate the mechanisms governing the differentiation of naïve T cells triggered by weak APCs into Tregs as against an effector phenotype when activated by LPS matured DCs. Aside the ability of Tregs from the previously published work of our group to suppress naive T cell proliferation, significantly inhibit heart transplant rejection as well as ear swelling, both Tregs and T_{eff} cells expressed similar levels of CD25 and CD69 and the only difference observed between both cell types was the significantly higher level of CD62-L in TofB compared to TofDC after 72h of activation (Reichardt et al. 2007). The ease of the system for generation of TofB and TofDC with 72h of co-culture thus leant itself as a model for the kinetic study of the dynamics of Treg generation from naïve T cells. Tregs are a very unique subset of T cells as they modulate the amplitude of the response from other T cells thus occupying a special niche in adaptive immunobiology. The concept, activity and mechanism of action of Tregs has been extensively studied and described (Bilate and Lafaille 2012;Josefowicz et al. 2012). However, despite all that is presently known about Tregs, not much is known about the underlying early cellular signaling events that occur in the process of transformation from naïve T cell to iTregs.

Aside the regulatory subset of T cells, there are also reports of regulatory subsets in other immune cell types such as regulatory DCs which have been shown to induce Tregs, inhibit T cell proliferation as well as induce anergy in T cells (Qian et al. 2012). Regulatory B cells as a subset have also discovered and described (Yang et al. 2013;Yoshizaki et al. 2012). This understanding brings into perspective the fact that regulatory subsets are perhaps intrinsically linked to the immune system in a checks and balance manner to prevent exaggerated immune responses which lie at the root of autoimmune conditions among other conditions which distort homeostatic regulation of biological processes. In view of the lack of insight into the underlying early signaling events driving iTreg development, this study set out to bridge this gap using a kinetic study of developmental dynamics from naïve T cells to iTregs with the CD4+CD25+Foxp3- phenotype profile. This work built upon previous work from our group where B cells were used as APC to trigger antigen specific T cells to produce the regulatory TofB and incorporated the more physiologically relevant iTreg produced by T cell triggering with iDC (TofiDC) (Etemire et al. 2013). To test the robustness of our concept and study, we replicated our data in 2 genetically different mouse strains on the Balb/C and C57Bl/6 backgrounds with very similar results in both strains thus excluding the chances of our observations being skewed by strain specificities or differing genetic composition.

Using our model cells we found that naïve T cells differentiating into Tregs utilized similar pathways as that leading to effector T cell induction, the only difference being the temporal modulation of signaling intensity of the PI3K/Akt pathway. In both iTregs of this study i.e. TofB and TofiDC, there was a transient reduction of activity in the PI3K/Akt signaling pathway between 6 and 24h, specifically at Akt Ser473 site while the activation loop at Thr308 was unaffected by this defect. This phosphosite-specific defect in Akt Ser473 has been reported in Foxp3+ Tregs (Crellin et al. 2007) and has been determined to be due to the activity of the novel molecule, PHLPP1 (Patterson et al. 2011). PHLPP is a phosphatase consisting of 2 isoforms; PHLPP1 and PHLPP2 which both control the extent and temporal effects of Akt signaling by catalyzing the dephosphorylation of the hydrophobic Ser473 motif which is a C-terminal phosphorylation switch that plays a vital role in the control of the Akt kinase (Brognard and Newton 2008). While the work of Crellin and co-workers identified defective Akt Ser473 in fully established Foxp3+ Tregs, this work has identified this same molecular signature during the process of development of naïve T cells into potent Foxp3- Tregs.

Akt is downstream of the PI3Kinase and is thus influenced by the activity of PI3K following T cell activation. While in effector T cells the role of PI3K activity is much more understood (Han et al. 2012), its role in Treg development is still subject to intense discussion as there are divergent reports on the function of this pathway in Treg development. While it has been published that PI3K activation inhibits Treg development (Littman and Rudensky 2010), there is also data indicating the opposite (Soond et al. 2012). Data from our study provide new insight into these discrepant reports as our data indicate that PI3K is important for and compatible with Treg induction. In our model, weak APCs expressing sub-optimal levels of co-stimulatory molecules relative to DCs were able to drive naïve T cells into the Treg lineage by inducing low intensity Akt signaling for the first 24h after commencement of co-culture which later increased to levels same as observed in effector T cells. Co-stimulation as an extra signaling input from APC to T cells while not a direct downstream effector of the TCR has been reported as being relevant for fully potent and optimal PI3K activation (Garcon et al. 2008). Further exploring the role of CD28 i.e. co-stimulatory input in our study, we observed that augmentation of CD28 levels in cultures with weak APCs activating T cells indeed established a role for co-stimulation in Treg development. Addition of CD28 to cultures with weak APC and T cells skewed differentiation away from regulatory cell fate towards an effector lineage as seen with TofB and TofiDC incubated with soluble CD28

which showed with significant loss of inhibitory capacity when tested functionally in T cell proliferation. These results indicate that low levels of CD28 signaling coupled with TCR triggering generate a signal sufficient enough to drive T cell differentiation which favours acquisition of regulatory function and is in agreement with other published data (Pletinckx et al. 2011). Interestingly, this model of low co-stimulatory input as being important for Treg development might be only applicable to iTreg generation as thymus developing Tregs have been demonstrated to require CD28 input for proper development and functioning (Guo et al. 2008;Zhang et al. 2013) and iTregs have been demonstrated to require the absence of CD28 for their induction (Semple et al. 2011). This divergent role of CD28 is therefore dependent on the type of Treg being developed i.e. iTreg vs nTreg. Aside the requirement of co-stimulation for conferment or inhibition of regulatory functionality, CD28 is also important for Treg survival and homeostasis (Guo et al. 2008). Thus we postulate that weak APCs deliver enough co-stimulatory input to not only induce Tregs but to ensure their survival as both iTregs in this study were viable cells as gauged by their high expression of CD25. The fact that PI3K activity in both iTregs as measured by its downstream effector Akt reached values similar to that in effector T cells after an initial time of reduced activity, strongly suggests that there exists a time dependent window of sensitivity in triggered T cells within which cell fate decisions driving generation of Tregs are made. This window of opportunity could be a time within which cellular machinery necessary for sub-serving regulatory function is mobilized and set in place.

