Investigating the immuno-regulatory roles of beta2 integrins

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Abstract

The balance of the immune system between activation versus tolerance is essential for mounting responses to pathogens whilst preventing excessive or inappropriate responses that cause host damage. The β_2 integrins (CD11a/b/c/d) are leukocyte-specific adhesion receptors that play essential roles in regulating immune responses. Many studies have focussed on the roles of β_2 integrins in contributing to immune activation including: leukocyte migration, immune synapse formation and phagocytosis. However, there is evidence that β_2 integrins can also play immuno-regulatory roles. The overall aim of this thesis was to explore the regulatory roles of β_2 integrins in dendritic cells (DCs) and γδ T cells.

Previous studies have shown in the absence of functional β_2 integrins, DCs display enhanced activation and are capable of inducing greater T cell responses. I identified that specifically, the absence of CD11a or CD11c, but not CD11b or CD11d, leads to increased co-stimulatory molecule expression on DCs generated *in vitro* and greater numbers of DCs in the spleen *in vivo*. CD11a knockout (KO) DCs were also capable of inducing enhanced T cell responses. These findings are the first to highlight CD11a and CD11c as negative regulators of DC function.

In studies using β_2 integrin-deficient mice, the development of skin and oral mucosal inflammation are associated with an expansion of γδ T cells therefore, I hypothesised that β_2 integrins are a novel regulator of γδ T cells. I characterised γδ T cells in β_2 integrin (CD18) KO mice and found an increased number of $V\gamma6V\delta1^+$ γδ T cells in the spleen, blood, lungs and uterus with enhanced IL-17 production. Thymic development of $V\gamma6^+$ cells was unaffected by integrin loss but development of $V\gamma4^+$ cells was impaired. $V\gamma6^+$ cells showed similar proliferative capacity however, this subset was associated with reduced expression of apoptotic markers, indicating the accumulation of these cells is likely due to increased survival. Together, this data highlights a previously undescribed role for β_2 integrins in promoting $V\gamma4^+$ γδ T cell development and regulating $V\gamma6^+$ γδ T cell numbers in the periphery.

These findings emphasise the importance of β_2 integrins in regulating immune responses. Further work to understand the downstream pathways of this regulation will identify potential therapeutic targets for diseases where DC or γδ T cell responses are dysregulated.

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Author’s Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Definitions/Abbreviations

+ve ctrl	Positive control
7AAD	7-Aminoactinomycin D
APC	Antigen presenting cell
Bcl-xL	B-cell lymphoma-extra large
Bcl2	B-cell lymphoma 2
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BrdU	Bromodeoxyuridine
Btnl	Butyrophillin
CCL	Chemokine ligand
CCR	Chemokine receptor
cDC	Conventional dendritic cell
CDK	Cyclin-dependent kinase
CDP	Common dendritic cell precursor
CFA	Complete Freund's adjuvant
CFSE	Carboxyflurescein succinimidyl ester
CNS	Central nervous system
cTEC	Cortical thymic epithelial cell
CTLA4	Cytotoxic T-lymphocyte associated protein 4
DAG	diacylglycerol
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Ep	Epithelium
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand

FCS	Foetal calf serum
FcγRI	Fc-gamma receptor 1
FMO	Fluorescence minus one
FTOC	Foetal thymic organ culture
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factors
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanine triphosphate
HBSS	Hanks' balanced salt solution
Het	Heterozygous
HEV	High endothelial venule
Hi	high
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hom	homozygous
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
JAM-A	Junctional adhesion molecule A
KO	Knockout
LAD	Leukocyte adhesion deficiency
LN	Lymph node
Lo	Low
LP	Lamina propria
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
medLN	Mediastinal lymph node
MF	macrophage

MHC	Major histocompatibility complex
MICA/B	MHC class-I-related chain A/B
mLN	Mesenteric lymph nodes
mo-DC	Monocyte-derived dendritic cell
MS	Multiple sclerosis
MZ B cell	Marginal zone B cell
NFκB	Nuclear factor kappa light chain enhanced of activated B cells
NK	Natural killer
Ns	Not significant
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
pDC	Plasmacytoid dendritic cell
pLN	Peripheral lymph nodes
PMA	Phorbol 12-myristate 13-acetate
RBC	Red blood cell
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute 1640
RT	Room temperature
SD	Standard deviation
SI	Small intestine
SLE	Systemic lupus erythematosus
SMAC	Supramolecular activation clusters
Sox	SRY-Box
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tfh	T follicular helper cell
TGFβ	Transforming growth factor beta

Th1	Type 1 helper T cell
Th17	T helper 17 cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
tolDC	Tolerogenic dendritic cell
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WT	Wild type

Chapter 1 Introduction

1.1 The Role of the Immune System

The primary function of the immune system is to identify and eliminate abnormal/foreign cells and pathogens, while remaining tolerant to self. The balance between activation of the immune system versus immune tolerance is important in maintaining responses to foreign pathogens while preventing inappropriate immunity against self-peptides[1]. In autoimmune diseases such as rheumatoid arthritis, psoriasis and diabetes, there is a loss of self-tolerance, however current therapies only target the resulting inflammation but not the underlying dysregulation of the immune response. Understanding the mechanisms of immune regulation and how this becomes aberrant in disease is required for development of the next generation of drugs which aim to re-establish immune tolerance[2,3].

1.2 Integrin Receptors

The control of immune cells occurs at many levels, one of which is through receptors expressed on the cell surface, such as integrin receptors. Integrin receptors are transmembrane αβ heterodimers that are expressed on the surface of a wide variety of cell types, including leukocytes. There are 18 α subunits and 8 β subunits that together form 24 different heterodimers[4] (Figure 1-1). These heterodimers differ in their cellular expression and in particular the β_2 and β_7 families are expressed exclusively on immune cells. Integrin receptors are involved in a range of cell functions including cell adhesion, leukocyte function, inflammation, cell migration, angiogenesis and wound healing[5–8]. Integrins therefore play an important role in regulating both homeostasis and responses to infection or disease.

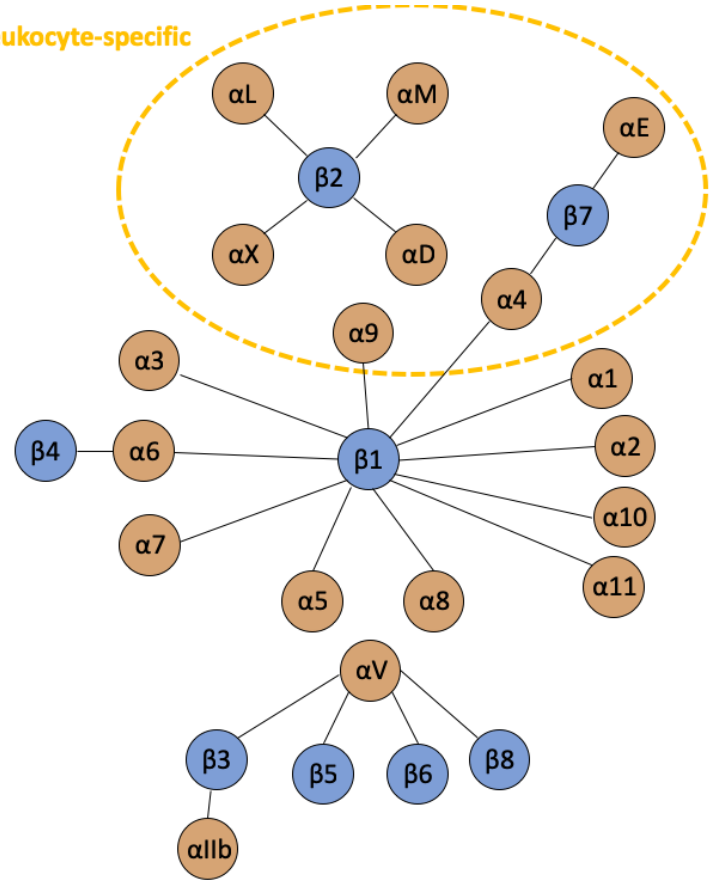

Figure 1-1 Integrin receptor families
Adapted from [9]. The 24 different integrin heterodimers that can form from combinations of the 18 α subunits and 8 β subunits. Leukocyte-specific integrin receptors are highlighted with a yellow dotted circle.

Integrins participate in bi-directional signalling termed 'inside-out' and 'outside-in' signalling which contributes to cellular function. Under steady state conditions they exist in an inactive form on the cell surface until receiving a stimulating signal that induces a conformational change enabling the receptor to bind extracellular ligands (inside-out signalling)[10]. Binding of extracellular ligands then induces intracellular signalling downstream of the integrin receptor that will modify aspects of cell behaviour such as proliferation, motility or gene expression (outside-in signalling) [7].

Integrin receptors exist in three different conformational states (Figure 1-2). In the inactive conformation the extracellular region is bent, with both α and β chains folded inwards towards the cell membrane and the ligand-binding region is therefore inaccessible, giving the receptor low affinity[11]. The close proximity of α and β cytoplasmic regions is thought to retain the receptor in its inactive state[12,13]. In the intermediate affinity state the extracellular domain extends out from the cell membrane, the ligand binding region displays intermediate affinity and there is an increased distance between the α and β intracellular regions[14]. Transition into the

high affinity conformation results in greater separation of the α and β chain cytoplasmic tails, altering the ligand-binding domain into its highest affinity[15].

These changes in the structure/affinity of integrin receptors are regulated by intracellular signalling cascades that alter interactions between integrin cytoplasmic tails through the binding of intracellular proteins. The triggering of these cascades that ultimately result in integrin activation can occur downstream of chemokine receptors, B- and T-cell receptors and selectins. Integrin receptors are maintained in an inactive state when bound to filamin, which stabilises the clasp between the cytoplasmic tails[16]. Rap1 is a GTPase that plays an important role in activating integrin receptors. The activation of Rap1 occurs in response to signals in the cell that induce the second messengers Ca^{2+} and diacylglycerol (DAG) or protein kinase C signalling, which activate guanine nucleotide exchange factors (GEFs), that in turn will activate the GDP to GTP exchange of Rap1[17]. Active Rap1 can then recruit and activate talin[18]. Talin binds to the β chain cytoplasmic tail, which disrupts the interaction between the α and β chains and alters the structural conformation of the receptor, to increase ligand affinity[19]. The subsequent binding of kindlin to a different region on the β chain tail further increases the affinity of the integrin receptor[20] and this activation of both talin and kindlin are essential for mediating cell adhesion[21]. Together, these intracellular signalling molecules contribute to inside out signalling by activating integrin receptors.

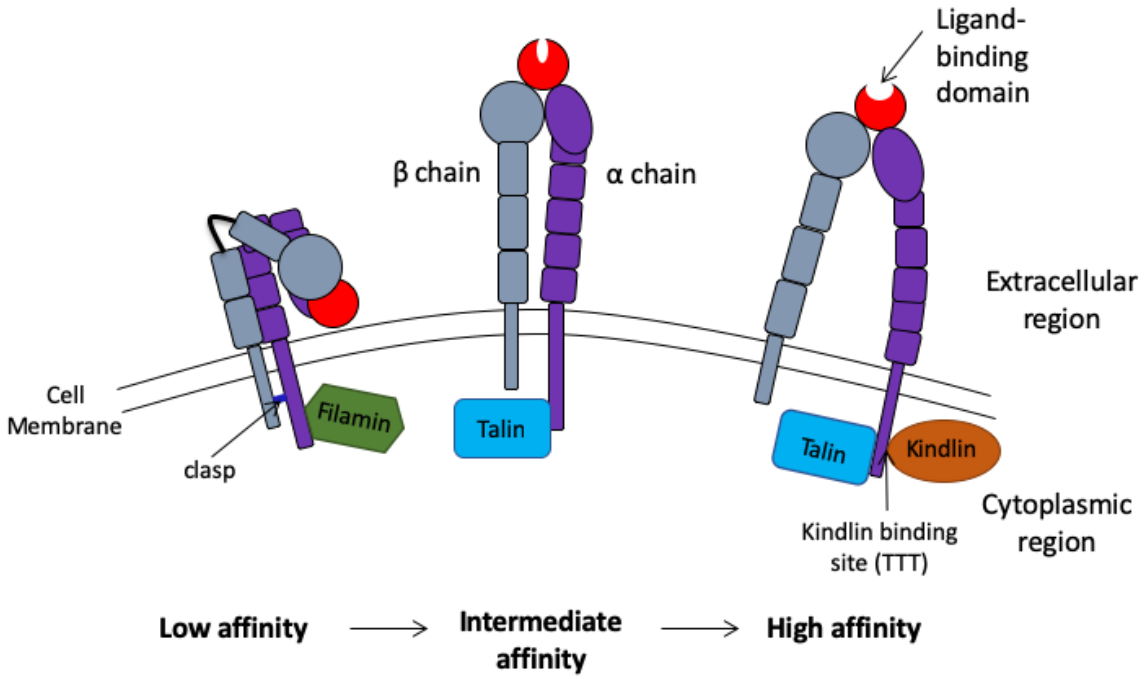

Figure 1-2 Structure and activation of integrin receptors.
Adapted from Lefort *et al.* 2012[14]. Integrin receptors can exist in three different confirmation states. In the bent confirmation, the receptors are bound to filamin and have low affinity for their ligand. They are held in this state by the protein-protein interactions (clasp) between the α and β chains. Activation from extracellular stimuli leads to the recruitment of talin which activates the extension into the intermediate affinity state and further recruitment of kindlin puts the receptor into its high affinity state, where it will then bind to extracellular ligands with high affinity.

1.3 β_2 Integrin Receptors

The β_2 are a family of leukocyte-specific integrins that all express the β_2 chain (CD18) hybridised to one of four different α chains: CD11a (α_L), CD11b (α_M), CD11c (α_X) or CD11d (α_D). These β_2 integrin family members show preferential binding to different but overlapping ligands, and they are expressed across all immune cells, but different family members predominate on different cell types (Figure 1-3). CD11a is highly expressed on B and T cells but is also found on dendritic cells (DCs), macrophages (MFs), and granulocytes. Whereas CD11b, CD11c and CD11d tend to be associated with myeloid cells and granulocytes. The functions of β_2 integrins can differ according to the cell type they are expressed on and this is discussed in more detail below.

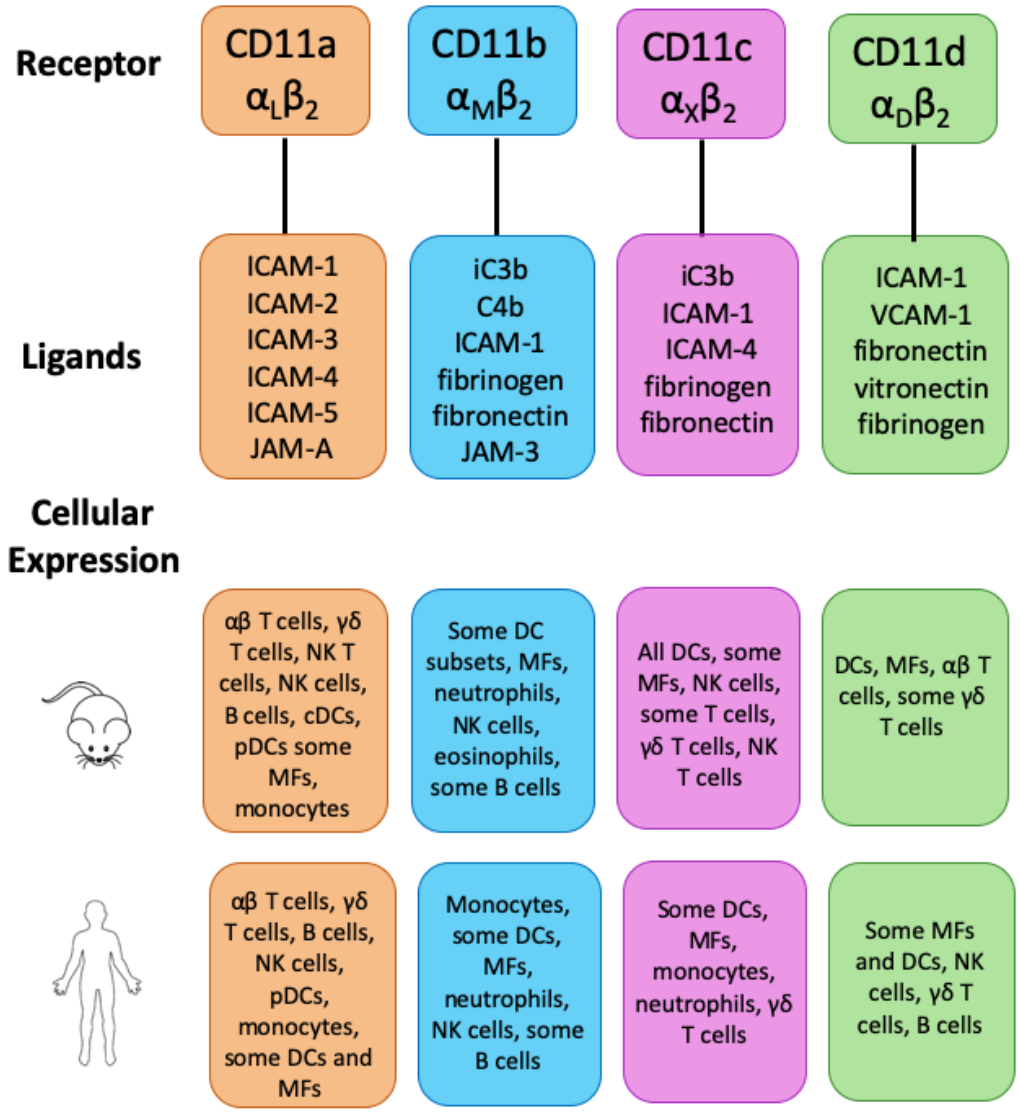

Figure 1-3 Beta2 integrin receptors, ligands and leukocyte expression
Abbreviations: natural killer (NK), macrophages (MFs), plasmacytoid dendritic cells (pDCs). CD11a predominantly binds to intracellular adhesion molecules (ICAMs) and junctional adhesion molecule-A (JAM-A), CD11b has over 40 reported ligands including the complement cleavage fragment iC3b and matrix proteins, CD11c has overlapping ligands with other integrins, CD11d also binds vascular cell adhesion molecule 1 (VCAM-1)[22–25]. In mice CD11a is highly expressed on αβ, γδ and NK T cells[26], expressed on cDCs with higher expression on CD8+ than CD8-[27] and on pDCs[28], also expressed on NK cells[29], monocytes[30], macrophages in the lungs but not peritoneum or spleen[31], B cells[32]. In humans CD11a is expressed on T and B cells[33], was shown to be higher on γδ than αβ T cells[34] and found on uterine γδ T cells[35], monocytes[36], monocyte-derived DCs[37], monocyte-derived MFs[38], plasmacytoid DCs[39] and NK cells[40]. CD11b expression is found on mouse neutrophils and eosinophils[41], peritoneal MFs and blood monocytes[42], conventional DC2s but not CD8+, CD103+ or pDCs[27,41,43], NK cells[44], and some B cells[45]. In humans CD11b expression is found on monocytes and macrophages[30,36], neutrophils[46], NK cells[47], monocyte-derived DCs[48], B cells[49]. In mice CD11c is expressed on all DCs including CD103+, CD11b+, with lower expression on pDCs[41,43,50], found on interstitial and alveolar macrophages[41], NK cells[51], NK and γδ T cells[52], a small percentage of T cells in the blood and small intestine[53]. Unlike in mice, only some human DCs express CD11c including monocyte-derived DCs[37,48] and cDCs, with higher levels on cDC2 than cDC1[54], but absent on plasmacytoid DCs[55]. Expression of CD11c is also found on human monocytes[30], neutrophils[56,57] and cervical tissue γδ T cells[52]. In mice CD11d expression has been found on peritoneal macrophages[42], low levels on T cells during foetal thymic development[58], expressed on some αβ T cells in the spleen and LNs but very low on γδ T cells[59]. In humans CD11d can be found on monocyte-derived MFs and DCs[60], is expressed highly on NK cells, moderate levels on γδ T cells and B cells but low or absent on αβ T cells[61].

1.3.1 Functions of β_2 Integrin Receptors

β_2 integrins are involved in a range of immune cell functions whereby they mediate: migration into lymph nodes (LNs) and tissues, interactions with other cells to form immune synapses (contacts between lymphocytes and antigen presenting cells (APCs) or target cells) or to initiate target cell killing, phagocytosis of pathogens/apoptotic cells and negatively regulate signalling downstream of toll-like receptors (Figure 1-4).

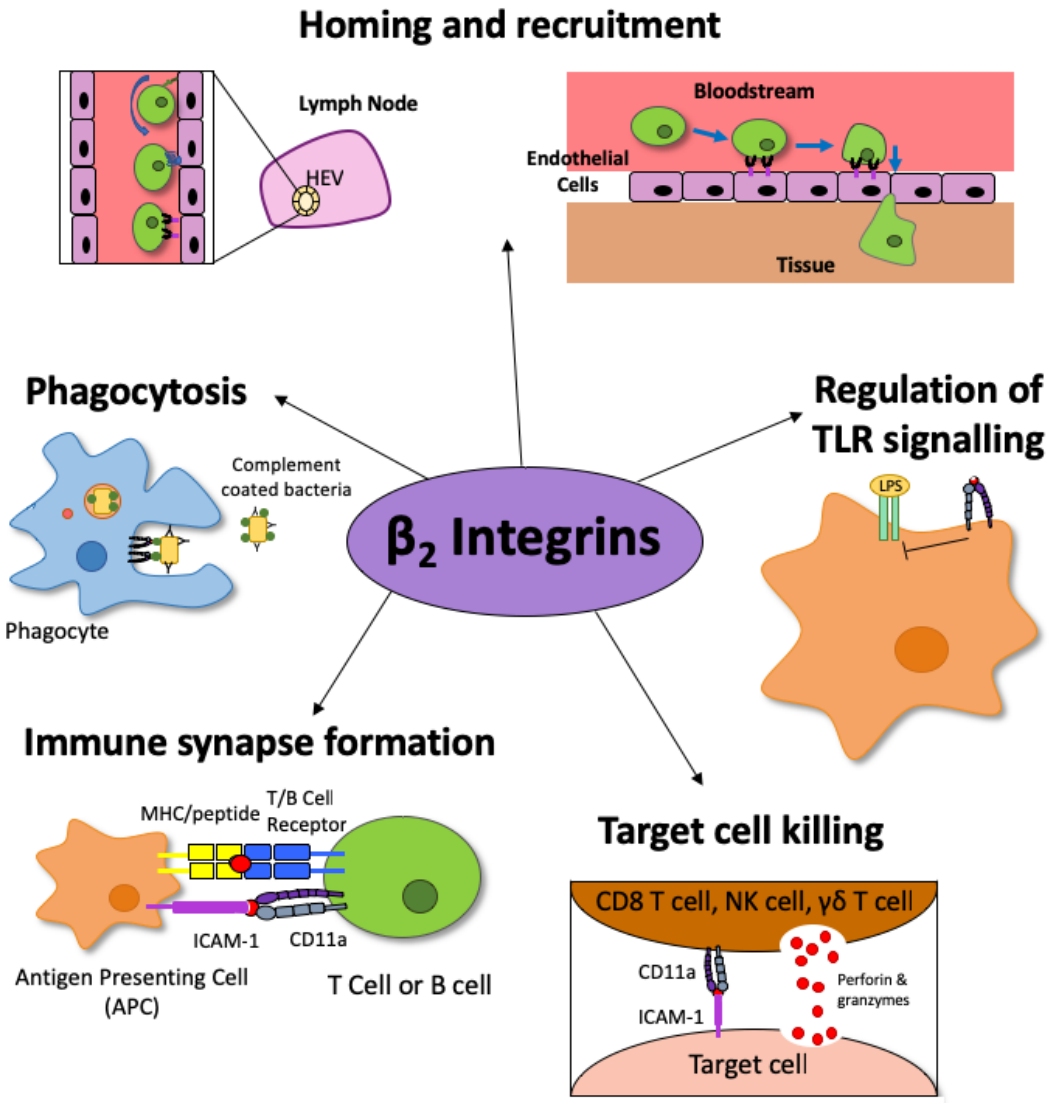

Figure 1-4 Functions of beta2 integrins
Adapted from[62,63]. β_2 integrins play a role in a range of immune cell functions. CD11a is important for migration of leukocytes into LNs and extravasation from the bloodstream into tissues. CD11a also mediates the formation of immune synapses to facilitate antigen presentation and the binding of CD8 T cells, NK cell and γδ T cells to target cells. CD11b/CD11c bind to opsonised particles to mediate phagocytosis by macrophages. CD18 and CD11b can also negatively regulate TLR-mediated signalling in macrophages and dendritic cells.

