

THE IMPACT OF CD45 ON DENDRITIC CELL ACTIVATION  
AND TOLEROGENIC RESPONSE

by

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## Abstract

Dendritic cells (DCs) are potent antigen presenting cells that orchestrate the immune system to mediate either a pro- or anti-inflammatory response, by provision of critical instructive signals to T cells. The leukocyte-specific tyrosine phosphatase CD45 can influence the immune response by its ability to act as either a positive or negative regulator of DC pro-inflammatory cytokine production. This dissertation explores the effect of CD45 in mediating a tolerogenic response in DCs.

The lack of CD45 in bone marrow derived dendritic cells (BMDCs) was found to cause a preferential production of IL-10 in response to LPS stimulation, despite the mature DC phenotype defined by elevated expression levels of MHCII and co-stimulatory molecules CD80 and CD86, and the unaffected ability of CD45 deficient BMDCs to drive naïve CD4<sup>+</sup> T cells or CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cell (Treg) proliferation *in vitro*. An important *in vivo* finding was that CD45 deficiency in RAG<sup>-/-</sup> mice provided greater protection against wasting disease in the Treg mediated prevention of experimental colitis. This survival advantage was found to correlate with an increased proportion of Tregs at the colonic lamina propria in CD45 deficient RAG<sup>-/-</sup> mice.

Culture of BMDC precursors with the anti CD45RB antibody generated BMDCs with reduced LPS-induced IL-12 production and T cell stimulatory capacity implicating CD45RB expression in the promotion of tolerogenic responses in DCs. Rapamycin, a pharmacological inhibitor of the mTOR pathway, was found to have a minimal effect on inducing a tolerogenic DC from BMDCs generated by culture in GM-CSF alone. However rapamycin exhibited a more profound suppressive effect on the ability of CD45 deficient DCs to drive T cell proliferation *in vitro*.

Overall this study suggests that the CD45RB isoform may specifically inhibit an inflammatory response in DCs but the loss of all CD45 isoforms in DCs may potentially mediate T tolerance through immune deviation, while the loss of CD45 in multiple innate immune cells may culminate in an environment that promotes Treg expansion or function *in vivo*.

## **Preface**

Ethics approval for this research was issued by the Animal Care Committee of The University of British Columbia Office of Research Services under the animal care certificate A11-0292.

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## List of Abbreviations

Symbol	Definition
23G2	MB23G2
7AAD	7-amino-actinomycin
Ab	antibody, antibodies
APC	antigen presenting cell
<i>APC</i>	allophycocyanin
ATCC	American Type Culture Collection
BM	bone marrow
BMDC	bone marrow derived dendritic cells
cDC	conventional dendritic cell
CFSE	carboxyfluorescein succinimidyl ester
CTLA-4	cytotoxic T-Lymphocyte antigen 4
DC	dendritic cell
DTR	diphtheria toxin receptor
EDTA	ethylene-diamine-tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Flt3L	Fms-like tyrosine kinase 3 ligand
Foxp3	forkhead box P3
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GSK	glycogen synthase kinase
HBSS	Hanks balanced salt solution
hr	hour(s)
i.p.	intraperitoneal
ICOS	inducible costimulator

ICOSL	inducible costimulator ligand
iDC	immature dendritic cell
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
iTreg	inducible regulatory T cell
JAK	janus kinase
LN	lymph node(s)
LP	colonic lamina propia
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MHCII	major histocompatibility complex class II
min	minute, minutes
MLN	mesenteric lymph nodes
MLR	mixed lymphocyte reaction
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene
NEAA	non-essential amino acids
NF- $\kappa$ B	nuclear factor kappa beta
NK	natural killer
nTreg	natural regulatory T cell
OVA	ovalbumin
PB	pacific blue
PBS	phosphate-buffered saline
PD-1	programmed cell death
pDC	plasmacytoid DC
PDL	programmed death ligand
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase

PLN	peripheral lymph nodes
PTP	protein tyrosine phosphatase
RA	retinoic acid
RAG	recombination-activating gene
RBC	red blood cell
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error mean
SFK	Src family kinase
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TCS	tissue culture supernatant
TGF- $\beta$	transforming growth factor beta
TH	T-Helper
TLR	Toll-like receptor
TNF $\alpha$	tumour necrosis factor alpha
Tr1	T regulatory type-1 cell
Treg	regulatory T cell
wk	weeks(s)

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## CHAPTER ONE: INTRODUCTION

### 1.1 Dendritic Cells

#### *1.1.1 Dendritic cells and activation of the adaptive immune response*

Dendritic cells (DCs) play a pivotal role in the induction of adaptive immunity by the provision of crucial instructions for lymphocyte activation and function <sup>1,2</sup>. DCs act as sentinels and continuously survey the body for the threat of infection <sup>2-4</sup>. Found throughout the body in peripheral tissue or at mucosal sites, DCs at steady state possess an immature, non-immunogenic state and are referred to as immature DCs (iDC) <sup>2,5</sup>. Detection of infection or inflammatory mediators leads to DC activation whereupon iDCs undergo a maturation process and migrate to the lymph nodes (LN) <sup>6</sup>. Mature DCs are now highly functional T cell activators and upon arrival in the LN, present major histocompatibility complex (MHC) complexed antigen to patrolling naïve T cells <sup>2</sup>. Naïve T cells circulating through LN transiently bind to DCs and sample the antigens presented by the large number of MHC molecules, for their specific T cell receptor (TCR)-binding peptide <sup>2</sup>. Rare encounters of antigen recognition by strong binding of the TCR to the MHC: peptide complex on DCs is termed signal 1 of T cell activation <sup>7</sup>. In order to initiate antigen-specific clonal expansion of naïve T cells, the second signal of co-stimulation is required <sup>2</sup>. Upon full activation with the receipt of signal 1 and 2, naïve T cells re-enter the cell cycle and undergo an intense period of cell division to generate a large number of progeny <sup>2,3,8</sup>. Finally signal 3 of T cell activation, is responsible for the determining the specific effector function of activated T cells <sup>3</sup>. To optimize the immune response against the array of potential pathogens and infectious agents that invade the

body, specialized classes of immune responses are developed to best control infection by specific pathogens<sup>3</sup>. These specialized classes of immune defence are driven by the development of effector CD4<sup>+</sup> T cells which can be further subdivided into different T helper (TH) cell subsets<sup>2,3,9</sup>. DCs, along with tissue-derived factors, help to determine the T cell response through the release of polarizing cytokines that promote the development of specific TH cell subsets e.g. TH1 cells are equipped to combat intracellular infection<sup>2,3,9</sup>. Correct integration of the signals present in the environment allows DCs to determine the most appropriate response necessary for a successful host immune response<sup>3,9</sup>.

### ***1.1.2 Dendritic cell subsets***

The antigen presenting cells (APC), DCs are not a homogenous population but are comprised of different subtypes which express the generic DC-associated integrin CD11c<sup>3,10</sup>. The heterogeneous DC population can be divided into two major populations: (1) the non-lymphoid tissue migratory and lymphoid tissue-resident DCs and (2) the plasmacytoid DCs (pDCs) which serve as part of the anti-viral defence and are characterized by their ability to promote the anti-viral response through copious production of type 1 interferons (IFN)<sup>11</sup>. In this study, the term “conventional” DCs (cDCs) will refer to all non-pDCs, both those present in the lymphoid or non-lymphoid tissues.

DCs present in non-lymphoid tissues are generated in the bone marrow and migrate as precursor cells to: sites of potential entry of pathogens such as mucosal sites (lung, gut, skin), filtering sites (kidney and liver), and sterile tissues (e.g. heart)<sup>10</sup>. Non-lymphoid tissue DCs are responsible for sampling antigens in their tissue environment



and migrating via the afferent lymphatics to the T cell zones of LN to either induce tolerance or in the presence of inflammatory signals, initiate immunity <sup>6</sup>.

### ***1.1.3 Overview of immunological tolerance***

Recombination of the TCR allows the generation of a T cell repertoire able to recognize a vast array of antigens <sup>2</sup>. Inevitably, some of these receptors will recognize antigens within the body also known as ‘self antigens’. These lymphocytes pose a threat as their accidental activation will initiate a damaging inflammatory attack on the body<sup>2,5,12</sup>. To prevent autoimmune responses the immune system has evolved mechanisms of tolerance <sup>12</sup>. Immunological tolerance is often considered to occur by two processes, namely central tolerance and peripheral tolerance <sup>12</sup>.

Central tolerance operates mainly in the sites of lymphocyte development namely the thymus and bone marrow<sup>2,12</sup>. Most of the auto-reactive T cells and B cells are screened in either the thymus (T cells) or the bone marrow (B cells) for hyper-reactivity to self-antigens, and if found to be autoreactive, are deleted at an immature stage of their development by deprivation of survival signals<sup>5,8,12</sup>. Despite this screening, potentially harmful self-reactive lymphocytes can still stochastically avoid the mechanism of central tolerance and can be found in the peripheral tissues<sup>5</sup>. Peripheral tolerance acts as a safeguard to prevent activation of any self-reactive lymphocytes that escaped deletion in the thymus or bone marrow<sup>5</sup>. In addition, peripheral tolerance is essential to prevent inappropriate immune response at immune-privileged sites such as the brain as well as towards innocuous foreign antigens, such as dietary antigens and non-pathogenic commensal organisms dwelling on mucosal surfaces<sup>12</sup>.

Regulatory T cells (Treg) represent a subset of T cells with immunosuppressive properties whose actions are crucial to the prevention of autoimmunity and chronic inflammation<sup>13</sup>. Tregs may be divided into categories based on their origin, cell surface marker expression and cytokine-secretion profile. Similar to conventional T cells, Tregs may be generated in the thymus before release into the periphery, as CD4<sup>+</sup> T cells expressing the Treg specific marker forkhead box p3 (Foxp3) and the IL-2R  $\alpha$  subunit CD25, and are referred to as natural regulatory cells (nTregs)<sup>14–16</sup>. These nTregs display a distinctly different TCR repertoire to conventional T cells with a bias for TCRs of high affinity peptides and are thought to be especially crucial in the prevention of autoimmunity by suppressing the action of autoreactive T cells<sup>17,18</sup>. Extrathymic generation of Tregs may also occur in the peripheral tissues where CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs can be induced from CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> naive T cells and are termed inducible (iTregs) or adaptive Tregs<sup>14,15,19</sup>. The induction of iTregs is carried out by DCs and *in vitro* and *in vivo* studies have described a requirement for strong TCR signalling and suboptimal co-stimulation along with transforming growth factor-beta (TGF- $\beta$ ) which is needed for the induction of Foxp3<sup>20,21</sup>. It is thought that the differentiation of iTregs, with the TCR repertoire of conventional T cells is protective against inappropriate immune responses against harmless non-self antigens like dietary antigens or allergens, and the commensal microbiota of the intestines<sup>22,23</sup>. Additionally, peripherally-derived Foxp3<sup>-</sup> Tregs have been identified and are distinguished on the basis of their production of immunoregulatory cytokines. These are the interleukin (IL)-10 secreting T regulatory type-1 (Tr1) cells, the TGF- $\beta$  secreting TH3 cells, double negative Tregs and the recently discovered IL-35<sup>+</sup> Tregs<sup>24</sup>. Tregs actively engage in the maintenance of immune

homeostasis and peripheral immune tolerance. Immunosuppression is carried out by Tregs in an antigen-dependent manner implying the selective suppression or removal of potentially harmful T cells<sup>19</sup>. Tregs are able to exert their immunoregulatory function through multiple cell-contact dependent or independent mechanisms<sup>19,25,26</sup>. These immunosuppressive mechanisms include: the secretion of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  by Tregs to directly dampen the inflammatory response from innate immune cells and lymphocytes<sup>5,27</sup>, the cytolytic activity of Tregs involved in the direct killing of effector T cells and APCs by Tregs to limit the immune response<sup>28</sup> and modification of DC phenotype and cytokine profile<sup>19,26</sup>. Ligation of the co-inhibitory molecule cytotoxic T-Lymphocyte antigen 4 (CTLA-4) on Tregs with the B7 molecules (CD80/86) on DCs results in the downregulation of DC-expressed co-stimulatory molecules<sup>26</sup>, expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) on DCs<sup>19,29</sup> and induction of TGF- $\beta$ , IL-10 and IL-27 production by pDCs; cytokines important for driving Tr1 development<sup>24,29,30</sup>.

#### ***1.1.4 Dendritic cells and peripheral tolerance***

DCs are potent stimulators of the primary immune response against pathogens but it has become increasingly evident that DCs also play a crucial role in mediating peripheral tolerance as demonstrated by the development of fatal autoimmune disease upon the ablation of DCs<sup>5,10,31</sup>. Unlike their immunogenic counterparts, tolerogenic DCs mediate the anti-inflammatory immune response by several means including: immune deviation (i.e. skewing of T helper cells to phenotype), induction or stimulation of regulatory immune cells (i.e. induction of Treg) in immune regulation and in *bona fide* tolerance, (i.e. deletion or anergy)<sup>5,24,27,32</sup>.

Under steady-state (i.e. in the absence of any detectable infection or overt inflammation), the immature phenotype of DCs is characterized by expression of low surface levels of MHC class II (MHCII) and co-stimulatory molecules and weak ability to stimulate T cell activation<sup>2,3,5</sup>. Tissue resident DCs continuously migrate to the local draining lymph nodes where sub-optimal priming of T cells in the absence of strong co-stimulation by iDCs promotes *in vivo* tolerance to self or other innocuous environmental antigens by T cell deletion or anergy<sup>2,5</sup>. Additionally, iDCs are also capable of expanding Tregs<sup>5,32</sup>.

Initially, tolerogenicity of DCs was attributed solely to iDCs, while mature DCs were considered immunogenic however it is now clear that tolerogenic potential is not strictly dependent upon maturation phenotype<sup>5,33</sup>. For example, disruption of E-cadherin-mediated DC–DC interaction promotes phenotypical maturation of DCs including upregulation of MHCII, co-stimulatory molecules and chemokine receptors however, these DCs fail to secrete pro-inflammatory cytokines and instead secrete high levels of IL-10 that induce a tolerogenic response<sup>34</sup>. Additionally, mature DCs with high co-stimulatory molecule expression are able to effectively induce Treg expansion *in vitro* and *in vivo*<sup>35,36</sup>. The ability of DCs to shape the T cell response is dependent not only on the production of TH polarizing cytokines but also on interactions between DC co-stimulatory molecules and their T cell expressed ligands. While ligation of co-stimulatory molecules, such as the APC expressed B7 molecules to T cell bound CD28, is quintessential to the development of the T cell immune response, the expression of negative co-stimulatory or co-inhibitory, molecules conversely limits effector T cell activation or biases T development towards Treg differentiation<sup>33,37</sup>. Hence the balance

of co-stimulatory and co-inhibitory molecules on the surface of DCs, is a crucial determinant for the outcome of DC-T cell interaction<sup>33,37,38</sup>.

Inducible costimulator (ICOS) (also known as B7H) is a member of the CD28 family and like CTLA-4, is expressed by murine T-cells after T-cell activation<sup>39-41</sup>. Its ligand, inducible costimulator ligand (ICOSL) (also known as B7H), has been identified to be a homolog of CD80 and CD86 molecules and is expressed on APCs such as DCs, macrophages and B cells<sup>39</sup>. However, ICOSL expression is inducible in some non-hematopoietic cells like endothelial cells, under inflammatory conditions<sup>42</sup>. ICOS plays a key role in stimulating T-cell activation and proliferation *in vitro*, and reports indicate that ICOSL-ICOS interactions fine-tune effector T cells function and is required for the development of IL-10 producing Tr1 cells<sup>39,43,44</sup>. More recently identified B7 family members include programmed death ligand (PDL-1) (also known as B7-H1 or CD274) and programmed death ligand 2 (PDL2) (also known as B7 DC or CD273)<sup>37</sup>. PDL-1 is ubiquitously expressed on hematopoietic cells<sup>45</sup> as well as on non-lymphoid tissues on endothelial cells and epithelial cells<sup>46</sup>. In contrast, expression of PDL-2 has so far shown to be more restricted being expressed on DCs, macrophages and B cells in an inducible manner<sup>45</sup>. The receptor of PDL-1 and PDL-2, programmed death-1 (PD-1) is as widely expressed and found on DCs, B cells, natural killer T cells, activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as on resting Tregs<sup>38,39,47</sup>. Interactions between the inhibitory receptor PD-1 and its ligands, PD-L1 and PD-L2, regulate both the induction and maintenance of peripheral T-cell tolerance<sup>37</sup>. Signalling through the PDL-1-PD1 axis has several different mechanisms to mediate tolerance including : the induction of T cell anergy by limiting the formation of the immunological synapse between APCs and T cells<sup>38</sup>, by

the inhibition of DC maturation through reverse signaling<sup>38,40</sup> and by enhancing the pool of Foxp3<sup>+</sup> Treg, although it is unclear whether the ligation of PD-1 solely converts naïve CD4<sup>+</sup> Foxp3<sup>-</sup> T cells to Foxp3<sup>+</sup> Treg, expands Foxp3<sup>+</sup> Treg, or selectively reduces Foxp3<sup>-</sup> T-cell populations<sup>48</sup>. A clear role for PDL-1 in iTreg generation has been shown by studies like that conducted by Wang *et al.*<sup>49</sup> that showed the blocking of PD-L1, but interestingly not of PD-L2, hindered the *in vitro* induction of Foxp3<sup>+</sup> iTregs by splenic iDCs<sup>49</sup>. Furthermore it has been shown that the induction of iTregs by tumours required DC-derived PD-L1<sup>49</sup>. Engagement of PDL-1 on DCs by soluble PD-1 has also been shown to trigger IL-10 production in DCs and likely via this mechanism enhance DC tolerogenic function by selectively enhancing the development of Tr1 cells<sup>40,50</sup>. The co-inhibitory molecule PDL-2, although not as extensively studied as PDL-1 has also been implicated as a negative regulator of the T priming<sup>37,51</sup>. Studies by Salama *et al.*<sup>52</sup> have found that blockade of PDL-2 but not PDL-1 exacerbated experimental autoimmune encephalomyelitis suggesting a role as an inhibitor of T cell activation<sup>52</sup>. Additionally, Zhang *et al.*<sup>51</sup> found that PDL-2 negatively regulated the T cell response as PDL-2<sup>-/-</sup> APCs exhibited more potent ability to activate T cells *in vitro* and showed that T cell tolerance to orally administered ovalbumin (OVA) antigen was abrogated by the lack of PDL-2<sup>51</sup>.

The best characterized co-stimulatory molecules are CD80/86 and are well-known for their key function in the provision of positive co-stimulation to T cells to initiate a primary immune response<sup>37</sup>. However, the interaction of CD80/86 on DCs with CD28 also plays a major role in tolerance by influencing the Treg expansion and homeostasis<sup>33</sup>. Not only does the thymic development of Foxp3<sup>+</sup> Treg depend on the interaction of

CD28 on Treg precursors with CD80/86<sup>53,54</sup> but recent evidence by Bar-On *et al.*<sup>55</sup> supports the idea that a CD80/86 dependent mechanism is critical for the maintenance of peripheral Treg homeostasis as the specific ablation of CD80/86 on DCs resulted in a reduction in the frequency and number of Treg in the periphery but not thymus<sup>55</sup>. Additionally, there are reports that IL-10 producing pulmonary DCs express high levels of CD80 and CD86 and are able to induce IL-10<sup>+</sup> Tr1 cells from naive CD4<sup>+</sup> T cells implying a further role for CD80/86 in Treg induction<sup>44</sup>.

### ***1.1.5 Influence of environment on tolerogenic dendritic cells***

DCs can be classified into distinct subsets, based on their phenotype, micro-environmental localizations, and functions<sup>10,56</sup>. Certain DC subsets have been identified as being particularly efficient in the induction of T cell tolerance<sup>5</sup>. Steady state pDCs were thought to possess inherent tolerogenic ability as immature pDCs have been shown to favour Treg generation<sup>57</sup> and mediate oral<sup>58</sup> and allo-antigen tolerance<sup>59</sup>. These pDCs reside in non-lymphoid tissue such as in the gut, airways and liver play critical roles in tolerance at these sites to foreign antigens<sup>3,57</sup>. Additionally, a subset of immature and mature CD123<sup>+</sup> DCs in humans were found to express IDO and hamper T cell *in vitro* proliferation<sup>60,61</sup>. However, further work into the field of tolerogenic DCs has dispelled the notion that tolerogenic potential is restricted to particular subsets, as an imperfect concept, as it has come to light that the local environment plays a large role in directing the immunogenic or tolerogenic capacity of DCs<sup>10,27,33</sup>.

Mucosal interfaces such as the gut are especially geared to the default creation of a tolerogenic environment<sup>62</sup>. In the MLN and lamina propria (LP) DCs promote the induction of Foxp3<sup>+</sup> iTregs from a naïve T cell population<sup>63–65</sup>. This tolerogenic capacity

of intestinal DCs is due to their conditioning with epithelial derived factors such as TGF- $\beta$ , the active vitamin A metabolite-retinoic acid (RA), IL-10, vasoactive intestinal peptide and thymic stromal lymphopoietin which are richly found within the gut environment<sup>66-68</sup>. Intestinal epithelial cells (IEC) are crucial in the creation of the local cytokine milieu that cultivates the tolerogenic function of intestinal APCs and T cells<sup>27</sup>. IEC are not only a rich source of multiple tissue factors, but are also able to induce the expression of retinal dehydrogenases in DCs, thus enabling DCs to produce RA<sup>27</sup>. Bacterial components can also shape the DC response as shown by the suppression of IL-12 production and simultaneous elevation of IL-10 production in DCs exposed to filamentous hemagglutinin from *Bordetella pertussis*, allowing DC to drive the differentiation of Tregs<sup>69</sup>. Among the intestinal CD11c<sup>+</sup> DCs, the CD103<sup>+</sup> DC population has the most potent ability to induce Tregs in a mechanism dependent on RA and TGF- $\beta$ <sup>65,70,71</sup>. However, the tolerogenic ability of CD103<sup>+</sup> DCs appears to be plastic, as this DC subset loses their tolerogenic properties under inflammatory conditions to acquire a more inflammatory function as indicated by the impaired ability to induce Foxp3<sup>+</sup> Tregs by CD103<sup>+</sup> DCs taken from colitic mice<sup>72</sup>. The influence of locale can also be seen in the observation that murine pDCs from the mesenteric lymph nodes (MLN) but not the spleen, are efficient in stimulating Treg response even after TH1 polarizing stimulus<sup>3,57</sup>.

The therapeutic potential of tolerogenic DC to treat autoimmune or hyper-inflammatory conditions, has driven the search for methods to control DC tolerogenicity. Knowledge of the influence of environmental signals on DC function has lead to the use of various anti-inflammatory and immunosuppressant agents to manipulate DCs and enhance their tolerogenic potential. These agents may use biologics such as, IL-10, TGF-



$\beta$ , vitamin A, vitamin D3, prostaglandin E2, among others, to emulate physiological conditions in the body that induce tolerogenic DCs <sup>27</sup>. Additionally, immunosuppressive pharmacological agents such as glucocorticoids, tacrolimus and rapamycin have been used to modify DC immunogenicity, often through inhibition of DC maturation <sup>32,73</sup>.

## **1.2 Protein Tyrosine Phosphatase CD45**

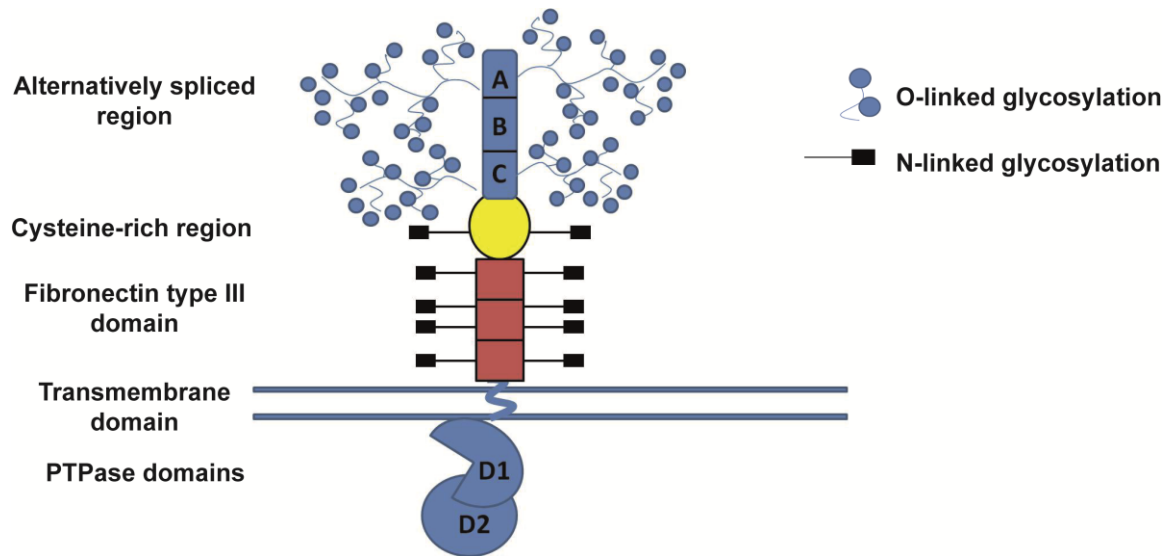
### ***1.2.1 The structure of protein tyrosine phosphatase CD45***

The leukocyte common antigen, CD45, is a protein tyrosine phosphatase (PTP) abundantly expressed on the surface of all nucleated hematopoietic cells <sup>74,75</sup>. CD45 is a type 1 transmembrane glycoprotein comprised of: a large extracellular domain, a single transmembrane domain, and a cytoplasmic portion containing the PTP domains of which only one domain is catalytically active <sup>74,76</sup>. CD45 may be expressed as multiple isoforms due to the alternative splicing of exons 4, 5 and 6 that encode the three extracellular regions (designated A, B and C respectively) residing at the most N-terminal domain <sup>74</sup>. These alternatively spliced domains possess multiple sites for O-linked glycosylation and the different levels of glycosylation at this site results in a drastic range in molecular weight and charge between the isoforms <sup>74-76</sup>. The highest molecular weight isoform contains exons 4/A, 5/B and 6/C (CD45ABC) (Figure 1.1) while the low CD45RO does not contain any exons <sup>74</sup>. Following the variable N-terminus, are the N-linked glycosylated cysteine-rich globular domain and the three fibronectin type III domains <sup>74</sup>. Despite the fact that cells can express the same CD45 isoform, the glycosylation pattern is different depending on the hematopoietic cell lineage <sup>74</sup>. CD45 isoform expression is highly regulated and varies depending on cell lineage, development and activation status

but the contribution of these individual isoforms to CD45 function still remains poorly defined <sup>74</sup>.

### ***1.2.2 The function of CD45 in dendritic cells***

The function of CD45 in the cells of the myeloid lineage is, in comparison to investigation in lymphocytes, an under-researched arena. Recent studies focusing on the function of CD45 in DCs, have shown dysregulated cytokine production in DCs and demonstrated a role for CD45 as a regulator of Toll-like receptor (TLR) signalling <sup>74,75</sup>. Piercy *et al.* <sup>77</sup> added to these findings by demonstrating that CD45<sup>-/-</sup> splenic and bone-marrow derived dendritic cells (BMDCs) produce elevated levels of IL-6 and tumour necrosis factor-alpha (TNFα) in response to TLR 3 and TLR 9 stimulation and increased nuclear factor kappa beta (NF-κB) activity <sup>77</sup>. The investigation on CD45 function in pDCs carried out by Montoya *et al.* <sup>78</sup> showed that expression of CD45-in particular the isoforms CD45RABC and CD45RO, was needed for to produce type 1 IFN in response to lymphocytic choriomeningitis virus infection <sup>78</sup>. Cross *et al.* <sup>79</sup> showed that in BMDCs, CD45 acted as either a negative or positive regulator of TLR-induced cytokine response in a myeloid differentiation primary response gene (88) (MyD88) dependent manner <sup>79</sup>. The pro-inflammatory response induced by MyD88- dependent TLR 2 and TLR9 were negatively regulated by CD45 while IFN-β production induced by TLR 3 or 4 stimulation was positively regulated <sup>79</sup>. The impact of dysregulated cytokine production was reflected in the reduced ability of CD45<sup>-/-</sup> BMDCs to polarize natural killer (NK) and T cells to a TH1 phenotype in response to stimulation with TLR3 and TLR4 <sup>79</sup>.



**Figure 1.1. Structure of CD45.** CD45 is expressed in multiple isoforms as a result of alternative splicing of variable CD45 exons. *D1*, domain 1 (catalytically active); *D2*, domain 2 (catalytically inactive).

### 1.2.3 CD45 and immunological tolerance

As CD45 isoforms only differ in the composition and glycosylation patterns of the extracellular region, it has been proposed that the purpose behind multiple isoforms is the differential binding of yet unknown extracellular ligands<sup>74,75</sup>. However, a role for the CD45RB isoform in mediating tolerance is implicated from findings of strong expression of CD45RB on a CD11c<sup>lo</sup> tolerogenic DC subset characterized by an immature plasmacytoid morphology and the secretion of high levels of IL-10 upon stimulation which allows for enhanced differentiation of Tr1 cells<sup>80,81</sup>. Further evidence suggesting the involvement of CD45RB in mediating tolerance in DCs is the use of the anti-CD45RB monoclonal antibody (mAb) as a novel immunotherapy drug to prevent allograft rejection in the field of transplantation<sup>82-84</sup>. A short course of anti-CD45RB mAb lead to the prolonged acceptance of allografts in animal renal and cardiac allograft

models as well as a striking prolongation of renal allograft survival in a primate transplantation model <sup>83</sup>. Evidence from *in vivo* studies suggests that *in vivo* anti CD45RB mAb is likely acting on several immune cell types <sup>83</sup>. So far anti CD45RB mAb therapy has been shown to affect NK function <sup>85</sup>, B cell activation <sup>85</sup> T cell anergy and deletion <sup>82,86</sup>, CD3/TCR signalling <sup>82</sup>, chemokine <sup>87</sup>, and to alter the ratio of CD45RB<sup>hi</sup> naïve/effector T cells and CD45RB<sup>lo</sup> Treg <sup>88</sup>. Other studies have proposed that the antibody plays a role in inducing regulatory B cells <sup>89</sup>. More recently, it has been suggested that the anti CD45RB antibody acts to alter the immunogenic function of DCs <sup>50,90,91</sup>.

### 1.3 Thesis Objectives

Emerging studies have shown a distinct role for CD45 in the regulation of cytokine production in DCs in response to activation signals through TLRs. This implication that CD45 can regulate the integration of environmental signals and subsequent DC response raises the question of whether CD45 is also able to modulate the integration of extra-cellular tolerogenic signals and alter the tolerogenic response of DC.

The successful use of the anti CD45RB mAb to prolong allograft survival and function in transplantation studies, implicates that the CD45RB isoform in particular regulates tolerance *in vivo*. However, it is unclear all the mechanisms by which this antibody- induced tolerance is mediated. Considering the potential role of CD45 in the regulation of DC response and the strong expression of CD45RB isoform subset of Tr1- promoting tolerogenic DCs, we hypothesize that the anti CD45RB antibody may also in part function to prolong allograft survival by the induction of a tolerogenic DCs.

My primary research question hence seeks to investigate how CD45 impacts the development of a tolerogenic phenotype, and function in DCs in response to immunogenic signal (TLR agonist), a tolerogenic signal (rapamycin) and to determine if the anti CD45RB mAb may mediate immunological tolerance by the induction of tolerogenic DCs.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Mice

Congenic CD45.2 C57BL/6J mice (hereafter referred to as CD45<sup>+/+</sup>), C57BL/6 recombinaase-activating gene (*rag*)-1 deficient mice (RAG), and CD45<sup>-/-</sup> exon 9 targeted mice<sup>92</sup> and CD45<sup>-/-</sup> exon 6 targeted mice (hereafter referred to as CD45<sup>-/-</sup>), TCR-transgenic mice specific for OVA peptide (323-339) (OVA<sub>323-339</sub>) (OT-II) and Balb/c mice were purchased from Jackson Laboratory (Bar Harbour, ME) and all mice, excluding Balb/c, were bred in-house. CD45<sup>-/-</sup> exon 9 targeted mice were backcrossed for a total of nine generations onto the CD45<sup>+/+</sup> background before homozygous matings were established. CD45<sup>-/-</sup> exon 6 targeted mice were backcrossed for a total of six generations onto the CD45<sup>+/+</sup> background before homozygous matings were established. Experiments used CD45<sup>-/-</sup> cells obtained from exon 9 targeted CD45<sup>-/-</sup> mice unless otherwise specified. RAG<sup>-/-</sup> mice and CD45<sup>-/-</sup> mice were backcrossed to create a homozygous CD45<sup>-/-</sup>RAG<sup>-/-</sup> double KO (45-RAG). Foxp3-green fluorescent proteins (GFP) - diphtheria toxin receptor (DTR) mice (on the C57BL/6J background) were generously provided by Dr. Hung-Sia (University of British Columbia (U.B.C.), Vancouver, BC). Experimental mice were sex and age matched and used between 6 to 12 weeks (wk) of age. Balb/c mice were used from 8-20 weeks of age. Mice were housed and bred under specific pathogen-free conditions at the University of British Columbia Wesbrook Animal Unit (Vancouver, BC, Canada) or at the Center for Disease Modelling (Vancouver, Canada, BC). Animal experimentation was conducted in accordance with

approved protocols of the University of British Columbia (UBC) Animal Care Committee and Canadian Council of Animal Care.