One distinguishing character of the iTregs in this study was the re-expression of CD62-L after an initial shedding phase which occurred vis a vis full activation as measured by CD25 expression and T cell proliferation. There was an initial TACE-mediated shedding phase in iTreg development as in TofDC but starting at 24 hours the cellular processes began to differ as while the iTregs re-expressed CD62-L, in effectors this molecule was consistently down-modulated (Etemire et al. 2013). An interesting observation in this study was the fact that commencement of CD62-L re-expression began at about the same time period when PHLPP1 mediated pAkt Ser473 defective signaling was most observable. The concept of PI3K/Akt attenuation being relevant for the generation of Tregs has been proposed by researchers in the field (Soond et al. 2012; Ouyang et al. 2012). Soond and co-workers propose that this attenuation of PI3K/Akt signaling allows for the re-expression of the transcription factor Foxo1 which then binds onto the Foxp3 locus to induce Treg induction.

In the context of our study, Foxo1 was indeed found to be in the nucleus of Tregs where we suspect that it binds onto the KLF-2 locus to drive CD62-L upregulation. Interestingly and contrary to expectations, we also observed the presence of Foxo1 in the nucleus of the effector TofDC. This finding was unexpected as CD62-L which is Foxo1 driven remained downregulated in this cell model. Foxo1 has been reported as being permanently excluded from the nucleus by PI3K/Akt activity following TCR activation (Fabre et al. 2005). However, this study performed imaging for 7 hours and while our data concur with this nuclear exclusion at this timepoint, we observed a re-appearance of Foxo1 at the later timepoints of 24 and 72h even in the presence of consistent PI3K/Akt signaling as determined by continual expression of phospho-Akt. This finding thus caused us to postulate that perhaps Foxo1 is subject to some form of epigenetic regulation with respect to CD62-L dynamics. Exploring epigenetic influence on CD62-L in terms of DNA methylation and histone modification using the pharmacological agents Decitabine and TSA respectively, our data hint that DNA methylation but not histone modification does play a role in CD62-L regulation as DNA hypomethylation under the influence of Decitabine doubled the amount of CD62-L positive cells in the effector T cell pool in our study but not in the iTregs. A search of literature revealed that there has been no reported/published study on the role DNA methylation or histone modification in CD62-L regulation to date. Thus in view of this, this current work offers some new insight into a mechanism that could play a role albeit undetermined in T cell migration and localization.

Despite the dearth of information on the possible role of DNA methylation in CD62-L regulation, Syrbe et al. 2004 have demonstrated that genomic hypomethylation by Decitabine genome induced a 20 fold increase in P-selectin expression levels of effector T cells activated with APC thereby establishing a role for epigenetic modification in the regulation of homing activity of T cells. The fact that CD62-L expression could be upregulated under hypomethylating conditions might be of clinical relevance as the drug Decitabine is widely used in cancer treatment regimens (Christman 2002;Ganesan et al. 2009) and T cell distribution has been described to play a role in cancer prognosis (Gu-Trantien et al. 2013;Matkowski et al. 2009;Naito et al. 1998). Another epigenetic factor investigated still in the context of CD62-L regulation was the miRNA let-7b which has been reported to upregulate CD62-L (Guenin-Mace et al. 2011). Not much is known nor has been reported in literature about the role of miRNAs in CD62-L dynamics and in this study, we could exclude differential expression of let-7b as a factor driving the divergent CD62-L characteristics of iTregs and effectors. Ultimately, these data hinted at a role for epigenetic regulation in some

aspect of CD62-L regulation but the precise location of this modification is yet unclear as investigation of the methylation status of 2 identified CpG islands on the KLF-2 locus onto which Foxo1 binds in the nucleus using MSP showed no differential methylation patterns. This then opens up the possibility that perhaps the epigenetic modification might be located on the Foxo1 protein itself. There is published data showing that Foxo1 does have its DNA binding capacity epigenetically altered following PI3K/Akt signaling or (Matsuzaki et al. 2005;Van Der Heide et al. 2004). Both reports also identified acetylation and phosphorylation as common modifications of Foxo1 but there are no reports yet of methylation modification in this protein.

Another interesting epigenetic linked phenomenon in our study was the absence of Foxp3 expression in both the TofB (Reichardt et al. 2007) and TofiDC. Foxp3 expression in Tregs is epigenetically regulated (Huehn et al. 2009;Polansky et al. 2008) and is driven by Foxo proteins (Harada et al. 2010). Roncarolo and co-workers have shown that immature DCs when used as APCs, convert naïve T cells in an allogeneic setting into Foxp3- $CD25^{low}$ Tr1 like Tregs under the influence of IL-10 (Levings et al. 2005). These examples bring to bear the plethora of possibilities and avenues via which Tregs can be induced and it remains to be determined what *in vivo* scenarios may promote or predispose the use of each of these systems for the generation of Tregs. There could also be the possibility that based on location *in vivo* as well as secretions from cells types in the locality as well as infection or nature of immunogenic impulse, different Treg types might be induced. The lack of Foxp3 expression in certain Treg populations also brings into focus the changing concept of "master regulator factors" for each T cell subset as data accumulate that T cell differentiation exists and function in conditions beyond the present one master regulator concept (Oestreich and Weinmann 2012). In a recent review, Sakaguchi and co-workers question the designation of Foxp3 as the master transcription factor for Tregs based on the fact that there have been discovered to be Foxp3+ T cells which do not possess regulatory function (Kitagawa et al. 2013). Examples of data challenging the prevalent master regulator concept include reports of $Foxp3^{+}$/T-bet^{+} and $Rorc^{+}$/T-bet^{+} T cells (O'Shea and Paul 2010) as well as such works of that of Hegazy and co-workers who demonstrate that terminally differentiated Th2 cells when adaptively transferred into mice infected with lymphocytic choriomeningitis virus (LCMV) become reprogrammed to $Gata3^{+}$T-bet^{+} and IL-4^{+}IFN-γ^{+} T cells and maintain this new Th1/Th2 phenotype for months (Hegazy et al. 2010).