1.3.1.1 CD11a

CD11a is expressed on almost all leukocytes and its main role is to mediate cell adhesion to endothelial cells, target cells or other immune cells. The adhesion of circulating leukocytes to ICAM-1 on the endothelium via CD11a mediates their transendothelial extravasation from the bloodstream into tissues[64]. Following initial interactions with the endothelium mediated by selectins and chemokine receptors, CD11a mediates cell arrest and subsequent diapedesis across the endothelial layer[65]. CD11a KO neutrophils displayed reduced adhesion to endothelial cells under shear stress *in vitro,* and *in vivo* were less able to migrate to the site of TNF-induced inflammation[66], indicating that neutrophil firm attachment to endothelial cells is dependent on CD11a. In contrast, monocyte recruitment to sites of inflammation occurs independently of CD11a, as the infiltration of monocytes into the peritoneum following thioglycolate injection occurred in the absence of CD11a[67].

The migration of T cells into LNs requires CD11a-mediated adhesion to the high endothelial venule (HEV), but migration to the spleen occurs independently of CD11a[68]. Lymphocytes from CD11a KO mice showed impaired trafficking to peripheral LNs and to a lesser extent to mesenteric LNs[64], likely due to the partial dependence on $\alpha_4\beta_7$ for migration to gut-associated tissues[69]. There is evidence to suggest that leukocyte migration within the LNs occurs independently of integrins, the migration of T cells in LN stroma was unaffected by the absence of CD11a expression[70]. It has been suggested that in tissues leukocyte migration is chemokine-dependent because in the absence of shear stress, integrins are not activated[71]. The main route of DC entry into LNs is from tissues rather than from the blood through HEVs and this process, at least for mature DCs, is reported to be β_2-independent[72,73].

The binding of CD11a to ICAM-1 mediates formation of the immunological synapse between T cells and APCs[74,75], B and T cells[76], and the target cell binding of CD8 T cells[77], NK cells[78] and γδ T cells[79,80]. The immune synapse is organised into supramolecular activation clusters (SMAC) and CD11a is located in the peripheral SMAC, which encircles and stabilises interactions between the TCR and peptide-MHC complex in the central SMAC[81,82]. In addition to its physical function in immune synapse formation, signalling downstream of CD11a has been shown to enhance TCR signal transduction[83], lower the threshold for TCR and BCR signalling[76,84],

enable T cell activation at a lower antigen dose[85] and facilitate the effective delivery of cytotoxic granules to mediate target cell killing[86,87]. In contrast to the role of CD11a in promoting immune activation when expressed on lymphocytes, expression on DCs has been shown to inhibit T cell responses[88].

There is also evidence that demonstrates the importance of CD11a in leukocyte generation and differentiation. A study using CD11a KO mice identified the requirement for CD11a in generating optimal numbers of common lymphoid progenitor cells in the bone marrow[89]. CD11a KO mice also display reduced numbers of Tregs in the thymus and spleen, suggesting that CD11a is required for optimal Treg generation[90]. The development and survival of follicular T cells (Tfh) was also found to require CD11a, as anti-CD11a antibody treatment depleted Tfh cells through enhanced apoptosis and CD11a KO mice have decreased numbers of Tfh cells[91].

1.3.1.2 CD11b

CD11b is primarily expressed on monocytes, macrophages (MFs), neutrophils and the cDC2 subset of DCs[41]. One of the ligands for CD11b is the complement cleavage product iC3b[92], which is released as a by-product of the classical complement pathway. The binding of CD11b to particles coated with iC3b enhances the phagocytic ability of the cell and mediates the uptake of pathogens, immune complexes and apoptotic cells[93]. It has been shown that apoptosis in neutrophils is induced following CD11b-mediated phagocytosis[94]. However, the activation of CD11b can prevent the spontaneous apoptosis of neutrophils by maintaining mitochondrial membrane potential, yet in the presence of pro-apoptotic stimuli (such as anti-Fas) the engagement of CD11b contributes to the induction of apoptosis[95].

CD11b can also bind to ICAM-1, therefore plays a potential role in extravasation and immune synapse formation. CD11b was shown to mediate DC binding to ICAM-1 on the lymphatic endothelium[96] and active CD11b on DCs can limit DC-induced T cell activation[97]. Neutrophil recruitment to the lungs during inflammation could be inhibited by blocking CD11b or ICAM-1[98], suggesting their interaction is important for mediating neutrophil recruitment. However, there is also evidence for CD11b inhibiting the recruitment of neutrophils to sites of inflammation. Using a TNFα air pouch model, neutrophil extravasation was increased in CD11b KO mice[66]. The

transmigration of monocytes through the endothelial layer also requires CD11b, but uses a ligand distinct from ICAM-1[99] and CD11b has been implicated in the retention of macrophages in fat tissue[100]. These studies highlight the cell- and context-dependent role of CD11b in mediating cell interactions and migration.

1.3.1.3 CD11c

The expression of CD11c is commonly associated with DCs in mice, but is also found on alveolar MFs[50,101]. A small proportion of T cells in the circulation and small intestine lamina propria can also express CD11c[53]. In humans CD11c is expressed on monocyte-derived DCs[48] and expression on conventional DC (cDC) subsets is higher on cDC2 than cDC1[54] but absent on plasmacytoid DCs[55]. As with CD11b, CD11c also binds to iC3b which mediates effective phagocytosis, but uses a different binding site[102]. In addition to this ligand, CD11c can bind to fibrinogen and ICAM ligands which play a role in leukocyte adhesion and migration in response to chemokine-stimulation[103–105]. The recruitment of monocytes/MFs to sites of inflammation is dependent on CD11c, demonstrated using a mouse model of atherosclerosis, where in the absence of CD11c expression, monocytes/MFs showed reduced accumulation in atherosclerotic lesions which correlated with a reduced disease severity[106]. It has been suggested that CD11c expressed on MFs in the spleen is important for the phagocytosis of red blood cells (RBCs)[107]. ICAM-4, a ligand for CD11c, is expressed on RBCs and the phagocytosis of RBCs by MFs could be blocked using antibodies against CD11c or ICAM-4[107].

Expression of CD11c has also been identified on some T cells and NK cells during cytomegalovirus infection, but the function in these cell types is unclear[108]. In humans, γδ T cells and CD8 T cells in the blood and cervical tissue can express CD11c[52]. The T cells in this study identified as $CD11c^+$ showed enhanced production of IFNγ compared to their CD11c negative counterparts and expressed many adhesion molecules including chemokine receptors and the gut-homing integrin $\alpha_4\beta_7$, therefore are thought to have mucosal tissue-homing properties. They also identified CD11c-expressing γδ T cells and NK cells in mice[52], although the function of the integrin receptor on these cell types was not explored.

1.3.1.4 CD11d

CD11d is less well characterised than the other β_2 integrin family members. Similar to CD11b and CD11c, it is predominantly expressed on myeloid cells. CD11d has been detected on murine macrophages[42] and human monocyte-derived MFs and DCs[60]. Engagement of the receptor in monocytes induced the production of inflammatory cytokines IL-8 and IL-1β as well as the monocyte chemoattractant protein MCP-1[60]. This suggests that CD11d may play a role in the recruitment and activation of other immune cells during an inflammatory response. CD11d may also play a role in the response to infection. The elimination of *Salmonella* bacterium both *in vitro* and *in vivo* was reduced in the absence of CD11d, indicating that CD11d plays a role in bacterial clearance[109]. However, the phagocytic ability of CD11d KO MFs was comparable to WT, suggesting that CD11d is involved in activating intracellular killing of the bacteria rather than phagocytosis. CD11d may also play a functional role in NK cells. Analysis of CD11d on cells from human blood found that NK cells expressed higher levels of CD11d than T or B cells[61], and in mice the activation of NK cells by neutrophils via CD11d-ICAM-3 interactions was shown to increase IFNγ production[110].

Despite CD11d being predominantly associated with innate cells, there is also some evidence for a role in T cells. Analysis of CD11d on human T cells found that γδ T cells from peripheral blood expressed greater levels of CD11d than αβ T cells, and this was upregulated in response to IL-2[61]. In mice CD11d expression has been described on γδ T cells from the spleen but not LNs[26] and CD11d was suggested to be involved in the early thymic development of αβ T cells, as transient expression was detected on T cells during foetal thymic development[58].

1.3.2 β_2 Integrins in Inflammation and Immune Regulation

The majority of β_2 integrin functions that have been described involve the activation/initiation of the immune response and can contribute to inflammation. This includes the uptake of antigens by APCs through phagocytosis, activation of adaptive immune responses mediated by interactions between APCs and T cells in LNs, recirculation of T and B cells into LNs during immunosurveillance and the recruitment of the induced effector cells to the site of an infection/inflammation. In T cells, CD11a signalling can directly contribute to activation, whereas there is accumulating evidence that, in other cell types, signalling downstream of integrin

receptors regulates leukocyte activation and has an important role in preventing unwanted inflammation.

It has been shown in both mouse models and human studies that β_2 integrin loss is associated with development of immune-mediated disease. In humans, mutations that cause loss of β_2 integrin expression or function lead to the development of leukocyte adhesion deficiency (LAD) type I and type III (LAD-I and LAD-III). In LAD-I, mutations in the CD18 gene cause a reduction or complete loss of CD18 expression. There are over 200 characterised mutations[111] and 323 published cases of LAD-I to date[112]. Patients commonly present initially with omphalitis (infection of the umbilical stump) or skin ulcers that develop in early life[113] and suffer from recurrent bacterial and fungal infections in the skin and mucous membranes throughout life[114]. The severity of disease correlates with the level of CD18 expression, which can range from <2% to >10% of normal levels, and in over 90% of cases with >4% expression patients will survive into adulthood[112]. In LAD-III, patients have a mutation in the gene encoding kindlin-3 (FERMT3)[115,116], which prevents the full activation of β_1, β_2 and β_3 integrins. This form of LAD is less common than LAD-I, with less than 40 reported cases[117]. Similar to LAD-I, patients with LAD-III present with recurrent bacterial infections but in addition to this they also a display bleeding disorder called Glanzmann thrombasthenia, due to defective integrin binding to collagen by platelets, required for blood clotting[118].

Defects in β_2 have also been associated with increased incidence of colitis, which is thought be related to defective neutrophil function[119]. There is an association between mutations in the CD11b (*Itgam*) and CD11c (*Itgax*) genes, and the development of systemic lupus erythematosus (SLE)[120]. β_2 integrins are required for the effective activation of neutrophils in response to immune-complex binding[121], therefore the impaired clearance of apoptotic cells may cause the release of intracellular components (e.g. DNA and other nuclear contents) that the immune system will then mount a response against.

Mouse models also support the role of β_2 integrins in inflammatory disease. The absence of β_2 expression in mice led to the development of chronic dermatitis, an inflammatory skin condition[5]. In a mouse model of colitis the absence of CD11a was protective whereas CD11b deficiency exacerbated disease and correlated with increased plasma cell infiltration[122]. The protective effect of CD11a loss was likely

due to impaired T cell function because the establishment of normal αβ T cell numbers in the intestine is dependent on β_2 integrin/ICAM-1 expression and the pathology in this model is T cell-mediated[123]. Using the K/Bxn serum transfer model of arthritis it was found that β_2 integrin KO mice were resistant to the development of disease[124]. Further analysis found that disease could not be induced in CD11a KO mice whereas the absence of CD11b amplified disease. These discrepancies in results in CD11a and CD11b KO mice may be explained by impaired T cell migration being responsible for the protective effect in CD11a KO mice, whereas the loss of CD11b is associated with an accumulation of neutrophils at inflammatory sites due to a defect in apoptosis[125], which may be the mechanism of exacerbated disease in CD11b KO mice. Another study using CD11b KO mice found that oral tolerance could not be induced in these mice, due to enhanced IL-6 production by APCs which increased the generation of Th17 cells and prevented the induction of oral tolerance[126].

This evidence clearly demonstrates that β_2 integrins play an important role in the regulation of immune responses and suggest that dysregulation of specific β_2 integrin subunit signalling could contribute to development and/or pathogenesis of autoimmune and inflammatory disease. Due to the association of β_2 integrin loss of function with autoimmune disease, it is essential that we understand how these receptors regulate immune cells in order to identify potential new therapeutic targets.

1.4 Dendritic Cells (DCs)

Dendritic cells (DCs) were first discovered by Ralph Steinman and Zanvil Cohn in the 1970s. They are the main APCs of the immune system and play an important role in initiating either the activation of an immune response or the induction of tolerance.

1.4.1 Development of DCs

DCs develop from committed precursors in the bone marrow. Plasmacytoid DCs (pDCs) and pre-conventional DCs (pre-cDCs) develop from common DC progenitors (CDP). The terminal differentiation of pDCs occurs in the bone marrow[127], but pre-cDC precursors will migrate via the bloodstream to lymphoid

organs and tissues where they will then develop into cDCs[128]. There are two subsets of cDCs, cDC1 and cDC2, that are defined by their differential transcription factor requirements. cDC1 depend on IRF8, BATF3 and ID2 for development, whereas cDC2 require IRF4 and ZEB2[129]. These cDC subsets can be identified by differential expression of surface markers: cDC1 express CD8α and CD103, whereas cDC2 express CD11b and CD172a[130]. The CD8α-expressing cDCs are located in lymphoid organs and the equivalent population in tissues express CD103[131]. The CD11b-expressing cDCs are predominantly lymphoid but can also be located in tissues[101]. CD172a-expressing cDCs are located in tissues including the spleen, lungs, liver, small intestine and colon[129].

There are also non-classical DCs, which derive from a distinct lineage than pDCs and cDCs. They develop from monocytes so are termed monocyte-derived DCs (moDCs)[132]. Similar to other DC subsets, moDCs can express MHC II and CD11c, as well as present antigens[130]. They can be distinguished from pDCs and cDCs by their expression of CD64 and Fc-gamma receptor 1 (FcγRI)[133]. Langerhans cells also develop from the monocyte lineage, and have been described as a population of tissue-resident DCs[134], as they are found exclusively in the epidermis. However more recently, gene expression analysis found more similarities between LCs and MFs than cDCs[135].

1.4.2 Functions of DCs

DCs are often referred to as ‘professional APCs’ as their primary function is to present antigen, of both self and foreign origin. They play an important role in bridging the innate and adaptive immune system, with the capability to induce immunogenic or immunoregulatory responses in CD4 and CD8 T cells.

1.4.2.1 Activation of the immune response by DCs

During a classical immune response, DCs in peripheral tissues will take up pathogens by phagocytosis and undergo maturation, then migrate to the draining LN where they present the foreign antigen to T cells, in order to generate an effective T cell response. Immature DCs have the ability to acquire antigen, but they do not gain the ability to effectively present antigen until their maturation is stimulated. The expression of MHC on the surface of immature DCs is low and many MHC molecules are not complexed to peptides[136], then during maturation newly

generated MHC-peptide complexes accumulate on the cell surface[137].This maturation of DCs occurs in response to 'danger' signals that come from either microbial products or tissue damage. These include: CPG motifs in bacterial DNA[138,139], double-stranded viral RNA[140], LPS[141] and necrotic cell products e.g. heat shock proteins[142]. During maturation, DCs will lose their phagocytic ability due to downregulation of antigen uptake receptors (e.g. Fc receptors) and upregulate expression of MHC and co-stimulatory molecules[143,144].

Following antigen uptake and maturation, the migration of DCs to LNs is essential for the generation of an effective T cell response. The chemokine receptor CCR7 expressed on mature DCs mediates their migration from tissues to lymphatics via binding to ligands CCL19 and CCL21[145], where they interact with T cells to initiate the immune response. In combination with the production of cytokines, DCs then activate the clonal expansion of T cells through antigen presentation and co-stimulation. These three signals are required to fully activate the T cell, with antigen presented in the context of MHC to the TCR as signal 1, co-stimulation through molecules such as CD80/86 binding to CD28 for signal 2, and cytokines provide signal 3, e.g. IL-12 for the induction of Th1 cells. DCs process exogenous antigens and present them on MHC Class II molecules to CD4 T cells, whereas endogenous antigens are presented on MHC Class I molecules to CD8 T cells. Additionally, some types of DC are also capable of presenting antigens acquired exogenously to CD8 T cells, in a process referred to as cross-presentation[146,147]. This is the main function of CD8α-expressing DCs and is important for cytotoxic responses to bacteria, tumours and viruses.

There is evidence that different subsets of DCs are specialised in inducing different types of immune response. $CD11b^+$ cDCs express high levels of MHCII, which enables them to induce robust CD4 T cell responses[148,149] and they can produce pro-inflammatory cytokines such as IL-23, which induces Th17 cells[150]. IL-12 production is important for the generation of Th1 cells[151] and $CD103^-CD11b^+$ DCs in the intestine prime both Th1 and Th17 cells[152]. $CD8\alpha^+$ DCs in the spleen cross-present antigen, which induces cytotoxic responses by CD8 T cells[153]. Whereas, pDCs are important for viral responses through production of type I interferons[154,155]. It is important to note that DCs display functional plasticity, and when exposed to different cytokines or pathogen-derived products in the microenvironment may perform altered functions.

The strategic location of immature DCs is also important for their function. $CD8\alpha^+$ DCs in the spleen are in the marginal zone where they can take up antigens from the blood[156]. Whereas DCs in LNs are located in the subcapsular sinus which is the site of entry for afferent lymphatic vessels that drain from tissues[157]. $CD103^+$ DCs are predominantly located in non-lymphoid tissues, where they will acquire antigen before migrating to the T cell zone in the draining LN. This physical localisation of DCs enables their uptake of antigens to present to T cells.

1.4.2.2 Induction of tolerance by DCs

DC also play an important role in the induction of tolerance. Tolerance is the non-responsiveness of the immune system against particular antigens. This is an active immune process, that is particularly important to prevent unwanted responses against factors such as self-peptides, commensal organisms and food antigens in the body. However, tolerance is also manipulated as an evasion strategy by cancer cells. DCs can contribute to the process of central tolerance through the presentation of self-peptides during the negative selection of T cells in the thymus, which will induce the death of any T cell that strongly binds to self-peptide MHC[158]. DCs can also contribute to peripheral tolerance, which involves the deletion or induction of anergy in autoreactive cells that were not removed during central tolerance and are present in peripheral tissues. Additionally, DCs contribute to peripheral tolerance by inducing regulatory T cells (Tregs), through the conversion of effector T cells into Tregs[159] and enhancing Treg proliferation[160]. DCs in the intestines were shown to be important mediators of immune tolerance, as the expansion of DCs by Flt3L enhanced the induction of oral tolerance[161]. Whereas the depletion of DCs in mice caused the development of autoimmunity, again highlighting the importance of DCs in inducing tolerance[162].

Under homeostasis, many DCs present in the periphery have an immature phenotype, associated with low level MHC and co-stimulatory molecule expression. The interaction of these DCs lacking co-stimulatory molecules with T cells is known to lead to tolerance induction[163,164]. Specifically, immature DCs can induce tolerance through T cell deletion and/or through the generation of Tregs[165]. In addition to this, the expression of inhibitory molecules on DCs can also suppress T cell responses. PD-L1 binds to PD-1 on T cells to provide inhibitory signals and reduce T cell proliferation[166]. PD-L1 is expressed on both mature and immature DCs[167] and

expression can be induced following toll-like receptor (TLR) stimulation[168], suggesting that this inhibitory signal is important for dampening immune activation following infection. The binding of CD80/86 to CTLA4 expressed on the T cell, in contrast to CD28, will also lead to T cell suppression[169]. Cytokine production by DCs also contributes to their tolerogenic function. The phagocytosis of apoptotic cells by DCs reduces their pro-inflammatory cytokine production[93] and the secretion of anti-inflammatory cytokines by DCs, such as IL-10[170] and TGFβ[171], can promote the induction of Tregs.

It is now known that tolerance is not only induced by immature DCs, but DCs matured under certain conditions can induce Tregs[172,173]. Factors present in the microenvironment contribute to the maturation of these so-called tolerogenic DCs. Treatment with IL-10 enables DCs to induce anergy in T cells[174]. It was also shown that IL-10 in combination with TGF-β induced DCs that stimulated lower levels of T cell proliferation and were capable of expanding Tregs[175]. Corticosteroids inhibited the ability of DCs to induce Th1 responses due to a reduced production of inflammatory cytokines IL-12, TNFα and IL-6, despite costimulatory molecule expression being unaffected[176]. There is also evidence that pathogen-derived factors can induce tolerogenic DCs, as a schistosome-derived phosphatidylserine was able to mature DCs capable of inducing Tregs[172].

These tolerogenic DCs (tol-DC) are required to both limit immune responses following infection and prevent inappropriate immune responses to self-antigens. They are characterised by low level MHC and co-stimulatory molecule expression, upregulation of inhibitory receptors such as PD-L1 and production of anti-inflammatory cytokines, e.g. IL-10 and TGF-β[177]. Due to their highly immunosuppressive functions, tolerogenic DCs are now being explored as a therapeutic for the treatment of autoimmune disease[3]. Monocytes are cultured from patient blood into DCs (using GM-CSF and IL-4) then treated with dexamethasone, rapamycin or vitamin D3 to induce the tolerogenic phenotype, characterised by low expression of CD83, CD86, HLA-DR and production of IL-10[178]. Tol-DCs are pulsed with self-antigens prior to patient transfer, with the aim to re-establish tolerance in diseases including multiple sclerosis[179], rheumatoid arthritis[180] and type 1 diabetes[181].