### ***2.1.2 Antibodies***

The following anti-mouse antibodies (Ab) were used in T cell purification and were all obtained from the U.B.C. Antibody Facility (Vancouver, BC) and included: biotinylated CD45RABC (RA3-6B2), biotinylated Ter119 (TER119), biotinylated Mac-1 (M1/70), biotinylated CD8 (53.67). Anti-biotin microbeads were purchased from Miltenyi Biotec (Auburn, CA). The following reagents and anti-mouse Ab were used in flow cytometry: streptavidin, rat IgG2b K isotype, TCR $\beta$  (H57-597), CD4 (GK1.5), CD25 (PC61.5), Gr-1 (RB6-8C5), CD11b (M1/70), CD45RB (16A), CD45RABC (B220), MHCII (I-A/I-E) (M5/114.15.2). CD11c (N418), PDCA-1 (129c), CD80 (16-10A1), CD86 (GL1), ICOSL (HK5.3) and CD273 (B7-DC) (122) and Foxp3 (FJK-16s) were conjugated to either fluorescein isothiocyanate (FITC) , allophycocyanin (APC), Pacific Blue (PB) phycoerythrin (PE), Alexa Fluor 647, PE-Cy7, PE-Cy5, or APC-Cy7 and were purchased from either BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA) or U.B.C. Antibody Facility (Vancouver, BC). The anti-mouse biotinylated Ab CD40 (HM40-3) and CD274 (B7-H1) (1-111A) were purchased from eBioscience (San Diego, CA).

FITC conjugated anti-Rat IgG (H+L) (mouse absorbed) polyclonal goat F (ab') was purchased from Cedarlane Laboratories (Burlington, NC). Hybridoma supernatant containing rat IgG2b against mouse CD45 (I3/2) and hybridoma supernatant containing rat IgG2b against mouse CD45RB (MB23G2; American Type Culture Collection (ATCC), Manassas, VA) were used to label pan CD45 (I3/2) and CD45RB respectively

(100 uL/ sample). Alexa Fluor 647-conjugated anti -CD45 (I3/2) (anti -CD45 (I3/2) Ab purified from tissue culture supernatant (TCS)) or unconjugated anti-CD45RB mAb (purified from MB23G2 TCS) were also used to label for pan CD45 and CD45RB respectively. The cell viability staining solution used in this study was 7-amino-actinomycin (7AAD).

## **2.2 Methods**

### **2.2.1 Flow cytometry.**

To detect cell surface molecules, cell samples ( $2 \times 10^5$  -  $1 \times 10^6$  cells/ tube) were first incubated in anti-CD16/32 (2.4G2; ATCC, Manassas, VA) TCS to block DC Fc receptors and subsequently labelled for 20 min with fluorochrome-conjugated Abs diluted in flow cytometry (FACS) buffer (1X phosphate buffered saline (PBS) solution, 4% fetal bovine solution (FBS), 2mM ethylene-diamine-tetra-acetic acid (EDTA). Cells were incubated for 5 min in a 1µg/mL 7AAD solution to exclude non-viable cells. Cells that were also fixed and permeabilized for Ab labeling of intracellular antigens were not incubated in 7AAD. After labelling with Ab and/or dye, cells were washed in 0.5-1 mL of FACS buffer where appropriate. For compensation settings, unlabeled and singly labelled cell samples for each fluorochrome or dye was used. To determine negative labelling, a fluorochrome-appropriate isotype control antibody or fluorescence-minus-one control (i.e. cells labelled with all fluorochrome-conjugated Ab except for the cell marker of interest) was used.

Samples to be analyzed for Foxp3 expression were permeabilized and fixed prior to labelling with anti-Foxp3 (FJK-16s) Ab, using the Foxp3 staining set from eBioscience (San Diego, CA). The fixation/ permeabilization process was carried out according to a



modified version of the manufacturer's protocol to label (nuclear) intracellular antigens; samples were incubated for at least 30 min with 0.5 mL of Foxp3 Fixation/Permeabilization working solution (1:4 ratio of Fixation/Permeabilization Concentrate: Fixation/Permeabilization Diluent) and washed when appropriate in 1 mL of 1X Permeabilization Buffer. Samples were incubated with APC- or Alexa Fluor 647-conjugated Foxp3 (FJK-16s) Ab for a minimum of 30 min.

To examine CD45 isoforms, cell samples incubated with I3/2 TCS, 23G2 TCS or unconjugated 23G2 mAb (10ng/mL) were not pre-incubated with 2.4G2 TCS. After 30-45 min incubation with I3/2 TCS, 23G2 TCS or unconjugated 23G2 mAb, cells were incubated for 20-30 min in FITC conjugated anti-Rat IgG (H+L) (mouse absorbed) polyclonal goat F (ab') and then labelled with fluorochrome-conjugated Abs against additional cell surface markers. Cells were washed twice in 0.5 mL FACS buffer, in between each of the described Ab labelling steps.

All incubation steps for the outlined flow cytometry preparation and Ab labelling of cells was performed on ice or at 4 °C. Data on labeled cells were acquired on the FACSLSR II (Beckton Dickinson) and analyzed using FlowJo (Tree Star Inc. ver.6.3.3 for MAC). Dead and autofluorescent cells were excluded from analysis where possible.

### **2.2.2 Culture of BMDCs**

BMDCs were propagated with granulocyte macrophage colony-stimulating factor (GM-CSF) according to Lutz *et al.*<sup>93</sup> (hereafter referred to as GM-CSF BMDCs). In brief, bone marrow (BM) cells were isolated from the femurs and tibias of mice and plated in non-tissue culture treated petri dishes at  $2 \times 10^5$  cells/mL in BMDC medium (RPMI-1640 (Invitrogen) supplemented with 10 % heat-inactivated FBS (Invitrogen), 2 mM L-

glutamine, 1X non-essential amino acids (NEAA) (Invitrogen) , 20 mM HEPES(Invitrogen), sodium pyruvate, penicillin/streptomycin, and 50  $\mu$ M 2-mercaptoethanol (Invitrogen) supplemented with 4% (v/v) of GM-CSF containing J558L supernatant<sup>94</sup>. GM-CSF supplemented BM cultures were cultured at 37 °C in 5% CO<sub>2</sub>. On day 3 and day 6, cells from 75% of culture were pelleted and re-suspended in an equal volume on fresh BMDC media and returned to original BMDC culture in petri dish. Fresh GM-CSF 4% (v/v) was added to the petri dishes. Non-adherent cells were harvested from the day 7 or 8 cultures and purified by positive selection for CD11c using MACS anti-CD11c immunomagnetic beads (Miltenyi Biotec) to > 95% purity. To assess purity of CD11c<sup>+</sup> cells in the culture, total BMDCs were harvested, counted by hemocytometry, and analyzed for CD11c expression by flow cytometry. For analysis of co-stimulatory and co-inhibitory molecule expression upon TLR 4 stimulation, day 7 or 8 BMDCs were CD11c-purified and transferred into petri dishes containing BMDC medium with or without 100 ng/mL Ultrapure LPS (*E. coli* 0111:B4) (Invitrogen). CD11c<sup>+</sup> BMDCs were analyzed by flow cytometry after 18-24 hr. For cytokine analysis, BMDC supernatants were obtained from the incubation of 1x10<sup>6</sup> cells/mL of CD11c-purified BMDCs in BMDC medium with or without 100 ng/mL of Ultrapure LPS (*E. coli* 0111:B4) (Invitrogen) for 18- to 24-hr. Supernatant was stored at -80°C for subsequent cytokine analysis via enzyme-linked immunoabsorbent assay (ELISA).

BMDCs were propagated with Fms-like tyrosine kinase 3 ligand (Flt3L) according to Brassel *et al.*<sup>95</sup>(hereafter referred to as Flt3L BMDCs). BM cells were isolated from the femurs and tibias of mice and plated in non-tissue culture treated petri dishes at 2 x10<sup>6</sup> cells/mL in BMDC medium (RPMI 1640 supplemented with 10% heat-

inactivated FBS, 2 mM L-glutamine, 1X NEAA, 20 mM HEPES, sodium pyruvate, penicillin/streptomycin, and 50  $\mu$ M 2-mercaptoethanol (all reagents for BMDC media obtained from Invitrogen)) supplemented with 20% (v/v) of Flt3L-containing TCS (supernatant obtained from CHO hybridoma transfected with murine Flt3L plasmid pEF-BOS/solmflk2-lig). Flt3L supplemented BM cultures were cultured at 37  $^{\circ}$ C in 5% CO<sub>2</sub>. On day 3, an equal volume of medium with fresh Flt3L was added to the plates. On day 6, half of the culture was removed, spun down, and replaced with fresh medium and Flt3L. Total non-adherent cells from BM culture were harvested for analysis on day 9 of culture. Cells from culture were harvested, counted by hemocytometry, and DCs in the BM culture identified based on CD11c expression.

### ***2.2.3 Rapamycin treatment of GM-CSF BMDCs***

CD11c-purified day 7 or 8 GM-CSF BMDCs were re-plated in BMDC medium and incubated at 37  $^{\circ}$ C either in the absence or presence of 10 ng/mL rapamycin purchased from Sigma-Aldrich (Oakville, ON). Rapamycin-treated and untreated control BMDCs were activated with LPS by the addition of Ultrapure LPS (*E. coli* 0111:B4) (Invitrogen) to BMDCs to a final concentration of 100 ng/mL, after 24 hr-rapamycin treatment. Rapamycin-treated BMDCs used as T cell stimulator cells were washed in BMDC medium prior to plating for use in T cell proliferation assay. Alternatively, rapamycin (Sigma-Aldrich) was added on day 2 of BM culture. On day 3 and 6 of BM culture, once BM cells were re-suspended in fresh BMDC medium 10ng/mL of rapamycin (Sigma-Aldrich) was added to the BM culture.

#### ***2.2.4 Anti CD45RB (23G2) mAb treatment of GM-CSF BMDCs***

CD11c-purified GM-CSF BMDCs were transferred into petri dishes containing of BMDC medium and incubated in the absence or presence of 10  $\mu\text{g/mL}$  of anti CD45RB mAb (23G2) for a 24 hr time period. 23G2-treated BMDCs were washed in BMDC medium prior to plating for use in T cell proliferation assays. Alternatively, BMDC were propagated from CD45<sup>+/+</sup> BM cells and 10  $\mu\text{g/mL}$  of anti CD45RB mAb (23G2) added on day 0, 3, and 6 of BM culture. On day 7 or 8, the cells were washed thoroughly and CD11c<sup>+</sup> BMDCs were purified to > 95% using anti-CD11c immunomagnetic beads (Miltenyi Biotec).

#### ***2.2.5 Isolation of splenic DCs***

Harvested spleens from were macerated by passing tissue through a 70  $\mu\text{m}$  nylon cell strainer (BD Falcon) to generate a single-cell suspension. Erythrocytes were depleted by hypotonic lysis using red blood cell (RBC) lysis buffer (10 mM Tris, 0.83%  $\text{NH}_4\text{Cl}$ , pH 7.25) for 5 min. CD11c and MHCII expression was used to identify splenic DCs in the single-cell suspension of splenocytes.

#### ***2.2.6 ELISA***

IL-12p70, IL-10 and IL-2 cytokine secretion by BMDC was quantified in supernatants from CD11c-purified BMDC (containing  $1 \times 10^6$  cells/mL) using the ELISA Ready-SETGo! kit (eBioscience) according to manufacturer's protocol.

#### ***2.2.7 Isolation and purification of CD4<sup>+</sup> T cells for in vitro T cell assays***

CD4<sup>+</sup> T cell subsets were isolated from the spleens and peripheral lymph nodes (PLN) of mice; PLN including the inguinal, axillary, brachial, and cervical lymph nodes. Single cell suspensions prepared by the maceration of freshly isolated spleens were

depleted of erythrocytes by hypotonic lysis using RBC lysis buffer (10 mM Tris, 0.83%  $\text{NH}_4\text{Cl}$ , pH 7.25) for 5 min. To enrich for  $\text{CD4}^+$  T lymphocytes, single cell splenocyte suspensions were depleted of  $\text{B220}^+$ ,  $\text{Ter119}^+$   $\text{MAC-1}^+$ , and  $\text{CD8}^+$  cells by negative selection. Splenocyte suspensions were labelled with biotinylated anti-mouse monoclonal antibodies against B220, Ter119, MAC-1, CD8 and then cells were isolated using anti-biotin microbeads (Miltenyi Biotec) and LS separation columns (Miltenyi Biotec) to obtain  $\text{CD4}^+$  T cell enriched cells. For instances in which only naïve T cells were to be obtained, biotinylated anti-mouse mAb against was included in the biotinylated Ab cocktail to negatively deplete  $\text{CD25}^+$  Treg cells from the  $\text{CD4}^+$  T cell population. To obtain Tregs,  $\text{CD25}^+$  cells were sorted by flow cytometry from the resultant  $\text{CD4}^+$  cell suspension:  $\text{CD4}^+$  T cells were labelled with PeCy7–conjugated anti-CD4, PE-conjugated anti-CD25 and sorted on the FACS Aria or Influx (Becton Dickinson) into the  $\text{CD4}^+\text{CD25}^+$  and  $\text{CD4}^+\text{CD25}^-$  T cell subsets. The purity of cell populations was  $> 90\%$  upon re-analysis.

### ***2.2.8 Isolation and purification of Tregs***

$\text{CD4}^+$  T cell subsets were isolated from the mouse spleens (C57BL/6J, Foxp3-GFP-DTR or Balb/c dependent on experiment) mice. Single cell suspensions prepared by the maceration of freshly isolated spleens were depleted of erythrocytes by hypotonic lysis using RBC lysis buffer (10 mM Tris, 0.83%  $\text{NH}_4\text{Cl}$ , pH 7.25) for 5 min. To enrich for  $\text{CD4}^+$  T lymphocytes, single cell splenocyte suspensions were depleted of  $\text{B220}^+$ ,  $\text{Ter119}^+$   $\text{MAC-1}^+$ , and  $\text{CD8}^+$  cells by negative selection. Splenocyte suspensions were labelled with biotinylated anti-mouse monoclonal antibodies against B220, Ter119, MAC-1, and CD8 followed by anti-biotin beads (Miltenyi Biotec) and sorted on an

AutoMACS (Miltenyi Biotec). The resulting CD4-enriched cells were stained with PeCy7–conjugated anti-CD4, FITC-conjugated anti-CD45RB (16A) Ab and PE-conjugated anti-CD25 and sorted on the FACS Aria or Influx (Becton Dickinson) into the CD4<sup>+</sup> CD25<sup>-</sup>CD45RB<sup>hi</sup> or CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> T cell subsets. The purity of cells was > 95%. Alternatively, CD4-enriched splenocytes obtained from Foxp3-GFP-DTR mice were labelled with PeCy7–conjugated anti-CD4, PE-conjugated anti-CD45RB mAb and APCCy7-conjugated anti-CD25 and sorted on the FACS Aria or Influx (Becton Dickinson) into the CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> T cell subsets.

### ***2.2.9 In vitro T cell proliferation assays:***

For T cell proliferation assays, purified BMDCs ( $5 \times 10^3$  cells /well) were plated in sterile 96-well round bottom plates. BMDCs were activated with 100 ng/mL Ultrapure LPS (Invitrogen). Prior to addition of T cells, BMDCs were spun, the supernatant aspirated and BMDC re-suspended in fresh BMDC media to remove LPS and/or BMDC treatment agents (rapamycin, 23G2 mAb). Purified CD4<sup>+</sup> T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) from Vibrant CFDA SE Cell Tracer kit (Invitrogen) according to the manufacturer's instructions (CFSE added to purified T cell suspension to a final concentration of 5  $\mu$ M). T cell proliferation was measured by CFSE dilution and was analyzed by flow cytometry.

### ***2.2.10 OVA: OT-II T cell assay***

CD4<sup>+</sup> T cells from OT-II transgenic mice (H-2<sup>b</sup>), express transgenic TCR (Valpha5Vbeta8) specific for chicken OVA<sub>323-339</sub> presented in the context of H-2<sup>b</sup><sup>96,97</sup>. OT-II CD4<sup>+</sup> T cells ( $5 \times 10^4$  cells/well) were co-cultured in BMDC media with  $5 \times 10^3$  CD45<sup>+/+</sup> or CD45<sup>-/-</sup> BMDCs that had been pulsed for 18-24 hr with 1 ug/mL of OVA<sub>323-</sub>

339 peptide and 100 ng/mL Ultrapure LPS (Invitrogen) in 96-well round-bottom plates (Corning Life Sciences) for 72 hr.

### ***2.2.11 Anti-CD3 T cell proliferation assay***

Naïve CD45<sup>+/+</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells (5 x 10<sup>4</sup> cells/well) were co-cultured with syngeneic CD45<sup>+/+</sup> or CD45<sup>-/-</sup> BMDCs plated at 5x10<sup>3</sup> cells/well in 96-well round-bottom plates (Corning Life Sciences) for 72 hr. Anti CD3 mAb (2C11) was added to a final concentration of 1 µg/mL per well activate T cells.

### ***2.2.12 Mixed leukocyte reaction***

In an autologous mixed leukocyte reaction (MLR), purified CFSE-labelled allogeneic Balb/c splenic CD4<sup>+</sup> T cells (5x10<sup>4</sup> cells/well) were co-cultured with purified CD11c<sup>+</sup> BMDCs (5x10<sup>3</sup>/well) in a 96-hr MLR using 96-well, round-bottom plates (Corning Life Sciences). Triplicate wells were plated per biological replicate. Protocol is a modification of that used by Jiang *et al.* <sup>98</sup>.

### ***2.2.13 Adoptive T cell transfer experimental colitis***

*Transfer of naïve T cells (experiment 1):* Experimental animals were sex and age-matched (RAG: (mean ± S.D.) Mean age 6.4 ± 1.1 wk, 45-RAG: Mean age 7.9 ± 1.3 wk). RAG and 45-RAG mice received intraperitoneal (i.p.) injection with either 4x10<sup>5</sup> syngeneic CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> naïve T cells suspended in PBS or with PBS alone. T cells were isolated from the spleens of female CD45<sup>+/+</sup> mice.

*Co-Transfer of naïve T cells and Treg:* Animals were all age and sex matched (RAG: (mean ± S.D.) Mean age of 8.3 ± 1.2 wk, 45-RAG: Mean age 8.8 ± 0.83 wk). RAG and 45-RAG mice were injected i.p. with either 4x10<sup>5</sup> syngeneic CD4<sup>+</sup>CD25<sup>-</sup>

CD45RB<sup>hi</sup> naïve T cells suspended in PBS or with a PBS-suspended mixture of  $4 \times 10^5$  syngeneic CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>hi</sup> T cells and  $1 \times 10^5$  syngeneic CD4<sup>+</sup> CD25<sup>+</sup> CD45RB<sup>lo</sup> Treg. Injected naïve T cells and Tregs were isolated from the spleens of male Foxp3-GFP-DTR mice. Experimental mice of the adoptive T cell transfer experiments were observed and weighed on a weekly basis. Any mice showing clinical signs of severe colitis disease and 20% weight loss from the initiation of the experiment were sacrificed according to the approved protocols of the University of British Columbia (UBC) Animal Care Committee and Canadian Council of Animal Care.

#### ***2.2.14 Cell preparation and flow cytometry analysis.***

The spleen, MLN and colonic LP were isolated from experimental mice sacrificed at either humane endpoint or pre-determined experimental end-point. Single cell suspensions to be used for flow cytometry analysis were prepared as follows:

**Spleen and MLN cell isolation:** Single cell suspensions were prepared by maceration of spleen or MLNs through 70  $\mu$ m filter cell strainers (BD Falcon) in sterile wash buffer (Ca- and Mg-free Hanks Balanced Salt Solution (HBSS) (Invitrogen), 5% FBS (Invitrogen)). Single cell suspensions were depleted of erythrocytes by hypotonic lysis using RBC lysis buffer (10 mM Tris, 0.83% NH<sub>4</sub>Cl, pH 7.25) for 5 min. Total live cell counts were performed by hemocytometry. Cells were then labelled with fluorochrome-conjugated Ab for analysis by flow cytometry.

**Lamina propria cell isolation:** Colons were removed from mice and transferred to cold wash buffer solution (HBSS (Invitrogen), containing + 5 % FBS (Invitrogen)). Feces were flushed from the colon and the colon transferred to fresh wash buffer where it



was cut into 0.5–1-cm pieces and washed extensively 3-5 times for 10-15 min in wash buffer solution or until remaining fecal matter cleared from the tissue . Washed colon tissue was incubated in an epithelial strip buffer (Ca- and Mg-free HBSS solution (Invitrogen), 5 % FBS (Invitrogen) and 2 mM EDTA) for six 15 min incubations at room temperature-a total of 90 min incubation. Following removal of epithelial cells the colon tissue was washed 2-3 times in RPMI-1640 medium (Invitrogen) containing 5 % FBS (Invitrogen) to remove EDTA. The remaining tissue was finely minced and further digested with collagenase (15000 U/mL; Sigma Aldrich) for 30-40 min at 37 °C. Cells from the LP were isolated by passing the collagenase digested colon tissue through a 70 µm filter cell strainers (BD Falcon). Total live cell counts were performed by hemocytometry. Cells were then labelled with fluorochrome-conjugated Ab for analysis by flow cytometry.

#### ***2.2.15 Microscopic examination and histological scoring.***

Colons were removed from sacrificed experimental mice and a 1 cm section of the distal colon was removed and fixed in 10 % paraformaldehyde. Tissues were stored in 70 % ethanol before being embedded in paraffin wax. Paraffin embedded cross-sections (7µm) were cut and treated with an hematoxylin and eosin stain and mounted on slides (WaxIt Histology Services, Vancouver, BC) Microscopic sections were mounted onto a slide and graded semi-quantitatively based on grading parameters of: (1) inflammatory cell (lymphocyte) infiltration , (2) epithelial cell hyperplasia, (3) mucin depletion, (4) transmural inflammation, and (5) ulceration. A grade of 0 (no change), 1(intermediate) or 2 (severe) was assigned to each parameter for a section, and the cumulative grades for the five parameters was used to assign an overall grade of intestinal inflammation to each

cross-section. A grade of 0 was comparable to the normal healthy colon tissue, grade 2 representing the highest intensity of damage for the histological parameter and grade 1 was assigned if the intensity of the parameter was less than that of grade 2 but more than grade 0. Colon cross-sections were scored in a blinded fashion. Photomicrographs were taken on a (Nikon Eclipse TS100) photomicroscope.

#### ***2.2.16 Statistical analysis***

Results are expressed as either the average of experimental means  $\pm$  standard error mean (SEM) or as the mean of biological replicates  $\pm$  standard deviation (SD). Data was analyzed on GraphPad Prism (version 5) for statistical significance using an unpaired or paired two-tailed Student *t* test as indicated. Values of  $p \leq 0.05$  were considered statistically significant (\*,  $p < 0.05$ ; \*\*,  $p \leq 0.01$ ; and \*\*\*,  $p < 0.001$ ).

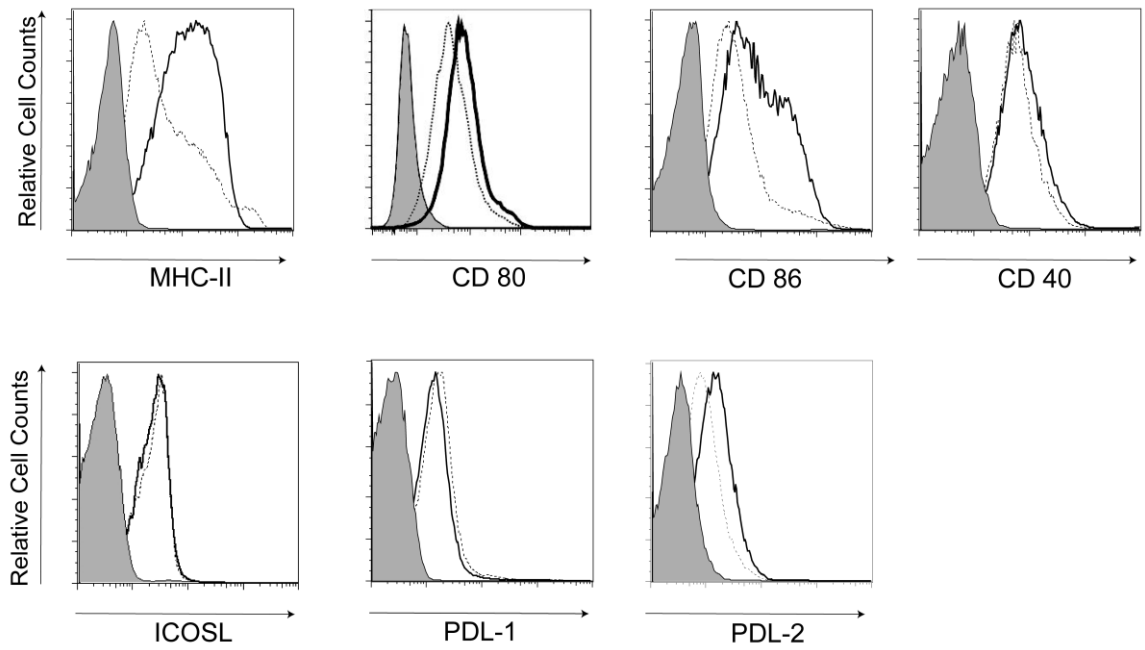
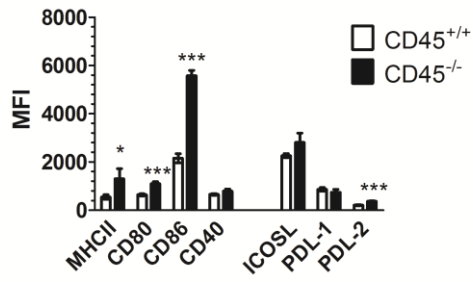
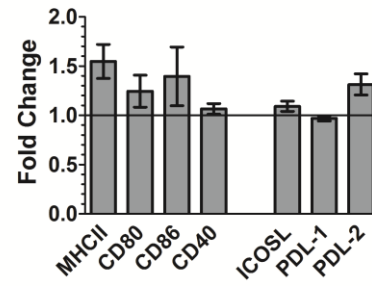
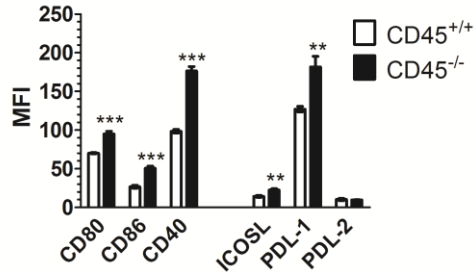
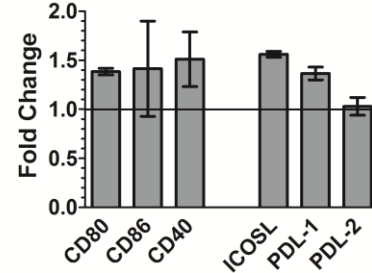
## CHAPTER THREE: THE IMPACT OF CD45 ON THE ACTIVATION STATUS OF DENDRITIC CELLS AND ON DISEASE OUTCOME

### 3.1 The Effect of CD45 Deficiency on the Activation Status of BMDCs

The activation status of DCs once thought to determine the tolerogenic capacity of BMDCs is now proving to also indicate the mechanism by which DCs may induce tolerance. Thus by examining the activation status of CD45-deficient GM-CSF BMDCs we will be able to determine if loss of CD45 is able to alter BMDC tolerogenic ability and gain insight into how the maturation status may play a role. This study will examine the effect of the loss of CD45 on the hallmark features of DC maturation in response to TLR ligation, namely by inspection of the upregulation of co-stimulatory and co-inhibitory molecules, production of pro- and anti-inflammatory cytokines and the capacity to stimulate proliferation in a naïve T cell, and Treg population.

#### ***3.1.1 CD45<sup>-/-</sup> BMDCs exhibit elevated expression levels of co-stimulatory and co-inhibitory molecules.***

Flow cytometry was used to determine the expression levels of co-stimulatory and co-inhibitory molecules and analysis found that day 7 CD45<sup>-/-</sup> BMDCs generally exhibited significantly increased levels of MHCII, CD80 and CD86 and equivalent levels of PDL-1 and ICOSL while there was a strong tendency for PDL-2 to be elevated in CD45 deficient BMDCs (Figure 3.1A-C). Unstimulated splenic CD11c<sup>hi</sup> MHCII<sup>hi</sup> cDCs isolated from CD45<sup>-/-</sup> mice also showed significantly elevated levels of the co-stimulatory molecules CD80, CD86 and CD40, as well as the co-inhibitory molecules ICOSL and PDL-1 (Figure 3.1D). Curiously, the fold increase in CD40, ICOSL and PDL-1 on CD45<sup>-/-</sup> splenic cDCs is greater than that observed on CD45<sup>-/-</sup> BMDCs (Figure 3.1E).

**A****B****C****D****E**

**Figure 3.1. Basal expression levels of co-stimulatory and co-inhibitory molecules on CD45<sup>+/+</sup> and CD45<sup>-/-</sup> GM-CSF BMDCs and splenic DCs.** Flow cytometric analysis was used to determine expression levels of MHCII, the co-stimulatory molecules (CD80,

CD86 and CD40) and co-inhibitory molecules (ICOSL, PDL-1 and PDL-2) on day 7 CD11c<sup>+</sup> CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs. **(A)** Data is presented as histograms of cell surface molecule expression. Expression levels are shown on log scale. Dotted line represents CD45<sup>+/+</sup> BMDCs, solid line represents CD45<sup>-/-</sup> BMDCs, filled histogram represents negative control. One representative biological replicate is shown in each histogram. **(B)** Flow cytometry data graphed with bars representing the average mean fluorescence intensity (MFI)  $\pm$  SD across three biological replicates. One representative experiment is shown of at least three independent experiments, each experiment using three mice per genotype. **(C)** Mean fold difference in basal levels of co-stimulatory and co-inhibitory molecule expression on CD45<sup>-/-</sup> BMDCs. Data is graphed as the average fold difference (MFI measured in CD45<sup>-/-</sup> BMDCs: MFI measured in CD45<sup>+/+</sup> BMDCs) of each shown cell surface molecule, across at least four independent experiments in which error bars represent the SEM and each experiment performed with 2-3 mice per genotype. **(D)** Basal expression levels of co-stimulatory and co-inhibitory molecules on CD45<sup>+/+</sup> and CD45<sup>-/-</sup> splenic DCs. DCs represented by the CD11c<sup>hi</sup> MHCII<sup>hi</sup> population. Graph depicts the mean MFI across three biological replicates in one experiment with error bars represent the SD. Shown is one representative of two independent experiments, with 3 mice used per genotype in each experiment. **(E)** Mean fold difference in basal levels of co-stimulatory and co-inhibitory molecule expression on CD45<sup>-/-</sup> splenic CD11c<sup>hi</sup> MHCII<sup>hi</sup> DCs. Data is graphed as the average ratio of (MFI measured in CD45<sup>-/-</sup> DCs: MFI measured in CD45<sup>+/+</sup> DCs) of each shown cell surface molecule, across two independent experiments. Error bars represent the SEM and each experiment was performed with 3 mice per genotype. Asterisks represent statistical significance as determined by unpaired Student's *t* test between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC MFI for each molecule.