With the observation that CD62-L is re-expressed on both iTregs in our study we wondered if CD62-L re-expression was more than just a phenotypic signature and was perhaps intrinsically associated with conferment of regulatory capacity in T cells triggered by weak APC. Recently, it was demonstrated by *in vitro* technique that activation of naïve CD8 T cells by a weak APC, liver sinusoidal epithelial cells led to similar CD62-L dynamics as observed in TofB and TofiDC i.e. initial downregulation and then re-expression after 24 hours (Bottcher et al. 2013). These CD8 T cells homed preferentially to lymph with same potency as naïve CD8 T cells which normally express high CD62-L levels. This report coupled with our data hint that weak APC might as a rule induce upregulation of CD62-L in T cells after interaction and it was thus necessary to begin probing the possible importance of re-expressed CD62-L. Additionally, addressing this question was important as there are numerous publications which have extensively linked the CD62-L transcription factor Foxo1 with Treg generation and development (Harada et al. 2010;Hedrick et al. 2012;Kerdiles et al. 2010) as well as being important for function (Samstein et al. 2012). CD62-L as a transcription product of Foxo1 has also been discovered and reported to be associated with several functions asides homing and migration.

CD62-L plays a role in memory T cell development and activity as it is associated with acquisition and maintenance of memory in these cells (Dang et al. 2009;Roberts et al. 2005). Additionally, CD62-L expression is linked with development of the key effector function; lytic activity in human tumour infiltrating T cells (Yang et al. 2011). Other CD62-L linked functions are assistance of virus clearing in CD8 T cells (Richards et al. 2008) as well as being vital for maturation and localization of hepatic NK cells during viral infections (Peng et al. 2013). Our data from TofDC generated with or without Ly294002 and Rapamycin helped to answer this question. Our results showed delineation between CD62-L upregulation and acquisition of regulatory function. Rapamycin treated TofDC, despite upregulated CD62-L could not inhibit proliferation of naïve T cells stimulated with DCs while the converse was the case with Ly294002 treated TofDC which just as the Rapamycin treated cells upregulated CD62-L but also showed strong regulatory behaviour unlike the Rapamycin treated sample. Our data thus indicate that acquisition of regulatory function is linked more to PI3K activity than mTOR signaling.

Patterson et al. converted CD4+ T cells into Tregs by TCR stimulation in the presence of IL-2 and TGF-β and report that addition of Rapamycin and Ly294002 to the culture induced PHLPP1 expression in parallel to Foxp3 induction. Based on this correlation between Foxp3

and PHLPP1 expression, they suggested that PHLPP1 upregulation plays a role in Treg development. This was experimentally confirmed by ChIP assays where they identified nine pututative binding sites on the PHLPP1 promoter for Smad-3. Smad-3 is indeed known to be activated during TGF-β induced Foxp3 expression (Tone et al. 2008). In our study, triggering of T cell using DCs as APC in the presence of mTOR or PI3K inhibitors led to a significant induction of PHLPP1 in only the PI3K inhibited TofDC while mTOR inhibited TofDC updated neither PHLPP1 nor exhibited regulatory function. While our data are in agreement with that of Patterson et al with regards to the involvement of attenuated PI3K/Akt signaling in Treg induction, we find and report that mTOR signaling attenuation is not required for induction of Tregs contrary to the report of Patterson et al. One critical point in analyzing this study is the fact that TGF-β was added in culture during the generation of these Tregs and given that TGF-β has been widely reported to be integral for iTreg differentiation in addition to TCR triggering (Zhou et al. 2009), it becomes difficult to disassociate the induction of Tregs from naïve CD4 T cells as being solely derived from the activity of this cytokine.

Others have reported on the requirement of mTOR along with PI3K and Foxp3 for induction of Tregs (Sauer et al. 2008). This study was conducted using antibody stimulation in contrast to our study which was performed using APCs which would in contrast to the strong signal associated with antibody stimulation deliver a more delicate input to T cells. There is an accumulating body of evidence that signal intensity via the TCR has far reaching and divergent consequences for T cell dynamics. Stimulation of the TCR with crosslinked CD3 has been demonstrated to induce strong stimuli with short lived but intense Erk activation while antibodies immobilized on beads induced more tonic signaling with long lasting and less intense Erk signaling which was found to induce T cell proliferation and survival at a significantly higher level (Arndt et al. 2013; Poltorak at al. 2013). These studies proposed immobilized antibody signaling dynamics as being more akin to APC induced signaling dynamics and offer new perspectives into possible reasons for the disparity between published data and some of the data from our study while highlighting the dynamic range of possibilities/outcomes following common TCR triggering in different conditions. One striking observation in this study was the fact that Rapamycin failed to induce regulatory function in triggered T cells as was obtainable with Ly294002. This finding was highly unexpected as Rapamycin is widely used clinically and in basic scientific research for expansion of Tregs from CD4+CD25+ cell populations (Battaglia et al. 2005;Battaglia et al. 2006;Battaglia et al. 2012). Apart from Patterson et al. who showed Treg induction albeit in the presence of TGF-β and Rapamycin, Chen et al. 2010 were able to induce Tregs from naïve CD4 T cells in a 6 day

co-culture prep with mitomycin treated B cells in the presence of Rapamycin. Based on similarities with our study, it is possible that the induction of regulatory function was to some extent dependent on the presence of B cells in the T-B co-culture prep. Our results agree with other published reports on the requirement of PI3K inhibition for iTreg generation and pose new questions with regards to the role of mTOR in this process. Data from mouse T cells with frap gene deletion thus lacking mTOR yields some more insight into the role of mTOR in iTreg generation. The frap1 gene encodes the mTOR protein in T cells. These T cells could be activated normally and expressed IL-2 but failed to differentiate into Th1, Th2 and Th17 cells due to deficiency in STAT activity. These mice could generate Tregs but specific T cells affected by frap gene deletion which lacked TORC1 and TORC2 showed a pronounced inability to differentiate into Tregs (Delgoffe et al. 2009). This result supports our data and strongly indicates that the generation of Treg occurs in the presence of intact mTOR activity and not in its absence or under conditions of inhibition while also highlighting the central role for mTOR in differentiation of T cells.