Overall, it is clear that the regulation of DCs is essential for controlling the outcome the T cell response, whether that is the effective activation to eradicate pathogens, or inhibition to prevent self-reactive responses. It is therefore essential to understand the mechanisms underlying DC activation and localisation, to potentially manipulate this process for the treatment of diseases where there is an inappropriate activation of T cells that leads to pathology.

1.4.3 β_2 Integrins and DCs

It was recently discovered that β_2 integrin receptors are important regulators of DCs. For example, β_2 integrins have been shown to be responsible for the negative regulation of TLR responses in DCs. Bone marrow-derived DCs (BMDCs) deficient in β_2 integrins were hyperresponsive to stimulation of TLR2, 4 and 9, demonstrated by enhanced production of IL-6, TNFα and IL-12[182]. This was also true for BM-derived macrophages (BMDM), and the mechanism of enhanced cytokine production in the absence of β_2 integrin receptor expression was through increased activation of NFκB[182]. A similar pathway was also described in a study where BMDMs lacking CD11b were stimulated via TLR3, 4 or 9, produced higher levels of TNF, IL-6 and IFNβ[183]. It was identified that in β_2 integrin-sufficient cells, the activation of Syk downstream of the integrin receptor phosphorylates and induces degradation of the TLR downstream adapter proteins MyD88 and TRIF[183]. This prevents further signals that induce pro-inflammatory cytokine production and NFκB activation. As enhanced TLR-mediated cytokine production is also found in BMDCs[182], there may be a comparable mechanism of negative regulation of TLR-mediated signalling downstream of β_2 integrin receptors in DCs. In another study using CD11b KO BMDCs, they were found to enhance the activation of CD8 T cells when stimulated with the TLR9 ligand CpG[184]. However, there is contrasting evidence that suggests CD11b plays a role in the positive regulation of TLR4-mediated responses in BMDCs[185]. Ling *et al.* found that CD11b KO BMDCs produced lower levels of IL-6 and TNFα in response to LPS than WT, and this was due to an impaired endocytosis of the TLR4 receptor in the absence of CD11b. Further work is required to dissect the precise roles of β_2 integrins in regulating TLR-mediated responses in DCs – the interplay between integrin and TLR signalling may be context- and TLR-specific.

It has also been shown that β_2 integrins can regulate DCs even in the absence of TLR stimulation. Mice have been generated that express signalling-deficient β_2 integrin receptors (beta2$^{TTT/AAA}$ integrin knock-in mice), where a mutation in the kindlin binding site (TTT/AAA substitution; as shown in Figure 1-2) on the β_2 integrin tail prevents kindlin binding and therefore impairs integrin function[186]. Beta2$^{TTT/AAA}$ integrin knock-in BMDCs expressed higher levels of activation markers and produced more IL-12 in the absence of activation by TLR stimuli[73]. The knock-in DCs *in vivo* also had a more mature and migratory phenotype. This evidence supports the important role for β_2 integrins in regulating DC responses even in the absence of TLR stimulation.

In addition to the activation status of DCs, antigen uptake is another important function that will affect the ability of a DC to induce a T cell response. CD11a expressed on endogenous DCs acts as a receptor for exosomes[27], the uptake of which is a mechanism for acquiring antigen. In contrast to this, β_2 integrin signalling-deficient BMDCs did not show impaired uptake of fluorescently-labelled antigen, yet analysis of their transcriptome found that several genes associated with antigen uptake were downregulated compared to WT BMDCs[73]. These studies suggest that β_2 integrins can influence the ability of DCs to acquire antigen, which may affect their ability to then present antigen to T cells.

In order for a DC to initiate an effective T cell response, it requires an antigen to be presented via MHC, sufficient expression of co-stimulatory molecules, and also the proximity to T cells is necessary for their interactions. Therefore, the location of DCs is crucial for the activation of T cells, which requires the migration of DCs from tissues to LNs, and β_2 integrins have been implicated in this process. The chemokine receptor CCR7 is upregulated on DCs to mediate their migration to LNs[145] and BMDCs lacking functional β_2 integrin receptors expressed higher levels of CCR7[73]. This suggests that loss of β_2 integrin function may enhance DC migration via CCR7. In agreement with this, endogenous DCs were increased in the spleen and LNs of mice with signalling-deficient β_2 integrin receptors, due to a higher proportion of migratory DCs expressing CD103 and CD11b[73].These data suggest that in the absence of functional β_2 integrins, DCs are more migratory and therefore integrins may play a role in restricting DC migration *in vivo*, but β_2 integrin expression is not required for DC migration to LNs. Other studies have also suggested that the migration of DCs to LNs occurs independently of β_2 integrins. The adoptive transfer

of LPS-matured BMDCs demonstrated their ability to migrate into LNs and localise to the T cell zone, and DCs lacking multiple integrins including β_2 (β_1, β_2, β_3 and β_7) showed equivalent migration to WT DCs[72]. In contrast to this, the number of DCs in the lungs was reduced in CD18 KO mice, potentially suggesting a role for β_2 integrins in mediating DC migration to the lungs. However, as these mice had a global-deficiency in CD18 the indirect effect of another cell population on DC numbers cannot be ruled out. Overall the evidence in the literature suggests that DCs do not require β_2 integrins for migration to LNs but further work is required to determine whether specific DC subsets or migration to other tissues involves β_2 integrins.

It is clear that β_2 integrins can affect DC activation and may be involved in the antigen uptake and migration of DCs, therefore the next question is what impact this may have on their interactions with T cells. There is evidence to support the hypothesis that DC regulation by β_2 integrins affects their ability to prime T cells. The transfer of BMDCs lacking functional β_2 integrin receptors resulted in enhanced levels of T cell-derived IFNγ, compared to levels induced by integrin-sufficient BMDCs[73]. This suggests the loss of β_2 integrin expression causes BMDCs to induce greater Th1 responses. Another study using BMDCs that express a constitutively active form of CD11a showed that when cultured with CD4 T cells, these DCs were less able to induce T cell proliferation, expression of T-bet and production of IFNγ than BMDCs with normal CD11a expression[88]. This again indicates that β_2 integrins can negatively regulate DCs to limit the priming of Th1 cells. It was suggested by Varga *et al.* that CD11b, but not CD11a, is the β_2 integrin receptor responsible for inhibiting the priming of T cells[97]. They showed that inducing integrin receptor activation *in vitro* using magnesium enhances the binding of BMDCs to ICAM-1, and this effect could be blocked using antibodies against CD18 or CD11b but not CD11a or CD11c. When activated BMDCs were pre-incubated with anti-CD11b they induced lower levels of T cell proliferation[97]. In a study using human monocyte-derived DCs, the ligation of CD11b reduced their ability to induce Th17 cells through downregulation of cytokine production (IL-1β, IL-6 and IL-23)[187]. Similarly in mice, the ligation of CD11b on BMDCs decreased their production of IL-6, IL-12 and TNF and their ability to activate T cells[188]. These studies indicate that β_2 integrins act to negatively regulate the activation of DCs, which in turn contributes to restricting T cell activation. However, the specific β_2 integrin/s responsible for this effect and which signalling pathways they modulate are not yet fully characterised.

Overall, there is evidence to support the role of β_2 integrins in negatively regulating DCs under steady state conditions and following TLR activation. This integrin-mediated regulation also impacts on the ability of DCs to induce T cell responses. As DCs are essential mediators of immune activation versus tolerance, understanding the mechanism of β_2 integrin-mediated regulation in DCs may provide new targets for the treatment of diseases where there is inappropriate immune activation due to a loss of tolerance.

1.5 Gammadelta (γδ) T Cells

γδ T cells are innate-like lymphocytes that share features of both innate and adaptive immune cells. They are found predominantly at mucosal sites in the body, with a lower frequency present in the blood and LNs. They are the first T cells to be produced during embryonic development and have an important role in neonatal immunity, responses to infection and immune surveillance[189,190].

1.5.1 Development of γδ T cells

1.5.1.1 Murine γδ T cell development

Both αβ and γδ T cells develop from a common double negative ($CD4^-CD8^-$) precursor in the thymus[191,192]. The precise mechanisms that govern the decision between αβ and γδ T cell fates are not completely understood - it is debated whether T cell fate is pre-determined or induced by specific signals during thymic development[193]. There is also evidence that epidermal γδ T cells develop from yolk sac precursors independently of haematopoietic precursors[194]. The development of γδ T cells in mice occurs in waves during embryonic stages, each of which is associated with a distinct T cell receptor (TCR) Vγ chain and localisation to specific tissues (Table 1-1, Figure 1-5), reviewed in[195,196]. The first wave of γδ T cell development occurs from days E13-15, when Vγ5-expressing γδ T cells are generated. These cells migrate to the skin to become an epidermal-resident population[197]. Following this, $V\gamma6^+$ γδ T cells then develop from E16-18, which migrate to the lungs, uterus, tongue, dermis and peritoneal cavity[198–200]. $V\gamma4^+$ and $V\gamma1^+$ γδ T cells are produced from E18 into post-natal life, and predominantly localise to the spleen and lymph nodes[201]. $V\gamma7^+$ cells are found in the intestines[202], and can develop in the thymus[203] but there is also evidence for extra-thymic development, as Vγ7-expressing cells develop in athymic mice[204].

Each of the γδ T cell subsets described preferentially secrete either IFNγ or IL-17 but can also produce other cytokines (Table 1-1), an effector phenotype which they gain during thymic development[205]. This allows for rapid responses in the periphery, whereby γδ T cells produce cytokines without the need for clonal expansion. This is in contrast to αβ T cells that require antigen presentation via MHC to induce their clonal expansion before they can secrete high levels of effector cytokines. The subsets of γδ T cells that produce IFNγ versus IL-17 can be separated according to surface marker and transcription factor expression. IFNγ-producing γδ T cells are $CD62L^{hi}$ $CD44^{lo}$ $CD45RB^{+}$ $CD27^{+}$ and express T-bet, whereas IL-17-producing γδ T cells are $CD62L^{lo}$ $CD44^{hi}$ $CD45RB^{-}$ $CD27^{-}$ and express RORγt[199,206,207]. This cytokine signature of γδ T cell subsets is established during thymic development and is under epigenetic control that maintains the preferential cytokine production in the periphery[208]. Of note, is that $Vγ1^{+}$ and $Vγ4^{+}$ γδ T cells can develop as either IL-17-producers or IFNγ-producers and can be distinguished using the markers described above whereas, $Vγ5^{+}$ and $Vγ6^{+}$ cells produce IFNγ and IL-17, respectively[203].

It is still not well understood how the initial migration of different γδ T cell subsets to specific tissues is controlled, with the exception of $Vγ5^{+}$ cells. During thymic development, $Vγ5^{+}$ cells upregulate expression of CCR10, which is required for their migration to the skin[209], where keratinocytes produce CCL27 (the ligand for CCR10)[210]. It has also been shown that CCL25 is produced in the intestines[211], and the intestine-associated $Vγ7^{+}$ γδ T cells express the corresponding receptor CCR9[211]. Trafficking of $Vγ7^{+}$ cells to the small intestine was also found to be partially dependent on the integrin $α_4β_7$[212]. CCR6 expression on $Vγ4^{+}$ and $Vγ6^{+}$ γδ T cells is involved in their migration to the dermis under homeostasis[213], but it is unclear which chemokines and/or adhesion receptors direct the migration of Vγ6-expressing cells to the lungs and uterus.

There are some pathways that are described to play a role in the development of γδ T cells, but there is still much to be understood about the mechanisms underlying γδ T cell subset development, which is the subject of ongoing research. The development of the IFNγ-producing $Vγ5^{+}$ and $Vγ7^{+}$ subsets is dependent on butyrophillin (Btnl) and btnl-like molecules. Skint1 is an immunoglobulin superfamily member, with homology to btnl molecules[214]. Skint1, expressed on thymic epithelial cells and keratinocytes, is required for both the development and maintenance of

Vγ5$^+$ γδ T cells[214,215]. Binding of γδ T cells to Skint1 on thymic epithelial cells upregulates Egr3 in the γδ T cell, which induces differentiation into IFNγ-producing effectors[215]. Btnl1 and Btnl6, expressed on epithelial cells in the small intestine villus, are required for the expansion and maintenance of Vγ7-expressing γδ T cells in the gut[216], where Vγ7-expressing γδ T cells are thought to develop extrathymically. The receptor for Skint1 has not been identified, but there is evidence that the Vγ7$^+$ TCR recognises Btnl molecules[217]. Several Sox family transcription factors have been implicated in the development of IL-17-producing γδ T cells. Sox13 and Sox4 are required for the thymic development of Vγ4$^+$ cells, as this subset is absent in mice that lack the expression of Sox13 or Sox4[218,219]. However, the pathways that induce Vγ6$^+$ γδ T cell subset development and maintenance have not yet been characterised.

There is also evidence to suggest that TCR signal strength is one of the determining factors in the development of γδ T cell subsets. In SKG mice the loss of Zap-70 function, a kinase downstream of the TCR, reduced the numbers of IL-17$^+$ γδ T cells but not CD27$^+$ IFNγ-producers[220]. This suggests that the generation of IL-17-producing γδ T cells requires TCR signals. However, induction of TCR signalling using an anti-TCRγδ antibody inhibited the development of IL-17-producing γδ T cells, whereas inhibition of Erk signalling downstream of the TCR enhanced IL-17$^+$ γδ production while inhibiting IFNγ$^+$ γδ development[203]. The discrepancy between TCR signalling promoting or inhibiting IL-17$^+$ γδ T cell development in these studies may suggest that Zap-70 signalling is necessary for the generation of IL-17$^+$ γδ T cells but not necessarily strong TCR signals. In CD3 double-haploinsufficient mice, that have reduced TCR expression and downstream Erk signalling in γδ T cells, there is decreased development of Vγ6$^+$ but not Vγ4$^+$ γδ T cells[221], suggesting the effect of TCR signalling is specific to individual γδ T cell subsets. These studies imply that TCR-mediated signalling is involved in γδ T cell development, but when and how the strength of TCR signalling in the thymus is delivered remains to be fully elucidated.

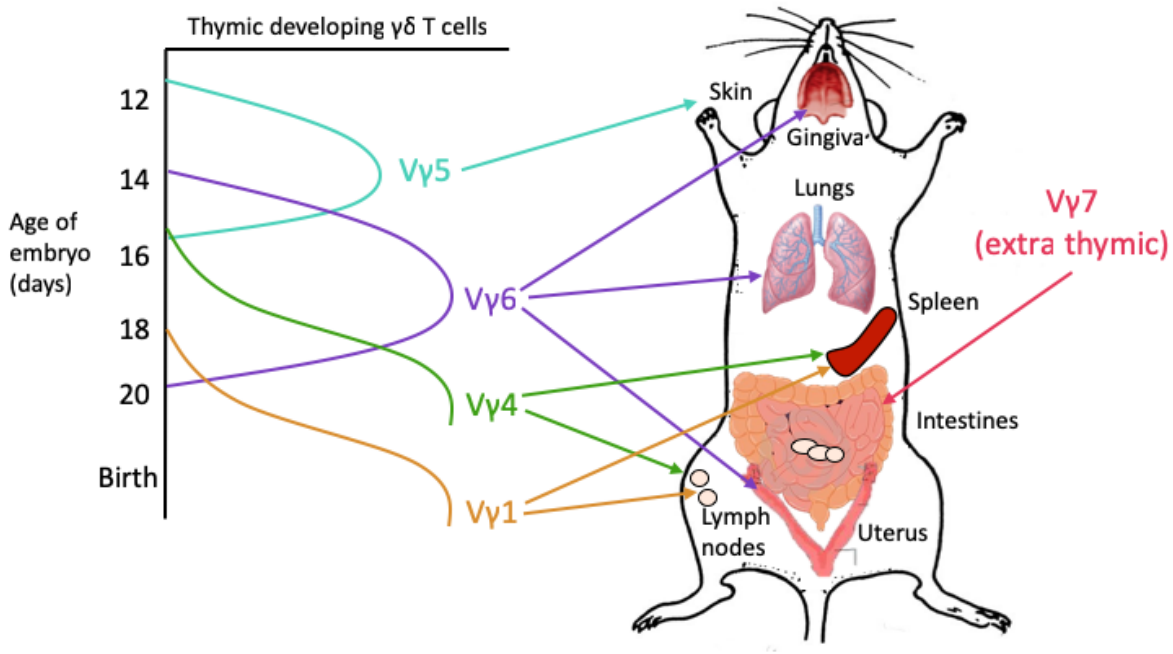

Figure 1-5 Murine γδ T cell development.
Adapted from Carding and Egan[222]. Murine γδ T cell subset 'waves' of development are depicted, together with the tissue(s) in which each subset resides.

Table 1-1 Summary of murine γδ T cell subsets

Subset	Age of development	TCR Repertoire	Tissue Localisation	Cytokine Production
Vγ1	E19 - postnatal	Diverse	Liver and lymphoid	IFNγ and TNF Can produce IL-17 and IL-4
Vγ4	E17 - postnatal	Diverse	Lymphoid, lungs and liver	IL-17 or IFNγ
Vγ5	E13 - E15	Invariant (Vγ5Vδ1)	Epidermis	IFNγ Can produce IL-22 and TNF
Vγ6	E15 - E18	Invariant (Vγ6Vδ1)	Uterus, lungs and tongue	IL-17 Can produce IFNγ
Vγ7	E19 - postnatal	Some variety	Intestine	IFNγ

Adapted from[223] showing γδ T cell subset development timings, TCR diversity, and specific tissue localisation and cytokine potential. Note that Vγ2 also exists at the mRNA level, however only a minority of sequences are in-frame and there is no correlation between in-frame Vγ2 transcripts and level of expression at the cell surface[224–226].

1.5.1.2 Human γδ T cell development

The development of γδ T cells in humans also occurs during embryonic stages, however the subsets of γδ T cells differ to those in mice. Human γδ T cells are broadly divided according to delta chain expression into Vδ1$^+$ and Vδ2$^+$ cells. Vδ2$^+$ cells are produced early during gestation[227], whereas Vδ1$^+$ cells are generated later during embryonic development, with the majority of γδ T cells expressing Vδ1 in cord blood[228] and in the paediatric thymus[229]. The Vδ2$^+$ cells then expand post-birth

and become the predominant population in the peripheral blood of adults[230]. Vδ2-expressing γδ T cells have a limited TCR repertoire[227,231], similar to the early developing murine γδ T cells, whereas Vδ1-expressing cells have a more diverse repertoire[232]. Unlike γδ T cells in mice with a more diverse repertoire that are located in the peripheral LNs, Vδ1$^+$ cells are predominantly located in tissues such as the intestine, skin and spleen[233,234].

There is evidence to suggest that, unlike in mice, human γδ T cells do not undergo functional pre-programming in the thymus. Comparison of γδ T cells from paediatric thymus and blood found that those in the thymus required activation by cytokines to enable their IFNγ production and to perform cytotoxic functions, whereas those from the blood readily produced cytokines[229]. γδ T cells that express T-bet and are capable of producing IFNγ are found in humans[227], but there is less evidence to support the presence of IL-17-producing γδ T cells in healthy individuals, unlike the presence of IL-17$^+$ γδ T cells in mice during homeostasis. However, IL-17-producing γδ T cells can be induced *in vitro* by IL-7 treatment[235] or a cocktail of cytokines in combination with phosphoantigens[236]. They have been detected in individuals with bacterial meningitis[236], HIV[237], psoriasis[238] and pulmonary tuberculosis[239]. This suggests that in humans γδ T cells may not be pre-programmed to produce IL-17 but under certain conditions during infection or inflammation these cells can be induced. In addition, most human studies use cells isolated from peripheral blood therefore, there may be IL-17-producing γδ T cells present in human tissues that are not readily accessible and rarely studied.

Although the subsets and characterisation of γδ T cells differs between mice and humans, the general localisation is similar, with γδ T cells found predominantly at barrier sites such as the skin, lungs and intestines, but also can be located in the blood and LNs[189]. They also display similar functions including: regulation of keratinocytes for wound repair[240], production of pro-inflammatory cytokines[241] and recognition of stress-induced ligands for cytotoxic responses to tumour cells[242]. Therefore, the use of mouse models to understand the development and functions of γδ T cells has translational capacity.

1.5.2 Survival and maintenance of γδ T cells

Following thymic development, γδ T cells become resident in peripheral tissues where they perform important effector functions. IL-17-producing γδ T cells are thought to develop exclusively in the embryonic thymus and are then retained in the periphery through self-renewal[243]. Using bone marrow chimeras it was shown that IL-17-producing γδ T cells cannot be generated in the adult thymus, regardless of whether the source of donor cells was from foetal or adult mice[244]. In line with these findings, following conditional depletion of γδ T cells, those with an IL-17-producing phenotype ($CD27^{-}CD44^{high}$), including $V\gamma4^{+}$ and $V\gamma6^{+}$ cells, did not fully reconstitute the spleen and peripheral LNs. In contrast, those with an IFNγ-producing phenotype ($CD27^{+}CD44^{low}$), including $V\gamma1^{+}$ cells in the spleen/LNs and $V\gamma7^{+}$ cells in the intestine, returned to normal levels after 7 weeks[245]. The exception was the IFNγ-producing $V\gamma5^{+}$ cells in the skin, that displayed a poor recovery, presumably due to their exclusive generation during embryonic development. Using another approach where γδ T cells were labelled with RFP in a tamoxifen-inducible system, IFNγ-producers lost their RFP expression 7 weeks post tamoxifen whereas IL-17-producers retained the RFP label and this was true for cells in the spleen, LNs and liver[245]. This suggests that IFNγ-producing γδ T cells are constantly replenished by newly generated cells from the thymus, whereas the turnover of IL-17-producers is much slower.

There are several cytokines and surface molecules that are thought to be involved in promoting the survival of γδ T cells. Studies have demonstrated the importance of IL-7 in γδ T cell survival. Following the labelling of proliferating cells with BrdU, in IL-7 KO mice there was a greater loss of $BrdU^{+}$ γδ T cells compared to heterozygous controls[246], indicating the importance of IL-7 for γδ T cell survival. γδ T cells have also been shown to upregulate the pro-survival molecules Bcl2, Bcl2-xL and Mcl-1 in response to IL-7[247]. In contrast to this, the administration of IL-2 *in vivo* enhanced γδ T cell death, based on expression of caspase-8[248]. Signalling via CD28 has also been implicated in γδ T cell survival, as blocking B7 molecules (ligands for CD28) enhanced γδ T cell apoptosis whereas activating CD28 inhibited cell death[249].

1.5.3 Functions of γδ T Cells

Unlike αβ T cells, γδ T cells are not restricted to recognising peptides in the context of MHC and can instead recognise non-peptide antigens. There are several characterised γδ TCR ligands including phycoerythrin[250], the non-classical MHC molecule CD1d[251], herpes simplex virus glycoprotein[252] and heat shock proteins[253]. In addition, the activation of γδ T cells can occur through other receptors, such as NKG2D, which recognises stress-related molecules expressed by epithelial cells[254]. The TCR repertoire of γδ T cells located at mucosal sites (e.g. Vγ5/Vγ6) are invariant, yet can respond to a variety of pathogens, suggesting that TCR recognition and signalling is not required for function[198,255]. The roles of γδ T cells in regulating homeostasis, activation of other immune cells and contribution to disease are detailed below.