The expression levels of co-stimulatory and co-inhibitory molecules on BMDCs in response to TLR 4 ligation were also measured. As illustrated in Figure 3.2, LPS stimulation of both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs resulted in upregulated levels of MHCII and co-stimulatory molecules CD80, CD86, CD40 as well as the co-inhibitory molecules ICOSL and PDL-1. Even with LPS stimulation, Figure 3.2 shows that expression levels of PDL-2, MHCII, CD80 and CD86 molecules remained higher in CD45<sup>-/-</sup> BMDCs than in their CD45<sup>+/+</sup> counterparts. However, the fold differences in expression levels of these molecules between the LPS-matured CD45<sup>-/-</sup> and CD45<sup>+/+</sup> BMDCs were less dramatic than those observed between unstimulated CD45<sup>-/-</sup> and CD45<sup>+/+</sup> BMDCs. It is interesting

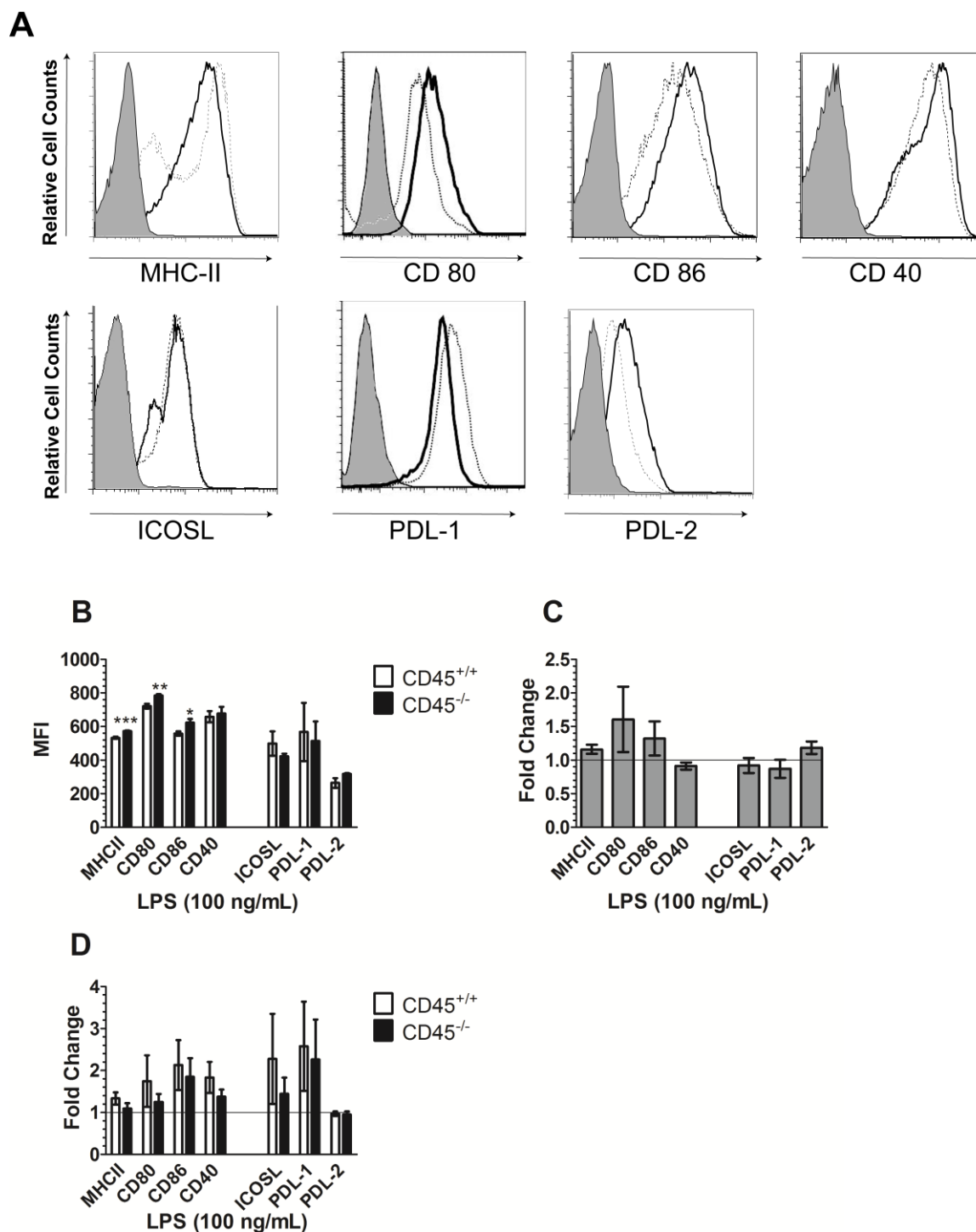
to also note that the fold increase of co-stimulatory and co-inhibitory molecules in response to LPS is greater in CD45<sup>+/+</sup> BMDCs than in their CD45 deficient counterparts.

### ***3.1.2 CD45 deficiency in BMDCs decreases IL-12 and enhances IL-10 LPS-induced cytokine production.***

Examination of BMDC cytokine response of CD45<sup>+/+</sup> BMDCs as illustrated in Figure 3.3 shows that 18-24 hr LPS stimulation (100 ng/mL) of day 8 CD11c<sup>+</sup> BMDCs induced production of both IL-12 and the prototypical anti-inflammatory cytokine IL-10. The production of IL-12 was greater than IL-10 in CD45<sup>+/+</sup> BMDCs but in contrast, CD45<sup>-/-</sup> BMDCs exhibited an opposite cytokine profile with greater production of IL-10 than IL-12. The IL-10 production by CD45<sup>-/-</sup> BMDCs (mean of 544 ± 70 pg/mL) was significantly enhanced compared to their CD45<sup>+/+</sup> counterparts (mean of 212 ± 57 pg/mL). On the other hand, there was a clear trend towards decreased IL-12 production from CD45<sup>-/-</sup> BMDCs (mean of 287 ± 108 pg/mL) in comparison to CD45<sup>+/+</sup> BMDCs (mean = 570 ± 104 pg/mL), although not statistically significant.

### ***3.1.3 CD45<sup>-/-</sup> BMDCs exhibit comparable ability to drive in vitro T cell proliferation in a naïve CD4<sup>+</sup> T cell population.***

In order to investigate the cumulative effect of the observed changes in CD45<sup>-/-</sup> BMDC co-stimulatory and co-inhibitory molecule expression and cytokine profile on lymphocyte activation, the effect of CD45 deficiency on the ability of BMDCs to drive T cell proliferation *in vitro* was measured.



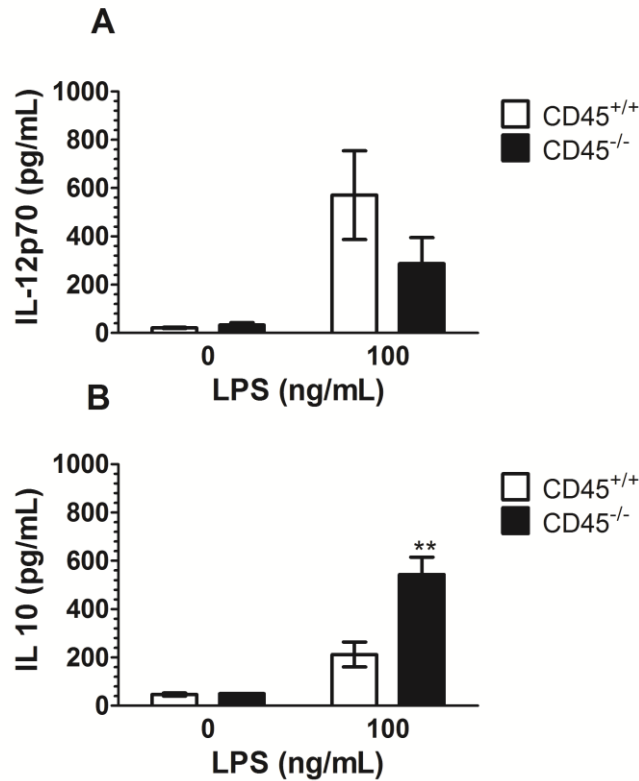
**Figure 3.2. LPS-induced expression levels of co-stimulatory and co-inhibitory molecules on CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs.** Expression levels of MHCII, co-stimulatory molecules (CD80, CD86, CD40) and co-inhibitory molecule (ICOSL, PDL-1 and PDL-2) expression levels on day 7 CD11c<sup>+</sup> CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs after 18-

24 hr stimulation with 100 ng/mL of LPS as determined by flow cytometry analysis. **(A)** Data presented as histograms of cell surface molecule expression. Expression levels are shown on log scale. Dotted line represents CD45<sup>+/+</sup> BMDCs, solid line represents CD45<sup>-/-</sup> BMDCs, and the filled histogram represents negative control. One representative biological replicate is shown in each histogram. **(B)** Flow cytometry data graphed with bars representing the average mean fluorescence intensity (MFI)  $\pm$  S.D. across three biological replicates. One representative experiment is shown of three independent experiments, each experiment using 3 mice per genotype. **(C)** Average fold difference in LPS-induced levels of co-stimulatory and co-inhibitory molecule expression of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs. Bars on graph for represent the average ratio of: (MFI measured in CD45<sup>-/-</sup> BMDCs) to (MFI measured in CD45<sup>+/+</sup> BMDCs) each cell surface molecule across three independent experiments in which error bars represent the SEM. **(D)** Mean fold difference in molecule expression (MFI) with 100 ng/mL LPS stimulation in CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs from basal levels. Average is across three independent experiments and error bars represent SEM. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC MFI for each molecule.

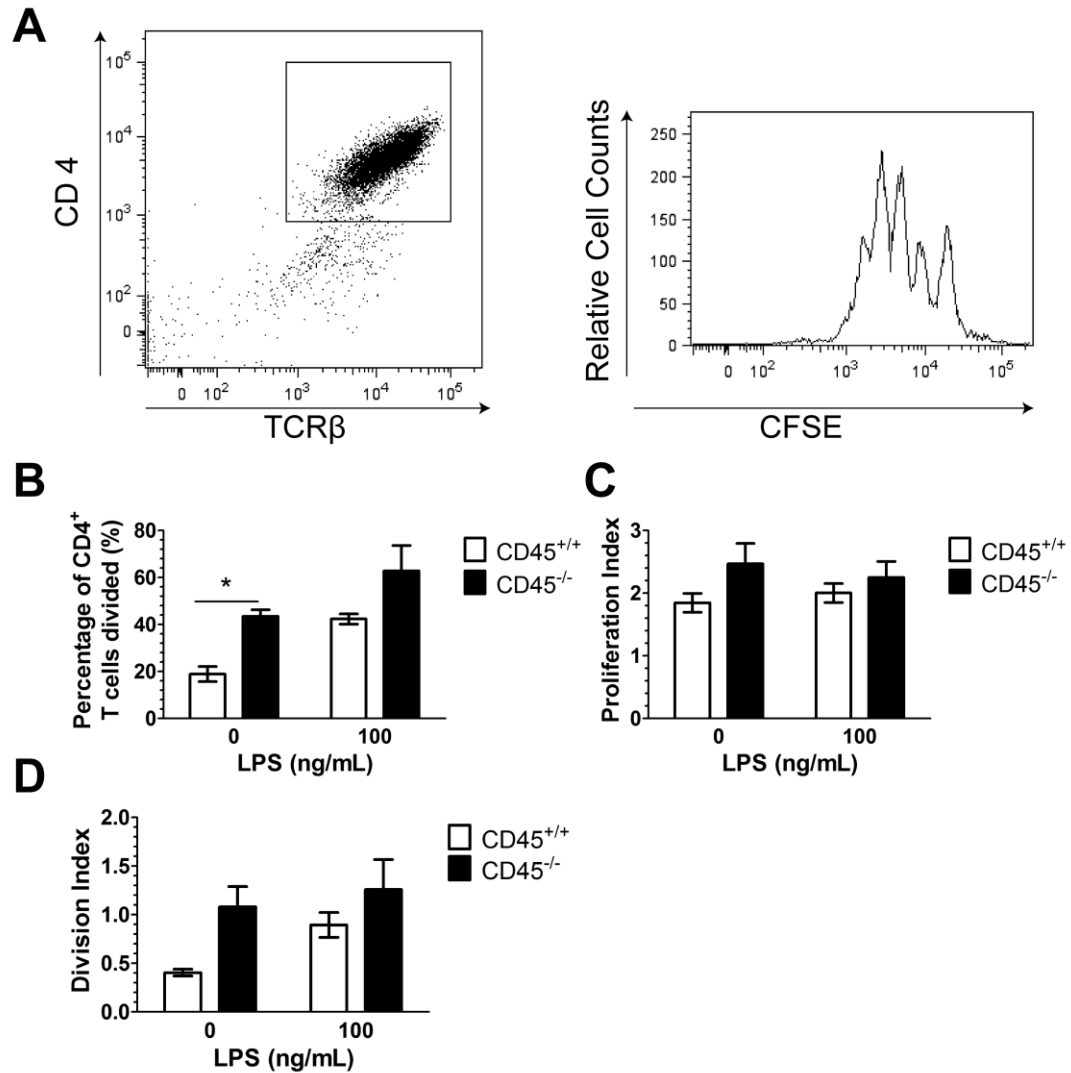
***Antigen-dependent (OT-II: OVA<sub>323-339</sub>) T cell proliferation:*** To assess the effect of CD45 deficiency in BMDCs on ability to drive DCs, CD4<sup>+</sup> T cells from OT-II transgenic mice (H-2<sup>b</sup>) were stimulated by OVA<sub>323-339</sub> pulsed BMDCs of *in vitro* co-cultures. In this study CFSE dilution of CD4<sup>+</sup> T cells was used to measure T cell proliferation. Figure 3.4A displays the gating scheme employed in flow cytometry analysis, by first gating on all TCR $\beta$ <sup>+</sup> CD4<sup>+</sup> cells, and then analyzing the subsequent proliferation of CFSE-labelled CD4<sup>+</sup> T cells using the software generated indices of proliferation of: proliferation index and division index. The percentage of T cells divided indicates the percentage of all the T cells which have undergone at least one division, the rate of T cell division is measured by the proliferation index and finally, the division index measures the amount of proliferation taking into account the undivided T cells. OT-II T cells were purified to obtain a naïve CD4<sup>+</sup> CD25<sup>-</sup> population to use in co-culture and Figure 3.4B demonstrates that in this model, there is a significantly increased ( $p = 0.029$ )



proportion of T cells which underwent cell division when stimulated by  $CD45^{-/-}$  BMDCs (mean of  $43.5 \pm 2.8\%$ ) compared to T cells stimulated by  $CD45^{+/+}$  BMDC counterparts (mean of  $18.9 \pm 3.2\%$ ).



**Figure 3.3. Cytokine production by  $CD45^{+/+}$  and  $CD45^{-/-}$  BMDCs in response to LPS stimulation.** Data graphed shows the mean concentration over four independent experiments of (A) IL-12 or (B) IL-10 produced by day 7 or 8 GM-CSF cultured  $CD11c^{+}$  BMDCs stimulated with 100 ng /mL of LPS for a 24 hr period. Cytokines were detected in the supernatant collected post 24 hr LPS stimulation from  $1 \times 10^6$  BMDCs. Individual experiments used 3 mice per genotype. Error bars represent SEM. Asterisks represent statistical significance as determined by paired Student's *t* test, between  $CD45^{+/+}$  and  $CD45^{-/-}$  BMDC cytokine production.



**Figure 3.4. Antigen-dependent T cell stimulatory ability of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> GM-CSF BMDCs.** Day 7 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> CD11c<sup>+</sup> BMDCs were pulsed with 1  $\mu$ g/mL of OVA<sub>323-339</sub> and activated with 100 ng/mL of LPS for 18-24 hr then plated with naïve (CD4<sup>+</sup>CD25<sup>-</sup>) OT-II T cells in a 72 hr co-culture. **(A)** Representative flow cytometry plots depicting the gating scheme used in flow cytometric analysis for the selection of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells within a BMDC: OT-II T cell co-cultures. Also shown is a representative histogram of the dilution of CFSE in TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells that have undergone division. **(B)** The percentage of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells divided **(C)** the rate of T cell division as measured by proliferation index and **(D)** the overall proliferation measured by the division index; the division index is the product of the percentage of T cells divided and the proliferation index. The bars of the graph represent the mean of the proliferation parameter  $\pm$  SEM. across two-three independent experiments, with 3 mice used in each individual experiment.

Although not statistically significant, there is a similar trend in the presence of LPS, for a greater proportion of T cells to divide under CD45<sup>-/-</sup> BMDC stimulation as shown by a greater mean of  $62.8 \pm 10.8$  % T cells divided compared to  $42.4 \pm 2.16$  % T cells divided in co-cultures undergoing stimulation by CD45<sup>+/+</sup> BMDCs. In Figure 3.4C the proliferation index values obtained in the absence of LPS stimulation, indicate a trend for proliferation of dividing cells to proceed at a faster rate in CD45<sup>-/-</sup> BMDC co-cultures (mean of  $2.5 \pm 0.3$ ) as compared to CD45<sup>+/+</sup> BMDC stimulated T cell co-cultures ( mean of  $2.0 \pm 0.2$ ) in the absence of LPS. However LPS stimulation led to a more similar rate of proliferation in both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC: T cell co-cultures as indicated by proliferation index of CD45<sup>+/+</sup> BMDC- (mean of  $2.0 \pm 0.15$ ) and CD45<sup>-/-</sup> BMDC- (mean of  $2.3 \pm 0.26$ ) stimulated CD4<sup>+</sup> T cells suggesting that the proliferation is occurring at a similar rate. Likewise, the division index, which takes into account the undivided cells, shows that a trend exists for higher T cell proliferation upon stimulation by CD45<sup>-/-</sup> BMDCs compared to CD45<sup>+/+</sup> BMDCs. Overall results suggest that CD45<sup>-/-</sup> BMDCs possess a greater capacity to stimulate T cell proliferation.

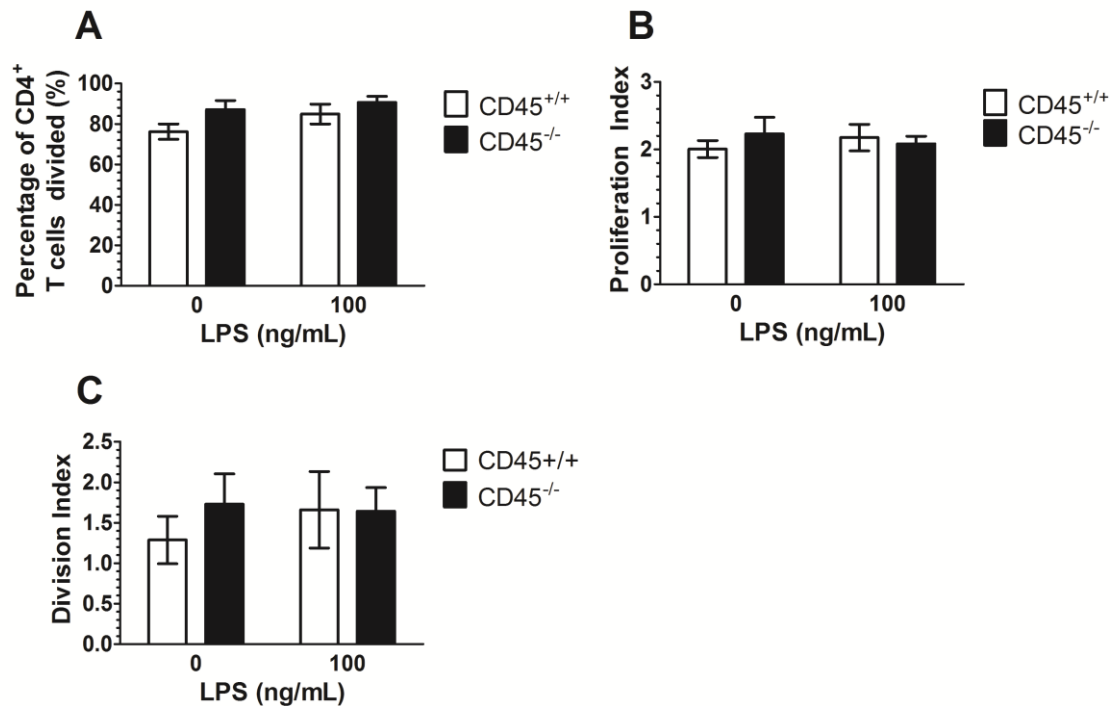
***Antigen independent (anti-CD3) T cell proliferation:*** An alternative assay, which functions independently of antigen, was used to measure T cell proliferation. This antigen-independent assay employed the anti-CD3 mAb to activate the TCR of T cells, while DCs supplied the necessary co-stimulation. In this assay, differences in DC antigen-processing or antigen presentation ability are overlooked as T cells are activated through the TCR by exogenous means with the anti CD3 mAb, and hence all T cells theoretically receive equal TCR stimulus. Figure 3.5 illustrates robust proliferation of CD4<sup>+</sup> T cells in the BMDC: T cell co-cultures, with both the CD45<sup>+/+</sup> and CD45<sup>-/-</sup>

BMDCs, interestingly even in the absence of exogenously added maturation stimulus.

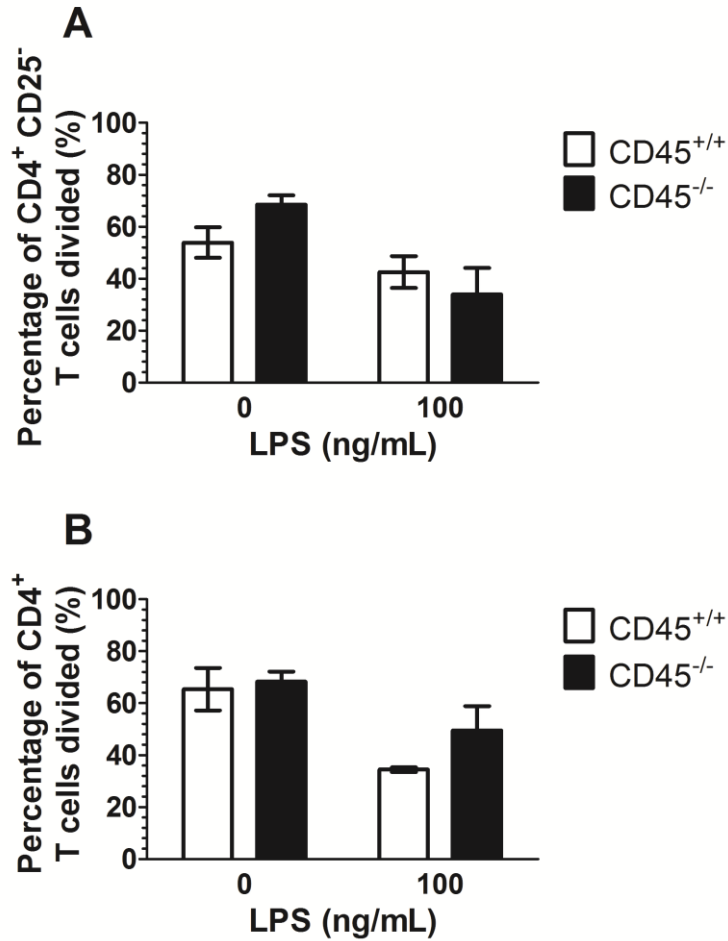
The process of DC purification and then the subsequent plating of BMDCs in the absence of GM-CSF may potentially cause some cell maturation<sup>99,100</sup>. The proportion of T cells induced to divide by unstimulated BMDCs showed a trend for the increased proliferation by CD45<sup>-/-</sup> BMDCs, with a mean proportion of T cells divided of  $76.2 \pm 3.8\%$  and  $87.1 \pm 4.5\%$ , for CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs respectively (Figure 3.5A). This increase was also seen in the proliferation index in which co-cultures containing unstimulated CD45<sup>+/+</sup> BMDCs (mean of  $2.0 \pm 0.1\%$ ) was reduced in comparison to CD45<sup>-/-</sup> BMDCs (mean of  $2.2 \pm 0.3$ ). The division index reflected the overall trend for increased T cell proliferation by CD45<sup>-/-</sup> BMDCs with a mean division index of  $1.7 \pm 0.4$  % induced by CD45<sup>-/-</sup> BMDCs compared to the value induced by CD45<sup>+/+</sup> BMDCs of  $1.3 \pm 0.3\%$ . However, LPS stimulation of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs did not reveal a significant trend towards greater induction of T cell proliferation by CD45<sup>-/-</sup> BMDCs.

***Mixed leukocyte reaction:*** In this study, an autologous MLR was established by using CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs (C57BL6J background, H-2<sup>b</sup> haplotype) as stimulators and measuring the proliferative response of allogeneic BALB/c (H-2<sup>d</sup> haplotype) CD4<sup>+</sup> T cells to evaluate the allostimulatory ability of BMDCs in the absence of CD45 expression. As the majority of T cells at the 96 hr time point had undergone several rounds of division, and clear peaks of varying CFSE intensity were not observed, T cell proliferation in the MLR assays was measured only by the percentage of T cells divided. In a co-culture with naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells (Figure 3.6A) CD45<sup>+/+</sup> BMDCs and CD45<sup>-/-</sup> BMDCs displayed no significant differences in T cell stimulatory ability regardless of stimulation with LPS although, the trend observed for the previous T cell

proliferation assays of unstimulated  $CD45^{-/-}$  BMDCs being able to drive a greater proportion of T cells into cell division remained ( $CD45^{+/+}$  BMDCs: mean of  $53.9 \pm 5.9$  %;  $CD45^{-/-}$  BMDCs: mean of  $68.5 \pm 13.6$  %). On the other hand, this trend was reversed in comparison of the percentage T cells divided with LPS stimulated  $CD45^{+/+}$  and  $CD45^{-/-}$  BMDCs ( $CD45^{+/+}$  BMDCs: mean of  $42.6 \pm 6.1$  %;  $CD45^{-/-}$  BMDCs: mean of  $34.0 \pm 10.1$  %).



**Figure 3.5. Antigen-independent T cell stimulatory ability of  $CD45^{+/+}$  and  $CD45^{-/-}$  GM-CSF cultured BMDCs.** Day 7  $CD45^{+/+}$  and  $CD45^{-/-}$   $CD11c^{+}$  BMDCs were activated with 100 ng/mL of LPS for 18-24 hr then plated with syngeneic (C57BL6J) naïve  $CD4^{+}CD25^{-}$  T cells in a 72 hr co-culture. Graph depicts (A) the percentage of T cells divided (B) the rate of T cell division as measured by the proliferation index and (C) the overall proliferation measured by the division index taking into account the percentage of T cells divided and the rate of T cell proliferation. Bars in graph are the mean of the proliferation parameter  $\pm$  S.E.M. over four independent experiments, with each experiment containing 3 mice.  $CD45^{-/-}$  cells were obtained from both exon 9 (two experiments) and exon 6 targeted  $CD45^{-/-}$  mice (two experiments).



**Figure 3.6.** The effect of CD45 deficiency on BMDC allostimulatory capacity. Day 7 or 8 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> CD11c<sup>+</sup> GM-CSF cultured BMDCs were either unstimulated or activated with 100 ng/mL of LPS for 18-24 hr then plated with (A) allogeneic (Balb/c) naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells (depleted of CD4<sup>+</sup>CD25<sup>+</sup> Tregs) or (B) with allogeneic (Balb/c) total CD4<sup>+</sup> T cells (containing Treg), in a 96 hr MLR. BMDC allostimulatory ability is quantified by the percentage of CD4<sup>+</sup> T cells divided and graphs depict mean percentage of T cells divided  $\pm$  SEM over three (0 ng/mL) and two (100 ng/mL LPS) independent experiments each containing 3 mice in each condition and for each mouse genotype. CD45<sup>-/-</sup> cells were obtained from both exon 9 and exon 6 targeted CD45<sup>-/-</sup> mice.

#### 3.1.4 CD45 deficiency in BMDCs does not affect BMDC ability to induce and expand *Foxp3*<sup>+</sup> Tregs

In order to evaluate whether CD45 deficiency in BMDCs may alter their interactions of with Tregs and subsequently affect BMDC T cell stimulatory ability, total

CD4<sup>+</sup> cells which were not depleted for CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs were used in a BMDC: T cell co-culture.

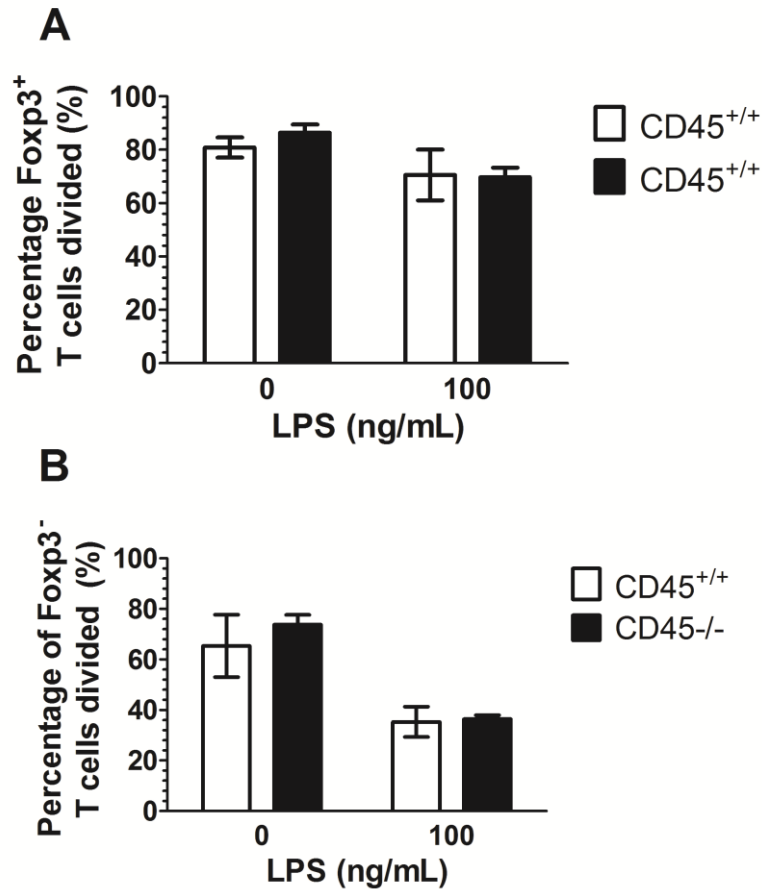
Figure 3.6B shows that when total CD4<sup>+</sup> T cells were used, similar T cell proliferation was observed to be induced by both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs in unstimulated conditions (CD45<sup>+/+</sup> BMDCs: mean of  $65.4 \pm 18.1$  %; CD45<sup>-/-</sup> BMDCs: mean of  $68.3 \pm 3.9$  %), but with LPS-stimulation, this time CD45<sup>-/-</sup> BMDCs displayed higher proficiency at stimulating T cell division than their CD45<sup>+/+</sup> counterparts (CD45<sup>+/+</sup> BMDCs: mean of  $34.5 \pm 0.9$  %; CD45<sup>-/-</sup> BMDCs: mean of  $49.5 \pm 9.3$  %). To further dissect proliferation within specific CD4<sup>+</sup> T cells populations, intracellular staining for the Treg specific transcription factor Foxp3, was employed. Results revealed that the Foxp3<sup>-</sup> T cells (non-Treg) and the Foxp3<sup>+</sup> (Treg) has undergone similar levels of T cell division between the CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC stimulated co-cultures (Figure 3.7) but with unstimulated CD45<sup>-/-</sup> BMDCs again stimulating slightly more T cell division in both the Foxp3<sup>-</sup> T cells (CD45<sup>+/+</sup> BMDCs: mean of  $65.4 \pm 12.3$  %; CD45<sup>-/-</sup> BMDCs: mean of  $73.8 \pm 3.9$  %) as well as Foxp3<sup>+</sup> T cells (CD45<sup>+/+</sup> BMDCs: mean of  $80.9 \pm 3.8$  %; CD45<sup>-/-</sup> BMDCs: mean of  $86.4 \pm 3.0$  %).

Additionally, assessment of the Foxp3<sup>+</sup> Treg frequency in the CD4<sup>+</sup> population at the 96 hr time point of BMDC: CD4<sup>+</sup> T cell co-culture would allow the assessment of whether CD45<sup>-/-</sup> BMDCs could differentially alter the expansion of Treg within a mixed Treg and effector T cell population. Typically, the proportion of CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs within the peripheral CD4<sup>+</sup> T cell population in C57BL/6J mice is 5-10% but Balb/c mice have been shown to contain a higher proportion of Tregs<sup>101</sup>. The original frequency of 14 % of CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs (Figure 3.8A) was usually found in the peripheral CD4<sup>+</sup>

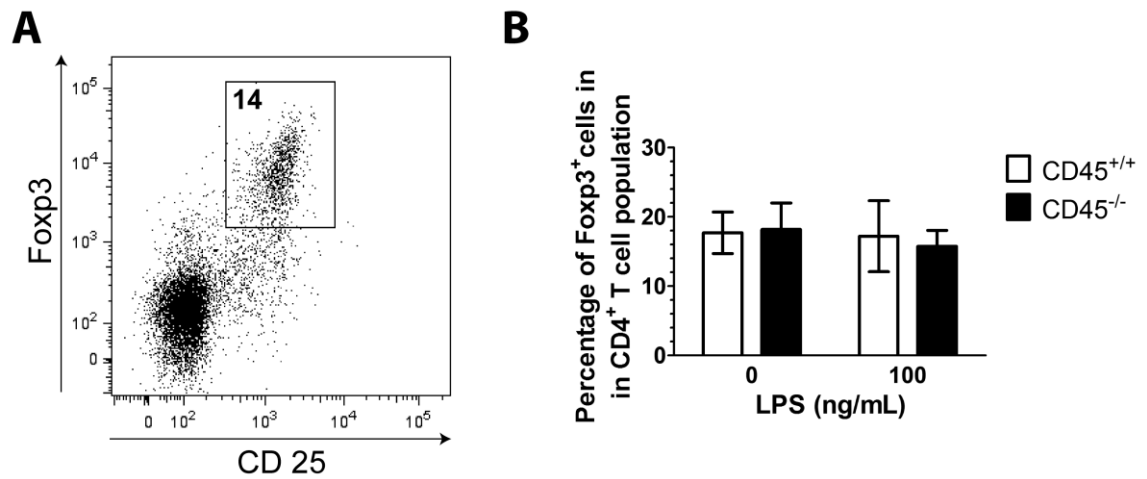
T cell population and if this percentage is maintained at the 96 hr time point within the BMDC:T cell co-culture, this would suggest that Treg were able to proliferate at the same rate as the non-Treg. Furthermore, examination of the Treg frequency post-co-culture may also indirectly offer some insight into the strength of the suppressive effect exerted by Treg *in vitro*, as a change in frequency, may indicate simultaneous Treg proliferation and suppression of non-Treg effector T cells. Figure 3.8B shows that similar proportions of Foxp3<sup>+</sup> T cells were maintained in co-culture containing unstimulated BMDCs, for both the CD45<sup>+/+</sup> and CD45<sup>-/-</sup>, with a mean percentage of  $17.7 \pm 3.0\%$  and  $18.2 \pm 3.8\%$  Foxp3<sup>+</sup> Tregs found in CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC MLRs, respectively.

CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs exposed to LPS yielded comparable frequencies of Foxp3<sup>+</sup> Tregs within CD4<sup>+</sup> population, with mean Foxp3<sup>+</sup> Treg percentages of  $17.2 \pm 5.1\%$  and  $15.8 \pm 2.3\%$ , respectively. Treg are known to be anergic to division *in vitro* unless provided with TCR and CD28 co-stimulation in the presence of IL-2<sup>102</sup>. This phenomenon is attributed to the fact that Tregs are unable to make high amounts of IL-2 like their effector T cell counterparts<sup>102</sup>. However it has been demonstrated that the highly stimulatory GM-CSF cultured BMDCs can drive Treg proliferation in the absence of non-Treg cells in high ratios of BMDC: Treg (1:1 or 1:2)<sup>102</sup>. Figure 3.9 shows that comparable Treg division could be induced at 1:1 BMDC: Treg ratio, by both LPS-stimulated CD45<sup>+/+</sup> BMDCs (mean  $36.3 \pm 4.6\%$ ) and CD45<sup>-/-</sup> BMDCs (mean  $31.0 \pm 5.0\%$ ).

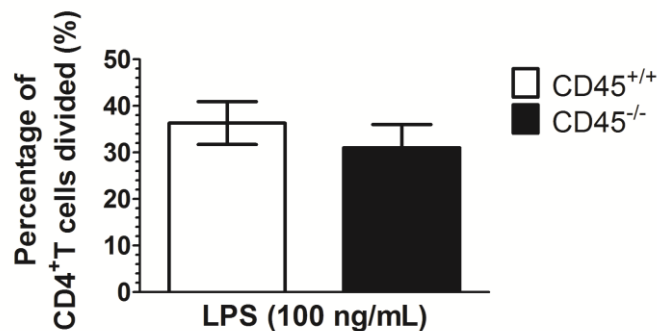




**Figure 3.7. Allostimulatory capacity of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs on Foxp3<sup>+</sup> and Foxp3<sup>-</sup> T cell population.** Percentage divided of (A) Foxp3<sup>+</sup> and (B) Foxp3<sup>-</sup> (bottom) in CD4<sup>+</sup> total T cell population. Day 7 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> CD11c<sup>+</sup> BMDCs were either unstimulated or activated with 100 ng / mL of LPS for 18-24 hr then plated with allogeneic (Balb/c) total CD4<sup>+</sup> T cells (containing Treg population) in a 96 hr MLR. T cell proliferation in the Foxp3<sup>+</sup> and Foxp3<sup>-</sup> populations of the co-culture was quantified by the percentage of T cells divided. Graph depicts mean percentage of T cells divided  $\pm$  SEM over four (0 ng/mL LPS) and two (100 ng/mL) independent experiments. Each experiment used 2-3 biological replicates for each condition and mouse genotype. CD45<sup>-/-</sup> cells were obtained from both exon 9 and exon 6 targeted CD45<sup>-/-</sup> mice.



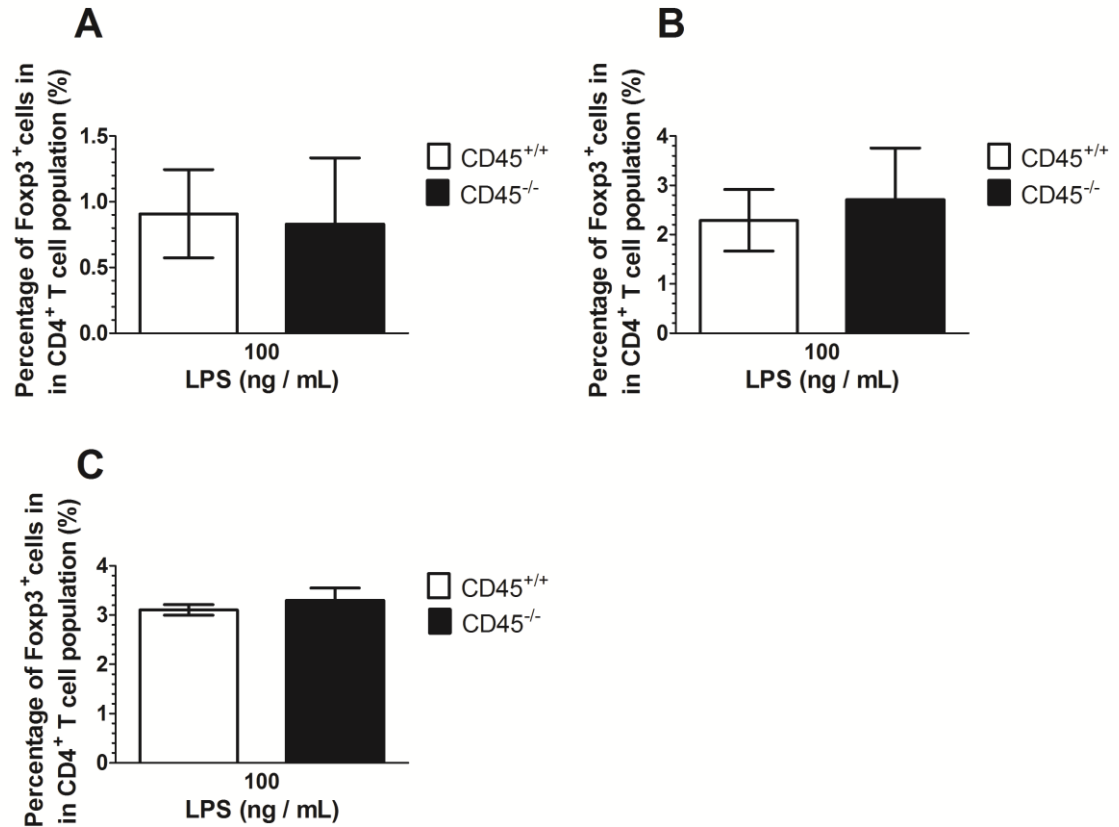
**Figure 3.8. Effect of CD45 deficiency on BMDC ability to expand an allogeneic Treg population.** (A) Flow cytometry plot depicting the frequency of Tregs present within the peripheral CD4<sup>+</sup> T cell population of Balb/c mice prior to co-culture with BMDCs (B) the frequency of Fop3<sup>+</sup> Tregs present in a total CD4<sup>+</sup> T cell population subsequent to 96 hr MLR with unstimulated or LPS-stimulated, CD45<sup>+/+</sup> or CD45<sup>-/-</sup> day 8 BMDCs. The frequency of Fop3<sup>+</sup> Tregs was determined according to the same flow cytometry gating scheme depicted in (A). Bars in graph represent the mean frequency of T cells over two independent experiments; each experiment used 3 mice per condition for each mouse genotype. CD45<sup>-/-</sup> cells were obtained from both exon 9 and exon 6 targeted CD45<sup>-/-</sup> mice.



**Figure 3.9. The effect of CD45 deficiency on the ability of BMDCs to stimulate T cell proliferation in allogeneic CD4<sup>+</sup>CD25<sup>+</sup> Treg.** Day 7 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> CD11c<sup>+</sup> GM-CSF cultured BMDCs were stimulated with 100 ng / mL of LPS for 18-24 hr then plated with allogeneic (Balb/c) CD4<sup>+</sup>CD25<sup>+</sup> Tregs (sorted by flow cytometry) in a 96 hr MLR with a BMDC: Treg ratio of 1:1. BMDC allostimulatory ability is quantified by T cell proliferation as measured by the percentage of T cells divided. Graph depicts mean percentage of T cells divided  $\pm$  SD across 3 biological replicates in one experiment. CD45<sup>-/-</sup> cells were obtained from exon 6 targeted CD45<sup>-/-</sup> mice in this experiment.

There is also the possibility that CD45<sup>-/-</sup> BMDCs have a differential ability to induce Foxp3<sup>+</sup> Tregs from a naïve CD4<sup>+</sup> T cell population. Thus Figure 3.10 shows the percentage of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells converted from a CD4<sup>+</sup> Foxp3<sup>-</sup> T cell population in various T cell proliferation assays. It was found that across the T cell proliferation assays, for CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs both un- and LPS- stimulated, induced a similar  $\approx$  1-3% conversion of naïve Foxp3<sup>-</sup> T cells into Foxp3<sup>+</sup> T cells.

Overall, these results show that in three different T cell proliferation assays, CD45<sup>-/-</sup> BMDCs do not display significant differences in their ability to stimulate T cell proliferation, although subtle trends towards increased naïve T cell proliferation under unstimulated conditions was observed. Additionally, CD45 deficiency in BMDCs does not alter their ability to expand the Treg population *in vitro*. Hence, the observed phenotypic and functional differences observed in CD45<sup>-/-</sup> BMDCs in relation to co-stimulatory and co-inhibitory molecule expression levels and pro-and anti-inflammatory cytokine in response to TLR4 ligation do not result in a significant change in the degree of T lymphocyte activation and subsequent proliferation.



**Figure 3.10. Effect of CD45 deficiency on the *in vitro* induction of Foxp3<sup>+</sup> Tregs by BMDCs.** Day 7 or 8 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> GM-CSF cultured BMDCs were stimulated for 18-24 hr with 100 ng / mL of LPS and plated in a 1:10 ratio of BMDC: T cells. Graphs show the mean percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in (A) a 72 hr OT-II T cell assay, (B) a 72 hr anti-CD3 T cell assay and (C) a 96 hr MLR. Error bars in represent SD across 3 biological replicates in (A). Error bars depict SEM in (B) across three independent experiments and (C) two independent experiments; each experiment used 3 mice per genotype. Cells were obtained from both exon 9 and exon 6 targeted mice for (B) and (C).

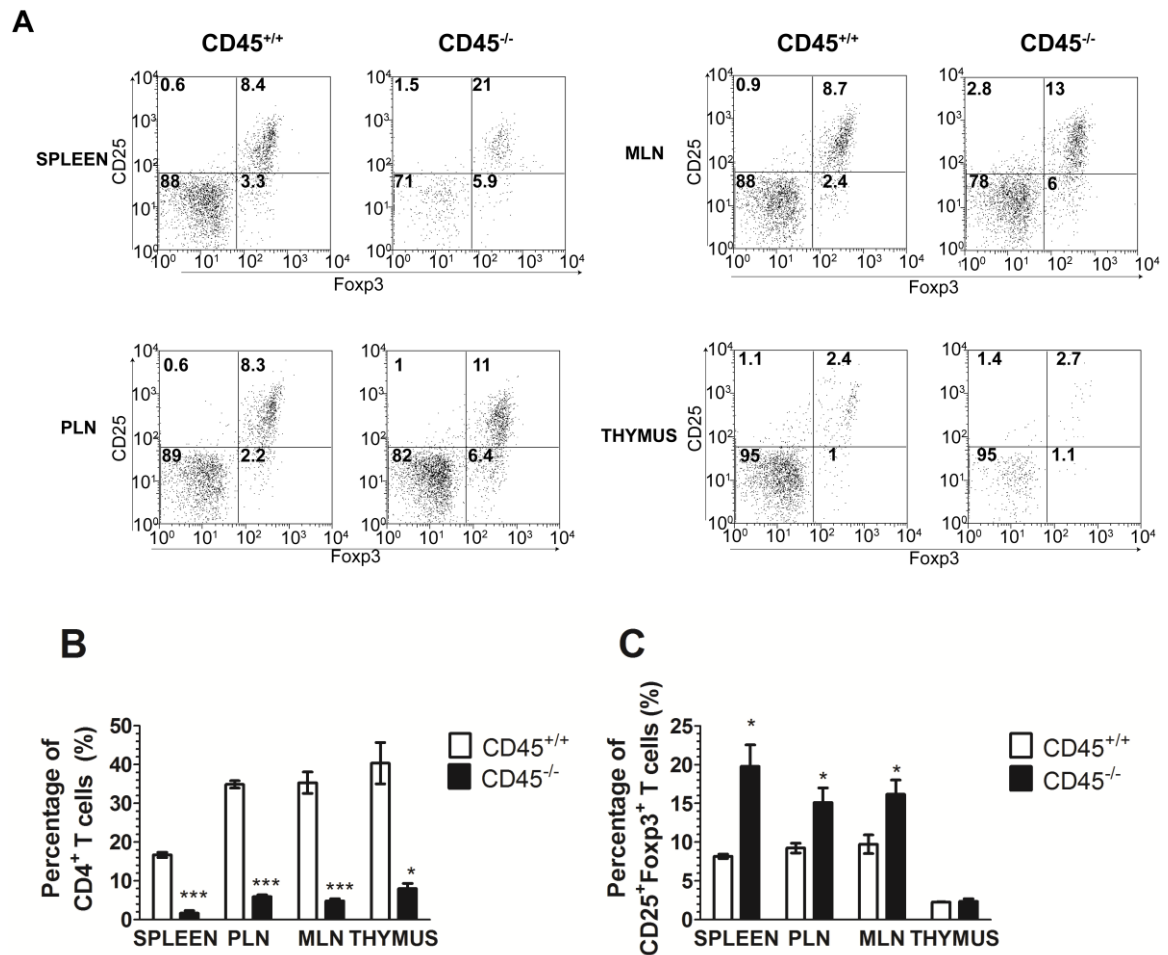
### 3.2 The Effect of CD45 Deficiency on Treg Interactions in vivo

Section 3.2 focussed on the *in vitro* phenotype and function of CD45<sup>-/-</sup>, GM-CSF BMDCs however *in vitro* observations, do not always fully correlate with *in vivo* phenomenon. Thus, we set out to compare CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs in CD45<sup>-/-</sup> and CD45<sup>+/+</sup> mice in the peripheral lymphoid organs.

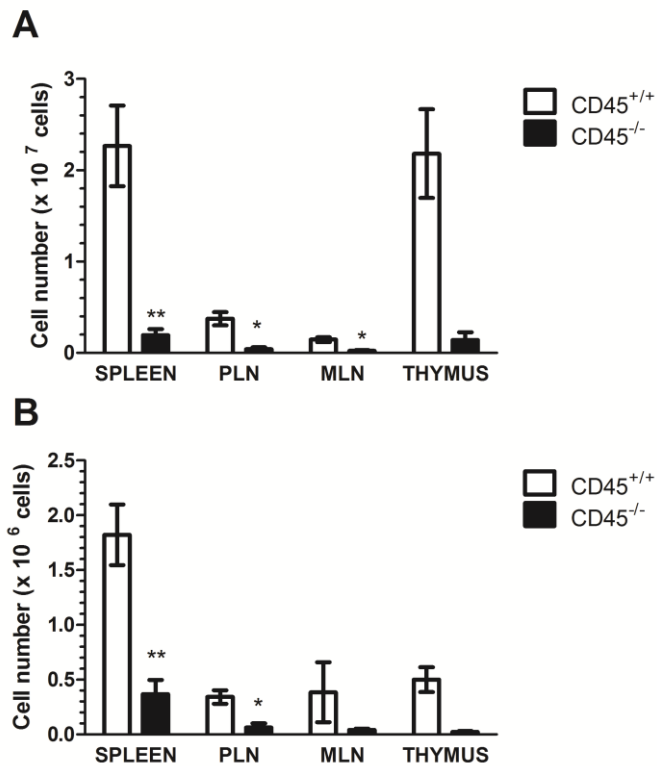
#### 3.2.1 CD45<sup>-/-</sup> mice display and enhanced *in vivo* frequency of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs within CD4<sup>+</sup> T cell population.

Flow cytometric analysis revealed that within the peripheral TCRβ<sup>+</sup> CD4<sup>+</sup> T cell population of CD45<sup>-/-</sup> animals, there was a significantly enhanced Treg frequency (spleen, p = 0.014 ; PLN, p = 0.042; MLN; p = 0.022) of  $\approx 10\%$  as illustrated in Figure 3.11. However, the diminished frequency of CD4<sup>+</sup> T cells in CD45<sup>-/-</sup> mice (Figure 3.11B) resulted in the overall numbers of CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs in CD45<sup>-/-</sup> mice being reduced compared to CD45<sup>+/+</sup> mice as shown in Figure 3.12. As an increased Treg frequency may stem from enhanced thymic output of nTreg the proportion of CD25<sup>+</sup> Foxp3<sup>+</sup> thymocytes was investigated. However, there was no significant difference in the frequency of

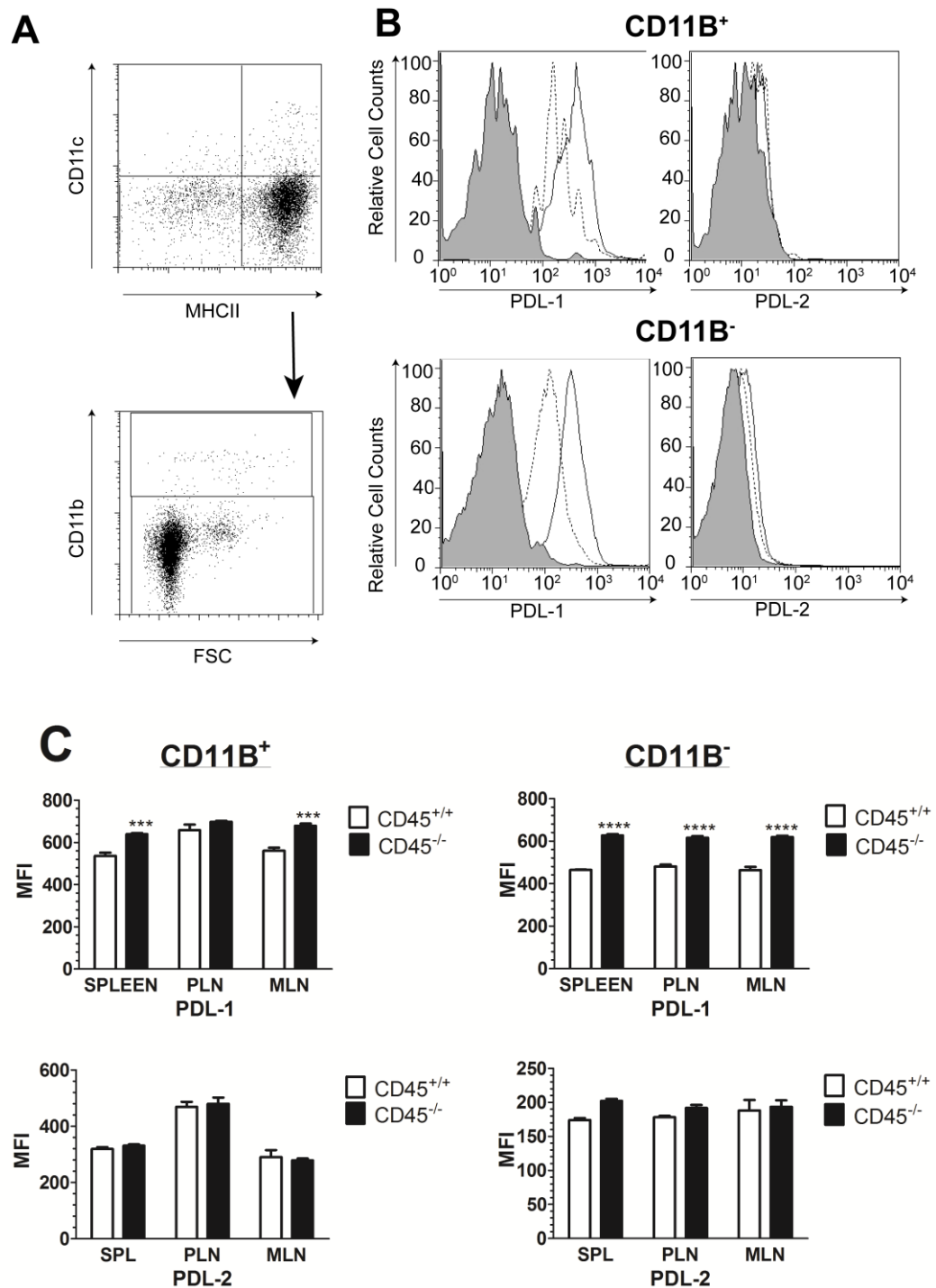
CD25<sup>+</sup> Foxp3<sup>+</sup> cells within the TCRβ<sup>+</sup>CD8<sup>-</sup>CD4<sup>+</sup> thymocytes (Figure 3.11) nor the TCRβ<sup>+</sup>CD8<sup>+</sup>CD4<sup>+</sup> thymocyte population (data not shown).



**Figure 3.11. Frequency of Tregs in CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.** Cells were isolated from the spleen, PLN, MLN and thymus of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice and cell populations determined by flow cytometry. **(A)** Representative flow cytometry plots compare the frequency of Tregs within the TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population in various lymphoid organs. The Treg frequency depicted the thymus was previously gated on a TCR $\beta$ <sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> thymocyte population **(B)** Comparison of Treg frequency in the spleen, PLN, MLN and thymus with data graphed to show the average percentage  $\pm$  SEM of CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs cells within the TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population (spleen, PLN, MLN) or TCR $\beta$ <sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> thymocyte population (thymus). **(C)** Frequency of CD4<sup>+</sup> T cells within the TCR $\beta$ <sup>+</sup> cell population in the spleen, PLN, MLN and thymus. Data obtained for (B) and (C) show the average frequency across four independent experiments (MLN), three independent experiments (spleen and PLN) and two experiments (thymus). Each experiment used 3 mice per genotype. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice. CD45<sup>-/-</sup> cells from the spleen, MLN, PLN and thymus were obtained from exon 9 targeted mice for all except one experiment from which cells were harvested from exon 6 targeted mice.



**Figure 3.12. Comparison of the CD4<sup>+</sup> and CD25<sup>+</sup>Foxp3<sup>+</sup> T cell number in lymphoid organs of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.** Data graphed as the average numbers of (A) CD4<sup>+</sup> T cells or (B) CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs within the CD4<sup>+</sup> T cell population in the spleen, PLN, MLN and thymus with error bars representing SEM. Data for the thymus is based on the TCRβ<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> thymocyte population (thymus). Data obtained for (B) and (C) show the average frequency across four independent experiments (MLN), three independent experiments (spleen and PLN) and two experiments (thymus). Each experiment used 3 mice per genotype. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice. CD45<sup>-/-</sup> cells from the spleen, MLN, PLN and thymus were obtained from exon 6 targeted mice for one experiment.



**Figure 3.13. Expression of PDL-1 and PDL-2 on non-DC cell populations in  $CD45^{+/+}$  and  $CD45^{-/-}$  mice.** The expression levels of the co-inhibitory molecules PDL-1 and PDL-2 on  $CD11c^{+}MHCII^{+}$  cells isolated from peripheral lymphoid tissue was measured by flow cytometry. (A) Representative flow cytometry plot depicting the gating strategy used on



total live cells based on the markers CD11c and MHCII. Subset outlined by black rectangular box represents the CD11c<sup>-</sup>MHCII<sup>+</sup> population which were further distinguished by CD11b expression. **(B)** Histograms of the expression levels of PDL-1 and PDL-2 on CD11c<sup>-</sup>MHCII<sup>+</sup> CD11b<sup>+</sup> and CD11c<sup>-</sup>MHCII<sup>+</sup> CD11b<sup>-</sup> cell populations in the spleen. Expression levels are shown on log scale. **(C)** Data graphed as bar charts, comparing the expression levels of PDL-1 and PDL-2 on the CD11c<sup>-</sup>MHCII<sup>+</sup> CD11b<sup>+</sup> (left) and CD11c<sup>-</sup>MHCII<sup>+</sup> CD11b<sup>-</sup> (right) cell populations in the spleen, PLN and MLN. The graphs show the mean fluorescence intensity (MFI) with error bars representing SD across three biological replicates. Graph shown is one representative of two independent experiments, with each experiment containing 3 mice per genotype. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.

It was interestingly noted that in CD45<sup>-/-</sup> non-DC cells (i.e. CD11c<sup>-</sup>), the levels of the co-inhibitory molecule PDL-1 was significantly enhanced in the CD11c<sup>-</sup> MHCII<sup>+</sup>CD11b<sup>+</sup> and CD11c<sup>-</sup>MHCII<sup>+</sup>CD11b<sup>-</sup> cell populations of CD45<sup>-/-</sup> mice in the spleen, PLN (for CD11c<sup>-</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> cells) and MLN (Figure 3.13). However, there was no significant change in the basal expression levels of PDL-2 on these two cell populations between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.

### **3.3 . The Effect of CD45 Deficiency in the Innate Immune System on the Outcome of T cell-mediated Colitis**

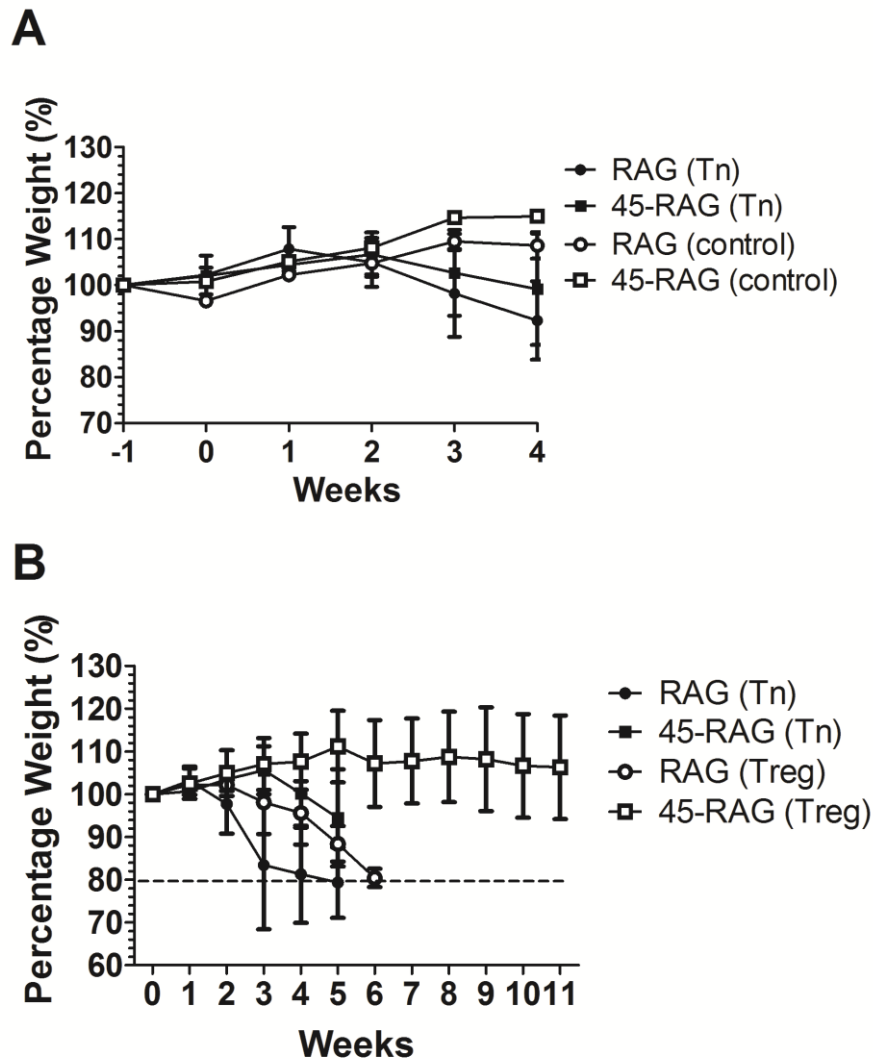
In section 3.2 a caveat in the investigation into the effect of CD45 deficiency on Treg homeostasis is that the use of CD45<sup>-/-</sup> animals does not allow us to delineate the contribution of CD45 deficiency in T lymphocytes to the increased peripheral Treg frequency. In order to investigate the involvement of CD45 deficiency in non-lymphocytes alone, an experimental T cell mediated model of colitis was employed. In this colitis model, CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>hi</sup> naïve T cells from CD45<sup>+/+</sup> mice were transferred into severe combined immunodeficiency (SCID) mice which causes intestinal inflammation and wasting disease over time<sup>103</sup>. The transferred naïve T cells will undergo enteric-antigen driven proliferation and activation of the naïve T cells to

differentiate into pathogenic TH1/TH17 subsets<sup>103,104</sup>. In this case, the SCID mice used were RAG mice and their CD45-deficient counterparts, 45-RAG. The use of RAG and 45-RAG mice, which lack both T and B lymphocytes<sup>2</sup>, allows us to determine the effect of CD45 deficiency in the cells of the innate immune system. To additionally examine how a CD45 deficient innate immune system interacts with CD45<sup>+/+</sup> Treg, this study also investigated the ability of RAG and 45-RAG mice to respond to Treg mediated prevention of colitis. These studies will provide an *in vivo* glimpse into the role of CD45 in innate immune cells and how it may affect T cell interactions.

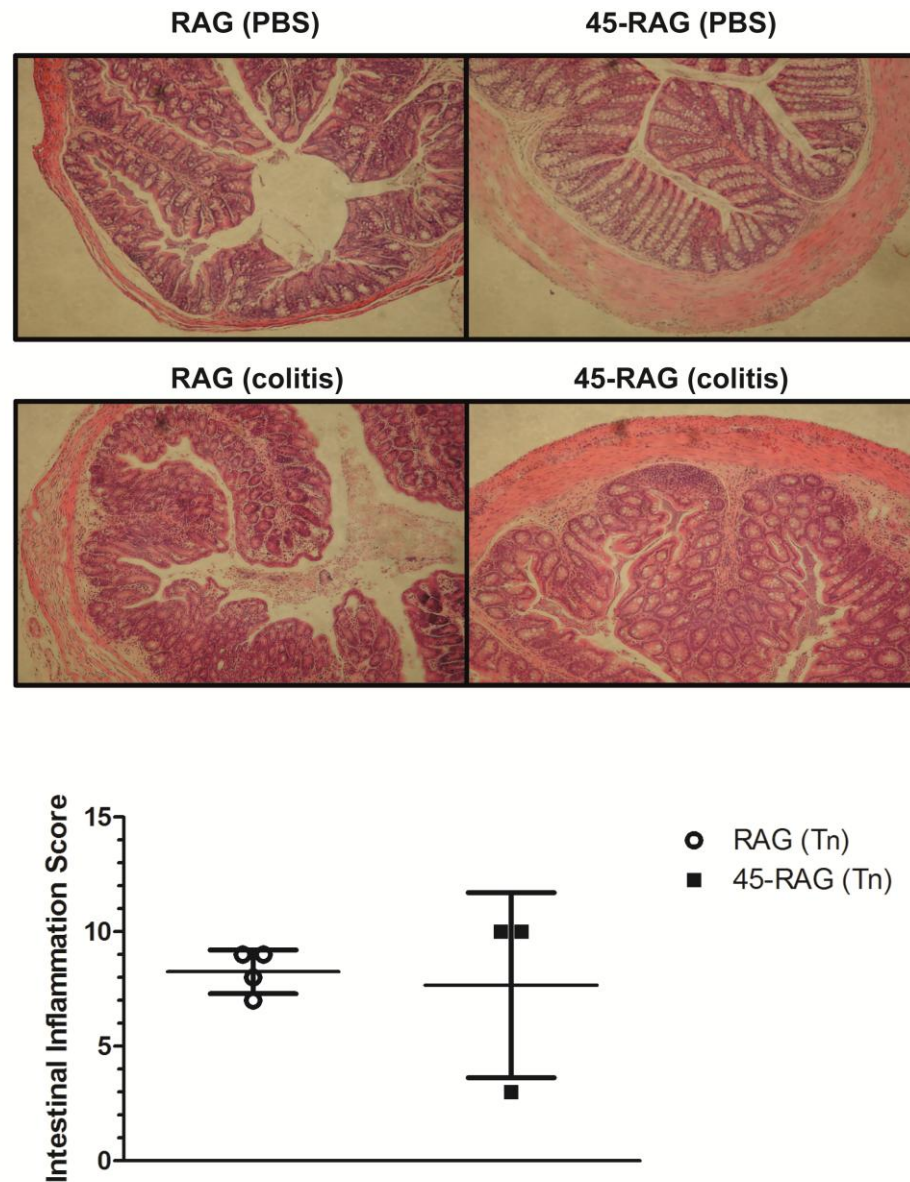
### ***3.3.1 CD45 deficiency provides greater protection against wasting disease during Treg mediated prevention of T cell colitis***

The development of characteristic wasting disease was quantified by weekly measurements of body mass post-colitis induction. In experiment 1, the PBS-injected controls gained weight throughout the course of the experiment (Figure 3.14A). In this study it was observed that RAG and 45-RAG mice transferred with CD4<sup>+</sup>CD25<sup>-</sup> CD45RB<sup>hi</sup> naïve T cells, (hereafter referred to as RAG (Tn) and 45-RAG (Tn) respectively), both developed wasting disease as shown by the onset of weight loss post-T cell transfer (Figure 3.14A). Although not statistically significant, wasting disease was delayed in 45-RAG (Tn) mice. Despite this slightly delayed onset of weight loss in 45-RAG mice, histological inspection revealed no obvious differences in the severity of intestinal inflammation experienced by RAG (Tn) and 45-RAG (Tn) mice. As shown in Figure 3.15, in comparison to control mice, the inflammation mediated by transferred naïve T cells caused an influx of lymphocytes to the intestinal LP and the disruption of normal epithelial cell structure and loss of goblet cells. In experiment 2, the outcome of

Treg mediated prevention of colitis RAG and 45-RAG mice was investigated by monitoring the development of wasting disease in RAG and 45-RAG mice co-injected with  $CD4^{+} CD25^{+} CD45RB^{lo}$  Tregs along with  $CD4^{+} CD25^{-} CD45RB^{hi}$  naïve T cells (hereafter referred to as RAG (Treg) and 45-RAG (Treg) respectively), from  $CD45^{+/+}$  Foxp3-DTR mice. Unexpectedly, RAG (Treg) lost weight post T cell transfer and by 6 weeks had lost 17-20 % of their weight (Figure 3.14B). In stark contrast, their 45-RAG (Treg) did not develop wasting disease and generally maintained their initial wk 0 body mass. While RAG (Treg) were only slightly protected and showed a 1-2wk delay in weight loss compared to RAG (Tn), from six weeks post T cell transfer there is a significant difference in weight loss in the 45-RAG (Treg) compared to 45-RAG (Tn) mice.