Investigation of the underlying dynamics of CD62-L control also involved gaining insight into the role of co-stimulation in this regard. While we had observed a lack of co-stimulatory input from weak APCs to be important for the induction of regulatory function, we also clarified the role of CD28 with respect to migratory potential as can be inferred from the level of CD62-L expression on primed T cells. We observed that augmented co-stimulation in both iTregs had no influence on CD62-L expression levels. This result placed side by side against the loss of regulatory function in iTregs generated with augmented co-stimulation indicates that there is some form or compartmentalization in the signaling mechanisms governing conferment of regulatory characteristic and homing potential. This dichotomy mirrors the divide in the process(es) between acquisition of regulatory function and upregulation of CD62-L in TofDC treated with Rapamycin or Ly294002. Mirenda et al. 2007 have reported that co-stimulation by CD28 is important for migration of memory T cells to the periphery while the data from Fuse et al. 2008 show that CD28 co-stimulation is important for priming and recall responses of memory CD8 T cells. Co-stimulation was also found to be important for responses to infections with murine gammaherpesvirus 68 (MHV-68) infection. These reports complement each other in driving this concept of compartmentalization on two levels; the first being that differences in co-stimulation requirement help to divide differentiation processes for different T cell subsets and secondly, differences in co-stimulation requirement enables T cells to navigate to their site of activity. In addition to these 2 points, the concept of compartmentalization also brings a new perspective to understanding the development and

functioning of extranodal regulatory T cells. Extranodal Tregs are distinct from nominal Tregs as they are CD62-L negative but express high amounts of E/P selectin binding ligands in addition to multiple adhesion molecules and chemokines linked with inflammation. These extranodal Tregs as the name indicates do not localize to the lymph node due to lack of CD62-L and were described to exhibit the most potent inhibitory activity against inflammatory processes in an antigen-induced arthritis disease model (Huehn et al. 2004). Based on the localization and described activity of these extranodal Tregs, it takes to reason that these cells developed in a manner wherein it acquired regulatory capacity via processes independent of CD62-L regulation.

In the last part of this thesis, we began exploring the impact of factors from the microenvironment within which T cell activation occurs. T cell activation and function is affected and modified based on microenvironmental factors. The input of the microenvironment in T cell activity is mostly addressed in the context of tumour-immunology with several publications to that effect (Carlson et al. 2013;Fisher et al. 2006;Santin et al. 2001;Weigelin et al. 2011). Our data already indicate that absence of the adhesion molecule ICAM 1 on lymph node stroma leads to decreased efficiency in T cell activation due to lack of sufficient residence time in the lymph node. Questions as to possible outcome in infectious conditions and T cell potency and activity under conditions of such altered microenvironments using several mouse models would be of interest and could answer questions as to whether or not adhesion molecules do more than just firm the interaction between T cells and APC and or surrounding structures doing T cell activation dynamics.