1.5.3.1 Role in physiological functions

There is increasing evidence to show that γδ T cells can play important roles in maintaining tissue homeostasis, in addition to their immune functions. In response to tissue damage γδ T cells promote epithelial cell regeneration to maintain physical barriers. These barrier sites include the skin, lungs, intestines and uterus, all of which are sites populated by γδ T cells. For example, γδ T cells in the skin induce the maturation of keratinocytes and mediate tissue repair through production of IL-13[256]. More recently, γδ T cells were identified in adipose tissue, where they regulate thermogenesis[257]. This function was demonstrated using TCRδ KO mice, which lacked the ability to restore their body temperature following exposure to cold due to the loss of IL-17 production[257]. IL-17-producing γδ T cells are also necessary for effective bone regeneration, as mice lacking either γδ T cells or IL-17 showed impaired generation of new bone following injury, and in WT mice most of the IL-17-producing cells in the bone marrow were γδ T cells[258].

1.5.3.2 Interactions with DCs and antigen presentation

When cultured with DCs, γδ T cells induced the upregulation of MHC, CD86 and CD83 on the DCs in a contact-dependent manner[259]. It was also shown that activated γδ T cells can enhance LPS-induced activation of DCs, resulting in increased production of IL-12 and an enhanced ability to induce Th1 responses[260].

In turn, DCs could induce γδ T cell proliferation and increase their production of IFNγ and TNF, though IL-17 was not measured[260].

In addition to activating and/or enhancing antigen-presenting functions of DCs, there is evidence that γδ T cells themselves can present antigen. Human γδ T cells upregulate MHC I and MHC II, CD80/CD86 and CCR7 in response to isopentenyl pyrophosphate (IPP), a ligand for the human Vγ9Vδ2 TCR, and this enabled them to induce proliferation of naïve CD4 T cells[261]. The expression of MHC II could also be induced on murine γδ T cells *in vitro* by stimulation with anti-CD3 and anti-CD28 antibodies, and these cells induced αβ T cell proliferation[262]. It remains to be determined whether γδ T cells can present antigen *in vivo*, although expression of MHC II was induced on γδ T cells in response to immunisation with CFA[263].

1.5.3.3 Interactions with B cells

There are several studies to suggest that γδ T cells are capable of supporting B cell responses. In mice that lack αβ T cells, germinal centres and class-switched antibodies were detected, suggesting that γδ T cells may be capable of providing help to B cells[264]. In the absence of γδ T cells, mice had reduced levels of IgE and IgG1 in response to immunisation with ovalbumin[265]. More recently, it was also shown that in response to DNA damage in the skin, γδ T cells promoted the production of IgE by B cells[266]. The B cell-promoting effect of γδ T cells may be indirect, for example by influencing T follicular helper (Tfh) cells which are the main cell population that provides help to B cells. γδ T cells have been shown to induce Tfh generation, as in TCRδ KO mice immunisation with OVA/CFA resulted in reduced germinal centre formation, Tfh generation and antibody production[267].

1.5.3.4 Responses to infection

γδ T cells can respond to infection or disruption of the epithelial barrier through recognition of stress-markers expressed on epithelial cells, such as keratinocytes found in the skin[268]. This recognition of stress markers expressed on epithelial cells has led to the description of γδ T cell function in lymphoid stress surveillance[269]. The activation of γδ T cells via NKG2D is induced by ligands upregulated on cells in response to infection or stress, such as RAE1 and H60 (in mice), MICA and MICB (in humans)[270]. This ability to recognise any cause of disruption to the epithelial barrier and respond immediately results in a protective response against several

different types of infection. The production of IL-17 by γδ T cells is required for the elimination of *Staphylococcus aureus* infection in the skin[271]. In response to oral *Listeria monocytogenes* infection, γδ T cells capable of producing IFNγ and IL-17 migrate to the intestine and enhance bacterial clearance following secondary infection[272]. γδ T cells can also influence the recruitment of other immune cells. They play a protective role in the response to chikungunya virus, where in the absence of γδ T cells the increased infiltration of other inflammatory cells exacerbates tissue damage[273]. Finally, in the lungs, γδ T cells induced by *Candida albicans* infection recruit neutrophils to enhance fungal clearance[274] and the same is true for bacterial clearance following *Streptococcus pneumoniae* infection[275].

1.5.3.5 Role in cancer

The role of γδ T cells in tumour surveillance was first implicated by the observation that mice lacking γδ T cells showed enhanced tumour development and burden in models using injection of PVD carcinoma cells and chemical carcinogenesis induced by DMBA[190]. Further research has since found that γδ T cells can be both protective and pathogenic towards tumours, depending on their subset (i.e. which cytokine they produce) and the type of cancer. The protective effect of γδ T cells has been shown in B cell lymphoma, melanoma and colon cancer[276–278]. IFNγ-producing γδ T cells are thought to mediate these anti-tumour effects through cytokine production, release of perforin to mediate cell-killing and by inducing class I MHC molecule expression on tumour cells that enables recognition by $CD8^+$ T cells[279–281].

Several cancer models in mice have demonstrated that, unlike IFNγ-producers, IL-17-producing γδ T cells can increase tumour formation and metastasis. In a murine breast cancer model system, IL-17 production by γδ T cells had a pro-tumour effect through the expansion of neutrophils that suppressed anti-tumour $CD8^+$ T cells[282]. In an ovarian cancer model, γδ T cell-derived IL-17 activated peritoneal macrophages that enhanced tumour cell proliferation[283]. Similarly, in a hepatocellular carcinoma model, myeloid-derived suppressor cells were induced by γδ T cell-derived IL-17, leading to enhanced tumour growth[284]. Conversely, there is also evidence that IL-17-producing γδ T cells can be protective in cancer. The anti-tumour effects of bladder cancer treatment using *Mycobacterium bovis* BCG were dependent on the recruitment of neutrophils mediated by $IL\text{-}17^+$ γδ T cells[285].

In humans, γδ T cells are also associated with both pro- and anti-tumour effects. Patients with breast cancer showed a positive correlation between γδ T cell infiltrate in the tumour and advanced tumour stages as well as lymph node metastasis, indicative of a pro-tumour effect[286]. Whereas analysis of the leukocyte infiltrate across 25 different cancers found that high levels of γδ T cells are associated with favourable outcomes, suggesting an important anti-tumour function[287].

Despite the contrasting roles for γδ T cells in cancer it is clear that γδ T cells capable of performing cytotoxic functions and producing IFNγ can mediate anti-tumour effects. This has led to the investigation of γδ T cell based immunotherapies for the treatment of cancer[278]. Most approaches involve the expansion of Vγ9Vδ2$^+$ γδ T cells, as these are readily available in peripheral blood and can be expanded *in vivo* and *in vitro* using aminobisphosphonates[223]. The safety of these approaches has been demonstrated in pilot studies and Phase I clinical trials, with evidence for therapeutic effects in some patients. In patients with metastatic breast cancer, the combined treatment of zoledronic acid and low dose IL-2 led to a correlation between the number of Vγ9Vδ2$^+$ cells and positive clinical outcomes (stable disease or partial remission)[288]. In another study using IL-2 with palmidronate, many patients with non-Hodgkin lymphoma or multiple myeloma showed stable disease or partial remission, again associated with increased Vγ9Vδ2$^+$ cells[289]. γδ T cells expanded in patients using these approaches display an effector phenotype based on CD69 expression, express HLA-DR indicating a potential for antigen presentation, and showed an enhanced capability to produce IFNγ[288–290]. There is contrasting data on the use of zoledronic acid and IL-2, as in patients with renal cell carcinoma the treatment reduced the percentage of Vγ9Vδ2$^+$ cells *in vivo* and they displayed impaired proliferation *in vitro* [291] highlighting the need for optimisation of expansion protocols to deliver consistent results.

Optimisation of these protocols to expand γδ T cells requires a better understanding of the pathways involved in γδ T cell proliferation and function. Vδ1$^+$ γδ T cells have not yet been used in clinical trials but there is evidence they have anti-tumour effects and are less susceptible to activation-induced cell death than Vδ2$^+$ cells[292]. The use of Vδ1$^+$ γδ T cells as a therapeutic requires the development of a protocol for selective expansion and activation. It is still not currently well understood how γδ T cells are regulated *in vivo*. This is important to understand how the γδ T cells migrate to the tumour/metastasis sites, how they interact with tumour cells and other

immune cells to mediate anti-tumour effects, what governs the stability of their phenotype when transferred or expanded (e.g. cytokine production), and what controls their survival. Elucidating the pathways involved in γδ T cell regulation will improve the success of γδ T cell therapeutics through the generation of cells of the correct phenotype with sufficient migratory capacity and survival *in vivo* for them to carry out effector functions.

1.5.4 β_2 integrins and γδ T cells

αβ T cells express high levels of CD11a and the function of this integrin has been well characterised to contribute to αβ T cell effector functions. In αβ T cells, CD11a has been shown to mediate cell-cell contacts for immune synapse formation and cytotoxic functions, as well as extravasation from the bloodstream into tissues and trafficking into lymph nodes[64,68,87,293]. The roles of CD11a may be different in γδ T cells.

β_2 integrins are expressed on γδ T cells in both mice and humans. In mice, γδ T cells express reduced β_2 integrins compared to αβ T cells, and γδ T cells have been shown to express highest levels of CD18/CD11a and to a lesser extent CD11b, CD11c and CD11d[59,294]. Whereas in humans, analysis of T cells from peripheral blood identified that γδ T cells express higher levels of CD11a[34] and CD11d[61] than αβ T cells. Although the role of different β_2 integrin receptors in γδ T cell function has not been extensively studied, there are several studies that have started to uncover their role.

Some studies have assessed the role of β_2 integrins in γδ T cell migration using disease models. In a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), pathogenic γδ T cells that infiltrated the central nervous system (CNS) upregulated β_2 integrins, whereas integrin expression on αβ T cells was unchanged[59]. This suggested that β_2 integrins may be important for γδ T cell migration into the CNS. However, subsequent work from the same group found that when transferred into mice with EAE, β_2 integrin-deficient γδ T cells were able to traffic effectively into the CNS[295], indicating that beta2 integrin receptors are not required for γδ T cell trafficking to the site of inflammation in this model. In line with this, it was shown that in mice with CD18 expression at 2-16% of WT levels (CD18^{Hypo}PL/J), inflammatory skin lesions that spontaneously develop showed a

greater γδ T cell infiltrate than controls[296]. Again, this suggests that the migration of γδ T cells to effector sites is not dependent on β_2 integrins. However, there is some evidence suggesting the involvement of β_2 integrins in γδ T cell migration. Following corneal wound abrasion, CD11a KO mice showed reduced γδ T cell numbers in the epithelium compared to WT, suggesting that the migration of γδ T cells to this site may be partially dependent on β_2 integrins. Alternatively, this may indicate the dependence of γδ T cells on β_2 integrins for migration is a tissue-dependent effect or an indirect effect through altered infiltration of other cells, that reduce the recruitment or retention of γδ T cells. When adoptively transferred, CD18 KO γδ T cells predominantly migrated to the liver and lungs, and were also found in the spleen but not peripheral LNs[297]. This again suggests that migration of γδ T cells to certain tissues does not require β_2 integrins, although they did not directly compare the migration with integrin-sufficient γδ T cells or characterise the subsets of γδ T cells that were transferred, which may influence where the cells migrate. Overall, evidence from the current literature supports the hypothesis that, in contrast to αβ T cells, γδ T cell migration occurs independently of β_2 integrins. However, there are currently no studies that directly test the contribution of β_2 integrins to γδ T cell migration under steady state conditions.

There is also evidence that suggests β_2 integrins are important for mediating the cellular interactions of γδ T cells. It was shown that γδ T cells can interact with endothelial cells via CD11a-ICAM-1 interactions[298]. However, the functional consequences of this interaction, and the specific requirement for individual integrin subunits, were not explored. Several studies describe the requirement for β_2 integrins for effective target cell killing by γδ T cells, likely also by binding to ICAMs. Imaging of γδ T cells bound to K562 leukaemia cells showed that CD11a localised to the region of contact between the two cells[299]. Using a blocking antibody against CD18, the cytotoxicity of γδ T cells against pancreatic cancer cells was inhibited[79], and against CD11a reduced the killing of Burkitt's lymphoma[80] and Daudi lymphoma cells[300]. The expression of the β_2 integrin ligands ICAM-1 and ICAM-2 were also found to be required for effective killing of pancreatic cancer cells by γδ T cells[301]. These studies indicate that, despite playing less of a role in γδ T cell migration, β_2 integrins are important for mediating the interactions of γδ T cells, and in particular are required their cytotoxic functions.

In addition to these studies of integrin contribution to γδ T cell migration and interactions, there is also evidence that the reduction or loss of β_2 integrin expression can cause an expansion of γδ T cells suggesting a role for β_2 integrins in γδ T cell proliferation. In mice with reduced CD18 expression there is an increased number of IL-17-producing γδ T cells in the skin-draining LNs which becomes exacerbated when they develop a spontaneous skin inflammation and γδ T cells migrate into the skin[296]. CD11a KO mice have been shown to develop periodontal disease with age, which also correlates with an infiltration of IL-17-producing γδ T cells[302]. Higher γδ T cell numbers have likewise been identified in β_2 integrin-deficient mice in the absence of overt signs of inflammation. An increased number of γδ T cells has been described in the cervical LNs of CD18 KO mice[297] and a higher percentage of IL-17-producing γδ T cells was identified in the spleen of these mice[303]. The mechanisms underlying γδ T cell expansion in these CD18-deficient mouse models was not identified and the number of γδ T cells in other mucosal tissues where γδ T cells perform essential functions, such as the lungs or intestines were not determined.

In summary, unlike for αβ T cells, there is less evidence to suggest that β_2 integrins are important for the migration of γδ T cells. Yet, similar to αβ T cells, the optimal cytotoxic function of γδ T cells via binding to target cells requires β_2 integrins. The increased number of IL-17$^+$ γδ T cells in the absence of β_2 integrin expression suggests a possible regulatory function, but it is unclear whether this is a direct effect on γδ T cells or mediated indirectly through another cell population.

1.6 Hypothesis and Aims

The aims of this thesis are based on the hypothesis that β_2 integrins are a negative regulator of DCs and γδ T cells.

It is well characterised that β_2 integrin receptors contribute to pro-immune functions. β_2 integrins are involved in the formation of contacts between immune cells[75,304] and enhance immune activation and signalling[84]. The migration of cells for entry into LNs[68] and transmigration into tissues[64] is dependent on β_2 integrins. The cytotoxic function of immune cells is also enhanced with β_2 integrin-mediated adhesion[77,79,87,301]. Despite these findings, in both mice and humans there is evidence that the loss of β_2 integrin expression is associated with inappropriate inflammation and autoimmunity. In humans with mutations in β_2 genes there is an increased incidence of colitis[119] and lupus[120], and in mice the loss of β_2 integrin expression can lead to spontaneous inflammation in the skin[5] and oral mucosa[302]. As the loss of β_2 integrin expression is associated with an increase in inflammation, this suggests that in addition to their roles in promoting immune responses, β_2 integrins can also act as immune regulators. Therefore, the overall aim of this thesis was to explore the immuno-regulatory roles of β_2 integrins. Immune regulation by β_2 integrins was explored in DCs and γδ T cells, based on evidence from the literature to suggest a regulatory role for integrins in these cell types.

DCs from CD18 KO mice were hypersensitive to TLR stimulation[182] and the loss of functional β_2 integrin receptors resulted in an increased activation of DCs under steady state conditions[73]. In chapter 3 I sought to determine the specific subunits involved in the negative regulation of DCs and the effect integrin-mediated regulation of DCs has on the outcome of the subsequent T cell response.

In mice with reduced or complete loss of CD18 expression, there is evidence of enhanced numbers of IL-17-producing γδ T cells in the spleen[303], skin[296] and cervical LNs[297]. As the loss of integrin expression causes an increase in γδ T cells, my hypothesis is that β_2 integrins act as a novel regulator of IL-17-producing γδ T cells. In chapter 4 I characterised the phenotype and localisation of γδ T cells in β_2 integrin-deficient mice and in chapter 5 I explored potential mechanisms underlying the expansion of γδ T cells in these mice.

Chapter 2 Materials and Methods

2.1 Mice

Details of all mouse strains used are listed in Table 2-1. For CD11a, CD11c and CD11d KO strains mice were initially bred from the sources listed. Upon discovery of an additional mutation in the *Dock2* gene present in these mice (detailed in Chapter 3), mice were crossed with C57BL/6 and selected for WT expression of *Dock2*. These *Dock2*^{WT} strains with retained integrin deficiency were used as indicated in Chapter 3 and in Chapter 4. Mice were used at age 6-14 weeks unless otherwise indicated. Mice were housed at the University of Glasgow and all protocols were conducted under licenses issued by the U.K. Home Office under the Animals (Scientific Procedures) Act of 1986 and approved by the University of Glasgow Ethical Review Committee.

Table 2-1 Details of Mouse Strains Used

Name of Strain	Description	Caging	Source
C57BL/6J	Wild type	Conventional	Envigo or transgenic negatives from CD18 KO line
CD11a KO (B6.129S7-*Itgal*$^{tm1Bll/J}$)	Global knockout for *Itgal*	Conventional	Jackson Laboratories (cat # 005257)
CD11b KO (B6.129S4-*Itgal*$^{tm1Myd/J}$)	Global knockout for *Itgam*	Conventional	Jackson Laboratories (cat # 003991)
CD11c KO	Global knockout for *Itgax*	Conventional	Gift from Prof Christie Ballantyne, Baylor College of Medicine, Texas, USA
CD11d KO (B6.129S7-*Itgad*$^{tm1Bll/J}$)	Global knockout for *Itgad*	Conventional	Jackson Laboratories (cat # 005258)
CD18 KO (B6.129s7-*Itgb2*^{tm2Bay}/J)	Global knockout for *Itgb2*	Individually ventilated cages	Jackson Laboratories (cat # 003329)
CD11a/CD11c Het	Heterozygous for *Itgal* and *Itgam*	Conventional	Bred in-house by crossing CD11a and CD11c KO strains
OT-II	T cells express a T cell receptor that specifically recognises chicken ovalbumin peptide (323-339) in the context of MHC Class II molecule I-A$^{d\ 305}$	Conventional	Bred in-house

2.2 Cell Isolation

Cells were counted by trypan blue exclusion using a haemocytometer.

2.2.1 Lymphoid Organs

Cells were isolated from the spleen, mesenteric lymph nodes, mediastinal lymph nodes peripheral lymph nodes (inguinal, brachial and axillary) and thymus by mashing the tissue between two pieces of 45μm nylon filtration fabric NITEX (Cadisch) in Hank's balanced salt solution (HBSS) (Gibco) supplemented 2% foetal calf serum (FCS) (Gibco). When DCs were going to be analysed, the organs were chopped into small pieces using a scalpel then digested in HBSS with 2% FCS and 2.68mg/ml collagenase D (Roche) in a shaking incubator at 37°C, 220rpm for 30mins prior to mashing. Cells were then pelleted by centrifugation at 400xg for 5min at 4°C. Splenocytes were then red blood cell (RBC) lysed using 1ml RBC lysis buffer (eBioscience) incubated at room temperature for 2.5min before washing in PBS.

2.2.2 Blood

Up to 300μl of blood was collected by cardiac puncture into 10μl of 0.5M EDTA. RBCs were lysed by adding 1ml RBC lysis buffer (eBioscience) for 2.5min at room temperature then cells were washed in PBS. This process was repeated 3-4 times, until the cell pellets were no longer visibly red.

2.2.3 Lungs

Lungs were digested as described previously[306]. Briefly, lungs were perfused with 10ml of 2mM EDTA prior to dissection then cut into small pieces. The tissues were incubated with 1.34mg/ml collagenase D (Roche) and 100μg/ml DNase (Sigma Aldrich) at 37°C, 200rpm for 40min then mashed through a 70μm cell strainer.

2.2.4 Small Intestine and Colon

SI and colons were washed in HBSS 2% FCS then fat and Peyer's patches were removed. The intestines were cut longitudinally, washed in HBSS 2% FCS to remove luminal contents then cut into 0.5cm segments. For SI, segments were incubated in 10ml of 2mM EDTA/HBSS at 37°C for 10mins then the supernatant

was collected by filtering through 45μM nylon filtration fabric NITEX (Cadisch; SI only). The SI supernatant contains cells from the epithelial layer. This was repeated a further two times then the segments were washed using 10ml of warmed HBSS. SI and colon segments were digested using 0.625mg/ml Collagenase V (Sigma Aldrich), 0.425mg/ml Collagenase D (Roche), 1mg/ml Dispase (Gibco) and 30ug/ml DNase (Roche) at 37°C, shaking at 160rpm for 25mins (SI) or 45mins (colon). Following digestion, the cell suspension was filtered through a 100μm followed by 40μm cell strainer.

2.2.5 Skin

Both ears were removed and cut into small pieces before incubating with 2mg/ml Collagenase IV (Sigma Aldrich), 2mg/ml Hyaluronidase (Sigma Aldrich) and 100 U/ml DNase (Invitrogen) for 40min at 37°C, shaking at 180rpm. After digestion, 2ml of complete RPMI was added to stop the reaction and the tissues were dissociated in gentleMACS C-tubes (Miltenyi Biotec) using the gentleMACs dissociator manufacturer program B.01 twice. Samples were then mashed through a 100μm cell strainer to obtain a cell suspension.

2.2.6 Gingiva

Gingival digest protocol was adapted from Mizraji *et al*., (2013)[307]. Briefly, the whole palette was collected and minced in PBS 2% FCS then digested using 2mg/ml Collagenase II (Lorne Laboratories) and 1mg/ml DNase I (Sigma Aldrich) for 20min, 37°C, shaking at 200rpm. 5mM EDTA was added and the gingivae were incubated for a further 10min. The samples were then filtered through a 70μm cell strainer.

2.2.7 Uterus

The uterus was digested using Multi Dissociation Kit 1 (Miltenyi Biotec) as per the manufacturer's instructions. Briefly, tissue was minced then incubated with 2.5ml incomplete RPMI (without added FCS or antibiotics), 100μl Enzyme D, 50μl Enzyme R and 12.5μl Enzyme A at 37°C, shaking at 200rpm for 20mins. Tissues were dissociated in gentleMACS C-tubes (Miltenyi Biotec) using the gentleMACs dissociator manufacturer program C then incubated for a further 20mins and the dissociation repeated. Samples were then mashed through a 70μm cell strainer to obtain a cell suspension.

2.2.8 Bone Marrow

Bone marrow cells were obtained by cutting the ends off the femoral and tibia bones and flushing PBS through using a 26G needle. Cells were then pelleted by centrifugation at 400xg for 5min at 4°C. Cells were then RBC lysed using 1ml RBC lysis buffer (eBioscience) incubated at room temperature for 2.5min before washing in PBS.