**Figure 3.14. The effect of CD45 deficiency on the outcome of wasting disease in experimental T cell mediated colitis.** Mean percentage weight of mice in (A) experiment #1 in which mice were either injected with naïve T cells (Tn) or PBS (control). RAG (Tn) (n = 7), 45-RAG (Tn) (n = 8), RAG (control) (n = 4) and 45-RAG (control) (n = 3) and (B) experiment #2 in which mice were injected with either naïve T cells or co-injected with naïve T cells and Tregs. RAG (Tn) (n = 3), 45-RAG (Tn) (n = 4), RAG (Treg) (n = 3) and 45-RAG (Treg) (n = 4) mice post-T cell induction. Error bars represent SD. across biological replicates. The dashed line indicates the humane end point of 20% weight loss (i.e. percentage weight 80%). Experimental colitis models were established with the assistance of Asanga Samarakoon.



**Figure 3.15. Effect of CD45 deficiency on degree of intestinal inflammation during experimental colitis.** Mice were sacrificed at 4 weeks post-colitis induction and sections of the colon removed for pathological scoring of the degree of inflammation observed in RAG (Tn) (n = 4) and 45-RAG (Tn) mice (n = 3). (Top) Intestinal cross section of the proximal colon taken from RAG (Tn) and 45-RAG (Tn) during T cell mediated colitis in experiment 1. (Bottom) Inflammation scores based on histopathology of intestinal cross-sections.

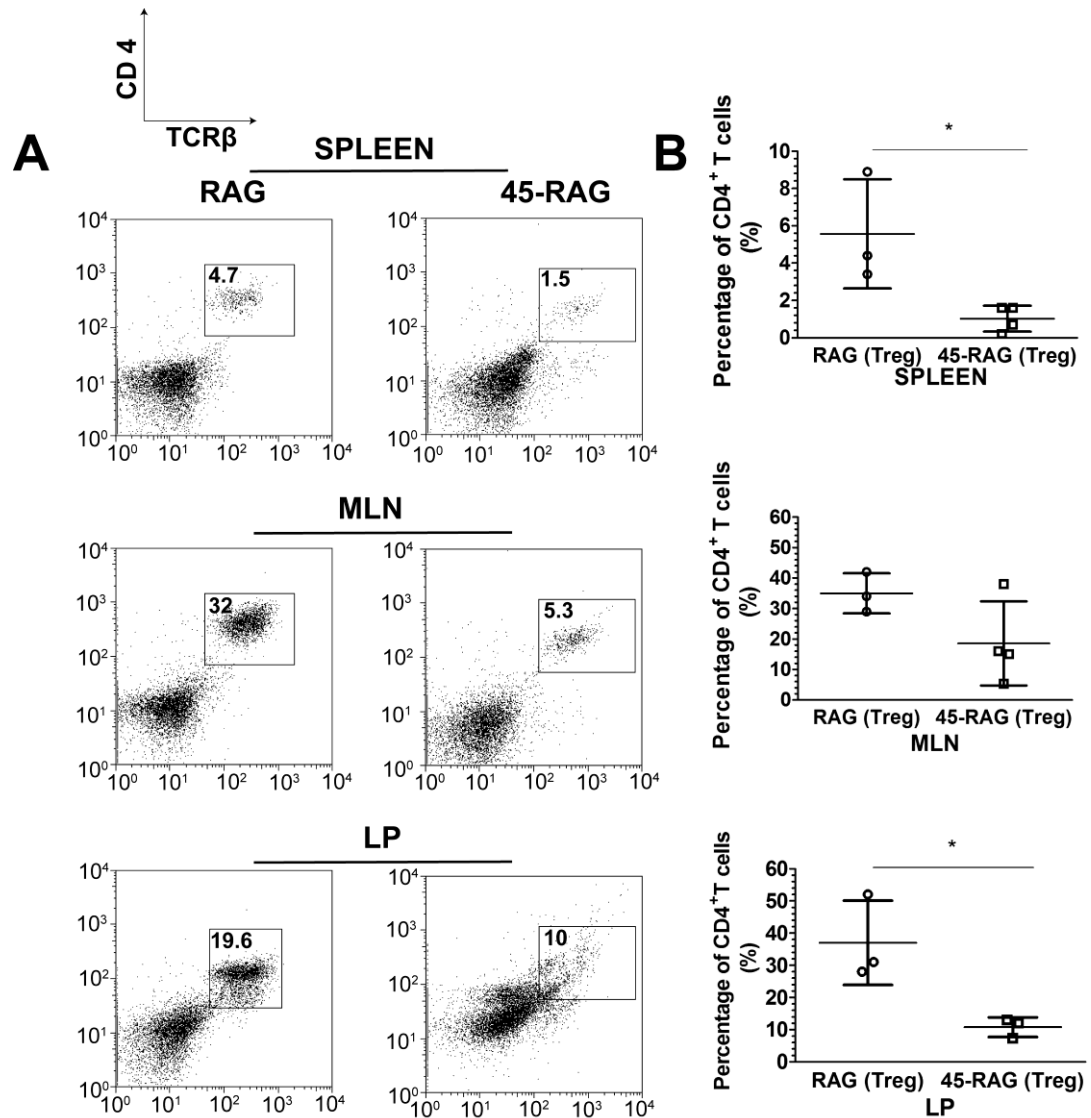
### ***3.3.2 A greater frequency of Treg is found in the LP of 45-RAG mice co-injected with naïve T cells and Tregs.***

Cell analysis of T cell populations was performed upon sacrifice of the mice: RAG (Treg) and 45-RAG (Treg) mice were sacrificed at 6 wk and 11 wk post colitis induction respectively, as at 6 wk post-colitis induction the RAG (Treg) mice had reached their pre-determined humane endpoint.

Inspection of the cell population in the different organs as illustrated in Figure 3.16, revealed that there was a significantly increased ( $p = 0.0274$ ) percentage of  $CD4^+$  T cells recovered from the spleen in the RAG (Treg) mice (mean =  $5.6 \pm 2.9$  %) compared to the 45-RAG (Treg) (mean =  $1.0 \pm 0.7$  %). However, in both RAG (Treg) and 45-RAG (Treg) the frequency of  $CD25^+$  Foxp3<sup>+</sup> T cells [RAG (Treg) mean =  $15.8 \pm 13.4$  % and 45-RAG (Treg): mean is  $19.8 \pm 5.5$  %] was comparable to the 4:1 or 80 %: 20 % ratio of naïve T cells: Tregs initially injected (Figure 3.17). A similar pattern was observed in the MLN in which the  $CD4^+$  T cell frequency was greater in RAG compared to 45-RAG [RAG (Treg): mean =  $35 \pm 7$  % and 45-RAG (Treg): mean =  $18.6 \pm 13.8$  %]. Although the percentage of  $CD4^+$  T cells was 1.2 fold higher in RAG (Treg) mice, the proportion of Tregs within the  $CD4^+$  T cell population of RAG (Treg) remained approximately 20 % while a greater proportion of Tregs tended to be found in the 45-RAG (Treg) [RAG (Treg) mice: mean =  $20.3 \pm 4.9$  % and 45-RAG (Treg) mice: mean =  $28.8 \pm 11.5$  %]. At the LP, the site of inflammation, a significant 3.4 fold increase ( $p = 0.0277$ ) in the frequency of recovered  $CD4^+$  T cells was again observed in the RAG (Treg) mice [RAG (Treg) mice: mean =  $37.0 \pm 13.1$  % and 45-RAG (Treg) mice: mean =  $10.8 \pm 3.0$  %]. The most notable difference between RAG (Treg) and 45-RAG (Treg) mice occurred in

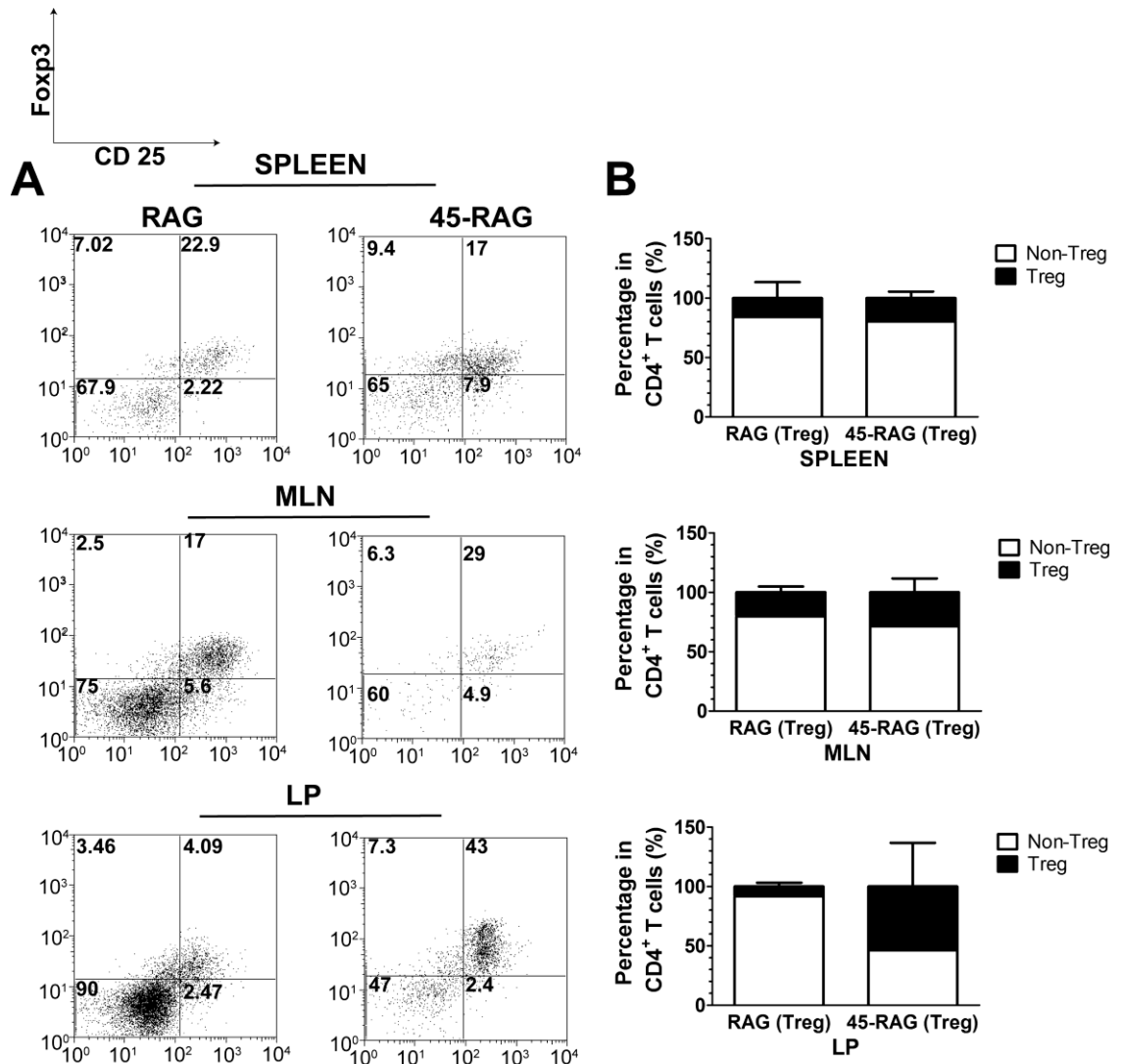
the proportion of Tregs found population at the LP, [RAG (Treg): mean =  $8.3 \pm 3.2$  % and 45-RAG (Treg): mean =  $53.7 \pm 36.8$  %], although this difference did not reach statistical significance due to biological variation among experimental 45-RAG (Treg) mice.

Figure 3.18 depicts the numbers of  $CD4^+$  T cells and  $CD25^+FoxP3^+$  Tregs found in the spleen, MLN and LP of RAG (Treg) and 45-RAG (Treg) mice, and shows that there was a trend for fewer  $CD4^+$  T cells to be recovered from 45-RAG (Treg) mice with this being significantly so in the MLN ( $p = 0.0011$ ; MLN  $CD4^+$  T cell numbers ( $\times 10^5$  cells): RAG (Treg) mice: mean =  $5.3 \pm 1.5$  and 45-RAG (Treg) mice: mean =  $0.3 \pm 0.3$ ). These decreased  $CD4^+$  T cell numbers lead to a significantly decreased Treg numbers in the MLN ( $p < 0.0001$ ; MLN Treg cell numbers ( $\times 10^5$  cells): RAG (Treg) mice: mean =  $1.0 \pm 0.2$  and 45-RAG (Treg) mice: mean =  $0.03 \pm 0.03$ ) and a trend for decreased Treg numbers in the spleen, while in the LP, there was a tendency for the mean Treg numbers in 45-RAG (Treg) to be greater than those in RAG (Treg) although not to a statistically significant level.

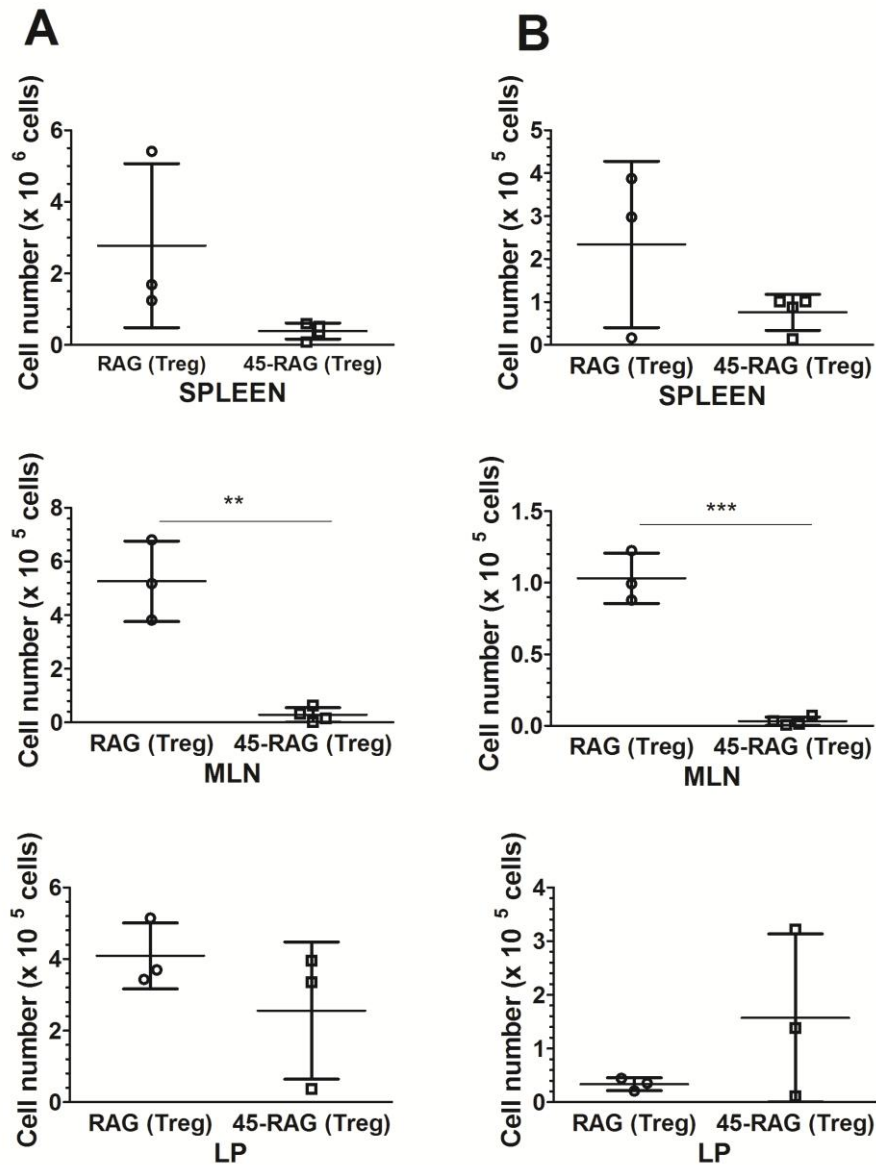


**Figure 3.16. Frequency of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells recovered from RAG (Treg) and 45-RAG (Treg) mice.** RAG (Treg) and 45-RAG (Treg) mice were sacrificed at 6 and 11 weeks post colitis induction respectively and the TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population in various organs were analyzed by flow cytometry. **(A)** Flow cytometry plots depict the TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell gating scheme and TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> cell frequency found in the spleen, MLN and LP. **(B)** Data is graphed to show the frequency of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells among the cells recovered from the spleen, MLN and LP from RAG (Treg) (n = 3) and 45-RAG (Treg) (n = 4 in spleen and MLN, n=3 in LP) mice. Errors bars of the graph represent SD. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.

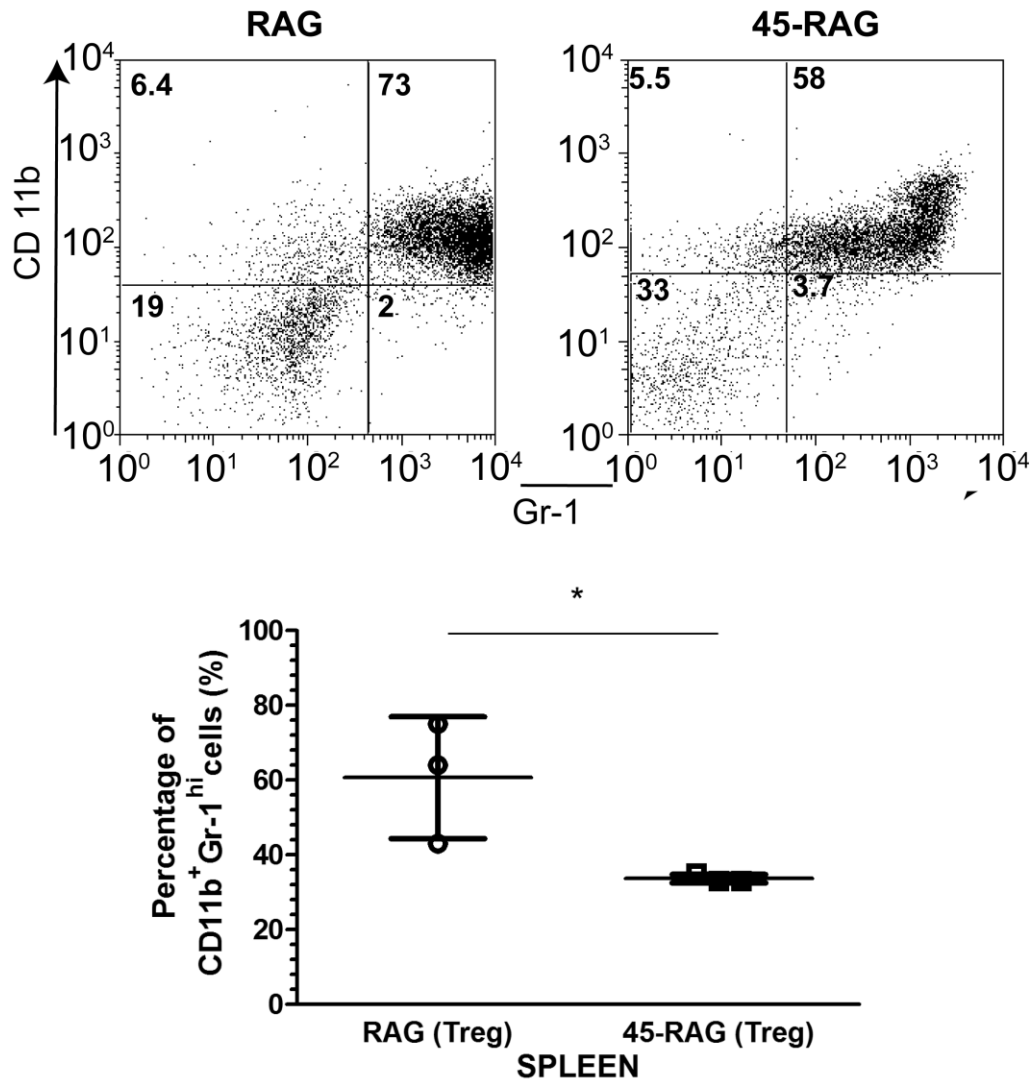




**Figure 3.17. Frequency of Treg within the CD4<sup>+</sup> T cell population of various organs from RAG (Treg) and 45-RAG (Treg) mice.** RAG (Treg) and 45-RAG (Treg) mice were sacrificed at 6 and 11 weeks post colitis induction respectively and the cell populations of various organs analyzed by flow cytometry. (A) Flow cytometry plots depict the CD25<sup>+</sup>Fcpx3<sup>+</sup> T cell gating scheme on a previously gated TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population found in the spleen, MLN and LP. (B) Data is graphed as the ratio of; mean percentage of Treg to mean percentage of non-Treg, within the TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population recovered from the spleen, MLN and LP of RAG (Treg) (n = 3) and 45-RAG (Treg) (n = 4) mice. Errors bars represent SD of Treg frequency.



**Figure 3.18. Numbers of CD4<sup>+</sup> T cells and CD25<sup>+</sup>Foxp3<sup>+</sup> Treg in various organs from RAG(Treg) and 45-RAG(Treg) mice.** RAG (Treg) and 45-RAG (Treg) mice were sacrificed at 6 and 11 weeks post colitis induction respectively and the cell populations of various organs analyzed by flow cytometry. Data is graphed as the mean number of TCRβ<sup>+</sup>CD4<sup>+</sup> T cells (**column (A)**) and the mean number of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg (**column (B)**) within the TCRβ<sup>+</sup>CD4<sup>+</sup> T cell population, recovered from the spleen, MLN and LP of RAG (Treg) (n = 3) and 45-RAG (Treg) (n = 4) mice. Errors bars represent SD. Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.



**Figure 3.19. Inflammatory cell populations in RAG (Treg) and 45-RAG (Treg) mice during experimental colitis.** (Top) Representative flow cytometry plot depicting the gating scheme used during flow cytometry analysis data depicting gating scheme of CD11b<sup>+</sup>Gr-1<sup>hi</sup> from total cell population isolated from the spleen and (bottom) mean percentage of CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells within the total recovered splenic cell population from RAG (Treg) (n=3) and 45-RAG (Treg) (n=4) mice. Errors bars represent SD. RAG (Treg) and 45-RAG (Treg) mice were sacrificed at 6 and 11 weeks post colitis induction respectively; differences in flow cytometric analysis on these two respective time points is the cause of the differences in gating schemes used in RAG (Treg) and 45-RAG (Treg) mice shown in (A). Asterisks represent statistical significance as determined by unpaired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>-/-</sup> mice.

Despite the survival advantage of the 45-RAG (Treg) mice, flow cytometry analysis of cell populations revealed that inflammation was not absent in the 45-RAG mice. A CD11B<sup>+</sup> Gr-1<sup>hi</sup> cell population, typically comprised of activated neutrophils<sup>105</sup> and recruited inflammatory monocytes<sup>106</sup> during inflammation, is observed in both the RAG (Treg) and 45-RAG (Treg) mice in the spleen although the frequency is significantly diminished ( $p = 0.045$ ) in the 45-RAG (Treg) mice (Figure 3.19).

### 3.4 Discussion

Similar to the previous reports, like that of Cross *et al*<sup>79</sup> we found that examination of CD45<sup>-/-</sup> BMDCs reveals a noticeably more activated phenotype in terms of co-stimulatory expression profiles. In this study, it was shown that among the previously unexamined co-inhibitory molecules ICOSL, PDL-1, and PDL-2, CD45<sup>-/-</sup> BMDCs displayed significantly elevated levels of PDL-2 under conditions of no maturation stimulus and this difference in PDL-2 expression between CD45<sup>+/+</sup> and

CD45<sup>-/-</sup> BMDCs is maintained upon LPS maturation. This data suggests that CD45 does not globally regulate the expression of co-inhibitory molecules but plays a role as a negative regulator of PDL-2 expression. Without the signalling pathways leading to expression of the many different co-stimulatory and co-inhibitory molecule expression being fully defined, at this point in time, it is difficult to speculate where CD45 or CD45 substrates may be acting in this process. Although the enhanced co-stimulatory molecule expression on CD45<sup>-/-</sup> BMDCs would typically be associated with an immunogenic DC phenotype, this is difficult to discern based on this parameter alone as it has become clear that phenotypically mature or semi-mature DCs are equally as capable of inducing peripheral tolerance although not necessarily by the same mechanism as immature DCs

<sup>5,33</sup>. In freshly isolated splenic DCs the observed difference in the expression of co-inhibitory molecule ICOSL and PDL-1, as well as the co-stimulatory molecules CD80, CD86, and CD40, was larger than that seen in GM-CSF BMDCs. This naturally raises the question as to the accuracy with which *in vitro* GM-CSF cultured BMDCs represent *in vivo* DC subsets and whether CD45 deficiency equally affects the co-stimulatory and co-inhibitory molecule expression on all DC subsets. It must be taken into account however, that while the BMDC population is a homogenous CD11c<sup>+</sup>CD11b<sup>+</sup> CD8α<sup>-</sup> population <sup>107</sup>, in the spleen the conventional CD11c<sup>+</sup> DC population can be further subdivided into subsets based on expression of CD4 and CD8α cell surface molecules<sup>107-109</sup>. Different DC subsets can express different levels of co-stimulatory molecules as demonstrated by the findings of Sundquist *et al.* <sup>110</sup> showed higher expression levels of CD86 on CD8α<sup>+</sup> DCs at steady state compared to their CD8α<sup>-</sup> counterparts <sup>110</sup>. It is interesting to note that there is a trend for the fold increase of co-stimulatory and co-inhibitory molecules in response to LPS to be greater in CD45<sup>+/+</sup> BMDCs than in their CD45 deficient counterparts. The fold change with LPS may be less exaggerated in CD45<sup>-/-</sup> BMDCs due partly to the already higher basal expression levels of co-stimulatory molecules and PDL-2 on CD45<sup>-/-</sup> BMDCs.

The production of pro-inflammatory cytokines by TLR activated DCs is vital for driving T cell polarization of the immune system leading to specific inflammatory or immunosuppressive responses <sup>3,5</sup>. Meanwhile, environmental signals determine the tolerogenic capacity of semi-mature and mature DCs <sup>3,5,33</sup>. Reports from past studies that have identified a possible role for CD45 substrate Src family kinases (SFK) in TLR-driven cytokine production, such as a role for Lyn in TLR4 signalling in monocytes and

for Hck in TLR-induced production of pro-inflammatory cytokines, points to a role for CD45 in cytokine regulation<sup>111–113</sup>. To prevent excessive and damaging inflammation, activation of DCs and macrophages by recognition of PRRs triggers the synthesis and secretion of IL-10 in addition to pro-inflammatory cytokines<sup>98,114,115</sup>. Our data demonstrates that CD45 deficiency in BMDCs skews the DC cytokine response to the TLR4 agonist LPS towards an anti-inflammatory profile. CD45<sup>-/-</sup> BMDCs produced significantly elevated production of IL-10 with a concomitant decrease in the TH1 polarizing cytokine IL-12. The previous report by Cross *et al.*<sup>79</sup> demonstrated that the pro-inflammatory cytokine response in CD45<sup>+/+</sup> BMDCs is highest for 100 ng/mL LPS stimulation, hence this concentration was chosen to use throughout this study. Cross *et al.* also observed that the LPS-induced BMDC pro-inflammatory cytokine response varied in a dose-dependent manner in which 100 ng/mL LPS stimulation resulted in a significant decrease in the production of the pro-inflammatory cytokines, IL-12, IL-6 and TNF $\alpha$ , in CD45<sup>-/-</sup> BMDCs as compared to their CD45<sup>+/+</sup> counterparts<sup>79</sup>. On the other hand, stimulation with 10 ng/mL LPS saw a significant enhancement in IL-12 and IL-6 but not TNF $\alpha$ , while stimulation with 1000 ng/mL LPS revealed no obvious differences<sup>79</sup>. Hence the results obtained in this study are only a reflection of the effect of loss of CD45 on the particular set of LPS-driven mechanisms operating at 100 ng/mL LPS stimulation.

The 2003 study by Napolitani *et al.*<sup>116</sup> reported that LPS induced the activation of the SFKs, c-Src and Lyn, in human DCs. Inhibition of these SFKs depressed LPS-induced pro-inflammatory cytokine production without affecting up-regulation of co-stimulatory molecules or ability to drive T cell proliferation, but impaired TH1 polarization of CD4<sup>+</sup> T cells<sup>116</sup>. Napolitani *et al.*<sup>116</sup> went on to reveal that the SFK-

inhibited phenotype and function of DCs was due to improper phosphorylation and consequent accumulation of c- Jun leading to reduced formation of AP-1 complexes upon LPS stimulation. These results are similar to the phenotype and function of CD45<sup>-/-</sup> BMDCs. Additionally, the hyper-phosphorylation of Lyn at the negative regulatory site (tyrosine 507) and Lyn inability to undergo activation upon LPS stimulation suggest that the altered phenotype and cytokine profile of CD45<sup>-/-</sup> BMDCs may be due to alterations in levels of AP-1 complex through dysregulation of Lyn by CD45. As IL-10 is known to suppresses the production of pro-inflammatory cytokines such as IL-12, the increased IL-10 may be exerting an autocrine effect on the CD45<sup>-/-</sup> BMDCs to suppress IL-12 production<sup>114,117</sup>. This is assuming that CD45<sup>-/-</sup> BMDCs respond normally to IL-10 as CD45 deficiency in other innate immune cells has been shown to negatively regulate the Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) signalling pathway<sup>118</sup>; the IL-10 effect is mediated by the ligation of IL-10 to the IL-10 receptor (IL-10R) and subsequent JAK/STAT 3 signalling<sup>114,117</sup>.

Despite the increased expression of PDL-2, the heightened MHCII, and co-stimulatory profile of CD45<sup>-/-</sup> BMDCs would suggest that CD45 deficiency in BMDCs would exhibit a greater ability to drive T cell proliferation, however the elevated secretion of the anti-inflammatory IL-10 by LPS-stimulated CD45<sup>-/-</sup> BMDCs suggests an opposite outcome in the ability to drive lymphocyte activation and proliferation. To resolve this contradiction, we investigated whether the changes in phenotype and cytokine profile in CD45 deficient BMDCs were able to significantly alter the functional capacity of BMDCs to activate T cells in three different *in vitro* T cell proliferation assays. Overall, the results from all three T cell proliferation assays showed that there

was a consistent tendency for CD45<sup>-/-</sup> BMDCs to drive greater proliferation of naïve CD4<sup>+</sup> T cells than their CD45<sup>+/+</sup> counterparts. In the antigen-dependent assay using OT-II T cells, the ability to induce T cell proliferation is dependent on the presentation of the antigen, OVA peptide, via MHCII. As such, the greater expression of MHCII of CD45<sup>-/-</sup> BMDCs suggests that there may be enhanced levels of MHCII: peptide complexes and hence greater T cell stimulatory ability. While the results presented no obvious differences in the T cell proliferation induced by LPS-stimulated CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs, an increased proportion of divided T cells was observed in CD45<sup>-/-</sup> BMDC-stimulated CD4<sup>+</sup> T cell co-cultures under conditions of no LPS stimulation. The allogeneic immunologic response in a MLR has been shown to bear specificity and memory<sup>119-121</sup>, is dependent on the expression of high densities of MHC class I and II products, and co-stimulatory molecules CD80 and CD86, and appears to be regulated by the same mechanism found in antigen-specific T cell activation<sup>121</sup>. Although CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs did not display any statistically significant differences in the degree of T cell proliferation induced from naïve CD4<sup>+</sup> T cells, there was again a trend for CD45<sup>-/-</sup> BMDCs to bring about more T cell proliferation in the absence of LPS. This trend was similarly observed when comparing the T cell stimulatory ability of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs in the anti-CD3 T cell assay. The observed inclination for enhanced T cell proliferation in co-cultures of naïve CD4<sup>+</sup> with CD45<sup>-/-</sup> BMDCs may possibly be a manifestation of the cumulative effect of the increased levels of MHCII and co-stimulatory molecules, CD80 and CD86, which is characteristic of CD45<sup>-/-</sup> BMDC despite the significantly enhanced levels of the co-inhibitory molecule PDL-2. As the difference in BMDC activation status, based on co-stimulatory and co-inhibitory



molecule expression, is more prominent in the absence of LPS, it correlates with the observation of greater difference in T cell stimulatory capacity observed in the absence of LPS stimulation.