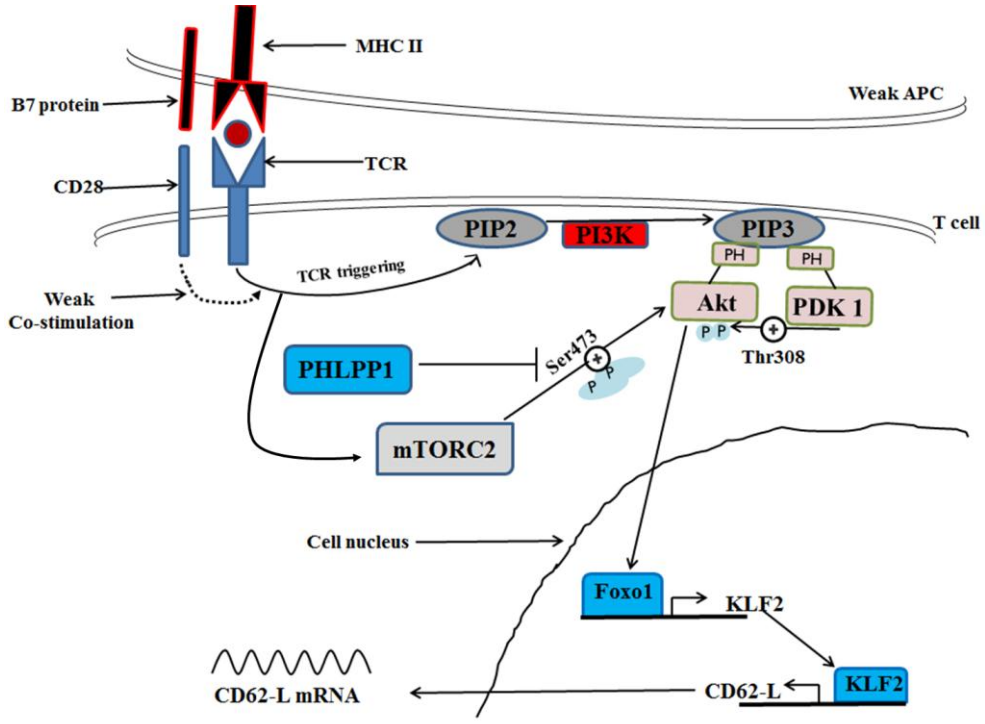
5.0 CONCLUSION AND OUTLOOK

This thesis set out to investigate the early signaling processes which drive conversion of naïve T cells into regulatory T cells and presents data showing that T cells differentiating into either Tregs or effector T cells make use of similar signaling patterns. The major difference in terms of signal transduction was the tuning of the PI3K/Akt signaling which was found to be differentially regulated between iTregs and effector T cells with the phosphatase PHLPP1 playing a key role in this tuning. Previous work has identified this phosphatase as being important for functioning of established Tregs (Patterson et al. 2011) but our data extend this finding by suggesting that PHLPP1 is also important for driving the induction of Tregs from a naïve T cell pool. PI3K/Akt signaling has been implicated as playing a role in Th17 differentiation (Kim et al. 2013;Nagai et al. 2013), Th22 differentiation (Mitra et al. 2012) as well as the generation of other CD4 T cell subsets (Han et al. 2012). In the case of iTreg differention vis a vis generation of the effector TofDC in this study, the differential outcomes were based on signaling thresholds with attenuated PI3K activity for the first 24 hours of Treg induction following which PI3K activity rose to levels commensurate with that of TofDC at later timepoints. In further works on elucidating the role of PHLPP1 in iTreg development, it will be important to knockdown this phospshatase by RNAi technique in naïve T cells and then study the dynamics and functionality of T cells triggered the same as in this study.

This study was also able to delineate the roles of the PI3K and mTOR signaling cascades in the acquisition of regulatory function with our data strongly hinting that regulatory activity is more dependent on PI3K activity and independent of mTOR signaling. Along this line, this study also established that CD28 co-stimulation plays no role in the induction and homing capacity of Tregs but that its absence is required for acquisition of regulatory function. In terms of regulatory function the presence of Foxo1 in the nucleus of both iTregs and effector T cells requires more thorough investigation in order to understand the precise process underlying this phenomenon. While in Tregs, CD62-L a target gene of Foxo1 is transcriptionally active while in TofDC, it is not. This pre-supposes that there is some inhibitory factor involved in this process. It would be interesting to by pull-down experiments investigate the proteins and factors complexed with Foxo1 in both cell lineages as this might shed new insight. Foxo1 is a master transcription factor with over 300 known target genes and drives many cell intrinsic processes (Ouyang et al. 2012). Given that Foxo1 is not a CD62-L dedicated/restricted transcription factor, it is important to understand in more details how it is interacts with each of its numerous target genes in different differentiation states/stages. In

the context of our study, we hypothesize that Foxo1 is excluded following T cell activation and while it out of the nucleus, the chromatin is remodeled to selectively bind Foxo1 upon its re-appearance at later timepoints. This might account for differential expression of CD62-L between iTregs and effector T cells generated and analyzed in this present work.

a)



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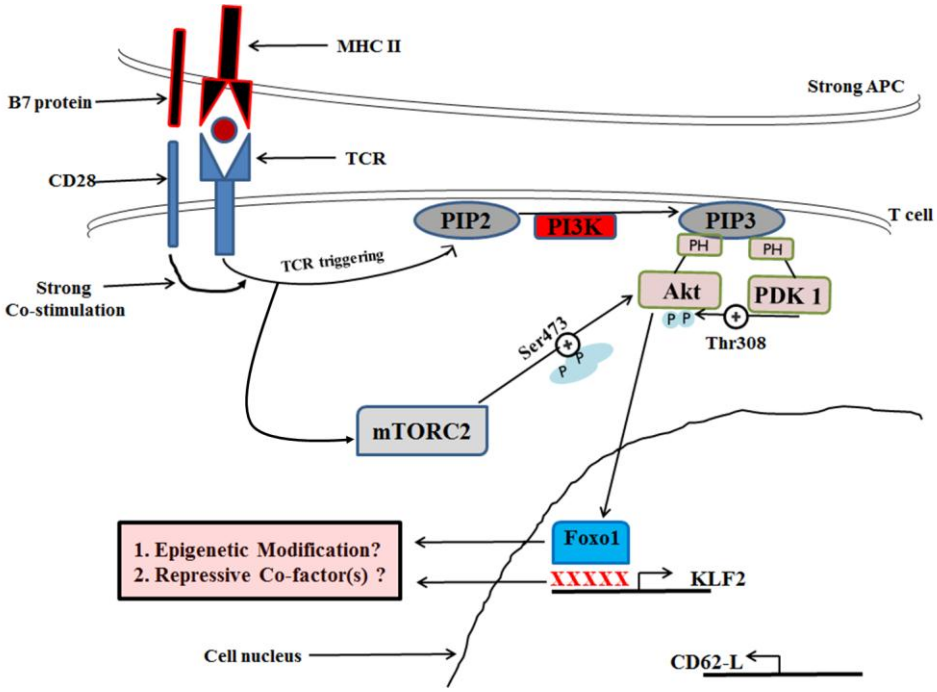

Fig 5.1 Model for signaling dynamics following activation of naïve T cells by weak or strong APC. (A) Schematic depicting signaling early events in T cells triggered by weak APC. PHLPP1 is upregulated after TCR triggering, thus reducing PI3K driven Akt Ser473 phosphorylation levels. Foxo1 after an initial nuclear exclusion phase re-appears at about 24h in the nucleus and drives CD62-L transcription and the T cells overall differentiate into Tregs. (B) Schematic depicting signaling early events in T cells triggered by strong APC. Akt Ser473 activity is sustained at a high level under the influence of PI3K activity following TCR triggering and Foxo1 after an initial nuclear exclusion phase re-appears in the nucleus but fails to drive CD62-L transcription by mechanisms possibly involving DNA methylation and other yet unelucidated factors. T cells thus triggered exhibit effector phenotype and function.