2.3 Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated by culturing bone marrow cells obtained from femoral and tibia bones in 10ng/ml GM-CSF (Peprotech) and 20ng/ml IL-4 (Peprotech). Total bone marrow suspensions were cultured without RBC lysis. Cells were plated into 10cm non-treated tissue culture plates and fed on days 3 and 6 of culture. BMDCs were harvested using 4mM EDTA incubated for 10min at 37°C. To activate BMDCs 50ng/ml LPS was added to the culture overnight. For flow cytometry analysis, BMDCs were gates as shown in Figure 2-1.

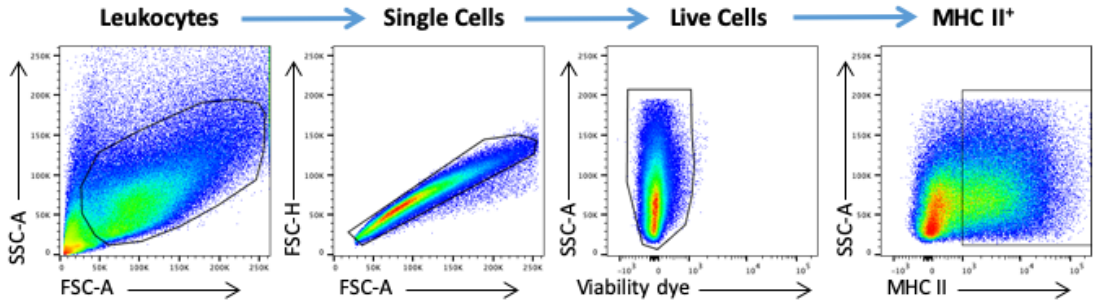

Figure 2-1 Gating strategy for BMDCs
Representative plots from WT BMDCs cultured without LPS.

2.4 BMDC-T-Cell Co-Culture

BMDCs were either stimulated with 50ng/ml LPS or left unstimulated overnight then pulsed with 5µg/ml $OVA_{323-339}$ peptide (Sigma-Aldrich) for 90mins. Lymph nodes from OT-II mice were pooled and the tissues disrupted using the end of a 2.5ml syringe in a 40µm cell strainer to produce a single cell suspension. Cells were pelleted by centrifugation at 380xg for 5min at 4°C then re-suspended in PBS. Using the same process spleens from OT-II mice were also pooled then re-suspended in RBC lysis buffer (1ml per spleen) and incubated at room temperature for 2.5mins

then washed in PBS. Lymph node and spleen cells were pooled then CD4+ T cells purified by negative selection using EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. This provided $CD4^+$ T cells of a 95-97% purity. The isolated $CD4^+$ T cells were then labelled with 1μM CFSE (Life Technologies) for 20mins at 37°C. Unbound dye was removed by washing cells twice in complete RPMI. Peptide-loaded BMDCs were cultured with the CFSE-labelled $CD4^+$ OT-II T cells in a ratio of 1:10 (using approx. $0.7x10^6$ T cells per well) in a total volume of 1ml in 24 well plates. Cells were harvested for flow cytometry analysis on day 1 and day 3 of culture.

2.5 Flow Cytometry

To control for background staining, fluorescence minus one (FMO) controls were used as indicated in the figures. For β_2 integrin α chain staining, CD18 KO mice were used as a negative control.

2.5.1 Extracellular Staining

Cells were pelleted by centrifugation at 400xg for 5min at 4°C then re-suspended in PBS and transferred into round-bottomed 96 well plates for staining. Typically, $1x10^6$ cells were used for BMDCs and $5x10^6$ cells for *ex vivo* cell suspensions. Cells were washed in PBS, incubated in viability dye for 30mins at 4°C prior to extracellular staining then washed again in PBS. Cells were then re-suspended in 50μl Fc block (made in-house using supernatant from 2.4G2 cell line) and incubated with appropriate antibodies in FACS Buffer (PBS 2% FCS, 1mM EDTA) for 30min at 4°C, with the exception of CCR7 staining that was done at 37°C for 15min. Antibodies used are listed in Table 2-2. Cells were washed in PBS then re-suspended in 200μl FACS Buffer before running on BD LSRII, BD LSR Fortessa or BD FACSCelesta. Data was analysed using FlowJo software (Treestar).

2.5.2 Intracellular Staining

For detection of intracellular cytokines, cells were stimulated with 10ng/ml PMA (Sigma Aldrich), 500ng/ml ionomycin (Sigma Aldrich) and GolgiPlug 1:1000 (BD Biosciences) for 4h at 37°C. Cells were fixed in 100μl Cytofix/Cytoperm (BD Biosciences) at 4°C for 20min then washed with 100μl Perm/Wash (BD Biosciences). Cells were then stained with appropriate antibodies in Perm/Wash for

1h at room temperature. Cells were washed in PBS then re-suspended in 200µl FACs Buffer before acquisition.

For intracellular staining of transcription factors, cells were fixed using 100µl Foxp3 Transcription Factor Fixation/Permeabilization buffer (eBioscience) for 1h at room temperature. Cells were then washed with 100µl permeabilization buffer (eBioscience) and stained with appropriate antibodies in permeabilization buffer for 1h at room temperature. Cells were washed in PBS then re-suspended in 200µl FACS Buffer before acquisition.

2.5.3 Apoptosis Marker Staining

For staining with Annexin V and 7AAD, single cell suspensions were washed in PBS following extracellular staining, then stained in Annexin V Binding Buffer (Biolegend) with Annexin V and 7AAD antibodies in a total volume of 100µl for 15mins at room temperature. 200µl of annexin buffer was then added to samples prior to running.

2.5.4 Phosphoflow

For staining of phosphorylated proteins, 50µl of single cell suspensions containing $4x10^6$ cells from the spleen, and each lungs sample was re-suspended in a total of 200µl, were exposed to 50µl of stimulus for the appropriate time in incomplete RPMI at 37°C. Cells were immediately fixed by adding 60µl of 4% paraformaldehyde (Sigma Aldrich) (final concentration of 1.5%) and incubating at room temperature for 10mins, then washed twice using FACS buffer. The cells were then permeabilised with 100µl of ice-cold 100% methanol at 4°C for 30mins, then washed twice in FACS buffer. Extracellular and intracellular markers were then stained using appropriate antibodies in FACS buffer for 2h at room temperature. Cells were washed in FACS buffer then re-suspended in 200µl FACS Buffer before acquisition.

Table 2-2 Antibodies used for flow cytometry

Marker	Fluorochrome	Clone	Conc. Used	Manufacturer
7AAD	-	-	5µl/sample	BD Biosciences
Annexin V	APC	-	5µl/sample	Biolegend
B220 (CD45R)	APC	RA3-7B2	1:200	BD Biosciences
Bcl2	FITC	Bcl/10C4	1:100	Biolegend
BrdU	FITC	B44	1:10	BD Biosciences
CCR7 (CD197)	PE Cy7	4B12	1:100	eBioscience
CD11a	APC	M17/4	1:200	Biolegend
CD11b	PE Cy7	M1/70	1:200	eBioscience
CD11b	Superbright 600	M1-70	1:200	eBioscience
CD11c	PerCP Cy5.5	N418	1:200	eBioscience
CD11c	APC eFluor780	N418	1:200	eBioscience
CD121a (IL-1βR)	APC	JAMA-147	1:200	Biolegend
CD127 (IL-7R)	BV510	A7R34	1:200	Biolegend
CD18	PE	M18/2	1:200	Biolegend
CD19	eFluor 450	eBio103	1:200	eBioscience
CD24	PE Cy7	M1/69	1:200	BD Biosciences
CD25	APC Cy7	PC61	1:200	Biolegend
CD27	PE Dazzle594	LG.3A10	1:200	Biolegend
CD27	BV421	LG.3A10	1:200	Biolegend
CD3	AlexaFluor 700	17A2	1:200	BioLegend
CD3	BV785	17A2	1:100	BioLegend
CD4	eFluor 450	RM4-5	1:200	eBioscience
CD4	PerCP Cy5.5	GK1.5	1:200	Biolegend
CD40	APC	1C10	1:200	eBioscience
CD44	PerCP Cy5.5	IM7	1:200	eBioscience
CD44	APC	IM7	1:200	eBIoscience
CD44	eFluor 780	IM7	1:400	eBioscience
CD45	PE	30-F-11	1:200	BD Biosciences
CD45RB	APC/Cy7	C36-16A	1:200	Biolegend
CD54 (ICAM-1)	PE/Cy7	YN1/1.74	1:200	Biolegend
CD54 (ICAM-1)	FITC	3E2	1:200	BD Biosciences
CD62L	PE	Mel-14	1:200	BD Biosciences
CD64	BV421	X54-5/7.1	1:200	Biolegend
CD69	PerCP Cy5.5	H1.2F3	1:200	eBioscience
CD8	PE Cy7	53-6.7	1:200	eBioscience
CD80	FITC	16-10A1	1:200	BD Biosciences
CD86	PE	GL1	1:200	BD Biosciences
Fas (CD95)	PE Cy7	Jo2	1:200	BD Biosciences
Gr1 (Ly-6G)	APC	IA8	1:200	Biolegend
IFNγ	PE/Cy7	XMG1.2	1:100	BioLegend
IL-17A	PE	TC11-18H10	1:100	BD Biosciences
IL-17A	BV605	TC11-18H10	1:100	BD Biosciences
IL-17F	PE	eBIO 18F10	1:100	eBioscience
IL-23R	PE	12B2B64	1:200	Biolegend
Ki67	PE/Cy7	16A8	1:100	BioLegend
MHC II	APC Cy7	M5114	1:200	eBioscience
NK1.1	AF700	PK136	1:200	Biolegend
NK1.1	e450	PK136	1:200	eBioscience
pSTAT3 (pY705)	AlexaFluor 647	4/P-Stat3	5µl/sample	BD Biosciences
pSTAT5 (pY694)	PE	47/Stat5	5µl/sample	BD Biosciences
RORγt	APC	AFKJS-9	1:100	eBiosciences
Tbet	PE Cy7	4B10	1:100	eBiosciences
TCRβ	APC	H57-597	1:200	BD Biosciences
TCRβ	PerCP Cy5.5	H57-597	1:200	eBioscience
TCRγδ	FITC	UC7-13D5	1:200	eBioscience
TCRγδ	BV650	GL3	1:200	BD Biosciences
Viability	eFluor 506	-	1:1000	eBioscience
Vγ1.1 (Vγ1)	BV421	2.11	1:200	BD Biosciences
Vγ2 (Vγ4)	FITC	UC3-10A6	1:200	BioLegend
Vγ2 (Vγ4)	PE	UC3-10A6	1:200	Biolegend
Vγ3 (Vγ5)	APC	536	1:200	BioLegend

2.6 Fluorescence-Activated Cell Sorting (FACS)

For isolation of γδ T cells for RT-PCR analysis, cells from CD18 KO spleens were first pre-enriched for T cells using EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies), providing $CD3^+$ cells of 90% purity with 10-40% expressing TCRγδ. Cells were then sorted using a FACS Aria IIU or FACS Aria III (BD Biosciences). Samples were sorted in FACS buffer gated on live, $CD3^+TCR\gamma\delta^+$ then $V\gamma1^-V\gamma4^-$ cells into PBS supplemented with 50% FCS.

2.7 BrdU Labelling and Staining

Mice were injected intraperitoneally with 1mg bromodeoxyuridine (BrdU) (Sigma Aldrich) in 200µl of PBS. From the day following injection, mice were fed 0.8mg/ml BrdU in the drinking water supplemented with 20% sucrose to make it more palatable. Fresh drinking water containing BrdU was given every other day and mice were culled after 7 days.

Tissues from BrdU-treated mice were processed as described in the cell isolation section, and then stained as described for viability and extracellular markers. After washing in PBS, cells were fixed using 100µl cytofix/cytoperm (BD Biosciences) for 20mins on ice. Cells were washed in 100µl permwash (BD Biosciences) then incubated in 100µl cytoperm permeabilization buffer plus (BD Biosciences) for 10mins on ice. After washing in 200µl permwash, samples were re-fixed in 100µl cytofix/cytoperm for 5mins on ice and washed again in permwash. To expose the incorporated BrdU, cells were incubated with 100µl of DNase (Sigma Aldrich) for 1h at 37°C. Samples were then washed in permwash and stained with anti-BrdU and any other intracellular antibodies for 1h at room temperature. After washing again with permwash, cells were re-suspended in 200µl FACS Buffer for acquisition.

2.8 ELISAs

Cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA) from serum and tissue supernatant samples. Serum was isolated from mouse blood by allowing the blood to clot at room temperature for up to 6h then samples were centrifuged at 133,000rpm for 5min and the serum removed. Tissue supernatants were collected by incubating the spleen, lungs or uterus in 2-300µl of complete RPMI at 37°C for 4h then the tissue was removed.

For all ELISAs, absorbance was measured at 450nm and 570nm using a Sunrise Absorbance Reader (Tecan). The reading A_{570} was subtracted from A_{450} and the background was removed by subtracting the blank reading from all samples. Cytokine concentration was determined by extrapolating the unknowns from a standard curve using GraphPad Prism software. The limits of detection were: IL-17A 62.5pg/ml, IL-1β 16pg/ml, IL-7 6.3pg/ml and IL-23 8pg/ml.

2.8.1 IL-17A

For detection of IL-17A, Costar 96 well high-binding assay plates (Corning) were coated with 50µl of 1µg/ml IL-17A capture antibody (BD Biosciences) in PBS overnight at 4°C. Plates were washed twice in PBS-T (PBS 0.05% Tween-20) then blocked with 200µl of 1% BSA (Sigma Aldrich) in PBS for 2h at room temperature. Plates were washed twice in PBS-T then 50µl of samples and standards added for 2h at room temperature. The top standard was added at 2000pg/ml and 2-fold serial dilutions performed across the plate. Plates were washed 3 times in PBS-T then 50µl of 1µg/ml IL-17A biotin detection antibody (BD Biosciences) in 0.5% BSA/PBS was added and incubated for 1h at room temperature. Plates were washed 3 times in PBS-T then 50µl of Extravidin-peroxidase (Sigma Aldrich) diluted 1:10,000 in PBS was added per well for 45mins at room temperature. Plates were washed 4 times in PBS-T then 50µl of SureBlueTMB substrate (KPL) was incubated in the dark. Once the blue colour developed, typically in 10min, the reaction was stopped by adding 50µl of 10% H_2SO_4.

2.8.2 IL-1β and IL-23

Levels of IL-1β and IL-23 were detected using Mouse IL-1β ELISA MAX™ Deluxe and Mouse IL-23 ELISA MAX™ Deluxe kits (Biolegend), respectively, according to the manufacturer's instructions.

2.8.3 IL-7

Levels of IL-7 were detected using Mouse IL-7 Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.9 Adoptive Transfer of Cells

2.9.1 OT-II T cell and BMDC Transfer

BMDCs and $CD4^+$ OT-II T cells were prepared as described in sections 1.3 and 1.4. $2x10^6$ $CD4^+$ OT-II T cells were transferred intravenously into WT recipients. BMDCs were stimulated with 50ng/ml LPS overnight then pulsed with 5µg/ml $OVA_{323-339}$ peptide (Sigma-Aldrich) for 90mins. On the day following the T cell transfer, $1.5x10^6$ BMDCs were injected subcutaneously into one footpad of recipient mice and the contralateral was left as a control. The popliteal lymph nodes were then analysed 1 or 3 days following the BMDC transfer.

2.9.2 Total T Cell Transfer

T cells were isolated from CD18 KO spleens using EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. T cells were then labelled with 1µM CFSE (Life Technologies) in PBS for 20mins at 37°C. Unbound dye was removed by washing cells twice in complete RPMI. Labelled T cells were re-suspended in PBS then $2x10^6$ cells were transferred intravenously into WT or CD18 KO hosts. Mice were culled 1, 4 or 8 days following the T cell transfer.

2.10 Static Adhesion Assay

ICAM-1 (6µg/ml, R&D Systems), Fibronectin (10µg/ml, R&D Systems) or PBS were coated onto Costar 96 well high-binding assay plates (Corning) overnight at 4°C. The following day, the plate was washed twice in PBS then blocked with 1% milk in PBS for 1h 15mins at 37°C. The plate was again washed twice in PBS then put on ice. BMDCs were re-suspended at $0.4x10^6$ cells/ml in adhesion medium (RPMI, 0.1%BSA, 20mM HEPES pH 7.25 and 2mM $MgCl_2$) then 400µl was added per well in duplicate or triplicate and incubated on ice for 8mins to allow cells to settle. The plate was then incubated at 37°C for 15mins for cells to adhere before being placed upside down in 2.5L of PBS 2mM $MgCl_2$ for 50mins at RT to remove any un-adhered cells. Liquid was removed from the wells, leaving 50µl. Adherent cells were then lysed with 3mg/ml p-Nitrophenyl Phosphate (PNPP) (Calbiochem) in 1% Tx-100/50mM acetate buffer pH5 incubated at 37°C for 60mins then 50µl of 1M NaOH was added to stop the reaction. Absorbance was measured at 405nm using a

Sunrise Absorbance Reader (Tecan). A total plate was also prepared by pelleting 400µl of BMDCs at $0.4x10^6$ cells/ml by centrifugation at 300xg for 1min then re-suspending cells in 200µl PBS. 50µl of cells were added to the total plate in triplicate then they were lysed, and absorbance read as with the experimental plate. The absorbance from the total plate was used to calculate the percentage adhesion for each sample.

2.11 Polymerase Chain Reaction (PCR)

All PCR and cDNA reactions were carried out using an S1000 thermal cycler (BioRad) as detailed in the sections following

Table 2-3 Primers used for PCR

Primer	Sequence 5' to 3'
Dock2 forward	GAC CTT ATG AGG TGG AAC CAC AAC C
Dock2 reverse	GAT CCA AAG ATT CCC TAC AGC TCC AC
Vγ1 forward	ATGCTGCTCCTGAGATGGC
Vγ1 reverse	TTATGAACGTTGATCATCACAGGAC
Vγ2 forward	TTGGTACCGGCAAAAAACAAATCA
Vγ2 reverse	CAATACACCCTTATGACATCG
Vγ4 forward	ATGAAGAACCCTGGCTCACAAG
Vγ5 forward	ATGTCAACCTCTTGGCTTTTTCTTC
Vγ6 forward	ATATATGTTTAAACGCCGCCACCATGGGGCTTCTGCTACAAGTC
Vγ6 reverse	ATATATGCGGCCGCTTAGGATTTCTTCTCATTGCCACAG
Vγ7 forward	ATGCTGTGGGCTCTGGC
Vg common reverse	TTAGGATTTCTTCTCATTGCCACAG
Vδ1 forward	AATAGCAATTCTACTGATGGTGG
Vδ2 forward	AGTCCTCAGTCTCTGACAATC
Vδ3 forward	CCAGATTCAATGGAAAGTAC
Vδ4 forward	GTACAAACAGCAAGGAGGGCAGG
Vδ5 forward	CCAGACAGTGGCAAGCGGCACTG
Vδ6 forward	TCAAGTCCATCAGCCTTGTC
Vδ reverse	CGAATTCCACAATCTTCTTG

2.11.1 Genomic DNA Preparation

The protocol for isolation of genomic DNA was adapted from Truett *et al.,* 2000[308]. Clips of 1-2mm were cut from mouse ear or tail and incubated in 100µl alkaline lysis reagent (25mM NaOH, 0.2mM EDTA, pH 12) for 45mins at 95°C. 100µl of neutralisation reagent (40mM Tric-HCl) was then added and samples were stored at -20°C prior to use.

2.11.2 RNA Purification

RNA was purified from single cell suspensions using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions and eluted in 30µl of RNase-free water. Quantification of RNA was carried out using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Samples were stored at -20°C prior to use.

2.11.3 cDNA Synthesis

Synthesis of cDNA was carried out using High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. The quantity of RNA used for the cDNA reaction was normalised to the maximum that could be used (concentration in 9µl) of the sample with the lowest quantity of RNA. Samples were incubated for 60mins at 37°C, 95°C for 5mins, then held at 4°C. Resulting cDNA samples were stored at -20°C.

2.11.4 *Dock2*^{Hsd} PCR

The PCR to detect the mutated form of *Dock2* (*Dock2*^{Hsd}) was adapted from Mahajan *et al.,* (2017)[309] with primer sequences from Yasuda *et al.,* (2014)[310], listed in Table 2-3. Each reaction contained 1X ThermoPrime ReddyMix PCR Master Mix (Thermo Fisher Scientific), 5µM primers, 50mM $MgCl_2$ (Thermo Fisher Scientific), 2µl genomic DNA, made up to 25µl with nuclease-free water. Initial denaturation for 3min at 94°C, followed by 35 cycles of 94°C 30s, 54°C 45s, 72°C 45s and then final extension for 2min at 72°C. The expected band size for the *Dock2*^{Hsd} copy number variant is 305bp.

2.11.5 Vγ/Vδ Chain RT-PCR

Expression of Vγ and Vδ chains was determined from sorted γδ T cells by reverse transcription PCR (RT-PCR). Primer sequences (Table 2-3) for Vγ1, Vγ4, Vγ5 and Vγ7 were kindly provided by Pierre Vantourout (Kings College London) and for Vγ6 by Leticia Monin Aldama (Francis Crick Institute, London). Sequences for Vγ2 and all Vδ chains obtained from Andrew *et al.,* (2005)[311]. Primers were purchased from Integrated DNA Technologies (IDT). Each reaction contained 2µl template cDNA, 1X Phusion HF buffer (Thermo Fisher Scientific), 0.2mM dNTPs (Invitrogen), 0.3µM primers, 0.02U/µl Phusion DNA polymerase (Thermo Fisher Scientific), 3% DMSO, 2mM $MgCl_2$ (Thermo Fisher Scientific) for Vγ6 reaction only, made up to 20µl with nuclease-free water. The protocol for gene amplification was initial denaturation for 30s at 98°C, followed by 35 cycles of 98°C 10s, x°C 30s, 72°C 30s and then final extension for 10min at 72°C. The annealing temperatures (x°C) are listed in Table 2-4.

Table 2-4 Annealing Temperatures for Vγ and Vδ RT-PCR

Gene	Temperature (°C)
Vγ1	66
Vγ2	55
Vγ4	66
Vγ5	50
Vγ6	66
Vγ7	68
Vδ1	60.5
Vδ2	60.5
Vδ3	55
Vδ4	60.5
Vδ5	55
Vδ6	60.5

2.12 Agarose Gel Electrophoresis

Gel electrophoresis was used to separate DNA fragments. Gels were 1% UltraPure agarose (Invitrogen), 1X SYBR safe gel stain (Invitrogen) in 1X Tris Acetate-EDTA (TAE) buffer (Thermo Fisher Scientific). Gels were run at 120V for 30mins and fragments visualised using Gel Doc™ XR+ system (BioRad).

2.13 Statistical Analysis

Data represents mean + standard deviation, or where individual data points are shown the mean is displayed. All statistical analyses were performed using GraphPad Prism software. Student *t* test for comparison of 2 groups, one-way ANOVA for comparison of one variable for more than 2 groups and two-way ANOVA for comparison of two variables for more than 2 groups. P values shown as *$p<0.05$, ** $p<0.01$, ***$p<0.005$, ****$p<0.0001$.