The effect of DC restricted CD45 deficiency on DC interactions with Tregs was also investigated in an *in vitro* allogeneic setting. Despite the activated phenotype, the preferential IL-10 production of CD45<sup>-/-</sup> BMDCs in response to LPS resembles that of mature tolerogenic DCs which may preferentially expand or induce Tregs or promote Treg function<sup>5,33,122</sup>. However investigation of the interactions of CD45<sup>-/-</sup> BMDCs with Tregs *in vitro* suggests that CD45 does not regulate a tolerogenic effect via enhanced generation or expansion of Foxp3<sup>+</sup> Tregs. However, it is possible that the higher levels of CD45<sup>-/-</sup> BMDC-derived IL-10 may have functioned to gear the TH response towards either a TH2 profile or the Foxp3<sup>-</sup> Tr1 cells whose development requires an IL-10 rich environment and co-stimulation by CD80/86 via T cell specific CD28<sup>5,27,33,123</sup>. As the cytokine profile of the CD45<sup>-/-</sup> BMDC stimulated-T cells was not investigated and Foxp3 expression was used as the primary marker of Tregs in this study, the development of Tr1 cells may have gone undetected. It has already been observed by Cross *et al.*<sup>79</sup> that LPS-stimulated CD45<sup>-/-</sup> BMDCs exhibited a reduced ability to induce a TH1 response thus future studies should seek to determine whether the activated phenotype and IL-10<sup>hi</sup> profile of LPS stimulated CD45<sup>-/-</sup> BMDCs may hold greater tolerogenic potential than their CD45<sup>+/+</sup> counterparts through immune deviation of naïve T cells from a pro-inflammatory TH1 subtype towards a default Tr1 or TH2 response<sup>79</sup>.

IL-10 is a potent anti-inflammatory cytokine<sup>114</sup>, one would predict that the higher IL-10 production from LPS-stimulated CD45<sup>-/-</sup> BMDCs would impair BMDC ability to

drive T cell proliferation however, *in vitro* results did not show increased suppression of T cell proliferation in T cell co-cultures with CD45<sup>-/-</sup> BMDCs. A possible reason for the lack of T cell inhibition by LPS-stimulated CD45<sup>-/-</sup> BMDCs may be due to the removal of IL-10 as the supernatant of LPS-stimulated BMDCs was removed prior to the addition of T cells to remove the LPS present. As studies into the kinetics of LPS-induced IL-10 production by CD45<sup>-/-</sup> BMDCs were not carried out, the actual quantity of IL-10 present in the co-culture with CD45<sup>-/-</sup> BMDCs remains unknown. The main caveat of *in vitro* T cell proliferation studies is the inherent artificialities of *in vitro* systems which may not necessarily accurately mimic the equivalent *in vivo* situation. For instance, in the OVA: OT-II assay the employed final concentration of (1 µg/mL) of OVA<sub>323-339</sub> peptide, represents an antigenic load far in excess of physiologically relevant levels at which antigen may be found in the body, while *in vitro* co-cultures are often used with higher cell ratios, e.g. 1: 10 BMDC: T cell ratio, than would be typically found *in vivo* where DC stimulatory capacity has been reported to be sufficiently efficient to stimulate 100-3000 T cells from one DC alone<sup>1</sup>. Thus *in vitro* assays although useful in teasing apart mechanisms, may mask subtle difference due to inaccuracy in the reflection reflect physiological conditions.

The prevalence of CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs within the CD4<sup>+</sup> T cell population of peripheral lymphoid organs was characterized and the discovery was made of an increased frequency of Tregs within the CD4<sup>+</sup> repertoire of CD45<sup>-/-</sup> mice; however, this increase was not reflected in mature TCRβ<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> thymocytes. This suggests that factors within the periphery may be responsible for promoting the conversion of naïve T cells into Treg, or expansion of n Treg. This may potentially be due to interactions of T

cells with DCs as an increasing number of studies are discovering that DC are crucial for Treg homeostasis in a manner dependent on MHCII as well as the co-stimulatory molecules CD80 and CD86<sup>5,33,124</sup>. As CD45 deficiency in BMDCs and splenic DCs show heightened levels of MHCII and CD80/86 molecules, interactions with CD45<sup>-/-</sup> DCs via these cell surface molecules may represent one potential mechanism by which n Treg frequency is being enhanced *in vivo*. However, speculation of possible reasons belying the enhanced Treg frequency is limited from this observation alone, because CD45 expression is deleted from all hematopoietic cells in CD45<sup>-/-</sup> animals. The increased Treg prevalence in the periphery may be due the lack of CD45 in the T cells and/or in the innate immune compartment. Additionally, other innate immune cells defined by a CD11c<sup>-</sup> MHCII<sup>+</sup> CD11b<sup>+</sup> and CD11c<sup>-</sup> MHCII<sup>+</sup> CD11b<sup>-</sup> phenotype, were observed to have increased levels of the co-inhibitory molecule PDL-1 but not PDL-2. These cell populations may represent macrophages and B cells respectively, and their increased expression of PDL-1, may ultimately contribute to the outcome of an immune response as PDL-1 ligation in DCs may mediate immune tolerance through increased ability to expand Tregs<sup>38,122</sup>. As the impact of CD45 deficiency on *in vitro* generated and *in vivo* macrophage subsets has still not been clearly defined, future studies to characterize this cell type in different lymphoid and mucosal tissue would prove useful in dissecting the role of CD45 in the innate immune system.

To more strictly examine the impact of CD45 deficiency in only the innate immune system, specifically during state of disease, a naïve T cell transfer model of experimental colitis was employed. In the two experiments conducted in this study, CD45 deficiency was not observed to alter the outcome of wasting disease to a significant

degree, as has been previously seen [unpublished data, Asanga Samarakoon], however the weight loss in 45-RAG (Tn) mice was delayed compared to that of RAG (Tn) mice. But it was the kinetics of the Treg mediated prevention of colitis where a clear CD45 dependent difference in the immune outcome was observed. RAG (Treg) mice unexpectedly still developed colitis and eventually succumbed to the disease albeit at a slightly delayed rate compared to RAG (Tn) mice. In stark contrast, all the 45-RAG (Treg) mice survived and did not lose weight post-colitis induction. The development of wasting disease in RAG (Treg) was unexpected and this departure from previously published findings<sup>103</sup> may be due to an insufficient number of Tregs employed in the co-transfer. Mice in this study were injected with a 4:1, naïve T cell: Treg ratio of CD4<sup>+</sup> T cells similar to previous studies employed by Mottet *et. al.*<sup>125</sup>. However previous experiments within the animal facilities used in this study, have shown that RAG mice develop T cell mediated colitis more rapidly than in other facilities with disease onset typically observed by 3 weeks (Asanga Samarakoon, personal communication, 2011) in contrast to the more typical 5-6 weeks onset in the literature<sup>125</sup>. Differences in the severity and kinetics of colitis in the T cell transfer model can vary with animal facilities possibly due to differences in endogenous flora<sup>126–128</sup>. The reasons for our discordant findings could reflect aspects of the endogenous flora in different colonies and/or the intensity of the pathogenesis. In the face of this more rapid disease onset in our facilities, it is plausible that a higher proportion of Tregs is required to fully protect against colitis. Despite the surprising morbidity of the RAG (Treg) mice, these results raise interesting questions as to why CD45-deficiency conferred RAG mice with a survival advantage in experimental T cell mediated colitis. Regardless of the lack of weight loss, 45-RAG

(Treg) mice did exhibit signs of systemic inflammation such as the presence of a splenic CD11b<sup>+</sup> Gr-1<sup>hi</sup> population which most likely represents a mixed population of activated neutrophils and monocyte-derived inflammatory DCs<sup>105,106</sup>. However, the presence of this splenic CD11b<sup>+</sup> Gr-1<sup>hi</sup> population in 45-RAG (Treg) mice was significantly reduced compared to RAG (Treg) mice. The data suggests that although the 45-RAG (Treg) mice were subject to lower intensity of systemic inflammation, which may explain the observed weight maintenance in 45-RAG (Treg) mice instead of weight increase over time. The 45-RAG (Treg) mice were still sufficiently, although not completely, protected allowing them to survive the ongoing inflammation. These observations of milder systemic disease in 45-RAG (Treg) may be an extrapolation of the delayed disease previously seen in the absence of Treg. The immunosuppressive effect of the Tregs in the 45-RAG mice may have been sufficient to enable some colitis protection and survival even at a sub-optimal Treg: naïve T cell ratio, due in part to the fact that 45-RAG mice tend to exhibit a slightly delayed onset of T cell colitis. But as the observed delay in this study is slight and not statistically significant, it is likely that there are additional underlying mechanisms due to altered interactions of Treg in a CD45<sup>-/-</sup> environment.

The greater proportion of Tregs in the colon of the 45-RAG (Treg) mice but not in the spleen and MLN also raises interesting possibilities that the CD45 deficient innate immune cells create an intestinal environment that may promote Treg recruitment, function, induction, or proliferation. In the Treg co-transfer model, the transferred Treg cell populations expand considerably *in vivo*, and most maintain Foxp3 expression<sup>127</sup>. Certain growth factors and cytokines, such as TGF- $\beta$  and IL-10 are required for maximal suppressive activity of Tregs and the cure of colitis has been shown to be at least partially

dependent on these cytokines<sup>126,127,129</sup>. In particular, in C57BL6-RAG<sup>-/-</sup> hosts, CD4<sup>+</sup> CD25<sup>+</sup> Tregs are required to produce IL-10 for efficient protection from CD45RB<sup>hi</sup> T cell-induced wasting disease as well as for the control of peripheral lymphocyte numbers<sup>126</sup>. IL-10 is also important for maintaining Foxp3 expression once the Treg cells are differentiated and exposed to inflammation as inflammatory signals during colitis can downregulate Foxp3 expression<sup>127</sup>. Findings of Murai *et al.*<sup>127</sup> indicate that IL-10 from non-lymphoid cells, particularly CD11c<sup>+</sup> CD11b<sup>+</sup> DCs, is required for Treg function in the T cell transfer model of colitis prevention<sup>127</sup> while other studies find that IL-10 from LP macrophages is important for the induction of Foxp3 expression<sup>127,130</sup>. With a diversity of intestinal APCs helping to shape the gut environment, alterations in the cytokine profile of these intestinal innate immune cells caused by CD45 deficiency, may then change the gut cytokine milieu of 45-RAG mice. These changes could potentially promote Foxp3<sup>+</sup> expression and function of Tregs and hence render 45-RAG mice more susceptible to the prevention of colitis by Tregs. As the role of CD45 as a positive or negative regulator of cytokine response in BMDCs is TLR-dependent, it is difficult to predict the response of CD45<sup>-/-</sup> DCs or macrophages *in vivo* as this situation will subject these APCs to a simultaneous barrage of different TLRs in addition to other tissue-derived factors signals from IECs.

For the purpose of this study, we did not seek to discriminate between transferred FoxP3-GFP<sup>+</sup> Treg and Foxp3<sup>-</sup>-GFP<sup>+</sup> Tregs induced *in vivo* from the naïve Foxp3-GFP<sup>-</sup> T cell population during disease by use of different congenic markers of the transferred Tregs and naïve T cells. Thus the expansion of the transferred Foxp3<sup>+</sup> cells would be indistinguishable from *bona fide* induction of Treg cells from the introduced naïve T

cells. However, in previous work, no significant difference between RAG and 45-RAG mice was found in the frequency of induced Foxp3-GFP<sup>+</sup> Tregs at the LP at 2 weeks post colitis induction (data not shown) but 2 weeks post-colitis may represent a premature time point to examine for the accumulation of inducible Tregs. It should be noted that in Figure 3.16A, the FACS analysis of 45-RAG LP samples appear to contain high levels of background CD4<sup>+</sup> staining, possibly from contaminating CD4<sup>+</sup> phagocytes. This background, coupled with few T cells remaining in the LP of 45-RAG (Treg), in part hampers accurate gating of a TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cell population and hence limits the interpretation of the Treg proportions present in the LP.

In conclusion, this investigation of the impact of CD45 on the activation status of DCs suggests that CD45 plays a role as a negative regulator of PDL-2 expression and as a negative regulator of the anti-inflammatory cytokine IL-10 in response to the TH1 promoting TLR signal LPS. CD45 also negatively regulates the expression of MHCII, co-stimulatory molecules CD80 and CD86 and differences brought about by the loss of CD45 in BMDCs were only subtly reflected in a slight trend for enhanced T cell stimulatory capacity of CD45<sup>-/-</sup> BMDCs. While CD45 does not play a role in the regulation of a tolerogenic response through the *in vitro* expansion or induction of Foxp3<sup>+</sup> Tregs, *in vivo* findings suggests that CD45 may potentially act as a negative regulator of tolerogenic responses in the innate immune system.

## **CHAPTER FOUR: THE EFFECT OF CD45 ON THE INDUCTION OF TOLEROGENTIC DENDRITIC CELLS**

Chapter 3 of this thesis focused on determining how the loss of CD45 affected the phenotype and function of *in vitro* BMDCs in both unstimulated conditions and in response to LPS stimulus. Additionally, exploratory studies were initiated into the *in vivo* influence of CD45 deficiency on the creation of a tolerogenic environment. Much of the previous literature on determining the role of CD45 in DCs has focused on the integration of TLR- induced signals and subsequent pro-inflammatory response. However, the influence of CD45 on the ability of DCs to integrate tolerizing signals and elicit a tolerogenic response is still unknown. This chapter will explore whether CD45 plays a role in the induction of tolerance in DCs by: (a) the direct ligation of CD45 isoform with an anti-CD45RB antibody and (b) treatment of DCs with the pharmacological tolerogenic mediator rapamycin.

### **4.1 The Effect of Anti CD45RB mAb on the Phenotype and Function of BMDCs**

Evidence from previous reports suggests that the CD45RB isoform in particular plays a role as a positive regulator of the tolerogenic response in DCs. In this study, we hypothesize that the antiCD45RB mAb (23G2) activates signalling downstream of the CD45RB isoform which leads to a tolerogenic DC phenotype and function. The generation of tolerogenic DC represents one mechanism by which antiCD45RB may potentially establish tolerance in transplantation models.

#### ***4.1.1 Expression of CD45 isoforms on BMDCs***

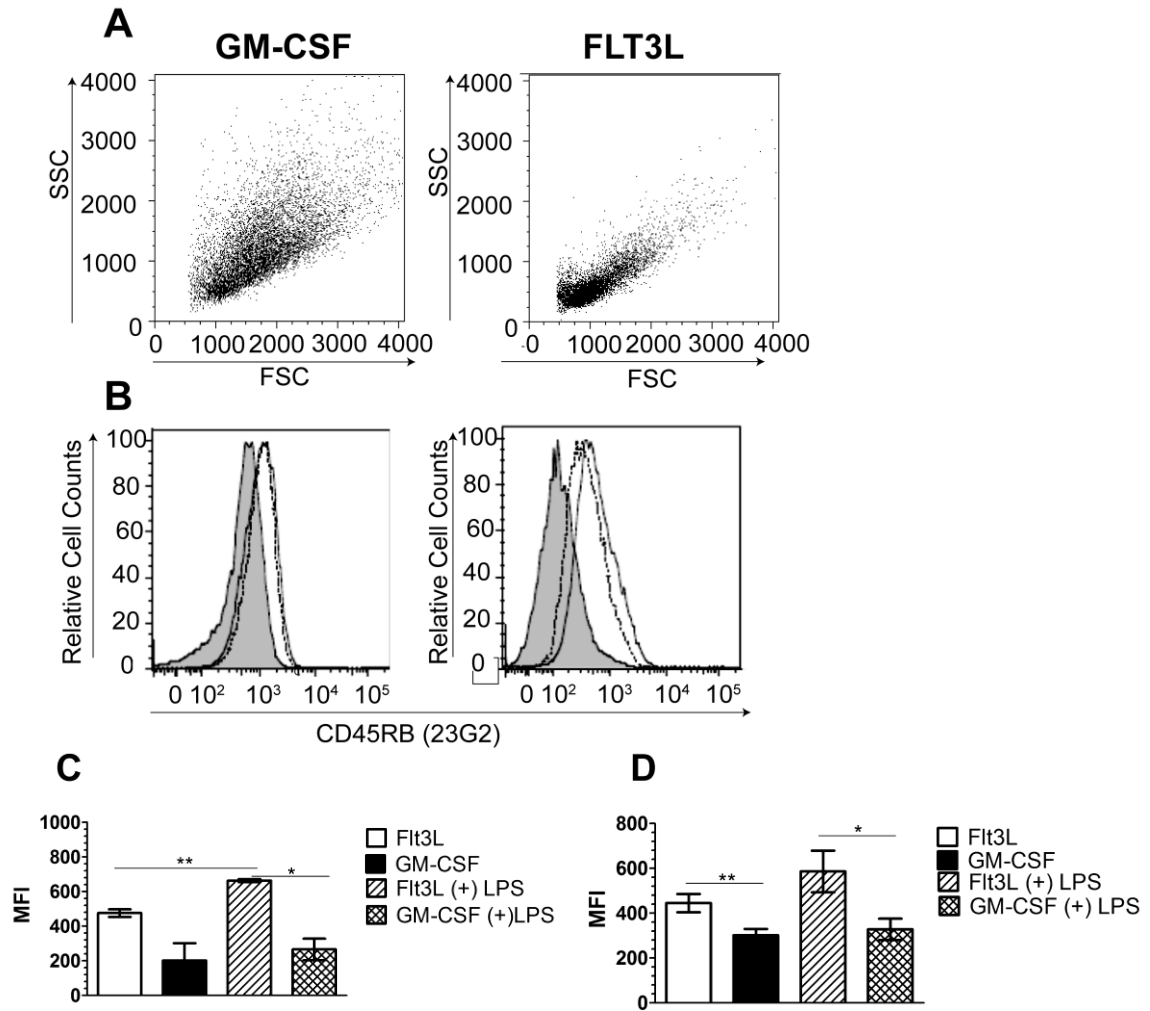
The first order of business was to determine the expression profile of CD45 isoforms on DCs. Using flow cytometry to assess the cell surface expression, we



observed that pan CD45 was expressed on freshly isolated splenic CD11c<sup>hi</sup>MHCII<sup>hi</sup> cDCs and among the CD45 isoforms tested (CD45RA, RB and RC), it was CD45RB that was the most prominently detected (data not shown) as Haidl *et al.*<sup>131</sup> have previously shown. Upon investigation of the basal expression profile of CD45RB on CD11c<sup>+</sup> GM-CSF BMDCs, uniformly low levels of CD45RB were observed (Figure 4.1). DCs generated from cultures in alternative cytokine cocktails can produce DCs with distinct characteristics<sup>107,132</sup> and hence CD45RB expression was also investigated on BMDCs generated from the *in vitro* culture of BM cells with the DC hematopoietic growth factor Flt3L, (hereafter known as Flt3L BMDCs)<sup>95</sup>. A noticeable difference in the size of GM-CSF and Flt3L-BMDCs was observed in which the latter was smaller in size and less granular based on the flow cytometry parameters of FSC and SSC (Figure 4.1A). This observation is in keeping with observations reported by other groups<sup>107</sup>. The CD11c<sup>+</sup> Flt3L-BMDCs also expressed CD45, as determined by binding of the pan-CD45 Ab (data not shown) but expressed a greater level of CD45RB compared to their GM-CSF BMDC counterparts as shown in Figure 4.1 B and C. Moreover, upon LPS stimulation, the Flt3L-BMDCs demonstrated a significant ( $p = 0.0086$ ) upregulation in CD45RB expression which was not observed in their GM-CSF BMDC counterparts (Figure 4.1C). Importantly, this trend was similarly shown when cells expressing the pDC marker PDCA-1 were excluded from the analysis (Figure 4.1D), as pDCs express the CD45RABC isoform against which the anti-CD45RB Ab cross-reacts. The CD45 isoforms, CD45RA and CD45RC were expressed on Flt3L CD11c<sup>+</sup> BMDCs but not significantly changed in response to stimulation by TLR4 agonist as was also the case for GM-CSF-BMDCs (Figure 4.2).

#### ***4.1.2 The effect of anti CD45RB antibody on the co-stimulatory and co-inhibitory molecule expression of GM-CSF BMDCs***

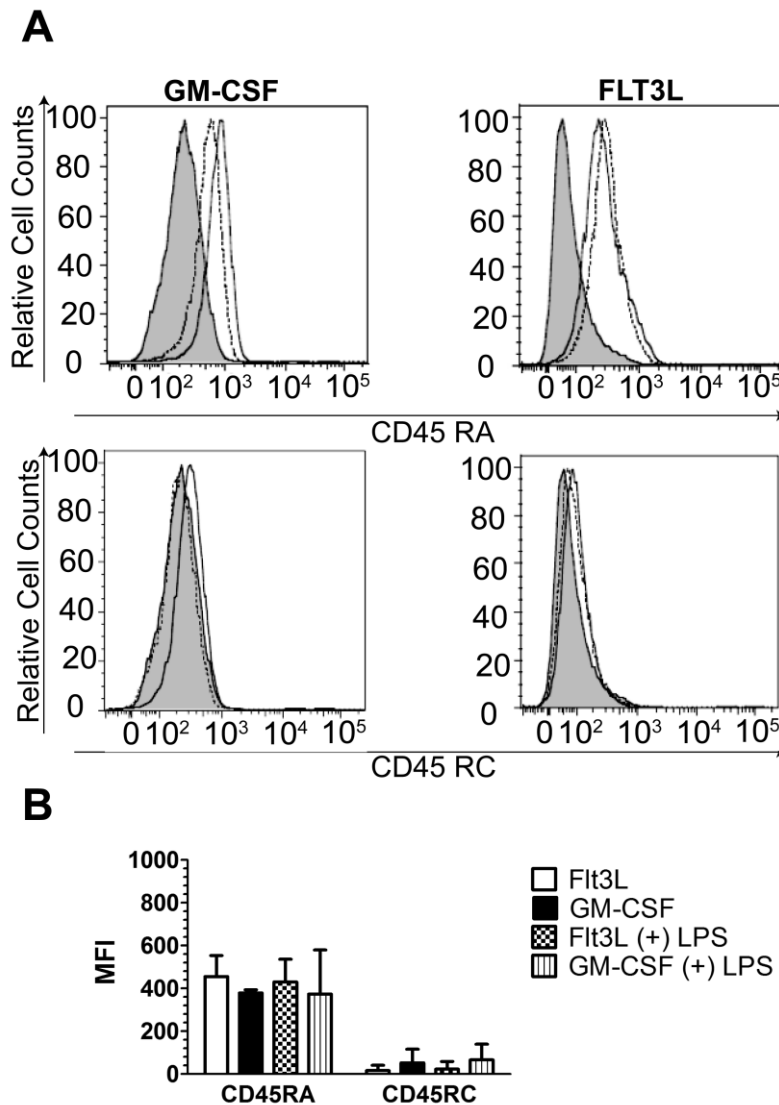
Anti CD45RB mAb was either cultured together with the GM-CSF- supplemented CD45<sup>+/+</sup> BM culture or, terminally differentiated day 7 CD45<sup>+/+</sup> CD11c-purified BMDCs were treated with anti CD45RB mAb for a 24 hr time period before further analysis was conducted. The culture of CD45<sup>+/+</sup> BM cells in the presence of anti CD45RB mAb was not found to significantly impact the total cell yield on day 7 of culture, despite the slight trend for anti CD45RB mAb cultured BMDCs (referred to as 23G2-cultured BMDCs) to have a lower number of cells (Figure 4.3A). Similarly the 24 hr treatment of purified day 7 CD11c<sup>+</sup> BMDCs with anti CD45RB mAb (referred to as 23G2-24 hr-treated BMDCs) did not reveal a significant effect of the antibody on the cell number (Figure 4.3), indicating that while anti CD45RB mAb may affect the apoptosis of naïve T cells <sup>133</sup>, it does not appear to have the same function in BMDCs. Additionally, neither culture nor 24 hr treatment of CD45<sup>+/+</sup> BMDCs with the anti CD45RB mAb caused a significant difference in the percentage of CD11c<sup>+</sup> cells present in the culture (data not shown).



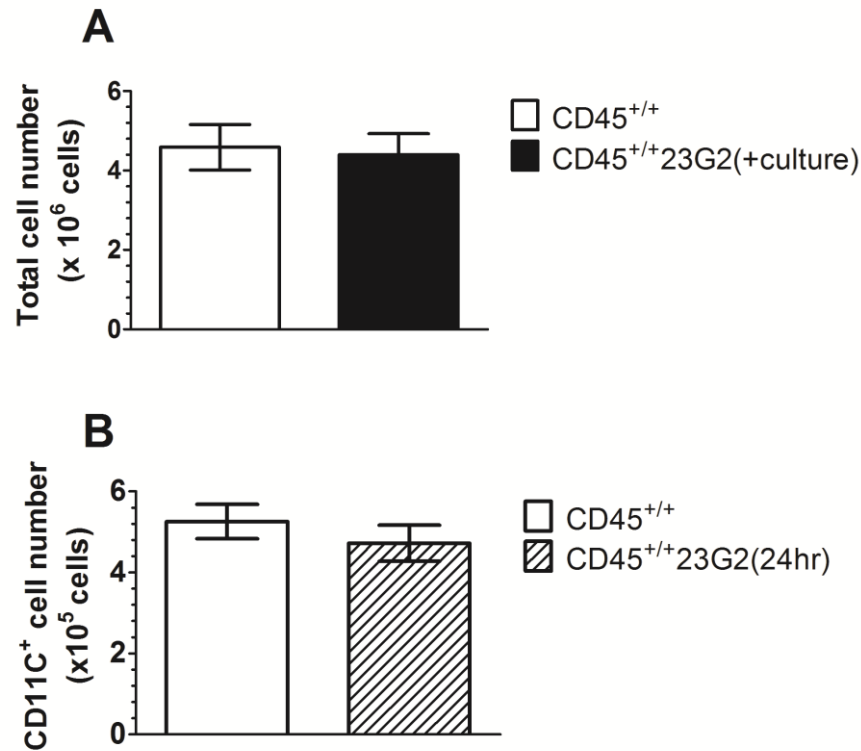
**Figure 4.1. Expression of CD45RB isoform on BMDCs in response to LPS stimulation.** BMDCs or freshly isolated splenocytes were stimulated for 18-24 hr with 100 ng/mL of LPS and then flow cytometric analysis performed to evaluate the response of CD45RB expression. **(A)** A representative flow cytometry plot depicting cell size of day 9 GM-CSF and Flt3L BMDCs based on FSC and SSC and **(B)** histograms of CD45RB expression shown for GM-CSF and Flt3L CD11c<sup>+</sup> BMDCs. The dashed line represents no LPS stimulation, the solid line represents LPS stimulation, and solid histogram represents the negative control. Expression levels are shown on log scale. Diagrams in (A) and histograms in (B) are representative of one biological replicate over three independent experiments, with 2-3 mice used per experiment. **(C)** Flow cytometry data graphed as the average mean fluorescence intensity (MFI) of CD45RB expression on CD11c<sup>+</sup> BMDCs from GM-CSF and Flt3L day 9 BMDC cultures, across two independent experiments, with three CD45<sup>+/+</sup> mice used per experiment. Errors bars represent SEM. **(D)** One representative of two independent experiments, displaying the average mean fluorescence intensity (MFI) of CD45RB expression on CD11c<sup>+</sup>MHCII<sup>+</sup>GM-CSF

BMDCs and CD11c<sup>+</sup>MHCII<sup>+</sup>PDCA-1<sup>-</sup> Flt3L BMDCs. Error bars represent SD of 3 biological replicates. Asterisks indicate statistical significance calculated using paired or unpaired Student *t*-test as appropriate. *Flt3L*, *Flt3L* BMDCs, *GM-CSF*, *GM-CSF* BMDCs, *LPS*, 18-24 hr 100 ng/mL *LPS* treatment.

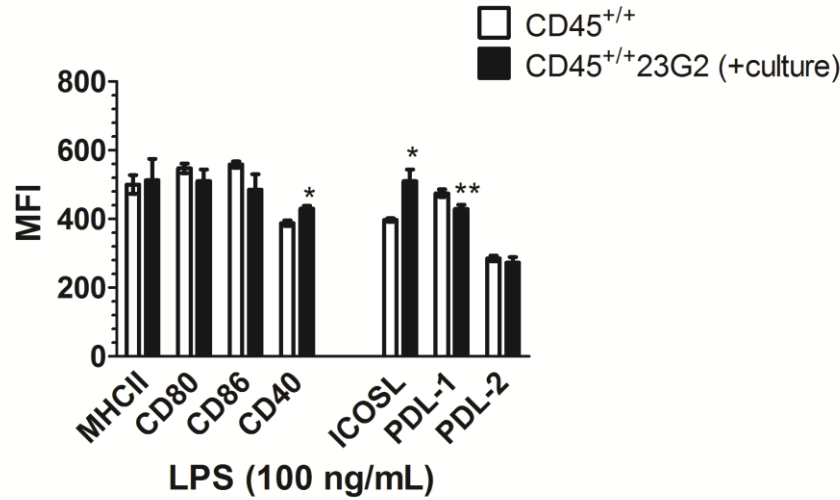
Culture of BMDCs with anti CD45RB mAb did not cause any significant alterations in the co-stimulatory and co-inhibitory molecule expression profile of CD45<sup>+/+</sup> BMDCs (data not shown). In response to LPS maturation, 23G2-cultured BMDCs did not exhibit a widespread suppression or elevation in MHCII, co-stimulatory or co-inhibitory molecules (Figure 4.4). Instead, particular molecules were significantly down regulated, such as PDL-1 ( $p = 0.0096$ ; CD45<sup>+/+</sup>: mean of  $475 \pm 11$ ; CD45<sup>+/+</sup> 23G2 (+culture): mean of  $430 \pm 12$ ), while others were slightly up-regulated, such as CD40 ( $p = 0.046$ ; CD45<sup>+/+</sup>: mean of  $388 \pm 8$ ; CD45<sup>+/+</sup> 23G2 (+culture): mean of  $430 \pm 8$ ). However, expression levels of ICOSL showed the most notable difference in 23G2-cultured BMDCs, in which ICOSL was significantly increased upon LPS stimulation ( $p = 0.0210$ ; CD45<sup>+/+</sup>: mean =  $397 \pm 5$  and CD45<sup>+/+</sup> 23G2 (+ culture): mean =  $510 \pm 34$ ).