Finally, we began investigating how the microenvironment modulates T cell activation *in vivo.* Using an ICAM knock out host, we could show that T cells were activated to the same levels as obtainable in wildtype hosts by measuring CD25 levels but our data showed that T cells in ICAM deficient hosts proliferated significantly less than in wildtypes. This suggests that the extent of the immune response might be slower in cases of ICAM deficiency on lymph node stroma. Given the nature of immune insults and the need for a prompt response, this will have strong functional relevance. Further works will involve recapitulation of this assay in other transgenic mice with defective integrins as well as performing viral and infectious studies in these mice to gain a better insight into how T cells respond to infections in altered microenvironmental conditions.

6.0 ZUSAMMENFASSUNG

Regulatorische T Zellen (Tregs) bilden eine eigene Untergruppe der T Zellen. Ihre Hauptaufgabe ist es, Immunantworten zu regulieren und damit auf einem, für den Organismus ungefährlichen Niveau zu halten. Prinzipiell kann man regulatorische T Zellen in zwei weitere Untergruppen aufteilen: Zum einen in die natürlich vorkommenden Tregs (nTregs), welche sich im Thymus entwickeln und zum anderen in induzierte Tregs (iTregs), welche aus dem naiven T Zellpool in der Peripherie heranreifen. Ein Hauptcharakteristikum von regulatorischen T Zellen ist die Expression des Transkriptionsfaktors Foxp3. Nichtsdestotrotz beschreiben neuere Arbeiten auch wichtige immunmodulatorische Funktionen von Foxp3-negativen Tregs. Obwohl die Gruppe der iTregs in den letzten Jahren Gegenstand vieler wissenschaftlicher Arbeiten waren und obwohl viele Details zu ihrer biologischen Funktion publiziert wurden existiert eine deutliche Wissenslücke, was die initialen Signalkaskaden angeht, welche die Konversion von naiven T Zellen zu iTregs einleiten. Unter Zuhilfenahme eines transgenen T Zellmodells (pOVA stimulierte T Zellaktivierung von OT-I/II T Zellen) wurden in der vorliegenden Arbeit die Prozesse charakterisiert, welche an der Ausreifung von naiven T Zellen zu CD4+ CD25+ Foxp3- iTregs nach Stimulation des T Zellrezeptors (TCR) durch schwache Antigen präsentierende Zellen (APC), wie z.B. B Zellen, beteiligt sind.

Die ersten Resultate dieser Arbeit zeigten, dass iTregs nach Aktivierung durch schwache APC (B Zellen) zunächst das Oberflächenmolekül CD62-L verloren. Dieses Molekül wird von den Zellen benötigt, um effektiv ihren Weg in den Lymphknoten zu finden. Nach 24 h war eine Re-Expression des Moleküls nachzuweisen und nach 72 h entsprach das Expressionslevel dem naiver T Zellen. Im Gegensatz dazu verloren T Zellen nach starker Aktivierung durch reife Dendritische Zellen (DCs) ebenfalls CD62-L, allerdings blieb dieser Zustand über mehrere Tage erhalten.

Basierend auf pharmakologischen Vorarbeiten wurde impliziert, dass der PI3K/mTOR Signalweg eine zentrale Rolle bei der unterschiedlichen CD62-L Regulation zwischen iTregs und Effektor-T Zellen einnimmt. Hierzu konnten wir zeigen, dass das hydrophobe Motiv der Proteinkinase Akt, Ser473, in iTregs nach Stimulation durch schwache APC konstant phosphoryliert vorlag. Dieses Aktivierungsmuster ging mit einer Hochregulierung der erst kürzlich beschriebenen Phosphatase PHLPP1 einher. Von dieser ist wiederum bekannt, dass sie spezifisch die Phosphatreste am Ser473 der Akt angreift.

Bezüglich der Wichtigkeit der PI3K/mTOR Signalkaskaden fanden wir, dass nach einer pharmakologischen Inhibition der PI3K naive T Zellen zu regulatorischen T Zellen

ausdifferenzierten. Das Blocken der mTOR Kaskade hatte keine Messbaren Auswirkungen in diesem Kontext.

Als nächstes wurde die Rolle der CD28 Ko-Stimulation bei der Generierung von regulatorischen T Zellen untersucht. Hierbei fanden wir, dass ein Fehlen der ko-stimulatorischen Moleküle bei der Aktivierung von T Zellen durch schwache APC unabdingbar für die Ausbildung ihrer regulatorischen Funktionen war. Eine verstärkte CD28 Stimulation führte zur Aufhebung jeglicher immunregulatorischer T Zellfunktionen. Interessanterweise hatte diese Ko-Stimulation keine Auswirkung auf die CD62-L Expression in den Zellen. Dieser Befund erlaubt die Schlussfolgerung, dass eine sub-optimale Aktivierung naiver T Zellen durch CD28 Ko-Stimulation notwendig für die Ausbildung des immunregulatorischen Potentials von iTregs ist, dass durch dieses die migratorischen Fähigkeiten der Zelle aber nicht weiter beeinflusst werden.

Abschließend charakterisierten wir die Rolle von Adhäsionsmolekülen auf Stromazellen bei der T Zellaktivierung. Wir konnten zeigen, dass adoptiv transferierte OT-II T Zellen in WT Tieren und in ICAM (Intercellular Adhesion Molecule) k.o. Mäusen gleich gut, durch Peptid beladene, ebenfalls transferierte DCs aktiviert wurden (Messung des CD25 Levels). Der Prozentsatz an proliferierenden Zellen lag in ICAM k.o. Tieren hingegen signifikant reduziert vor. Daraus formulierten wir die These, dass T Zellantworten im Lymphknoten deutlich verzögert oder ineffektiv ablaufen, wenn das Stroma des lymphatischen Organs kein ICAM als „Zellanker“ aufwies.