Chapter 3 Characterisation of β_2 integrin-mediated regulation of dendritic cells and the impact on interactions with T cells

3.1 Introduction

DCs are innate leukocytes responsible for linking innate and adaptive immunity. The main function of DCs is to present antigen to T cells, resulting in either activation of an immune response or induction of tolerance[312,313]. This activation or inhibition is dependent on factors present in the DC microenvironment. In the presence of pro-inflammatory cytokines such as TNF-α or microbial factors such as pathogen-associated molecular patterns (PAMPs) DCs are likely to activate other immune cells[165]. The induction of tolerance tends to occur both in the presence of anti-inflammatory cytokines such as IL-10 and under steady state conditions (absence of pathogens or cytokines) when self-antigen is presented by immature DCs[165,314]. This balance between immune activation versus the induction of tolerance is particularly important in the context of autoimmune disease, where there is an inappropriate inflammatory response due to loss of tolerance to self-antigens. An increased knowledge of DC regulation is therefore required to improve our understanding of what controls DC activation. This will allow the manipulation of DC-induced activation versus tolerance, which can be used to improve responses to infection/cancer or dampen responses in autoimmune disease.

One way in which DCs are regulated is through their integrin receptors. In particular, the β_2 integrins are a family of leukocyte-specific adhesion molecules expressed on DCs. β_2 integrins all express the β_2 chain (CD18) hybridised to one of four α chains: CD11a (α_L), CD11b (α_M), CD11c (α_X) or CD11d (α_D), all of which are expressed on DCs[27,37,60,315]. β_2 integrins have been shown to negatively regulate TLR-mediated responses: Macrophages and DCs from CD18 deficient mice were hyperresponsive to stimulation by TLR2, 4 and 9, shown by enhanced production of IL-6 and IL-12[182]. Specific knockout of CD11b also resulted in an enhanced production of TNF, IL-6 and IFNβ in macrophages treated with agonists for TLR3, 4 and 9[183]. However, there is contrasting evidence that supports the role of CD11b in positive regulation of TLR4 responses shown by Ling *et al.*, that CD11b KO BMDCs had impaired cytokine production in response to LPS[185]. It has also been shown that β_2 integrins can regulate DCs in the absence of TLR stimulation. In mice that lack functional β_2

integrin receptors (beta2$^{TTT/AAA}$ integrin knock-in mice), BMDCs expressed higher levels of activation markers irrespective of their maturity state[73]. This was also true for knock-in DCs *in vivo,* as they expressed higher levels of CD86 both in the absence and presence of an inflammatory stimulus[73]. This evidence supports the important role for β_2 integrins in regulating DC responses.

In addition to their role in regulating DC activation, β_2 integrins are also implicated in the migration of DCs. BMDCs generated from mice with functionally-deficient β_2 integrin receptors expressed higher levels of the chemokine receptor CCR7, which mediates DC migration to LNs[73]. These beta2$^{TTT/AAA}$ integrin knock-in mice also displayed an increased percentage of DCs in the spleen, peripheral and mesenteric lymph nodes under steady state conditions[73]. The increase was due to a higher proportion of CD103$^+$ and CD11b$^+$ migratory DCs. This data suggests that in the absence of functional β_2 integrins, DCs are more migratory and therefore integrins may play a role in restricting DC migration *in vivo*. Other work has suggested that DC migration to LNs is independent of β_2 integrins. The transfer of WT and integrin-deficient LPS-matured BMDCs (lacking β_1, β_2, β_3 and β_7) showed equivalent migration to LNs and similar localisation within the T cell zone[72]. Based on this data, β_2 integrins can influence the migration of DCs, although potentially in a context-dependent manner, and may differ for immature versus mature DCs.

There is clear evidence showing that β_2 integrins directly influence the activation of DCs and may influence their migration. In addition to this, β_2 integrins expressed on DCs have been implicated in modulating the subsequent priming of T cells. The transfer of peptide-loaded BMDCs induces a Th1 response, demonstrated by IFNγ production, and this was enhanced when β_2 integrin signalling-deficient BMDCs were transferred compared to WT[73]. In line with this, the co-culture of DCs with constitutively active CD11a induced lower levels of CD4 T cell proliferation than WT DCs and reduced IFNγ production and T-bet expression, indicative of an impaired Th1 response[88]. Similarly, magnesium-induced activation of β_2 integrins on DCs impaired their ability to induce T cell proliferation and this effect was found to be CD11b-dependent[97]. Taken together, this data demonstrates that alterations in β_2 integrins on DCs can influence the outcome of the subsequent T cell response.

Chapter 3 Characterisation of β2 integrin-mediated regulation of dendritic cells and the impact on interactions with T cells

There is clear evidence that β_2 integrins play an important role in the regulation of dendritic cell function, but it is not yet understood which subunits are responsible for this regulation and how these individually impact the subsequent T cell response. The aims of this chapter were:

1. To determine which of the β_2 integrins (CD11a, CD11b, CD11c or CD11d) regulate DC activation *in vitro*.

2. Examine whether this β_2 integrin-mediated regulation of DCs also occurs *in vivo*.

3. Assess the impact of β_2 integrin loss on DC interactions with T cells and subsequent T cell priming.

3.2 Results

3.2.1 CD11a and CD11c KO BMDCs display enhanced activation markers and reduced ligand binding

It has previously been shown that the absence of functional β_2 integrin receptors leads to elevated activation marker expression on BMDCs, but the specific subunits responsible for this altered phenotype are unknown[73]. To determine the loss of which β_2 integrin subunit/s mediate this activated phenotype, BMDCs were generated from WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice. The BMDCs were either left unstimulated (resting/immature DCs) or stimulated with LPS overnight (activated/mature DCs) before assessing cell surface marker expression by flow cytometry.

First, integrin expression on BMDCs was measured to check whether the absence of one β_2 integrin receptor had an impact on expression of the others. The β_2 integrin family members have a level of redundancy due to their overlapping ligand repertoire, therefore another subunit may be upregulated to compensate for integrin loss. Levels of the common β_2 chain (CD18), α chains (CD11a, CD11b and CD11c) and the β_2 ligand ICAM-1 were measured. CD11d expression was not measured, as there are no murine antibodies currently available.

Expression of CD18 was significantly reduced in all immature β_2 integrin KO BMDCs, as expected, and in mature CD11a and CD11c KO BMDCs compared to WT (Figure 3-1). It is unclear why the levels of CD18 on mature CD11b and CD11d KO BMDCs are equivalent to WT, as CD11b is expressed highly on BMDCs, therefore it was expected the loss of this subunit would reduce CD18 expression. Levels of CD11a were similar in all genotypes other than CD11a KO BMDCs in the absence of stimulus, suggesting that CD11a expression is not altered by loss of other integrin subunits in resting BMDCs. However, after activation with LPS, CD11a expression was significantly increased in CD11b and CD11c KO BMDCs. This may suggest that in the absence of CD11b or CD11c, CD11a is upregulated as a compensatory mechanism in response to TLR4-mediated signalling. Surprisingly, expression of CD11b was significantly downregulated in all β_2 integrin KO BMDCs, regardless of activation status. Over 90% of WT BMDCs express CD11b[316] and my data implies that this expression may be partially controlled by other β_2 integrins. In

contrast to this, the expression of CD11c remained unchanged in the absence of CD11a, CD11b or CD11d.

The β_2 integrin ligand ICAM-1 is expressed on DCs and can mediate their interactions with T cells[73]. Levels of ICAM-1 were significantly higher on CD11a KO BMDCs regardless of maturation status and were upregulated on mature CD11c KO BMDCs. This could indicate a feedback loop between the receptor (CD11a or CD11c) and ligand (ICAM-1) expression. The increased expression of ICAM-1 is likely to mediate the binding of DCs to β_2 integrins expressed on T cells and may partially overcome the loss of interactions that occur due to the absence of β_2 integrins on DCs that would normally bind to ICAM-1 on T cells. Additionally, the expression of ICAM-1, CD18 and the α subunits were unchanged by LPS-induced maturation.

It can be concluded that the loss of any one β_2 integrin subunit in BMDCs results in a significant reduction in CD11b expression. Additionally, the expression of ICAM-1 was elevated in CD11a and CD11c KO BMDCs. These changes must be taken into consideration when interpreting the results from experiments using BMDCs, as altered expression of integrin subunits other than the one that is targeted by the KO could contribute to any changes found in their phenotype and/or function.

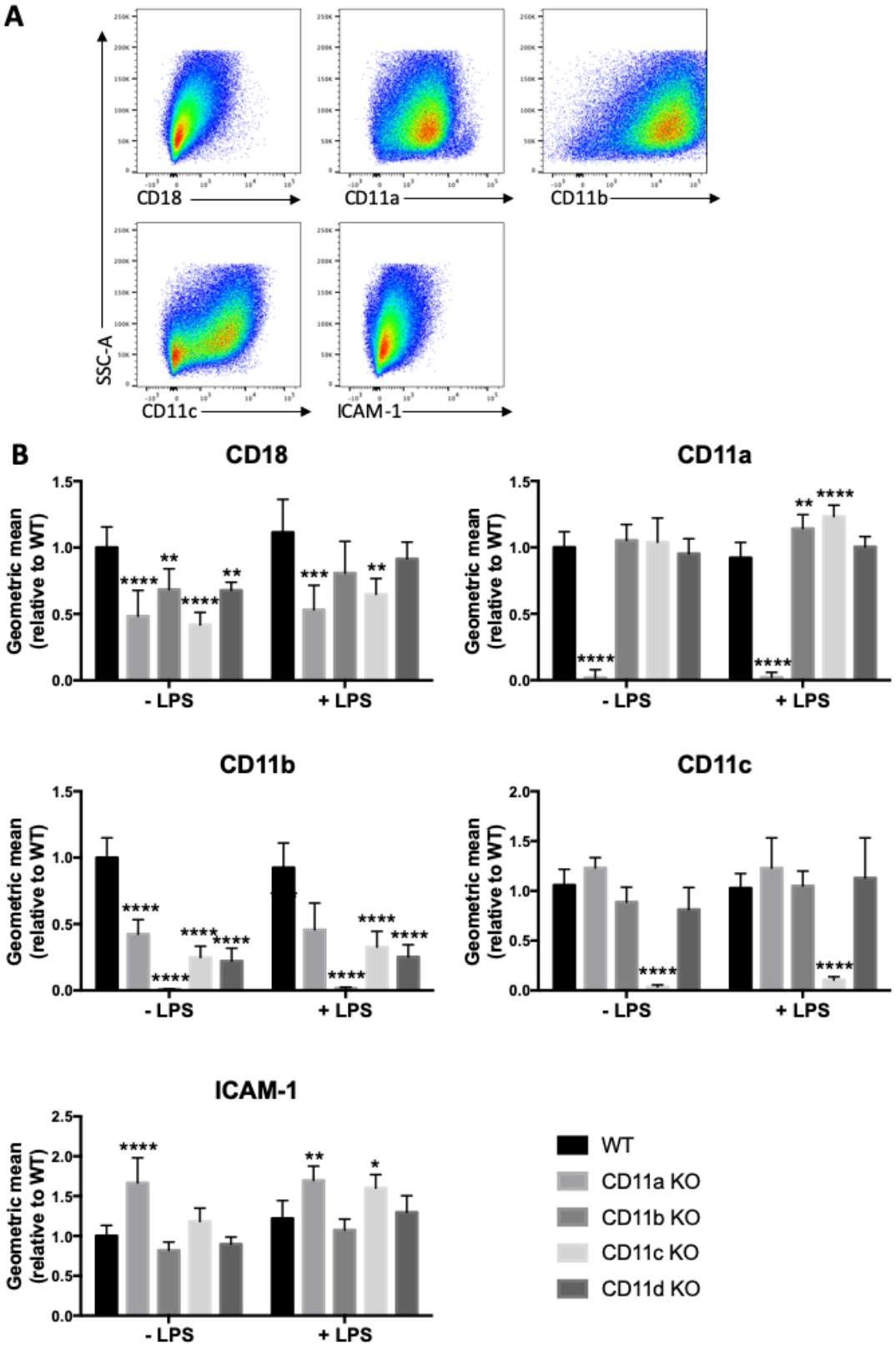

Figure 3-1 Expression of CD11b is reduced on all β₂ integrin-deficient BMDCs and the loss of CD11a or CD11c induces upregulation of ICAM-1.
WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO BMDCs were cultured with or without 50ng/ml LPS overnight then cell surface marker expression was measured by flow cytometry. Cells were gated on MHC II$^+$. **(A)** Representative plots of WT BMDCs -LPS. **(B)** Expression of β$_2$ integrins and ICAM-1 on BMDCs. Values shown are relative to WT -LPS from individual experiments. WT n=8, CD11a/b/c/d KO n=4. Data from a minimum of 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

After identifying that β_2 integrin-deficient BMDCs lack expression of the expected integrin subunit but also show reduced levels of CD11b, the activation status of the cells was then analysed. The aim was to characterise which β_2 integrin subunit/s are responsible for regulating BMDC activation. DCs present peptide to CD4 T cells via MHC II, which in combination with co-stimulatory signals via receptors such as CD40, CD80 and CD86 induces T cell activation. Assessment of these markers on β_2 integrin-deficient BMDCs showed no statistically significant difference in the expression of MHC II in any β_2 integrin KO BMDCs compared to WT (Figure 3-2). However, there were differences found in co-stimulatory molecule expression on CD11a and CD11c KO BMDCs: CD40 was upregulated in mature CD11a KO BMDCs compared to WT. CD80 expression was higher in both resting and activated CD11a and CD11c KO BMDCs. Higher levels of CD86 were also found in mature CD11a and CD11c KO as well as immature CD11a KO BMDCs. Interestingly, expression of CD80 was decreased in immature but not mature CD11b KO BMDCs, though none of the other markers of activation were differentially expressed in the CD11b KO BMDCs. The chemokine receptor CCR7 is upregulated on mature DCs to induce their homing to LNs. Expression of CCR7 was significantly upregulated on mature CD11a KO BMDCs but no differences were found on any other BMDCs compared to WT.

In summary, the absence of CD11a or CD11c in BMDCs resulted in enhanced expression of co-stimulatory molecules CD40, CD80 and CD86. This suggests that these β_2 integrin receptors are responsible for negatively regulating BMDC activation through control of co-stimulatory gene expression. Importantly, there was no difference in the activation marker expression on BMDCs lacking CD11b alone, therefore it is unlikely that the reduced expression of CD11b on the other integrin-deficient BMDCs has an impact on their activation.

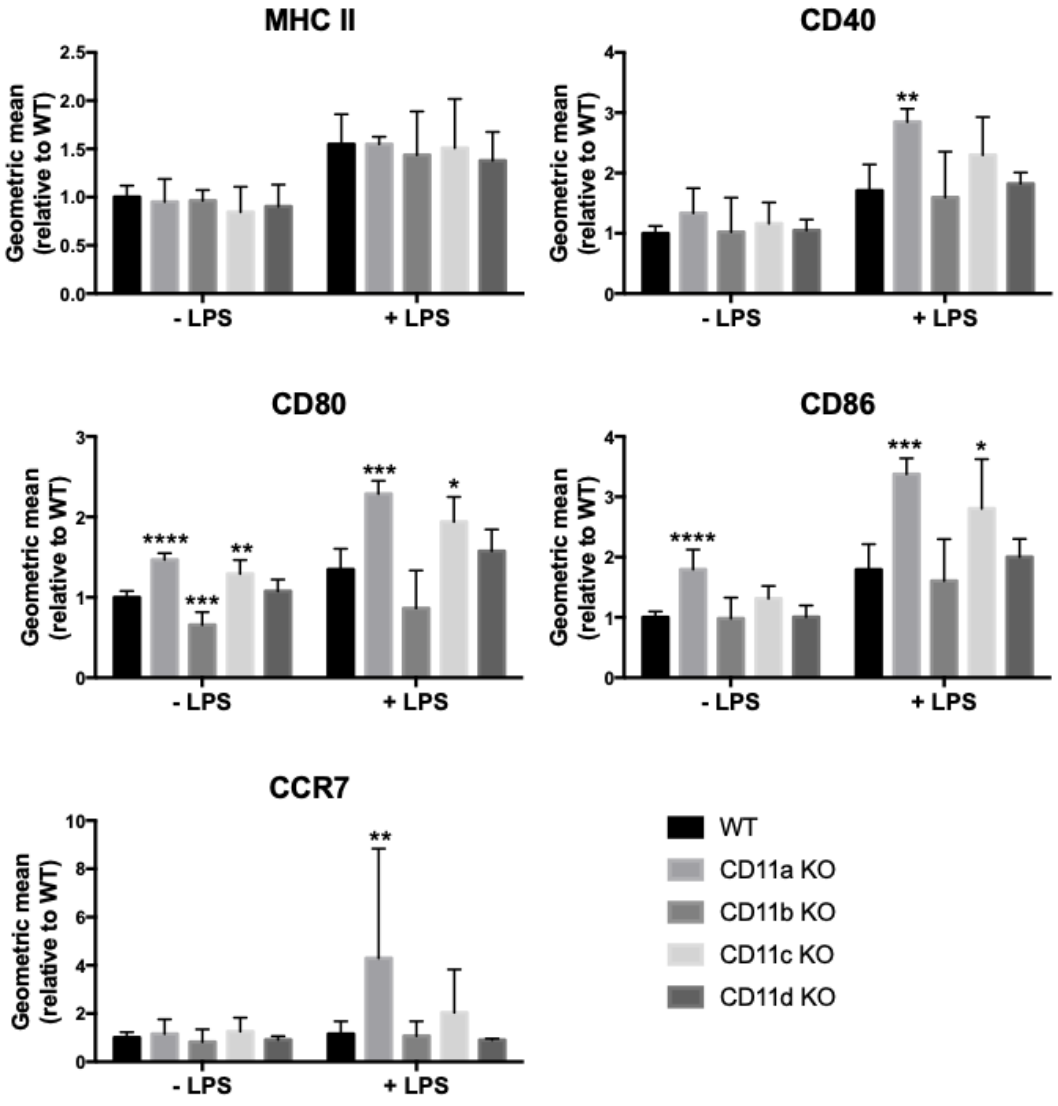

Figure 3-2 Increased expression of activation markers on CD11a and CD11c KO BMDCs. WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO BMDCs were cultured with or without 50ng/ml LPS overnight then cell surface marker expression was measured by flow cytometry. Cells were gated on MHC II^+. Values shown are relative to WT -LPS from individual experiments. WT n=8, CD11a/b/c/d KO n=4. Data from a minimum of 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

BMDCs lacking CD11a or CD11c expressed higher levels of co-stimulatory molecules that are important for mediating the activation of T cells. In addition to co-stimulation of T cells, the physical interaction between DCs and T cells is essential for mediating T cell activation. It is well characterised that CD11a expressed on T cells binds to ICAM-1 on APCs during immunological synapse formation, when T cells are activated via MHC II-TCR interactions[75]. It is likely that the reverse of this interaction also occurs, where integrin expression on the APC binds to ICAM-1 on the T cell[88,97]. DC integrin expression may, therefore, influence the outcome of the DC-T cell interaction and subsequently affect T cell activation/proliferation. It was

previously discovered that BMDCs lacking functional β_2 integrin receptors displayed reduced binding to ICAM-1 and formed weaker contacts with T cells[73]. To determine which of the β_2 integrin subunits mediate adhesion to ICAM-1, BMDCs from WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice were used in a static adhesion assay to measure their ability to bind to ICAM-1 (β_2 integrin ligand), with fibronectin (β_1 integrin ligand) as a positive control.

In the absence of CD11a or CD11c, immature BMDC binding to ICAM-1 was significantly reduced compared to WT (Figure 3-3A). There was no difference in the adhesion of CD11b, CD11c or CD11d KO BMDCs to ICAM-1. In mature BMDCs, the lack of CD11a significantly reduced binding to ICAM-1, whereas in the absence of CD11b or CD11c increased adhesion was seen compared to WT. Fibronectin is predominantly a β_1 integrin ligand so was used as a positive control, although it can also bind some β_2 integrins. The binding of immature CD11a KO BMDCs to fibronectin was decreased whereas CD11b and CD11c KO BMDCs showed significantly increased adhesion to fibronectin. Activation of these BMDCs with LPS abolished the differences in adhesion to fibronectin and no differences were found in the binding of immature or mature CD11d KO BMDCs to fibronectin.

Based on these results, it can be concluded that CD11a and CD11c are important for BMDC adhesion to ICAM-1. This reduced adhesion of BMDCs to ICAM-1 in the absence of CD11a or CD11c may impact on the strength and duration of interactions with T cells, and ultimately on the outcome of the T cell response.

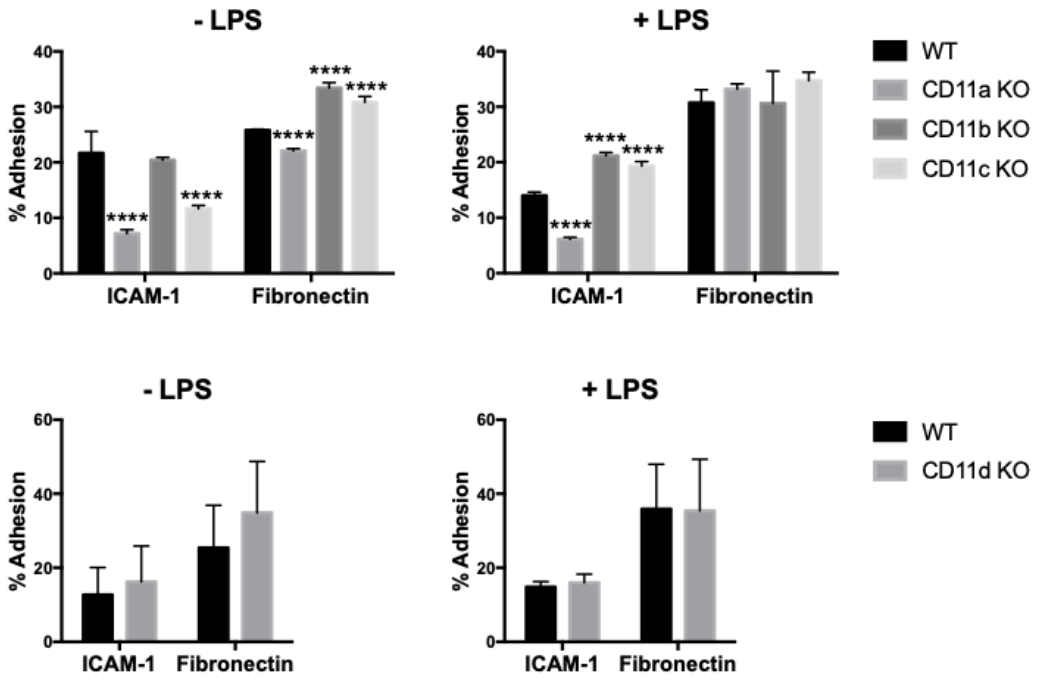

Figure 3-3 Decreased binding of CD11a and CD11c KO BMDCs to ICAM-1.
WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO BMDCs were cultured with or without 50ng/ml LPS overnight then binding to ICAM-1, fibronectin and milk blocking only measured by a static adhesion assay. Values calculated are relative to the total cells added per well. WT, CD11a/b/c KO n=4. WT, CD11d KO n=6. Data is pooled from a minimum of 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

3.2.2 CD11a KO BMDCs induce an enhanced T cell response

The loss of CD11a or CD11c expression in BMDCs resulted in an increased expression of co-stimulatory molecules but they displayed a reduced adhesion to ICAM-1. This could result in an increase in T cell activation if there are sufficient interactions between the integrin-deficient BMDCs and T cells. On the other hand, if the adhesion of BMDCs to T cells is impaired then the activation of T cells may be reduced, regardless of the increased levels of co-stimulatory molecules. To address these questions, the impact of β_2 integrin loss on the ability of BMDCs to prime T cells was assessed. BMDCs generated from WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice were cultured with $CD4^+$ OT-II T cells, then T cell proliferation (by CFSE dilution) and activation (expression of CD25, CD69 CD62L and CD44) were measured by flow cytometry after 1 or 3 days in culture.