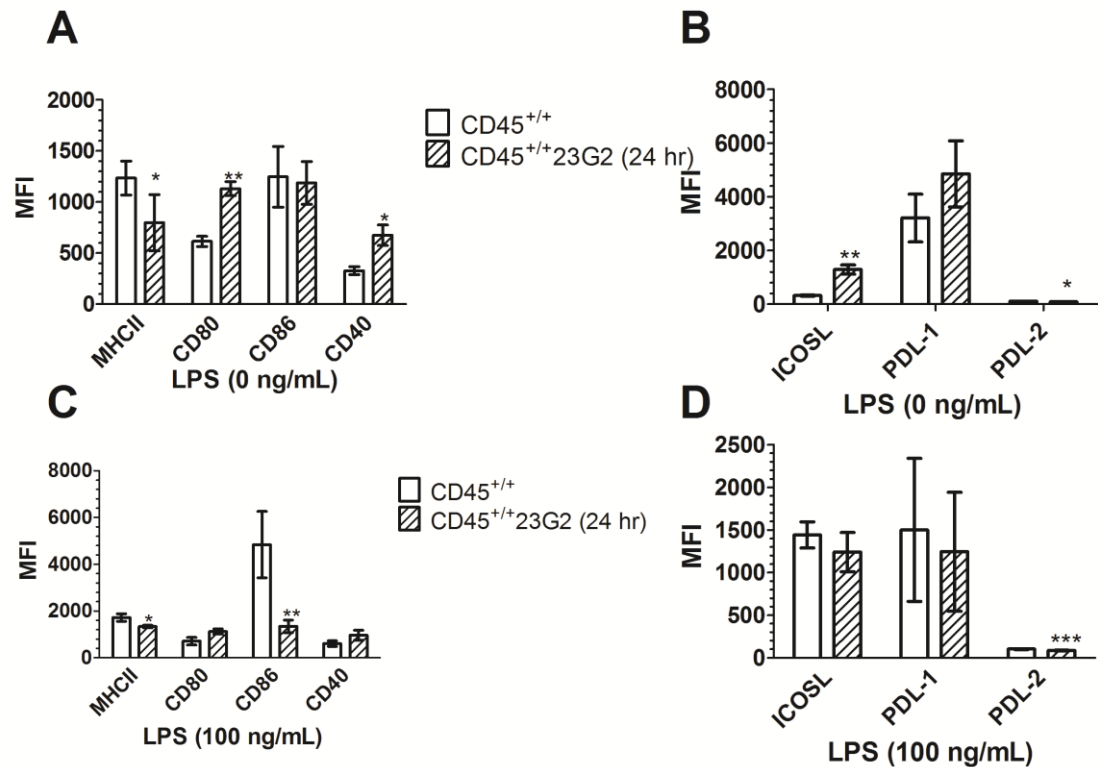
**Figure 4.2. Expression of non-CD45RB isoforms on GM-CSF and Flt3L-BMDCs.** (A) Histogram depicting the basal and LPS-induced expression levels of CD45 isoforms CD45RA (14.8) and CD45 RC (GL24) on GM-CSF and Flt3L BMDCs. Expression levels are shown on log scale. The dashed line represents no LPS stimulation, the solid line represents LPS stimulation, and the solid histogram represents negative control. Histograms are representative of one biological replicate over two independent experiments with 3 mice per experiment. (B) Graph shows flow cytometry data of non-CD45RB isoforms on *in vitro* cultured GM-CSF and Flt3L BMDCs upon LPS stimulation graphed as the average mean fluorescence intensity (MFI) over two independent experiments with three CD45<sup>+/+</sup> mice per experiment. Errors bars represent the SEM. *Flt3L*, *Flt3L* BMDCs, *GM-CSF*, *GM-CSF* BMDCs, *LPS*, 18-24 hr 100 ng/mL *LPS* treatment.



**Figure 4.3. The effect of anti CD45RB mAb (23G2) on the cell yield of a GM-CSF BMDC culture.** (A) The effect of culturing CD45<sup>+/+</sup> BM cells in a GM-CSF BM culture in the presence of anti CD45RB mAb (23G2) (10 ng/mL) on the total yield of day 7 non-adherent cells. The graphs shows the mean cell number across three independent experiments, with 3 mice per experiment. Error bars represent the SEM. (B) The effect of a 24 hr incubation of CD11c<sup>+</sup> GM-CSF BMDCs with anti CD45RB mAb (23G2) (10 ng/mL) on cell yield post-incubation. Graph depicts the mean number of CD11c<sup>+</sup> BMDCs obtained post antibody incubation across two independent experiments, with 3 mice used in each experiment. Error bars depict SEM. CD45<sup>+/+</sup>, untreated control; CD45<sup>+/+</sup> 23G2 (+ culture), BM cells of a 7-day GM-CSF BMDC culture cultured in the presence of treated of anti-CD45Rb mAb CD45<sup>+/+</sup> 23G2 (24 hr) BMDCs, day 7 CD11c<sup>+</sup> GM-CSF BMDCs treated with anti-CD45Rb mAb for 24 hr.



**Figure 4.4. The effect of culturing BMDCs with anti CD45RB mAb on the LPS-induced co-stimulatory and co-inhibitory molecule expression.** Day 7 GM-CSF BMDCs were either cultured in the absence or presence of anti CD45RB mAb (23G2) (10 ng/mL), then CD11c -purified BMDCs were stimulated with LPS (100 ng/mL) for 18-24 hr and expression levels of cell surface molecules determined by flow cytometry. Graph shows flow cytometry data graphed as the average mean fluorescence intensity (MFI) across 3 biological replicates within one experiment with errors bars representing SD. Shown is one representative of two independent experiments. Asterisks indicate statistical significance, calculated using paired Student *t*-test, between CD45<sup>+/+</sup> and CD45<sup>+/+</sup> 23G2 (+culture). *CD45<sup>+/+</sup>*, untreated control BMDCs; *CD45<sup>+/+</sup> 23G2 (+culture)* BMDCs, cultured for 7 days in the presence of treated of anti-CD45Rb mAb (23G2).



**Figure 4.5. The effect of 24 hr incubation of BMDCs with anti CD45RB mAb on LPS-induced expression of co-stimulatory and co-inhibitory molecules.** Day 7 CD11c<sup>+</sup> GM-CSF BMDCs were treated for a 24 hr time period with anti CD45RB mAb (23G2) (10 ng/mL) and then stimulated with LPS (100 ng/mL). Expression levels of cell surface molecules were determined by flow cytometry. The effect of anti CD45RB mAb (23G2) in the absence of LPS, on (A) co-stimulatory and (B) co-inhibitory molecule expression. The effect of anti CD45RB mAb upon LPS stimulation on (C) co-stimulatory and (D) co-inhibitory molecule expression. The graphs show the average mean fluorescence intensity (MFI) of 3 biological replicates within one experiment with errors bars representing SD. Shown is one representative of two independent experiments. Asterisks indicate statistical significance calculated using paired Student *t*-test between CD45<sup>+/+</sup> and CD45<sup>+/+</sup> 23G2 (24 hr). CD45<sup>+/+</sup>, untreated control BMDCs; CD45<sup>+/+</sup> 23G2 (24 hr) BMDCs, day 7 GM-CSF CD11c<sup>+</sup> BMDCs treated with anti CD45RB mAb for 24 hr.

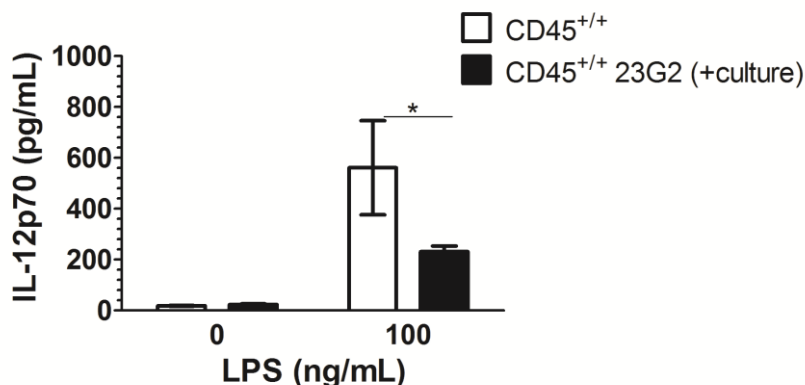
Examination of the co-stimulatory and co-inhibitory molecule profile of 23G2-24 hr-treated BMDCs also showed alterations in the expression of only specific molecules (Figure 4.5). Yet these alterations did not mirror those observed in 23G2-cultured BMDCs. In the absence of LPS there was a significant increase in levels of CD80 ( $p =$



0.0045; CD45<sup>+/+</sup>: mean =  $616 \pm 50$  and CD45<sup>+/+</sup> (24 hr): mean =  $1132 \pm 68$ ), CD 40 (p = 0.0332; CD45<sup>+/+</sup>: mean =  $328 \pm 38$  and CD45<sup>+/+</sup> (24 hr): mean =  $676 \pm 50$ ) and ICOSL (p = 0.081; CD45<sup>+/+</sup>: mean =  $326 \pm 24$  and CD45<sup>+/+</sup> (24 hr): mean =  $1295 \pm 171$ ). On the other hand, MHCII and PDL-2 were significantly decreased (MHCII: p = 0.00493; CD45<sup>+/+</sup>: mean =  $1234 \pm 166$  and CD45<sup>+/+</sup> (24 hr): mean =  $798 \pm 275$ ) and (PDL-2: p = 0.0129; CD45<sup>+/+</sup>: mean =  $116 \pm 3$  and CD45<sup>+/+</sup> (24 hr): mean =  $87 \pm 5$ ). With LPS stimulation, there was a significant decrease in the levels of MHCII (p = 0.0249; CD45<sup>+/+</sup>: mean =  $1725 \pm 161$  and CD45<sup>+/+</sup> (24 hr): mean =  $1335 \pm 53$ ) and PDL-2 (p = 0.0003; CD45<sup>+/+</sup>: mean =  $102 \pm 6$  and CD45<sup>+/+</sup> (24 hr): mean =  $86 \pm 5$ ). The most notable fold decrease however, occurred in CD86 expression level (p = 0.0036; CD45<sup>+/+</sup>: mean =  $4842 \pm 1418$  and CD45<sup>+/+</sup> (24 hr): mean =  $1352 \pm 266$ ).

#### ***4.1.3 GM-CSF BMDCs cultured with anti CD45RB mAb have reduced production of IL-12 production in response to LPS.***

As discussed previously, cytokine production plays a key role in determining the final T cell response, the effect of anti CD45RB mAb on cytokine production of BMDCs was examined by ELISA.

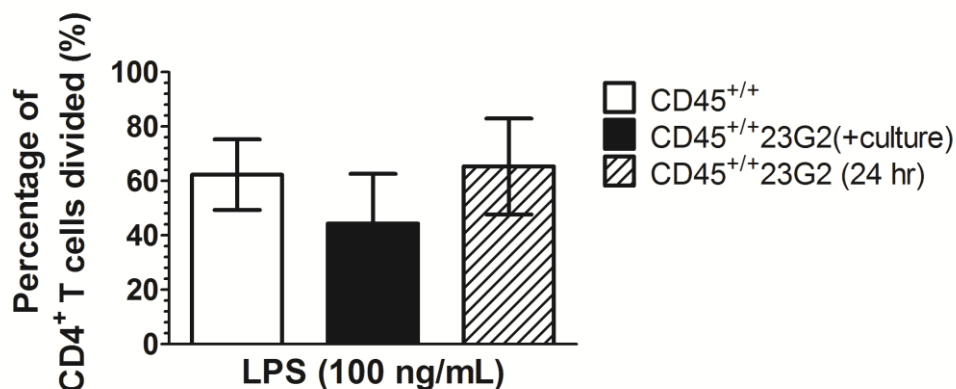


**Figure 4.6. The effect of culturing BMDCs with anti CD45RB mAb on BMDC LPS-induced IL-12 cytokine production.** Day 7 CD11c-purified GM-CSF BMDCs that were either cultured in the absence or presence of anti CD45RB mAb (23G2) (10 ng/mL) were then stimulated with LPS (100 ng/mL) for 18-24 hr and IL-12p70 production measured by ELISA. The graph shows the mean IL-12 concentration of 3 biological replicates within one experiment. Error bars represent the SD. *CD45<sup>+/+</sup>, untreated control BMDCs; CD45<sup>+/+</sup> 23G2 (culture), BMDCs from a 7 day GM-CSF BM culture in the presence of anti CD45RB mAb.*

Investigation revealed that 23G2-cultured CD11c<sup>+</sup> GM-CSF BMDCs produced significantly less of the biologically active form of IL-12, IL-12p70, ( $p = 0.0372$ ; 23G2-cultured: mean =  $561 \pm 184$  pg/mL) compared to untreated counterparts (untreated control: mean =  $231 \pm 22$  pg/mL) in response to 24 hour TLR 4 agonist stimulation (Figure 4.6). In contrast, 23G2-24 hr-treated BMDCs did not show significantly reduced IL-12 production, nor was IL-10 production significantly altered (data not shown).

#### ***4.1.4 GM-CSF BMDCs cultured with anti CD45RB mAb tend to display a decreased ability to drive in vitro CD4<sup>+</sup>T cell proliferation.***

The effect of anti CD45RB mAb on the functional ability of CD11c<sup>+</sup> GM-CSF BMDCs to drive T cell proliferation was assessed using an *in vitro* anti CD3 T cell proliferation assay performed by co-culturing either 23G2-cultured or 23G2-24 hr-treated BMDCs with anti-CD3 activated syngeneic CD4<sup>+</sup> CD25<sup>-</sup> naïve T cells.



**Figure 4.7. The effect of anti CD45RB mAb conditioning on the T cell stimulatory ability of BMDCs.** CD11c-purified day 7 GM-CSF BMDCs cultured with anti CD45RB mAb (23G2) (10 ng/mL) or day 7 CD11c-purified GM-CSF BMDCs treated for 24 hr with anti CD45RB mAb (23G2) (10 ng/mL), were stimulated with LPS (100 ng/mL) for 18-24 hr and co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> syngeneic naïve T cells activated by anti-CD3 mAb. The graph depicts T cell proliferation as the average percentage of T cells divided across three independent experiments (CD45<sup>+/+</sup> 23G2 (+culture)) and (CD45<sup>+/+</sup> 23G2 (24 hr)), or across four independent experiments (CD45<sup>+/+</sup>). (CD45<sup>+/+</sup>, n = 6; CD45<sup>+/+</sup> 23G2 (+culture), n = 5; CD45<sup>+/+</sup> 23G2 (24 hr), n = 3). Error bars represent SEM. CD45<sup>+/+</sup>, untreated control BMDCs; CD45<sup>+/+</sup> 23G2 (culture), BMDCs from a 7 day GM-CSF BM culture in the presence of 23G2 mAb; CD45<sup>+/+</sup> 23G2 (24 hr) BMDCs, day 7 BMDCs treated with 23G2 mAb for 24 hr.

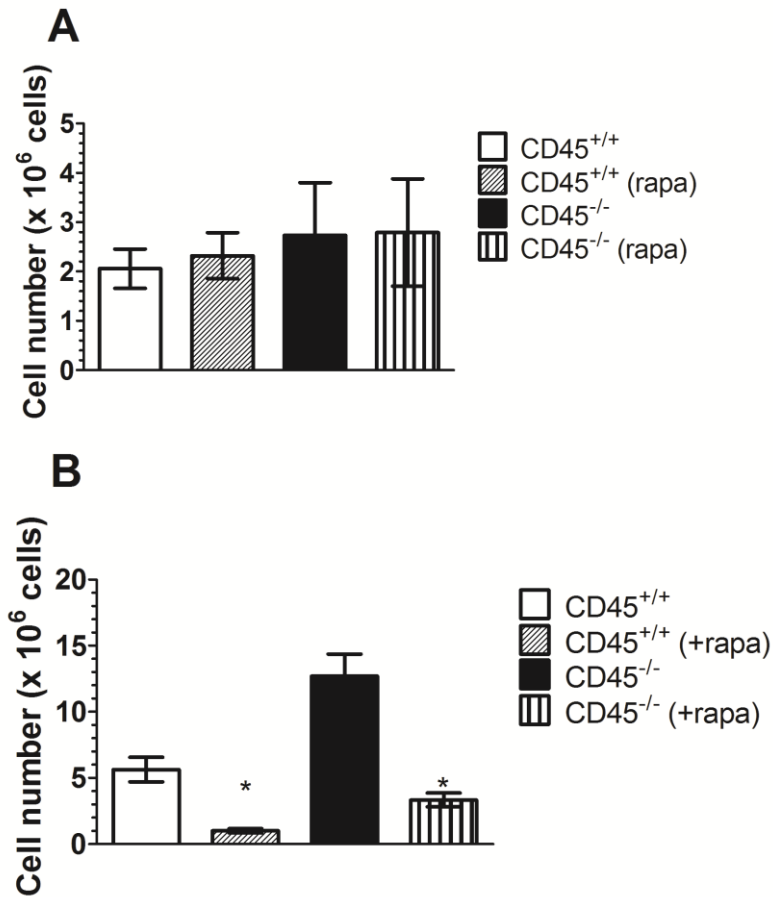
T cell proliferation was gauged as a percentage of the T cells divided on day 3 of the co-culture. Although the difference in the induced T cell proliferation between untreated control and 23G2-cultured BMDCs did not reach statistical significance, there was a trend for the 23G2-cultured BMDCs to exhibit diminished T cell proliferation (mean = 44.5 ± 18.3 %) compared to untreated counterparts (mean = 62.3 ± 13.0%) as shown in Figure 4.7. Meanwhile, co-culture of T cells with 23G2-24hr-treated BMDCs induced similar levels of T cell proliferation as untreated control BMDCs, with 23G2-24 hr-treated BMDCs co-cultures resulting in an average of 65.3 ± 17.6% T cells divided.

## 4.2 The Effect of CD45 Deficiency on the Rapamycin-Mediated Induction of Tolerogenic Dendritic Cells

Numerous reports have demonstrated that the tolerizing action of the clinically used and well known immunosuppressant rapamycin extends to DCs<sup>73,134,135</sup>. Evidence supports that rapamycin conditioning inhibits DC maturation and maintains an immature phenotype and tolerogenic function in DCs<sup>73,134,136</sup>. As we have previously looked at the effect of CD45 deficiency on BMDC maturation in response to the pro-inflammatory stimulus LPS, this section will focus on investigating whether CD45 plays a role in modulating the DC response to a tolerogenic signal, namely the inhibitor of mammalian target of rapamycin (mTOR), rapamycin.

### 4.2.1 *CD45 deficiency in BMDCs does not significantly alter the reduction of co-stimulatory and co-inhibitory molecule expression in response to LPS.*

On day 7 of GM-CSF BM culture, CD11c<sup>+</sup> BMDCs were purified from GM-CSF CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BM cultures and incubated with rapamycin (10 ng/mL) for 24 hr and then stimulated with LPS (100 ng/mL) for 18-24 hr. It was found that the incubation of day 7 CD11c<sup>+</sup> BMDCs with rapamycin did not adversely affect the total number of cells in comparison to control DCs (Figure 4.8A). However, the anti-proliferative properties of rapamycin<sup>137</sup> were observed when rapamycin was present in the BM culture from day 2 of culture Figure 4.8B, which resulted in a significantly diminished yield of CD11c<sup>+</sup> cell in both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs (CD45<sup>+/+</sup>: p = 0.0263; CD45<sup>-/-</sup>: p = 0.0243 ). Due to the limited number of cells obtained when rapamycin was included early in the BM culture, subsequent experiments used a 24 hr incubation of rapamycin on day 7 or 8 CD11c<sup>+</sup> BMDCs .

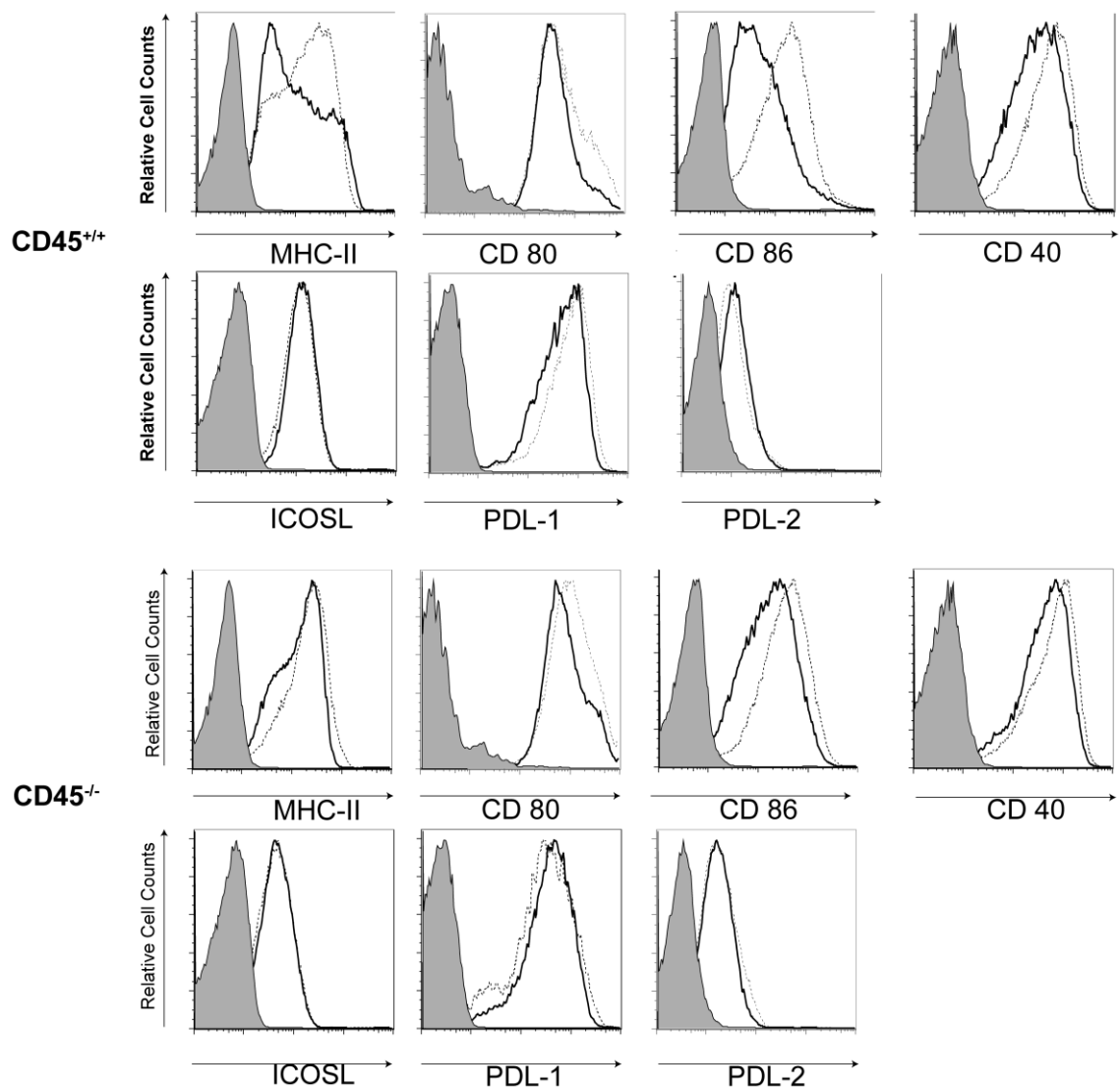


**Figure 4.8. The effect of rapamycin treatment on cell yield of CD11c<sup>+</sup> BMDCs. (A)** Data is graphed as the mean number of non-adherent cells obtained post- 24 hr rapamycin incubation of CD11c-purified day 7 BMDCs across three independent experiments, and each experiment performed with 3 mice per genotype. Errors bars represent the SEM. **(B)** The effect of the presence of rapamycin during a 7 day BM culture on the yield of day 7 CD11c<sup>+</sup> BMDCs. Graph shows the mean number of CD11c<sup>+</sup> cells obtained on day 7 of culture of across two independent experiments with errors bars representing the SEM (CD45<sup>+/+</sup>: n = 4, CD45<sup>-/-</sup> : n = 4). Errors bars represent SEM. Asterisks indicate statistical significance calculated using paired Student *t*-test between untreated and rapamycin-treated cells; (*rapa*), 24 hr-treated rapamycin BMDCs; (+ *rapa*), rapamycin-cultured BMDCs.

Rapamycin conditioned BMDCs have been reported to possess a phenotype similar to that of iDCs , with low expression levels of MHCII and co-stimulatory molecules, as well as resistance to upregulation of co-stimulatory molecules in the face of maturation stimulus<sup>73,136</sup>. Thus, we also evaluated the effect of rapamycin treatment

on the upregulation of co-stimulatory molecule expression of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs in response to LPS. Additionally, the effect of rapamycin on the LPS-induced expression of co-inhibitory molecule expression, which has not to current knowledge been determined, was also examined in this study.

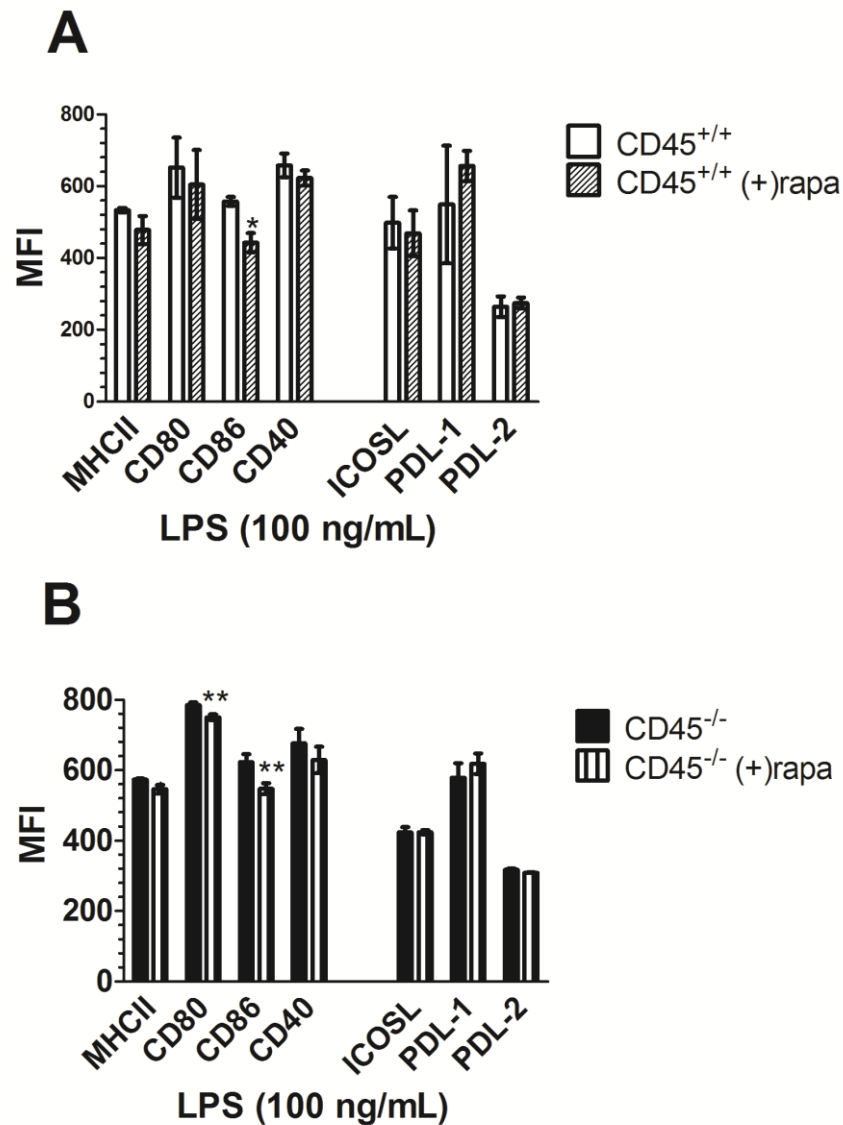
In the absence of LPS, rapamycin treatment did not bring about a significant change in the expression levels of MHCII, co-stimulatory molecules (CD80, CD86, CD40), or co-inhibitory molecules (ICOSL, PDL-1, PDL-2,) in CD45<sup>+/+</sup> BMDCs (data not shown ). The effect of rapamycin on expression levels of MHCII, CD40, ICOSL, and PDL-1 were similar for non-LPS-stimulated CD45<sup>-/-</sup> BMDCs (data not shown). However in CD45<sup>-/-</sup> BMDCs there was a slight but significant decrease in the expression of the co-stimulatory molecules CD80 ( $p = 0.0091$ ) and CD86 ( $p = 0.0012$ ), as well as the co-inhibitory molecule PDL-2 ( $p = 0.0023$ ) (data not shown).



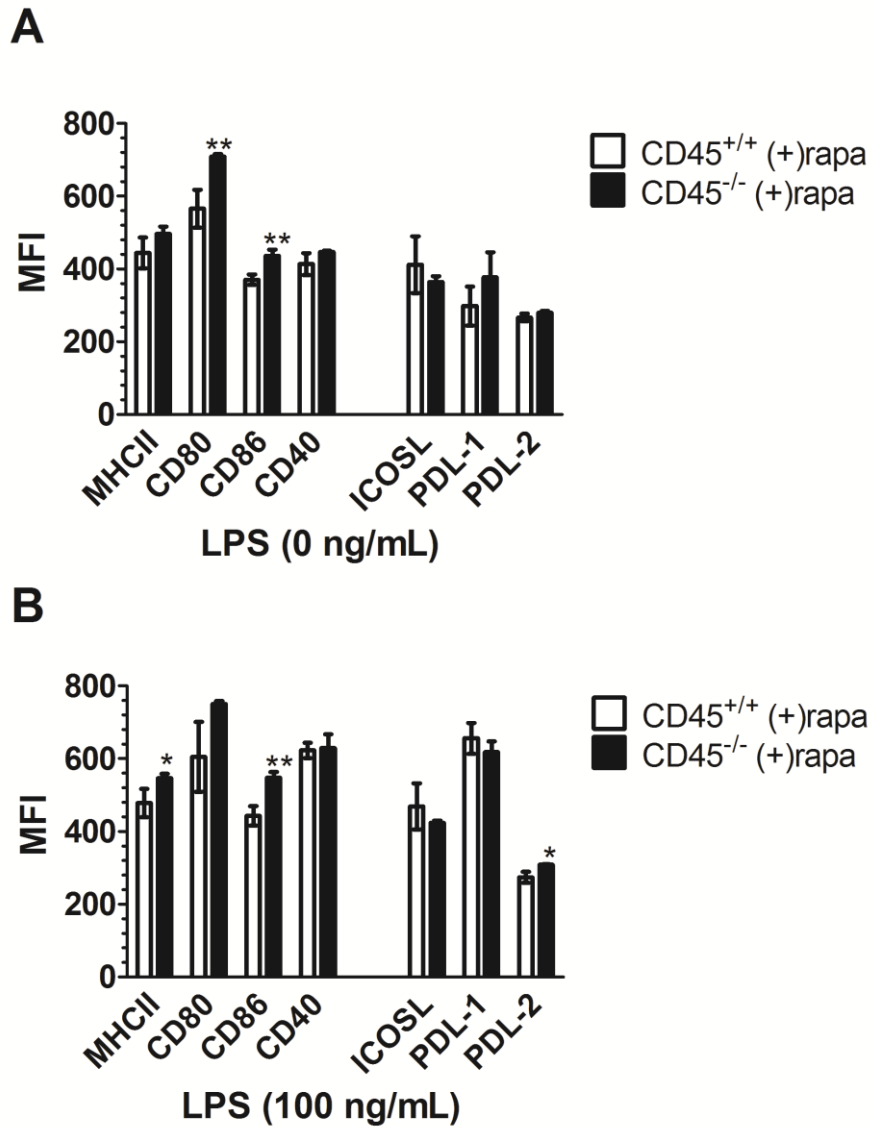
**Figure 4.9. The effect of rapamycin on the LPS-induced upregulation of co-stimulatory and co-inhibitory molecules on  $CD45^{+/+}$  and  $CD45^{-/-}$  BMDCs.** Expression levels depicted by histograms of MHCII, co-stimulatory (CD80, CD86, CD40) and co-inhibitory molecules (ICOSL, PDL-1, PDL-2) expression levels on day 7  $CD11c^{+}$   $CD45^{+/+}$  and  $CD45^{-/-}$  BMDCs after 18-24 hr stimulation with 100 ng/mL of LPS as determined by flow cytometry analysis. Expression levels are shown on log scale. Dotted line represents LPS-stimulated BMDCs, solid line represents LPS-stimulated, rapamycin treated BMDCs, and the filled histogram represents the negative control. One representative biological replicate is shown in each histogram across two independent experiments; each experiment used 3 mice per genotype.

The maturation stimulus LPS was used to determine whether CD45 deficiency in BMDCs was able to modulate rapamycin-induced inhibition of co-stimulatory molecule expression. Figure 4.9 and Figure 4.10 show that while there is a trend for rapamycin treatment of CD45<sup>+/+</sup> BMDCs to suppress the LPS-induced up-regulation of MHCII and the co-stimulatory molecules CD80 and CD40, only CD86 expression levels are diminished to a significant level ( $p = 0.0101$ ; CD86: CD45<sup>+/+</sup> BMDCs: mean =  $558 \pm 13$ ; CD45<sup>+/+</sup> (+) rapa: mean =  $443 \pm 27$ ). In LPS-stimulated CD45<sup>-/-</sup> BMDCs, there is a similar trend for decreased levels of MHCII and co-stimulatory molecules CD80, CD86, and CD40 and in this case the rapamycin-mediated decrease in LPS-induced expression levels of both CD80 and CD86 reached statistical significance (CD80:  $p = 0.0071$ ; CD45<sup>-/-</sup> BMDCs: mean =  $784 \pm 8$ ; CD45<sup>-/-</sup> (+) rapa: mean =  $750 \pm 8$ ) and (CD86:  $p = 0.005$ ; CD45<sup>-/-</sup> BMDCs: mean =  $623 \pm 23$ ; CD45<sup>-/-</sup> (+) rapa: mean =  $548 \pm 16$ ). Upregulation of CD86 expression in response to LPS was most noticeably decreased for both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs. Rapamycin treatment however did not significantly affect the LPS-induced levels of the co-inhibitory molecules ICOSL, PDL-1 and PDL-2 in either CD45<sup>+/+</sup> or CD45<sup>-/-</sup> BMDCs although a non-significant trend for increased PDL-1 expression with rapamycin treatment was observed in both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs (Figure 4.10). Rapamycin treatment did not ablate the higher expression of MHCII, CD80, CD86, and PDL-2 characteristic of the CD45<sup>-/-</sup> BMDC phenotype as shown in Figure 4.11.