Zusammenfassend kann gesagt werden, dass die vorliegende Arbeit Einblicke in früh ablaufende Signalwege bei der Generierung von iTregs aus naiven T Zellen gibt. Darüber hinaus beschreibt sie ein zeitliches Fenster nach T Zellrezeptorstimulation in welchem die Höhe der PI3K/Akt Aktivität bestimmt, ob sich eine T Zelle zu einer Effektorzelle oder zu einer Immunzelle mit regulatorischem Potential entwickelt. Darüber hinaus wird in der Arbeit die These formuliert, dass Adhäsionsmoleküle auf Stromazellen lymphatischer Organe eine wichtige Rolle bei der Einleitung von T Zellantworten haben.

7.0 REFERENCES

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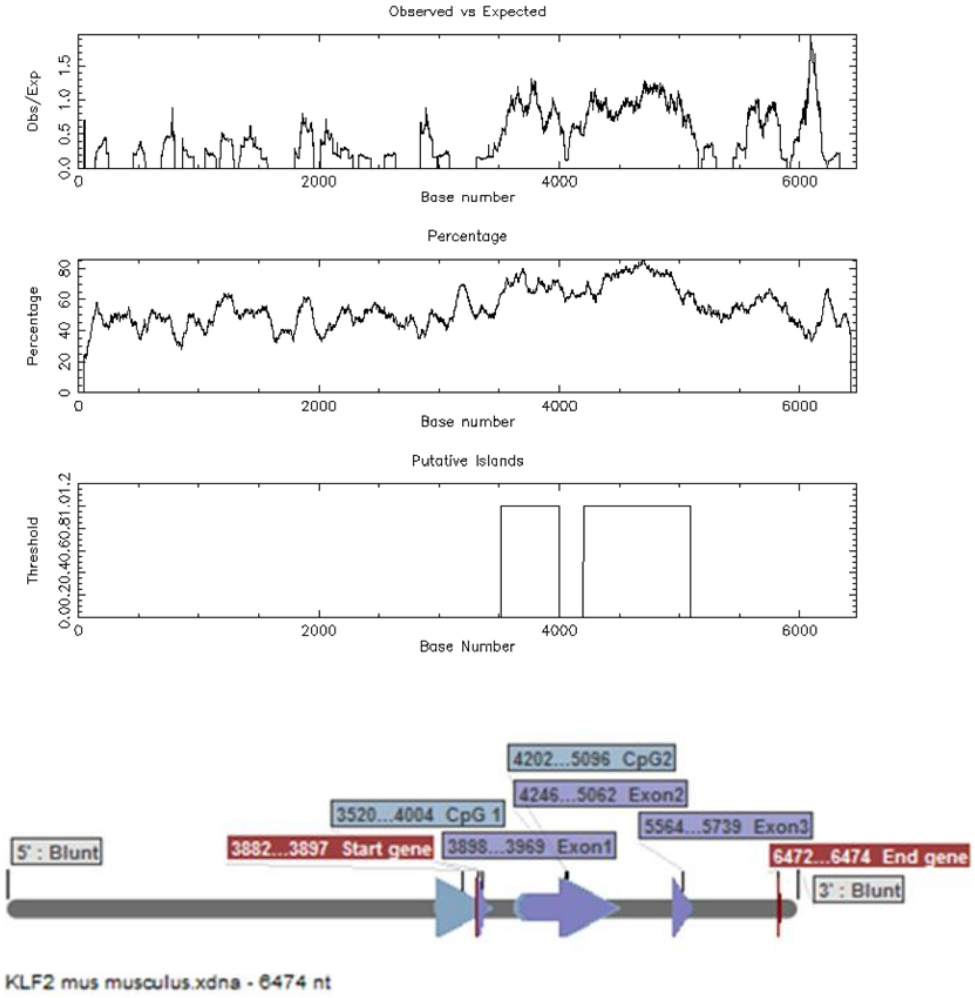
Supplementary Figures; Gene mapping of the mice KLF-2 locus

Figure 1. Mouse KLF-2 locus was analysis with GC contents and other determined characteristics.



CPGPLOT islands of unusual CG composition

EMBOSS_001 from 1 to 6474

Observed/Expected ratio > 0.60

Percent C + Percent G > 50.00

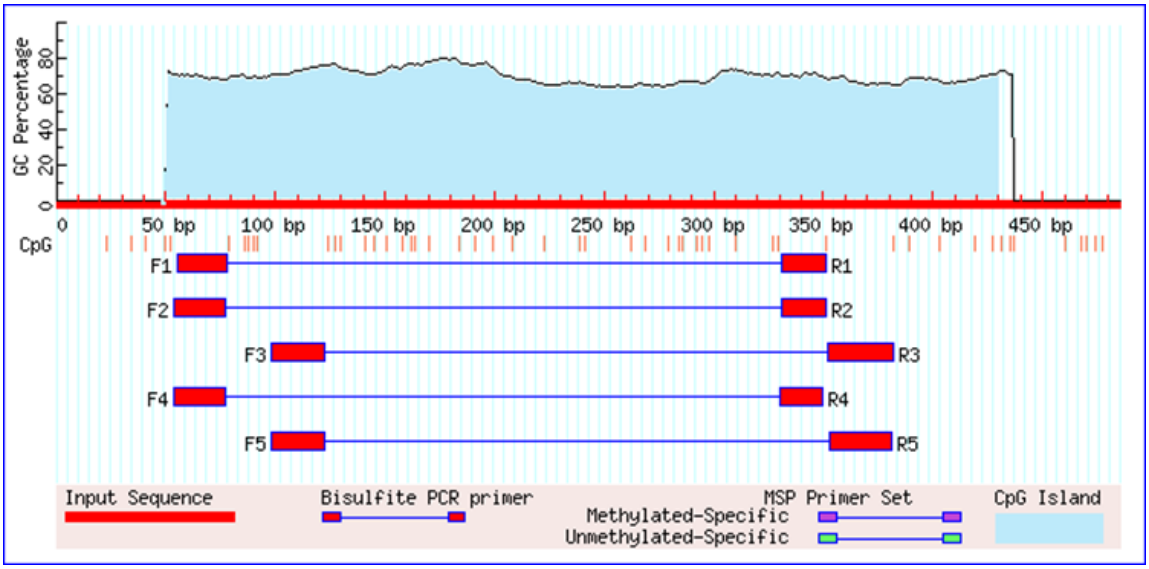
Length > 200

Length 485 (3520..4004)