The co-culture of $CD4^+$ OT-II T cells with either immature or mature BMDCs for 3 days induced their proliferation (Figure 3-4A). The peaks of CFSE expression allowed the detection of 4 divisions of the T cells. The percentage of T cells that

divided 1-4 times was variable between groups, but the majority of the T cells had divided 2 or 3 times (Figure 3-4B). The percentage of T cells that did not divide was similar across the cultures using immature BMDCs (Figure 3-4C). However, the co-culture of T cells with mature BMDCs showed a significant decrease in the percentage of undivided T cells when cultured with CD11a KO BMDCs compared to WT, but no difference when cultured with BMDCs deficient for CD11b, CD11c or CD11d. The proportion of T cells that divided 2 or 3 times were compared, because the majority of T cells that divided were in these gates (30-50%). There was no difference in the percentage of T cells that had undergone 2 or 3 divisions when cultured with immature BMDCs (Figure 3-4C). In the cultures with mature BMDCs, there was a significant increase in the percentage of T cells that divided 2 and 3 times when cultured with CD11a KO BMDCs compared to WT. No difference was found when T cells were cultured with mature CD11b, CD11c or CD11d KO BMDCs.

The loss of CD11a expression on BMDCs resulted in increased proliferation of CD4 T cells *in vitro*. This suggests that CD11a plays a role in inhibiting DC-induced proliferation of CD4 T cells.

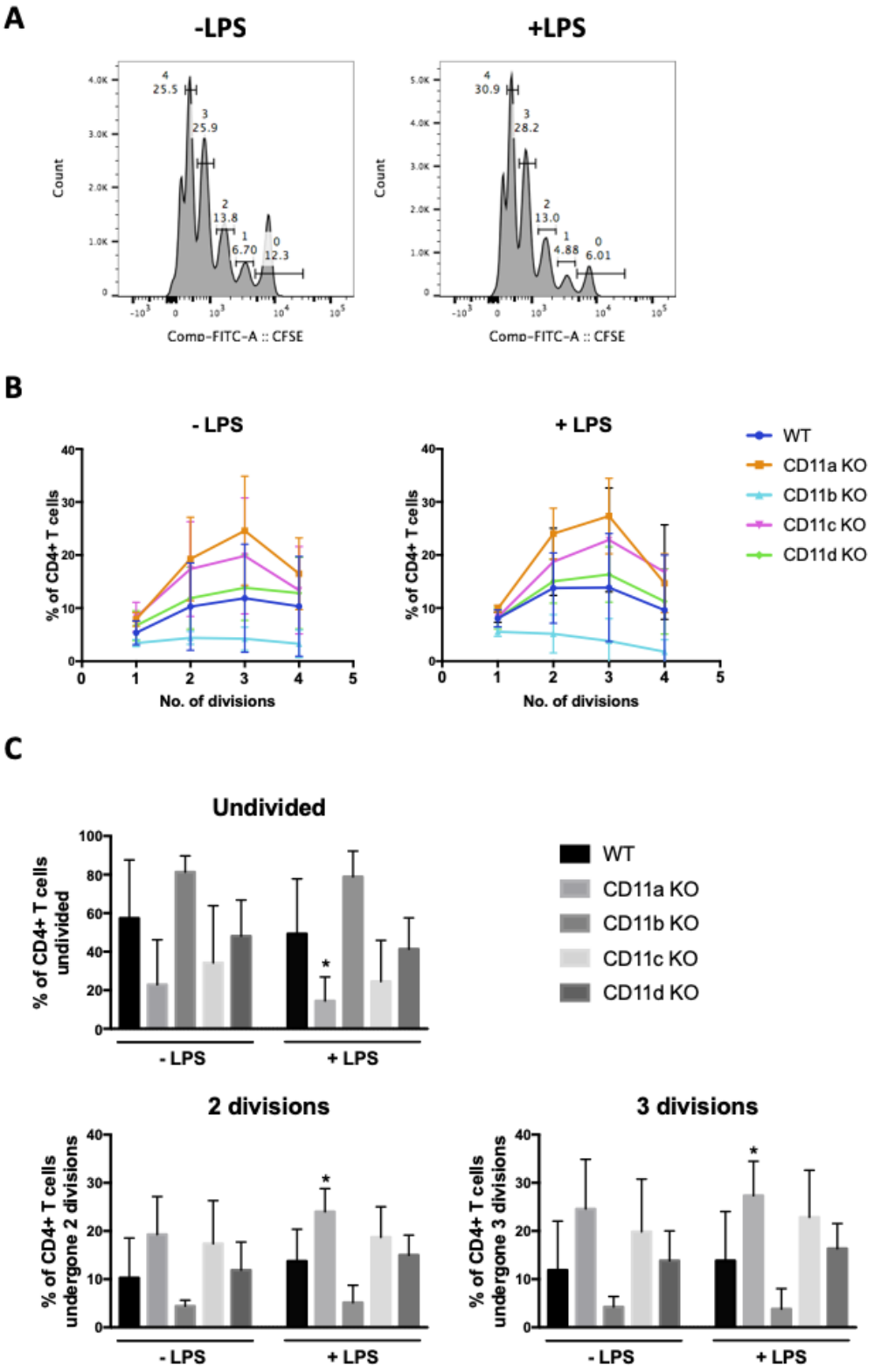

Figure 3-4 Increased T cell proliferation of OT-II T cells cultured with CD11a KO BMDCs. WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO BMDCs were cultured overnight with or without 50ng/ml LPS then pulsed with 5μg/ml $OVA_{323-339}$ peptide for 90mins before culturing with $CD4^+$ OT-II T cells *in vitro* at a ratio of 1:10. **(A)** Gating strategy for cell divisions based on CFSE fluorescence after 3 days. Representative plots from culture with WT BMDCs shown. **(B)** Percentage of OT-II T cells that divided 1-4 times. **(C)** Percentage of OT-II T cells that did not divide, underwent 2 or 3 divisions. n=5, data pooled from 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

In addition to measuring increased proliferation of T cells induced by CD11a-deficient BMDCs the expression of the early T cell activation markers CD25 and CD69 were assessed. After 1 day of culture, there was no significant difference in the expression of CD25 or CD69 on T cells cultured with immature BMDCs, regardless of genotype (Figure 3-5A). When cultured with mature CD11a KO BMDCs, T cells expressed significantly higher levels of both CD25 and CD69 compared to WT on day 1. CD25 expression was lower on T cells that were cultured with mature CD11b KO BMDCs compared to WT BMDCs but expression of CD69 was not significantly different. Similarly, both CD11c KO and CD11d KO BMDCs induced expression levels of CD25 and CD69 in T cells to the same extent as WT.

The differentiation of T cells from naïve into effector phenotype was determined by expression of CD44 and CD62L: $CD44^{lo}CD62L^{hi}$ (naïve) and $CD44^{hi}CD62L^{lo}$ (effector) T cells were quantified on days 1 and 3 of culture (Figure 3-5B). After 1 day in culture with immature WT BMDCs there was a large population of naïve (60%) and a smaller population of effector T cells (<20%) (Figure 3-5C). After 3 days of culture there was a reduced population of naïve T cells (40%) and expansion of effector T cells (>20%). The culture of T cells with immature CD11a KO BMDCs resulted in a significant decrease in the percentage of naïve T cells after both 1 and 3 days, whereas those with CD11b KO BMDCs retained significantly more naïve T cells compared to WT. Co-culture with immature CD11c or CD11d KO BMDCs resulted in the same proportions of naïve and effector T cells as with WT BMDCs. In cultures with mature BMDCs, there was a significant decrease in the percentage of naïve T cells after 1 and 3 days of culture with CD11a KO BMDCs and after 3 days with CD11c KO BMDCs compared to WT. Conversely, culture with CD11b KO BMDCs resulted in a higher percentage of naïve T cells and in cultures with CD11d KO BMDCs the percentage of naïve and effector T cells was similar to WT.

Overall, these results show that *in vitro,* CD11a KO BMDCs induce increased proliferation, expression of activation markers CD25/CD69 and differentiation of CD4 T cells into an effector phenotype. This indicates that despite their reduced ability to adhere to ICAM-1, CD11a KO BMDCs are able to sufficiently interact with T cells to induce activation and proliferation. Further experiments are required to quantify the dynamics of this interaction and confirm that the enhanced T cell

activation by CD11a KO BMDCs is due to their increased co-stimulatory molecule expression.

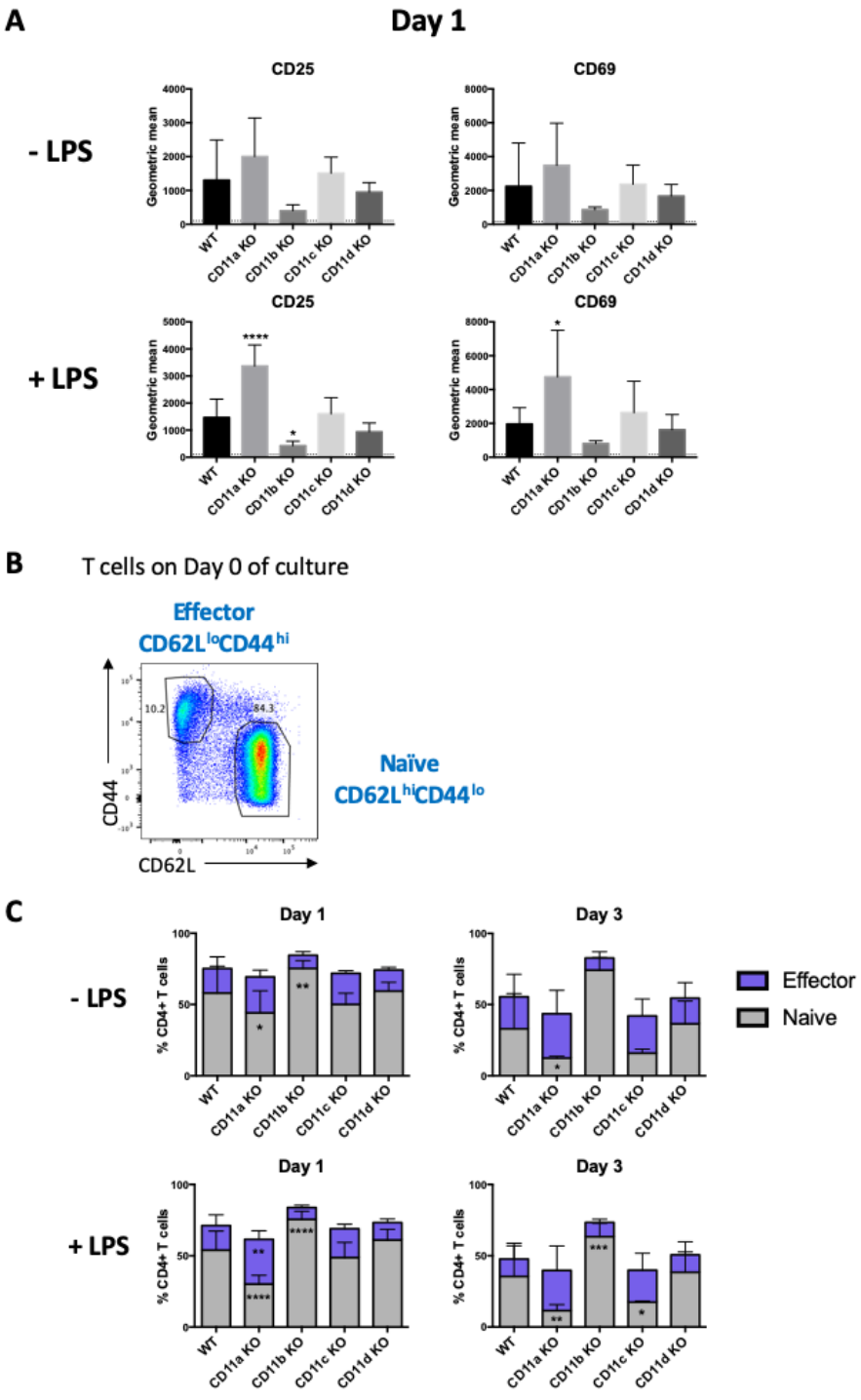

Figure 3-5 Increased activation of OT-II T cells cultured with CD11a KO BMDCs.
WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO BMDCs were cultured overnight with or without 50ng/ml LPS then pulsed with 5μg/ml $OVA_{323-339}$ peptide for 90mins before culturing with $CD4^+$ OT-II T cells in vitro in a ratio of 1:10. **(A)** Mean expression of T cell activation markers CD25 and CD69 after 1 day of culture. Dotted line represents mean expression on T cells prior to culture. **(B)** Gating strategy for naïve ($CD44^{lo}CD62L^{hi}$) and effector ($CD44^{hi}CD62L^{lo}$) T cells. Representative plot from T cells prior to co-culture (day 0). **(C)** Percentages of naïve and effector T cells following 1 and 3 days of co-culture. n=5, data pooled from 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

Having determined that CD11a KO BMDCs induce greater activation of CD4 T cells *in vitro,* the ability of CD11a KO BMDCs to stimulate CD4 T cell responses *in vivo* was then assessed. To investigate this, $CD4^+$ OT-II T cells (that express the congenic marker CD45.1) were transferred into WT mice i.v. and then, the following day, either WT or CD11a KO peptide-loaded BMDCs were injected into the footpad. The T cell response was measured in the draining (popliteal) LN 3 days post-transfer of BMDCs.

A population of OT-II ($CD45.1^+$) T cells was found in the LN draining the site where BMDCs were transferred, but not in the contralateral LN (Figure 3-6A). There was a significant increase in the percentage and number of transferred T cells in the draining LN of mice injected with CD11a KO BMDCs compared to WT BMDCs. However, when the expression of activation markers was analysed on these T cells there was a significant decrease in both CD25 and CD69 on T cells from mice injected with CD11a KO BMDCs. The expression of CD62L was similar on T cells that were stimulated with either genotype of BMDCs but CD44 expression was also lower with CD11a KO BMDCs compared to WT.

The *in vitro* co-culture of CD11a KO BMDCs with OT-II T cells resulted in greater T cell proliferation, enhanced expression of CD25/CD69 and an enhanced effector phenotype ($CD62L^{lo}CD44^{hi}$). The interaction of these same cells *in vivo* resulted in an increased number of T cells when mice were injected with CD11a KO BMDCs compared to WT. This suggests that CD11a KO BMDCs are able to induce greater T cell recruitment and/or proliferation *in vivo.* This could be confirmed by labelling the cells with CFSE prior to transfer, to allow quantification of proliferation. However, there was not the expected increase in activation marker expression in these T cells as was seen *in vitro* (Figure 3-5). Instead, expression of T cell CD25, CD69 and CD44 were all lower in mice injected with CD11a KO BMDCs compared to WT. As CD25 and CD69 are markers of early T cell activation, it is possible that the CD11a KO BMDCs may have induced higher expression of these markers prior to the 3-day timepoint. Further analysis of T cell activation over several time-points using CFSE-labelled T cells would be required to determine if T cell activation and proliferation *in vivo* is enhanced following priming by CD11a KO BMDCs.

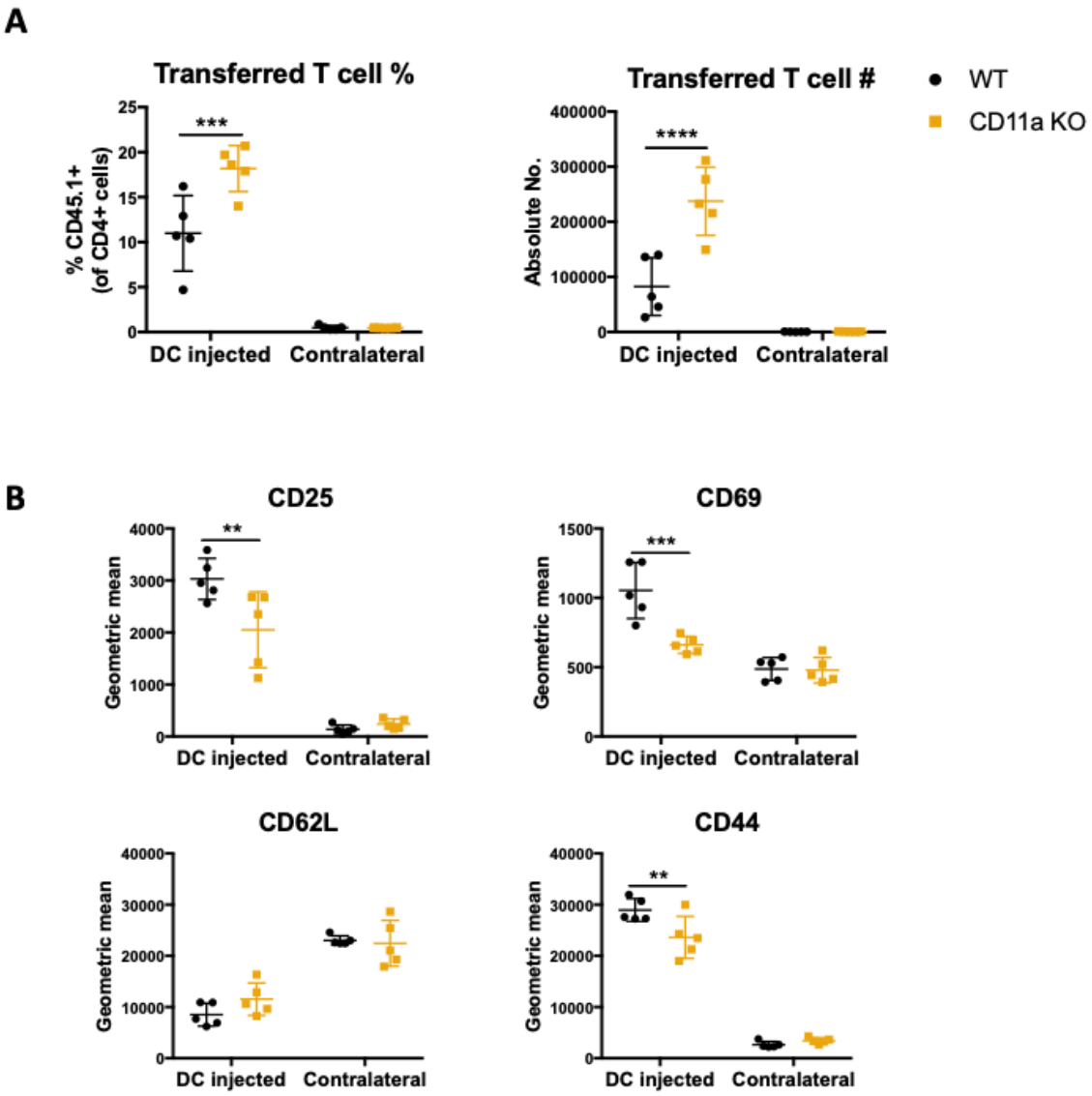

Figure 3-6 CD11a KO BMDCs induce higher numbers of CD4 T cells *in vivo* but these T cells show reduced activation marker expression.
$CD4^+$ OT-II T cells were transferred into mice i.v. then challenged the following day with OVA-peptide loaded WT or CD11a KO BMDCs s.c. in the footpad. 3 days following BMDC injection the draining LN (popliteal) from the injection site and the contralateral side were analysed by flow cytometry. Cells were gated on $CD4^+CD45.1^+$. **(A)** Percentage and numbers of transferred T cells. **(B)** Activation marker expression on transferred T cells. n=5, data from 1 experiment. Data represents mean ± SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

3.2.3 Increased percentage of DCs in CD11a and CD11c KO mice but no difference in activation marker expression

The results so far indicate that CD11a and CD11c subunits are responsible for regulating the activation status of *in vitro*-generated BMDCs. The next question was whether the loss of CD11a or CD11c expression results in enhanced activation of DCs *in vivo*. To test this, DCs were stained *ex vivo* from β_2 integrin-deficient mice. Cells were isolated from the spleen, mesenteric LN (mLN) and peripheral LN (pLN; inguinal, brachial and axillary LNs pooled) of WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice then analysed by flow cytometry to determine the expression of activation markers on the DCs.

CD11c is commonly used as a marker for DCs when analysing murine tissues by flow cytometry, but due to the use of CD11c KO mice this strategy could not be used. To overcome this, a series of dump gates were used to remove all possible cell types other than cDCs (macrophages/monocytes, B cells, T cells, neutrophils, NK/NK T cells, pDCs) then cells were gated on MHC II^+ and these were regarded as the DCs (Figure 3-7A). Using this gating strategy, the population of DCs analysed were consistently of 80-90% purity, based on CD11c expression in WT and β_2-deficient mice that expressed CD11c.

Analysis of DCs from the spleen, mLN and pLN showed an increased percentage of DCs in the lymphoid organs of CD11a and CD11c KO mice compared to WT, but no difference in CD11b or CD11d KO mice (Figure 3-7B). However, the absolute numbers of DCs in the lymph nodes and spleen of the β_2 integrin KO mice were similar to WT (Figure 3-7C). The similar number of DCs found across β_2 integrin-deficient mice indicates that DC migration to the spleen and LNs is unaffected by integrin loss. This supports the idea that β_2 integrins are not required for DC migration, as found previously[72]. There was a proportional increase in DCs from CD11a and CD11c KO mice, this may suggest there is a reduced number of another immune cell population causing this difference. Indeed, analysis of the total cell number in the spleen and LNs of β_2 integrin-deficient mice demonstrated a reduced cellularity in the organs of CD11a and CD11c KO mice but not CD11b or CD11d KO compared to WT (Figure 3-8). It is therefore likely that proportional differences in DCs found in CD11a and CD11c KO mice are due to a reduced number of another cell population. αβ T cells require CD11a for entry into LNs[68], therefore the lower

cell number in CD11a KO mLN and pLN is likely due to a reduced number of T cells. It is unclear what causes the lower cell number in the LNs of CD11c KO mice or in the spleen. A more global analysis of leukocyte populations present in these mice would be required to answer this question. Overall there was no difference in the number of DCs *in vivo* from β_2 integrin-deficient mice.