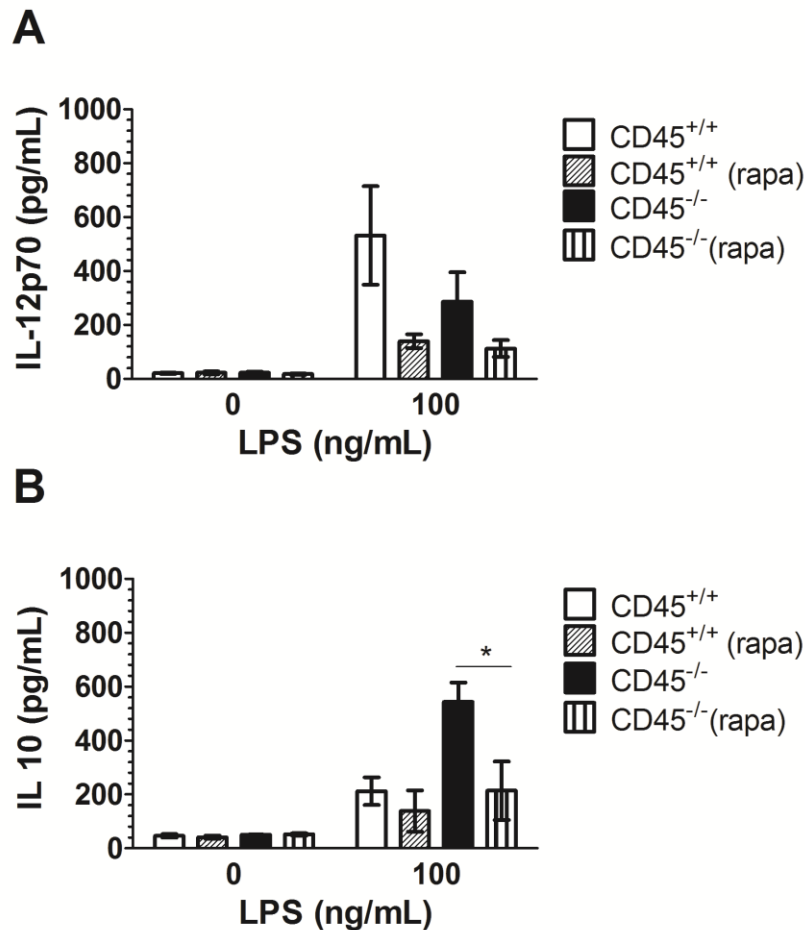
**Figure 4.10. The effect of rapamycin on LPS-induced upregulation of co-stimulatory and co-inhibitory molecule expression on CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs.** Day 7 CD11c<sup>+</sup> GM-CSF BMDCs were treated with rapamycin for a 24 hr incubation period and then stimulated with LPS (100 ng/mL). Cell surface molecule expression was determined by flow cytometry. Data is graphed to show co-stimulatory and co-inhibitory molecule expression on (A) CD45<sup>+/+</sup> and (B) CD45<sup>-/-</sup> BMDCs in the presence of LPS (100 ng/mL). Graph depicts the average mean fluorescence intensity (MFI) of three biological replicates within one experiment where error bars representing the SD. Shown is one representative of two independent experiments, each experiment used 3 mice per genotype. Asterisks indicate statistical significance calculated using paired Student *t*-test between untreated and rapamycin-treated BMDCs. (+) *rapa*, rapamycin 24 hr treated BMDCs.



**Figure 4.11. Comparison of the effect of CD45 deficiency on the co-stimulatory and co-inhibitory molecule expression of rapamycin-treated BMDCs.** (A) Cell surface molecule expression in the absence and (B) presence of LPS. Graphs depict the average mean fluorescence intensity (MFI) of three biological replicates within one experiment where error bars representing the SD. Shown is one representative of two independent experiments, with 3 mice per genotype used in each experiment. Asterisks indicate statistical significance calculated using unpaired Student *t*-test between CD45<sup>+/+</sup> (+) rapa BMDCs and CD45<sup>-/-</sup> (+) rapa BMDCs. (+) rapa, rapamycin 24 hr treated BMDCs.

#### ***4.2.2 Rapamycin impairs the LPS-induced production of IL-10 and IL-12 by CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs.***

To investigate cytokine production in this study, day 7 CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs were incubated with rapamycin (10 ng/mL) and then stimulated with LPS (100 ng/mL) for 18-24 hr period before IL-12p70 and IL-10 levels were measured by ELISA. Figure 4.12 depicts the effect of rapamycin treatment in the absence or presence of LPS on CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDC IL-12 and IL-10 production. Rapamycin treatment of both CD45<sup>+/+</sup> BMDCs resulted in a drastic although not statistically significant ( $p = 0.09$ ) reduction in IL-12 production (CD45<sup>+/+</sup>: mean =  $532 \pm 183$  pg / mL and CD45<sup>+/+</sup> (rapa): mean =  $140 \pm 25.4$  pg / mL). Rapamycin treatment also reduced IL-10 production from CD45<sup>+/+</sup> BMDCs, (CD45<sup>+/+</sup>: mean =  $212 \pm 51.2$  pg / mL and CD45<sup>+/+</sup> (rapa): mean =  $138 \pm 76.7$  pg / mL). In contrast to this, the magnitude of the reduction in IL-10 production caused by rapamycin treatment of CD45<sup>-/-</sup> BMDCs was significantly ( $p = 0.011$ ) impaired and reduced by 2.5-fold with rapamycin treatment (CD45<sup>-/-</sup>: mean =  $544 \pm 71.0$  pg / mL and CD45<sup>-/-</sup> (rapa): mean =  $214 \pm 109$  pg / mL). LPS-induced IL-12 production was also slightly impaired, but not significantly so, by rapamycin treatment in CD45<sup>-/-</sup> BMDCs (CD45<sup>-/-</sup>: mean =  $287 \pm 108$  pg / mL and CD45<sup>-/-</sup> (rapa): mean =  $113 \pm 30.9$  pg / mL).

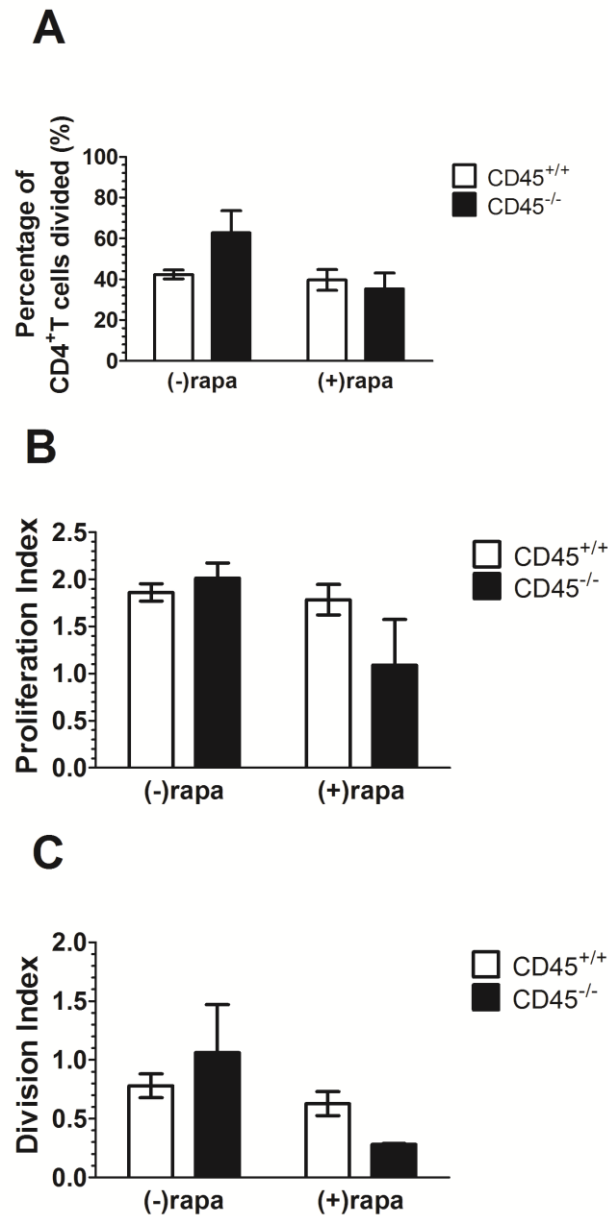


**Figure 4.12. The effect of rapamycin on the LPS-induced cytokine production of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs.** Data is graphed to show the mean concentration across four independent experiments; each experiment used 3 mice per genotype). Error bars represent SEM. Asterisks indicate statistical significance calculated using paired Student *t*-test. *Rapa*, rapamycin treated BMDCs.

#### 4.2.3 Rapamycin causes greater impairment of the T cell stimulatory capacity of CD45 deficient BMDCs.

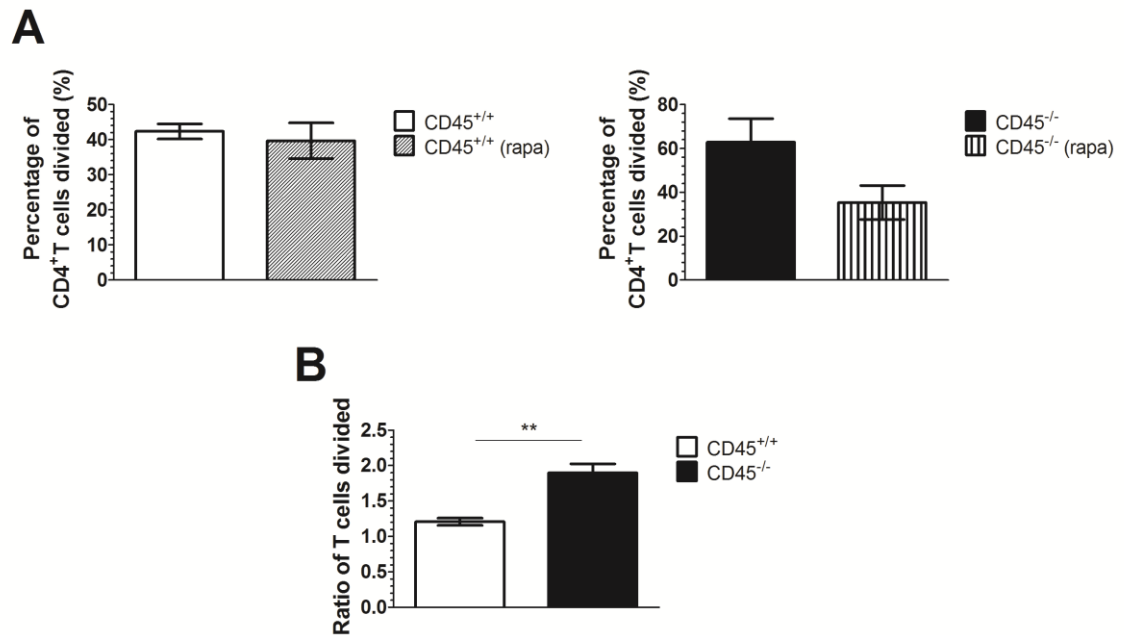
Another characteristic of rapamycin-conditioned DCs is that they are weak stimulators of T cells and induce hyporesponsiveness and apoptosis in alloreactive T cells<sup>73,136,138</sup>. Here, an *in vitro* antigen-dependent OT-II T cell proliferation assay was employed to gauge the T cell stimulatory ability of rapamycin-treated BMDCs. The BMDCs were incubated with rapamycin (10 ng/mL), and then simultaneously pulsed

with the peptide OVA<sub>323-339</sub> and stimulated with LPS (100 ng/mL), before co-culturing with CFSE-labelled CD4<sup>+</sup>CD25<sup>-</sup> OT-II T cells for a 72 hour period. We observed that CD45<sup>+/+</sup> rapamycin-treated BMDCs exhibited equal ability as untreated control CD45<sup>+/+</sup> BMDCs to drive T cell proliferation *in vitro* but interestingly, rapamycin had a more profound suppressive effect on the T cell stimulatory capacity of CD45<sup>-/-</sup> BMDCs. The percentage of T cells divided was similar for both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs (Figure 4.13 A), however, there was a non-significant trend for the rate of proliferation, as measured by the proliferation index, to be decreased in co-cultures stimulated by CD45<sup>-/-</sup> rapamycin-treated BMDCs (Figure 4.13B), and an overall trend for decreased T cell proliferation by CD45<sup>-/-</sup> rapamycin-treated BMDCs as seen by the division index values in Figure 4.13C. Figure 4.14 compares the effect of rapamycin on CD45<sup>+/+</sup> BMDCs and CD45<sup>-/-</sup> BMDCs by comparison of the T cells divided in the rapamycin treated BMDC co-culture to the T cells divided in the co-culture of the untreated BMDCs. This ratio reveals that rapamycin more profoundly dampened the ability of CD45<sup>-/-</sup> BMDCs to stimulate T cell proliferation as compared to CD45<sup>+/+</sup> BMDCs (Figure 4.14). This greater effect of rapamycin on CD45<sup>-/-</sup> BMDCs is due to the fact that normally, there is a tendency for CD45<sup>-/-</sup> BMDCs to stimulate CD4<sup>+</sup> CD25<sup>-</sup> naïve T cells to undergo greater proliferation than CD45<sup>+/+</sup> BMDC stimulated T cells. However, rapamycin treatment of CD45<sup>-/-</sup> BMDCs decreased T cell proliferation to similar levels as those seen to be induced by CD45<sup>+/+</sup> BMDCs (either untreated or rapamycin-treated).



**Figure 4.13. The effect of rapamycin on BMDC T cell stimulatory ability of BMDCs.**

Graph depicts (A) the percentage of T cells divided (B) the rate of T cell division as measured by the proliferation index and (C) the overall proliferation measured by the division index; taking into account the percentage of T cells divided and the rate of T cell proliferation. Bars on the graph are the mean value across two independent experiments with error bars representing SEM. Each experiment used 3 mice per genotype. (-) *rapa*, untreated BMDCs; (+) *rapa*, rapamycin 24 hr treated BMDCs.



**Figure 4.14. Comparison of the effect of rapamycin on the ability of LPS-stimulated CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs to drive T cell proliferation. (A)** The percentage of T cells stimulated to divide in BMDC: T cell co-cultures containing CD45<sup>+/+</sup> (left) or CD45<sup>-/-</sup> (right) BMDCs. **(B)** Ratio which equals: (percentage of divided T cells induced by untreated BMDCs) to (the percentage of divided T cells induced by rapamycin-treated BMDCs) for both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs. Bars on the graph represent the average across three independent experiments. Each experiment used 2 mice per genotype. Error bars represent SEM. Asterisks in (B) indicate statistical significance calculated using unpaired Student *t*-test. (*rapa*), rapamycin 24 hr treated BMDCs.

### 4.3 Discussion

The anti-CD45RB monoclonal antibody (23G2) has shown effectiveness in preventing allograft rejection in several animal transplantation models<sup>82–84,139</sup> implying a role for CD45 in the regulation of immune tolerance. However, *in vivo* administration of antibody allows it to act on the plethora of CD45-expressing leukocytes, including T and B lymphocytes, DCs, NK cells, macrophages, granulocytes and others<sup>83,89</sup>. This study sought to examine the effect of this anti-CD45RB mAb on DCs, using GM-CSF BMDCs as the prototypical DCs. Investigation revealed that GM-CSF BMDCs, although

expressing CD45, possessed low levels of the CD45RB isoform on the cell surface and expression of CD45RB remained unchanged upon LPS stimulation. On the contrary, Flt3L BMDCs not only expressed at higher expression levels of CD45RB than their GM-CSF counterparts, but also upregulated CD45RB in response to LPS; a pattern maintained with the exclusion of PDCA-1<sup>+</sup> pDCs from the Flt3L BMDC subset. Although the CD45RB expression was not studied on the individual CD8 $\alpha$ <sup>+</sup> or CD8 $\alpha$ <sup>-</sup> DC subsets of the Flt3L BMDCs, these observations may reflect differences in the regulation of CD45RB expression in different DC subtypes, as the DC subsets that differentiate *in vitro* in response to GM-CSF signalling are not identical to Flt3L-generated DCs<sup>107</sup>. The CD11c<sup>+</sup> DCs generated by BM culture with Flt3L were observed to be smaller and less granular than their GM-CSF BMDC counterparts as has been similarly seen by previous reports<sup>95,107,132</sup>. Research groups such as Xu *et al.*<sup>107</sup> report that GM-CSF DCs and Flt3L DCs represent different DC subsets, with GM-CSF DCs being the *in vitro* equivalent of monocyte derived “inflammatory” TNF $\alpha$  and inducible nitric oxide synthase DCs (also known as Tip DCs) whereas the Flt3L DCs better correspond to the steady state resident DCs, pDCs, CD8 $\alpha$ <sup>+</sup> cDCs and CD8 $\alpha$ <sup>-</sup> cDCs, found in the spleen *in vivo*<sup>107</sup>. Furthermore, the function of GM-CSF and Flt3L BMDCs may not be identical as Flt3L BMDCs have previously been described to stimulate vigorous *in vitro* T cell proliferation similar to GM-CSF BMDCs but various *in vivo* models demonstrated induced tolerance to antigen or tumours by Flt3L DCs<sup>140</sup>.

In this study we did not find a consistent downregulation of co-stimulatory molecules either in the absence or presence of LPS, to mimic an iDC phenotype for either 23G2-cultured BMDCs or 23G2-24 hr treated BMDCs and the lack of consistent clear-



cut results makes it difficult to interpret the effect of the antibody on DC expression of MHCII, co-stimulatory and co-inhibitory molecules. These results partially contrast those by Xia *et al.*<sup>91</sup> where it had been previously reported that culture with anti CD45RB mAb may arrest GM-CSF BMDCs in an immature state leading to decreased expression of MHCII and of the co-stimulatory molecules, CD40 and CD86, in response to TNF $\alpha$  and LPS stimulation. However, protocol differences between our study and that by Xia and colleagues must be taken into account as the latter employed both GM-CSF and IL-4 for the *in vitro* cultivation of BMDCs and used TNF $\alpha$  in addition to LPS to mature DCs. Another feature of tolerogenic DCs is reduced secretion of pro-inflammatory mediators and a poor ability to stimulate naïve T cells<sup>5,32</sup>. The observations of significantly reduced LPS-induced IL-12 production coupled with the trend for weaker ability to drive *in vitro* T cell expansion suggests that the 23G2-cultured BMDCs possess a less inflammatory DC with reduced immunogenic function. Despite no observation of an immature co-stimulatory molecule expression profile, the notably increased ICOSL expression on LPS-stimulated 23G2-cultured BMDCs may be important in shaping T cell response by promoting the production of IL-10 from CD4<sup>+</sup> T cells<sup>44,114,141</sup>. Our findings on the effect of anti CD45RB mAb (23G2) on DCs support the report of Qi *et al.*<sup>142</sup>, published during the completion of this study, that showed that DCs were required for the development of the anti-CD45RBmAb-induced immune tolerance<sup>142</sup>.

In this study it was observed that 23G2-cultured BMDCs resulted in a non-significant trend towards decreased ability to drive T cell proliferation in addition to a significantly diminished IL-12 production, while 23G2-24 hr treatment of CD11c<sup>+</sup> day 7 BMDCs affected neither the cytokine nor T cell stimulatory capacity. This suggests that

the developmental time point at which anti CD45RB mAb is administered (to BMDC precursor cells vs. terminally differentiated BMDCs) as well as the length of exposure to this antibody, is an important factor in determining the ability of anti CD45RB mAb to modulate DC function. However, as the anti CD45RB mAb is a whole Ab and retains the Fc portion, there is the possibility that binding of anti CD45RB mAb to Fc receptors on CD11c<sup>+</sup> DCs may have additionally altered BMDC response in a way independent of the effect on CD45RB isoform.

Anti CD45RB mAb has previously been shown to activate CD45RB tyrosine phosphatase enzymatic activity in T lymphocytes<sup>143</sup> and although it has not to date been explicitly shown whether the antibody exerts the same effect in DCs, the potential activating function of anti CD45RB mAb in combination with our findings, suggest that activation of CD45RB mediates a tolerogenic response in DCs. The association of CD45RB with tolerance may explain the results of CD45RB expression on *in vitro* BMDCs. GM-CSF BMDCs may already have differentiated to a Tip-DC like DC subtype and preferentially initiate a pro-inflammatory response and hence maintain low levels of CD45RB expression regardless of maturation stimulus, while the observed upregulation of CD45RB in response to LPS on Flt3L BMDCs may be occurring in the DC subset that helps to mediate *in vivo* tolerance. Additionally, the anti CD45RB mAb may act on DC precursors present in BM to inhibit BMDC inflammatory function by activating CD45RB isoform, which may subsequently negatively regulate the DC response to inflammatory or maturation signals. Our cumulative findings suggest that the CD45RB isoform may regulate the tolerogenic response of BMDCs.

Blockade of mTOR by rapamycin is documented *in vitro* to tolerize DCs such that DC maturation and ability to stimulate T cell proliferation is impaired<sup>73,135,138</sup>. In this study it was observed that there was a trend for rapamycin-treatment of GM-CSF BMDCs to inhibit LPS maturation in terms of upregulation of MHCII and co-stimulatory molecules but with no consistent effect on the expression of co-inhibitory molecules. The co-stimulatory molecule CD86 was the most susceptible to suppression by rapamycin as shown by the most consistently and prominently observed inhibition of CD86 expression. However, there was no obvious difference in the effect of rapamycin on BMDCs due to CD45 deficiency. Rapamycin treatment did not ablate the higher expression of MHCII, CD80, CD86, and PDL-2 characteristic of the CD45<sup>-/-</sup> BMDC phenotype. However, in the absence of LPS, rapamycin-treatment slightly but significantly depressed the expression of these same molecules, suggesting that CD45 may play a minor role in regulating mTOR-controlled pathways leading to MHCII, co-stimulatory and co-inhibitory molecule expression. These findings are generally in agreement with the effect of rapamycin on DCs<sup>73,138,144</sup> however protocol differences between this study and those in previous reports likely account for the less potent effect of rapamycin on LPS maturation of DCs observed in this study. Namely, in the experiments of this study rapamycin was not included throughout the duration of the BM cultures and only GM-CSF was provided as the DC growth factor. Findings of Hackstein *et al.*<sup>145</sup> showed that the late addition of rapamycin (added at day 5 of BM culture) inhibited a mature DC phenotype similarly to day 2 rapamycin-cultured BMDCs, but to a slightly lesser extent<sup>145</sup>.

The production of the cytokines IL-10 and IL-12 was observed to be diminished with rapamycin in both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs suggesting no effect of CD45 deficiency in the rapamycin-induced cytokine response. It has been shown that mTOR and glycogen synthase kinase (GSK3), both of which act downstream of phosphoinositide 3-kinase (PI3K), differentially regulate TLR-mediated IL-12 and IL-10 production by murine DCs<sup>146</sup>. Blockade of mTOR activity by rapamycin abrogates the negative regulation of IL-12 production and so enhances the release of IL-12<sup>48</sup>. This increase in IL-12 is due to a hindrance of the autocrine secretion of IL-10 that normally suppresses IL-12 production<sup>134,135</sup>. In contrast, GSK3 promotes IL-12 and suppresses IL-10 production thus, inhibition of GSK attenuates IL-12 production but increases IL-10 production by LPS stimulated DCs<sup>48,134,135</sup>. The more dramatic rapamycin-mediated impairment of IL-10 production in CD45<sup>-/-</sup> BMDCs, suggests that the distinctive LPS-induced IL-10<sup>hi</sup> cytokine profile observed in CD45<sup>-/-</sup> BMDCs may in part be dependent on an enhancement of mTOR dependent cytokine production pathways. A potential explanation for the observed reduction in the production of both IL-10 and IL-12 in both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs, is that the prolonged exposure to rapamycin which was present throughout the LPS stimulation, may have caused a global inhibition of the activity of factors responsible for initiation of translation, resulting in a global decrease of BMDC cytokine synthesis and secretion<sup>147</sup>. Results show that rapamycin-treated CD45<sup>+/+</sup> BMDCs did not exhibit any impairment compared to control CD45<sup>+/+</sup> BMDCs in the ability to drive T cell proliferation in an antigen-dependent OT-II T cell proliferation assay. On the other hand, the T cell stimulatory ability of CD45<sup>-/-</sup> BMDCs was significantly impaired by rapamycin treatment. This suggests that the slightly enhanced T

cell stimulatory ability of CD45<sup>-/-</sup> BMDCs may be in part due to an mTOR dependent mechanism. Our observed results on the effect of rapamycin on DCs differ from that of previous reports due to protocol differences as the immature phenotype and corresponding poor T cell stimulatory capacity of rapamycin-conditioned DCs is IL-4 dependent in murine systems and Hackstein *et al.*<sup>145</sup> found that in GM-CSF BMDCs cultured in the absence of IL-4, the suppressive effect of rapamycin on DC maturation was lost<sup>145</sup>. Nonetheless, the effect of rapamycin on CD45<sup>-/-</sup> BMDCs suggests a connection between CD45 and mTOR activity. Data from Cross *et al.*<sup>79</sup> found that the loss of CD45 in BMDCs did not find any obvious differences in the phosphorylation status of protein kinase B in CD45<sup>-/-</sup> BMDCs but the relationship between CD45 and downstream mTOR pathways is an area that has not been as yet exhaustively researched. This validates further investigation on the effect of CD45 deficiency on signalling pathways in BMDCs to elucidate the mechanisms responsible for the CD45<sup>-/-</sup> BMDC phenotype.

## CHAPTER FIVE: SUMMARY AND FUTURE PERSPECTIVES

### 5.1.1 Summary

The aims of this thesis were to discern whether CD45 could modulate the tolerogenic response of DC to extracellular signals. In this study, we observed that in the absence and presence of maturation stimuli CD45<sup>-/-</sup> BMDCs had an altered phenotype with elevated expression of MHCII, PDL-2, and the co-stimulatory molecules CD80 and CD86. In contrast to this seemingly activated status, CD45<sup>-/-</sup> BMDCs exhibited an altered cytokine profile in response to LPS with significantly greater production of IL-10 and a concomitant decrease in IL-12. However, the observed differences did not translate into obvious differences in the ability to drive T cell proliferation. Rapamycin was used as a tolerogenic cue to induce a tolerogenic phenotype and function similar to that of iDCs. Treatment of terminally differentiated CD11c<sup>+</sup> BMDCs with rapamycin resulted in a trend for slightly decreased co-stimulatory molecule expression, especially so for CD86, but with no obvious differences in the response of CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs. Despite the similar reduction in IL-12 and IL-10 cytokine production exhibited by both CD45<sup>+/+</sup> and CD45<sup>-/-</sup> BMDCs when treated with rapamycin, T cell stimulatory capacity of

CD45<sup>-/-</sup> BMDCs was more profoundly affected by rapamycin treatment. The trend for CD45<sup>-/-</sup> BMDCs to possess an enhanced ability to drive *in vitro* T cell proliferation that was abrogated by rapamycin treatment suggestive of altered mTOR activity in BMDCs in the absence of CD45.

Additionally, the regulation of specific CD45 isoforms may mediate the tolerogenic response in DCs as the administration of the anti CD45RB mAb (23G2) to

BM DC precursors was able to alter BMDC function by significantly decreasing IL-12 production with a trend for reduced T cell stimulatory capacity. This effect was not observed upon administration of anti CD45 RB antibody to fully differentiated CD11c<sup>+</sup> BMDCs suggesting that these BMDCs may already committed to a certain inflammatory response. Moreover the differential regulation of CD45RB isoform on GM-CSF BMDCs compared to their Flt3L BMDC counterparts in response to TLR4 stimulus LPS, suggests that CD45's role in DCs may be further complicated by different functions of CD45 in the diverse DC subsets in an isoform-specific manner.

Finally, along with the enhanced prevalence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the peripheral lymphoid tissues of CD45<sup>-/-</sup> mice, the most striking *in vivo* findings was the observation that CD45 deficiency in the innate immune system allowed for greater Treg mediated prevention of colitis in an adoptive T cell transfer model. This survival advantage conferred by CD45 deficiency was found to correlate with an increased proportion of Tregs at the LP in CD45 deficient RAG mice. This raises the question as to whether loss of CD45 in intestinal immune cells particularly DCs and macrophages, changes the gut environment in such a way as to result in altered interactions with Tregs promoting Treg survival, or immunosuppressive function.

In conclusion, these findings suggest that while the CD45RB isoform may specifically inhibit an inflammatory response in DCs, the loss of all CD45 isoforms in BMDCs may potentially mediate T tolerance through immune deviation to a Tr1 development in response to pro-inflammatory stimulus and the loss of CD45 in multiple innate immune cells may cumulate in an intestinal environment that promotes Treg expansion or function *in vivo*.

### 5.1.2 Future Perspectives

Many future avenues still remain to determine how CD45 regulates the DC tolerogenic response to stimulus. This includes *in vitro* studies to extend the findings of this dissertation and determine whether the LPS-induced IL-10<sup>hi</sup> profile of CD45<sup>-/-</sup> BMDCs is able to exert a tolerogenic function by promoting the development of IL-10 secreting Tr1 cells. Additionally, investigation into whether the enhanced IL-10 production of CD45<sup>-/-</sup> BMDCs is exclusively carried out by TLR4 or if stimulation with TLR3, a TLR that also utilizes the MyD88-independent pathway, can bring about this skew in IL-10 and IL-12 production in DCs, will refine the search for the CD45-regulated signalling pathway that leads to cytokine production. In light of the ability of Tregs to affect DC function, investigation of the effect of Tregs on CD45<sup>-/-</sup> BMDC phenotype and function is another potential aspect of exploration. While focus thus far has looked at the response of CD45<sup>-/-</sup> DCs, to TLR agonists, and here to rapamycin and the anti-CD45RB mAb, it may also be of benefit to look more closely at the role of CD45 in signalling downstream of cytokine ligation to respective receptors on DCs. In the mast cells, the hyper-activation of the cytokine-mediated JAK/STAT kinases due to loss of CD45 may be similarly affect DC response to DC-polarizing pro- and anti-inflammatory cytokines.

Ideally, to uncover how CD45 deficiency in DCs specifically is able to better mediate a Treg mediated prevention of colitis, future investigation would benefit from the use of a conditional genetic knockout mouse strain. The use of a conditional CD11c-specific CD45 knock out RAG strain in a naïve T cell colitis and Treg mediated prevention of colitis would enable researchers to determine the contribution of solely DCs to the altered outcome of colitis. Among the steps to uncover the underlying reason



of the greater protection from T cell mediated colitis gained by 45-RAG, is to determine exactly how the lack of CD45 in the innate immune system may affect Tregs whether that be Treg function, induction, expansion or survival. This may be achieved by variations of Treg mediated prevention of adoptive T cell colitis including: co-injection by of naïve T cells and Tregs of different congenic markers to allow the prevalence of Treg induction from naïve T cells to be quantified at several time points, the use of proliferation dye labelled Tregs to observe *in vivo* Treg proliferation, the use of flow cytometry to determine Foxp3 expression over the course of colitis and quantification of factors such as IL-10, TGF- $\beta$  and RA in CD45-deficient mice during colitis. Additionally, future examination of the effect of CD45 deficiency in the intestinal macrophage subsets as well as gut DCs will also help to unravel the role of CD45 in intestinal inflammation.

Findings in this study suggest that the CD45RB isoform may act as a positive regulator of the tolerogenic function of DCs by decreasing both IL-12 production and the ability drive T cell proliferation in response to the TLR stimulus LPS. Investigation of CD45RB shows that although present, the differential upregulation of the CD45RB isoform on GM-CSF BMDCs and FLt3L BMDCs, upon TLR 4 ligation suggests that CD45RB may not play the same role in every DC subset. Further studies examining the CD45RB isoform expression and effect of anti-CD45RB mAb on CD45 activity in different *in vitro* cultured and *ex vivo* DC subsets to elucidate whether CD45RB carries out a similar global function in DCs and if anti CD45RB mAb may exert a tolerogenic effect on all DC subsets.

Rapamycin treatment was observed to inhibit the trend for increased ability to stimulate CD4<sup>+</sup> T cell proliferation by CD45<sup>-/-</sup> BMDCs, implicating a connection

between CD45 function and pathways downstream of mTOR. Of note, PI3K- mTOR signalling downstream of Flt3L controls DC development and administration of rapamycin impairs DC development *in vitro*, especially the pDCs and the CD8 $\alpha$ <sup>+</sup> like cDCs<sup>109</sup>. Conversely, DC-specific hyperactivation of mTOR enhanced Flt3L-driven DC development in culture and caused an expansion of CD8 $\alpha$ <sup>+</sup> and CD103<sup>+</sup> cDCs *in vivo*<sup>109,148</sup>. This is an interesting observation in relation to CD45, as Monotoya *et al.*<sup>78</sup> reported increased numbers of splenic pDCs in CD45<sup>-/-</sup> mice and Cross *et al.*<sup>79</sup> observed an enhanced frequency of splenic CD11c<sup>+</sup> CD8 $\alpha$ <sup>+</sup> DCs<sup>78,79</sup>. In combination with the findings of this study, previous reports tentatively suggest a link between CD45 and mTOR signalling that warrants further investigation into the influence of CD45 and its substrates on pathways downstream of mTOR.

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