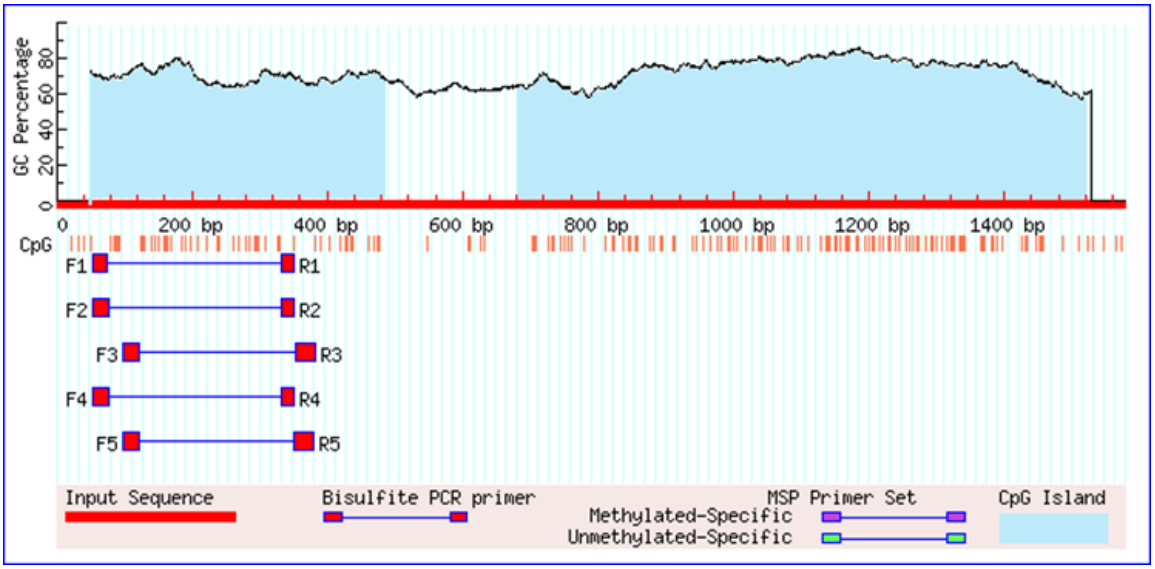
Length 895 (4202..5096

Figure 2. Identification of KLF-2 CpG islands and primer sequence target and lenght for MSP

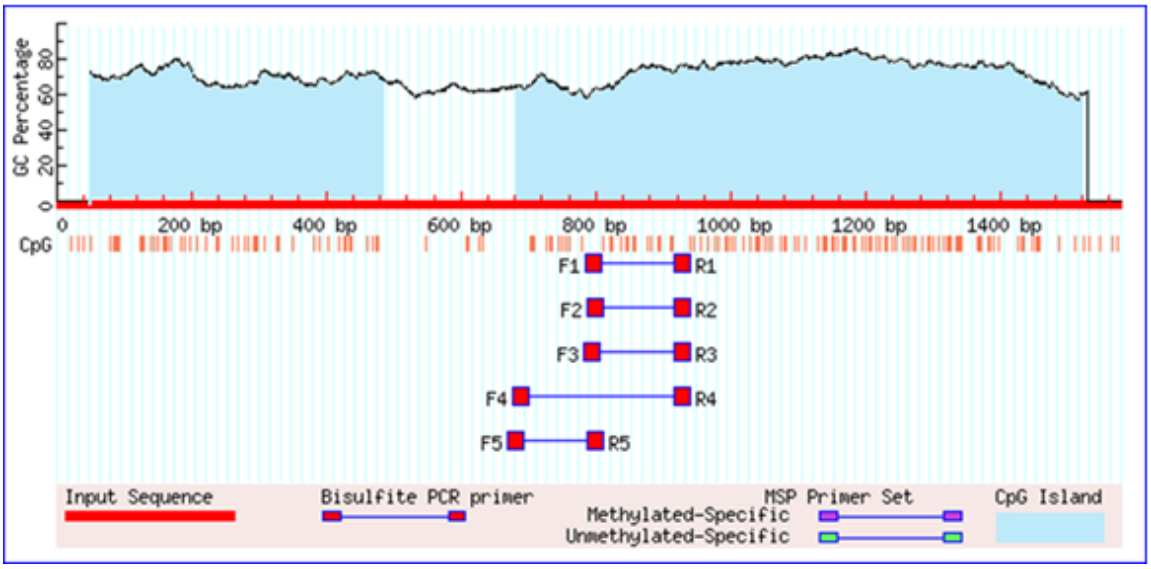
a) CpG Island 1



b) CpG Island 2



c)


GC Percentage
0
20
40
60
80
0
200 bp
400 bp
600 bp
800 bp
1000 bp
1200 bp
1400 bp
CpG
F1
R1
F2
R2
F3
R3
F4
R4
F5
R5
Input Sequence
Bisulfite PCR primer
MSP Primer Set
Methylated-Specific
Unmethylated-Specific
CpG Island

Acknowledgement

"Die Danksagung ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten".

Curriculum Vitae

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Erklärung

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat.-Fakultäten zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Molecular Mechanisms of Differential Activation of Naive T cells by Weak and Strong Antigen-presenting Cells“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Eloho Etemire befürworte.

Essen, den 05-08-2013 Prof. Dr. Matthias Gunzer

Erklärung

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat.-Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den 05-08-2013 Eloho Etemire

Erklärung

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat.-Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den 05-08-2013 Eloho Etemire