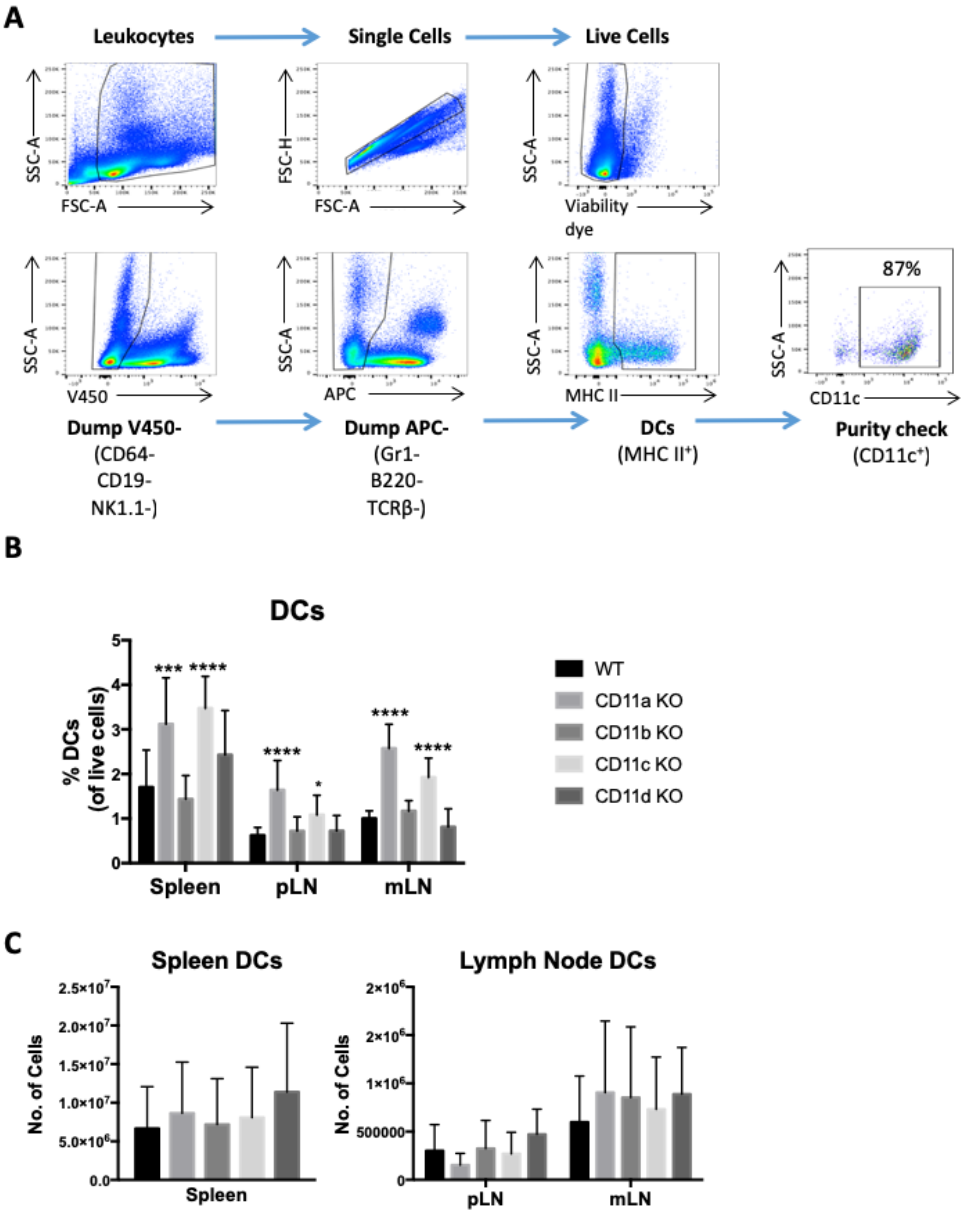

Figure 3-7 Increased percentage of DCs in lymphoid organs of CD11a and CD11c KO mice. Cells were isolated from the spleen, mesenteric LN (mLN) and peripheral LN (pLN: inguinal, brachial and axillary pooled) from WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice. DCs were analysed by flow cytometry. **(A)** Gating strategy to define DCs. **(B)** Percentage and numbers of DCs. Spleen - WT/CD11a KO/CD11b KO/CD11c KO n=12, CD11d KO n=6. LN – WT/CD11a KO/CD11b KO/CD11c KO n=16, CD11d KO n=4. mLN – WT/CD11aKO/CD11b KO/CD11c KO n=10, CD11d KO n=4. Data pooled from 2-7 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

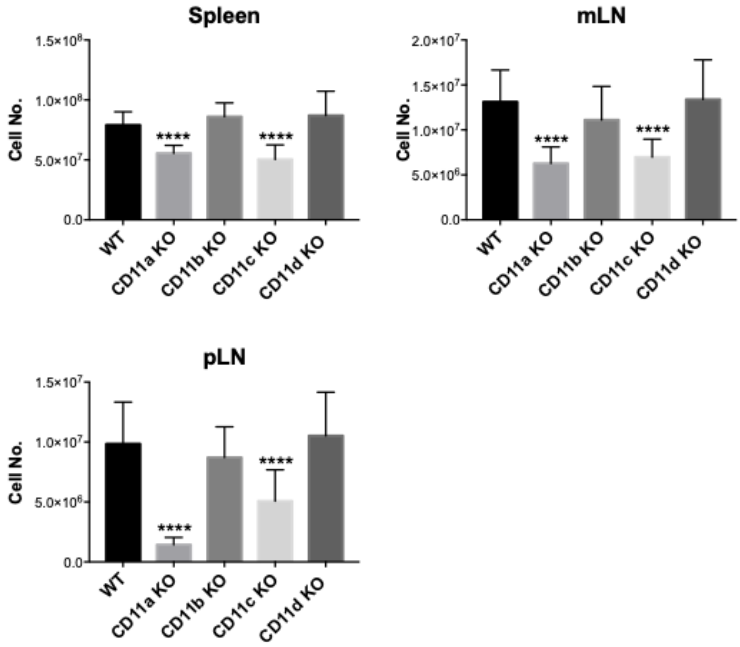

Figure 3-8 Decreased total cellularity in CD11a and CD11c KO spleen and lymph nodes. Cells were isolated from the spleen, mesenteric LNs (mLN), peripheral LNs (inguinal, axillary and brachial pooled; pLN) of WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice. Total cell numbers calculated are shown. n=16, data pooled from 6 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

Following analysis of DC numbers in β_2 integrin-deficient mice, the activation status of these DCs was then measured. Analysis of surface marker expression on DCs *ex vivo* showed that overall there were no significant differences in the expression of MHC II, the co-stimulatory molecules CD80, CD86, CD40 or the chemokine receptor CCR7. There was the exception of CD11c KO DCs from the spleen that expressed higher levels of CD86 compared to WT, and CCR7 expression was increased on CD11d KO DCs from the mLN. Although statistically significant, these differences were small.

Based on these results, it can be concluded that the increased expression of activation markers on BMDCs in the absence CD11a and CD11c *in vitro* is not replicated on *in vivo* DCs.

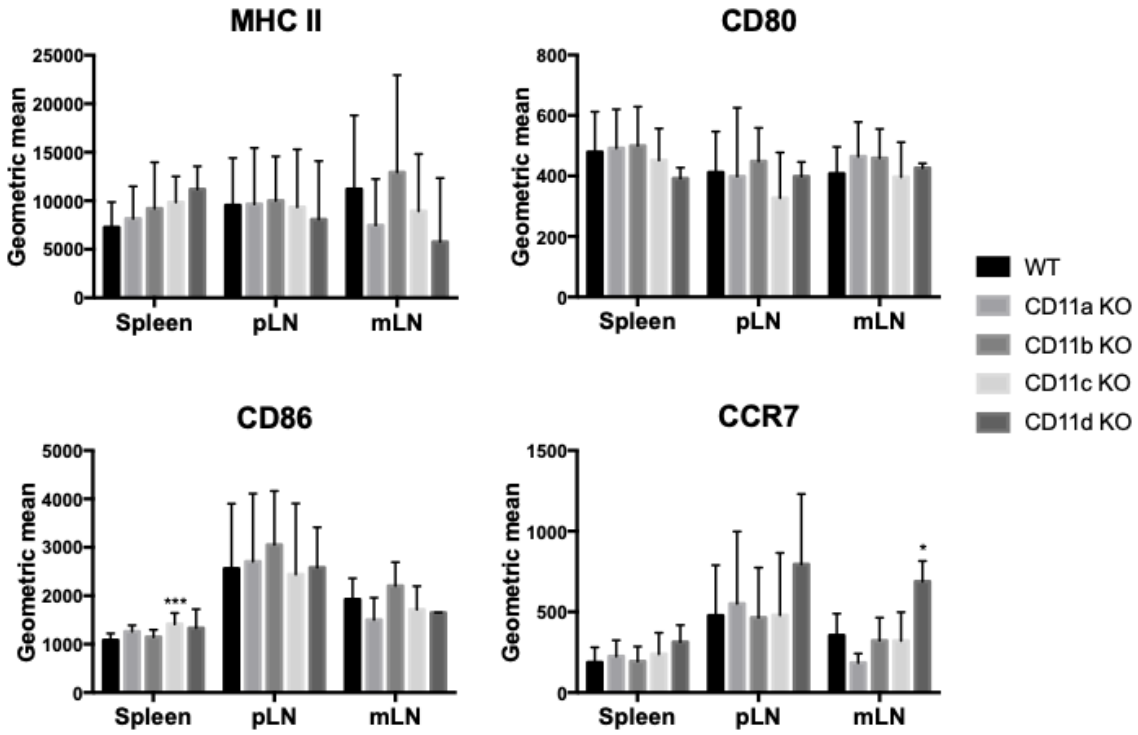

Figure 3-9 DCs from integrin-deficient mice show equivalent expression of activation markers.
Cells were isolated from the spleen, mesenteric LN (mLN) and peripheral LN (LN: inguinal, brachial and axillary pooled) from WT, CD11a KO, CD11b KO, CD11c KO or CD11d KO mice. Surface marker expression on DCs (identified as per the gating strategy outlined in figure 7A) was analysed by flow cytometry. Spleen - WT/CD11a KO/CD11b KO/CD11c KO n=12, CD11d KO n=6. pLN – WT/CD11a KO/CD11b KO/CD11c KO n=13, CD11a, CD11d KO n=7. mLN – WT/CD11a KO/CD11b KO/CD11c KO n=10, CD11d KO n=4. Data pooled from 2-6 independent experiments. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. **p*<0.05, ** *p*<0.01, ****p*<0.005, *****p*<0.0001.

3.2.4 CD4 T cells from CD11a and CD11c KO mice display enhanced activation and cytokine production *ex vivo*

In addition to understanding how the loss of β_2 integrin expression affects DCs, I also wanted to understand what impact this may have on the subsequent T cell response. The results from earlier *in vitro* experiments showed that CD11a KO BMDCs expressed higher levels of co-stimulatory molecules (Figure 3-2) and when co-cultured with T cells induced increased levels of T cell activation and proliferation (Figure 3-4, Figure 3-5). There was no difference in the activation status of DCs *ex vivo*, however this may be due to high variability in the data. This variability could be explained by the mixed population of DCs that were analysed (cDC1 and cDC2) compared with the less heterogeneous population of BMDCs generated *in vitro.* It is also possible that the increased proportion of DCs identified in CD11a and CD11c KO mice may affect T cell responses due to an increased ratio of DCs to T cells. To assess whether levels of T cell activation are enhanced *in vivo* in integrin-deficient

mice, the expression of activation markers was assessed on CD4 T cells *ex vivo*. Cells were isolated from the spleen, mLN and pLN of WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice, and then analysed by flow cytometry to determine the expression of activation markers on the CD4 T cells. The gating strategy is shown in Figure 3-10A.

On CD4 T cells isolated from the spleen of CD11a and CD11c KO mice there was a decreased expression of CD62L and increased expression of CD44 compared to WT (Figure 3-10B). The expression of CD25 was significantly lower on CD4 T cells from the CD11a KO spleen but no difference in CD69 expression across all genotypes. On T cells from the mLN, there was also a higher percentage of $CD44^+$ in CD11a and CD11c KO mice compared to WT, but there was no difference in expression of CD62L, CD25 or CD69 (Figure 3-10C). In the pLN, CD11a KO mice displayed decreased expression of CD62L on CD4 T cells, accompanied by increased levels of CD44, CD25 and CD69 relative to WT mice (Figure 3-10D). For T cells from CD11c KO mice the only difference in the pLN was a small but significant increase in CD44. There was no difference in the expression of any of the activation markers measured on CD4 T cells from CD11b or CD11d KO mice, in any of the tissues studied.

This data suggests that the loss of CD11a or CD11c expression in mice results in an increased activation status of CD4 T cells. This finding of enhanced T cell activation was true in all 3 lymphoid organs investigated (spleen, mLN and pLN), though there were subtle differences in marker expression between tissues. Further work is required to determine if this increased activation is an intrinsic property of the β_2 integrin-deficient CD4 T cells or due to alterations in another cell population. Based on the *ex vivo* data showing no difference in DC numbers or their activation marker expression, this effect is unlikely to be DC-dependent.

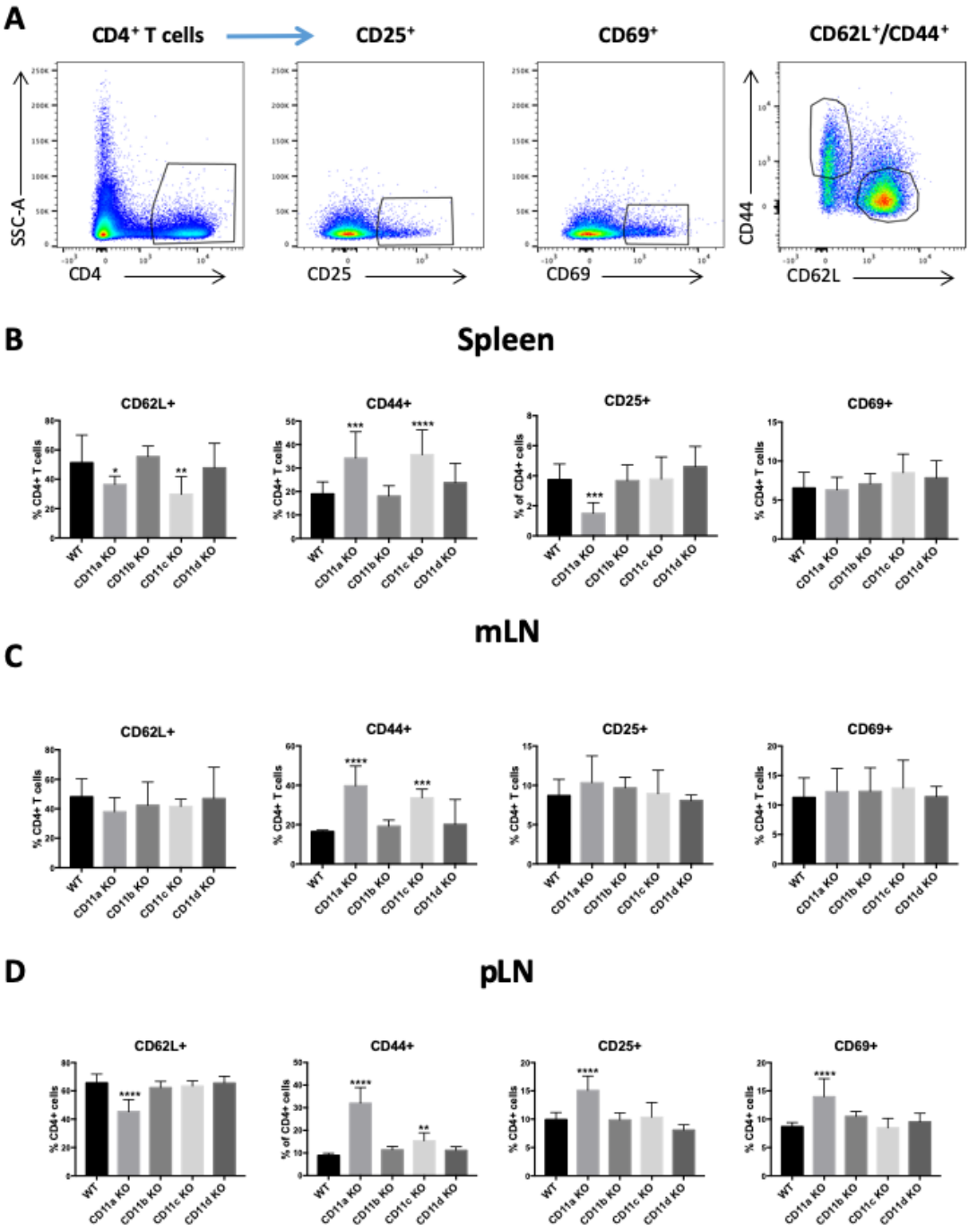

Figure 3-10 Increased activation marker expression on CD11a and CD11c KO CD4 T cells. Cells were isolated from WT, CD11a KO, CD11b KO, CD11c KO or CD11d KO mice and surface marker expression on CD4 T cells was analysed by flow cytometry. **(A)** Gating strategy from WT spleen, representative of other genotypes and tissues. Activation marker expression on CD4+ T cells from the spleen **(B)**, mesenteric lymph nodes (mLN) **(C)** and peripheral lymph nodes (pLN: inguinal, brachial and axillary pooled) **(D).** Spleen - n=12 (cKO), n=11 (WT, abdKO), n=6 (18KO), 4 expt. mLN – n=9 (cKO), n=8 (WT bdKO), n=6 (aKO), 3 expt. LNs – n=9, 3 expt. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001

After identifying that the absence of CD11a or CD11c results in enhanced T cell activation *in vivo*, the next question was whether integrin loss had an effect on the CD4 T cell functional response. To address this, cells from the spleen of WT and β_2

integrin-deficient mice were analysed by flow cytometry following *in vitro* stimulation with PMA/ionomycin to assess their potential to produce cytokines.

CD4 T cells from CD11a and CD11c KO mice showed enhanced production of both IFNγ and IL-17 compared to WT mice (Figure 3-11). Whereas cytokine levels were similar in T cells from WT, CD11b and CD11d KO mice. This indicates that, in addition to their increased activation status in CD11a and CD11c KO mice, CD4 T cells show an enhanced ability to produce IFNγ and IL-17.

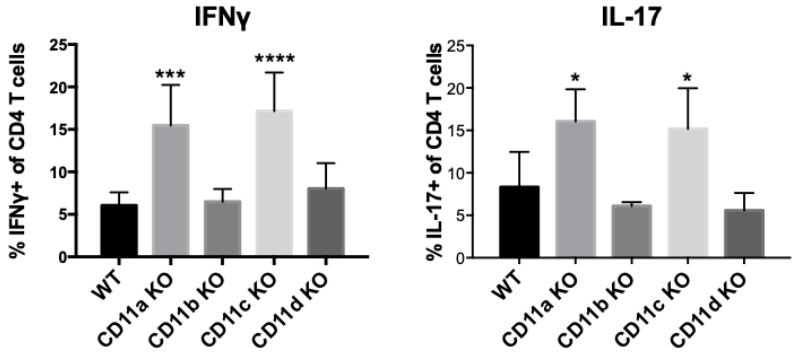

Figure 3-11 Increased production of IFNγ and IL-17 by CD4 T cells from CD11a and CD11c KO mice.
Cells isolated from the spleen of WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice were stimulated *in vitro* with PMA/ionomycin and levels of IFNγ and IL-17 were measured by flow cytometry. Cells were gated on CD4+. Percentage of CD4+ cells that expressed IFNγ or IL-17 are shown. IFNγ n=6, IL-17 n=4, data pooled from 2-3 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

3.2.5 Dock2 mutation found in CD11a KO, CD11c KO and CD11d KO mice

A publication by Mahajan *et al.* was brought to the attention of the lab in August 2016. The authors described an additional genetic mutation found in their line of sialic acid-deficient mice, in a gene called *Dock2*. The mutation in the *Dock2* gene was a frameshift mutation that causes the production of nonsense RNA, leading to a loss of functional *Dock2* expression. They traced this mutation to a line of 'WT' C57BL/6N^{Hsd} mice from Harlan Sprague Laboratories in the US (now Envigo Biosciences) and suggested the mutation in *Dock2* spontaneously arose in the C57BL/6N^{Hsd} colony. As the C57BL/6N^{Hsd} line is routinely used for backcrossing, it is likely that many GM strains generated in the US may exhibit phenotypes due to this unknown deficiency in the *Dock2* gene, in addition to the actual targeted gene of interest. This mutation in the *Dock2* gene is referred to as *Dock2*^{Hsd}.

It was decided to screen the β_2 integrin-deficient mice for *Dock2*^{Hsd} as they were originally generated in US labs and may have been backcrossed onto C57BL/6N^{Hsd} mice affected by the *Dock2* mutation. DNA from β_2 integrin-deficient mice was analysed for expression of *Dock2*^{Hsd} by PCR. Note that this PCR protocol detects *Dock2*^{Hsd} in genomic DNA, however it does not discriminate between heterozygous and homozygous expression.

For CD11a and CD11b KO lines, the original breeders (2 males and 2 females that were purchased from Jackson Laboratories) were analysed. The CD11c KO mice were a gift from Professor Christie Ballantyne (Baylor College of Medicine, Texas, USA). Ear clips were not kept from the original breeders so DNA from the first litter of mice that were then bred to continue the line were examined. Both the CD11d and CD18 KO lines originated from cryopreserved embryos purchased from Jackson Laboratories, so DNA from the mice born following implantation of these embryos was analysed.

The expression of *Dock2*^{Hsd} was identified in the CD11a KO, CD11c KO and CD11d KO mice, but was absent from CD11b KO, CD18 KO and WT control (Figure 3-12A). The implantation of cryopreserved embryos resulted in the birth of 3 mice for the CD11d KO line and only 1 for the CD18 KO line. Due to this low number, one of each line was bred with a C57BL/6 purchased from Harlan UK (negative for *Dock2*^{Hsd}). To assess whether the CD11d KO line retained the expression of *Dock2*^{Hsd} and to confirm the absence in the CD18 KO line, DNA from the breeders of these lines was examined. The expression of *Dock2*^{Hsd} was found in 7 out of the 12 CD11d KO breeders, whereas all of the CD18 KO breeders were negative for *Dock2*^{Hsd} (Figure 3-12B).

This indicates that CD11a KO, CD11c KO and some of the CD11d KO mice I used for previous experiments had a mutation in the *Dock2* gene in addition to their integrin deficiency. The expression of *Dock2*^{Hsd} is probably homozygous in the CD11a and CD11c KO lines, whereas many of the CD11d KO mice are likely to be heterozygous. This is because the CD11a and CD11c KO mice were obtained as live breeding pairs, whereas the CD11d KO was from an implanted embryo that was bred with a WT. Based on these findings, a careful re-examination of the data was

required to determine which immune phenotypes were due to the β_2 integrin deficiency and which were due to loss of Dock2 function.

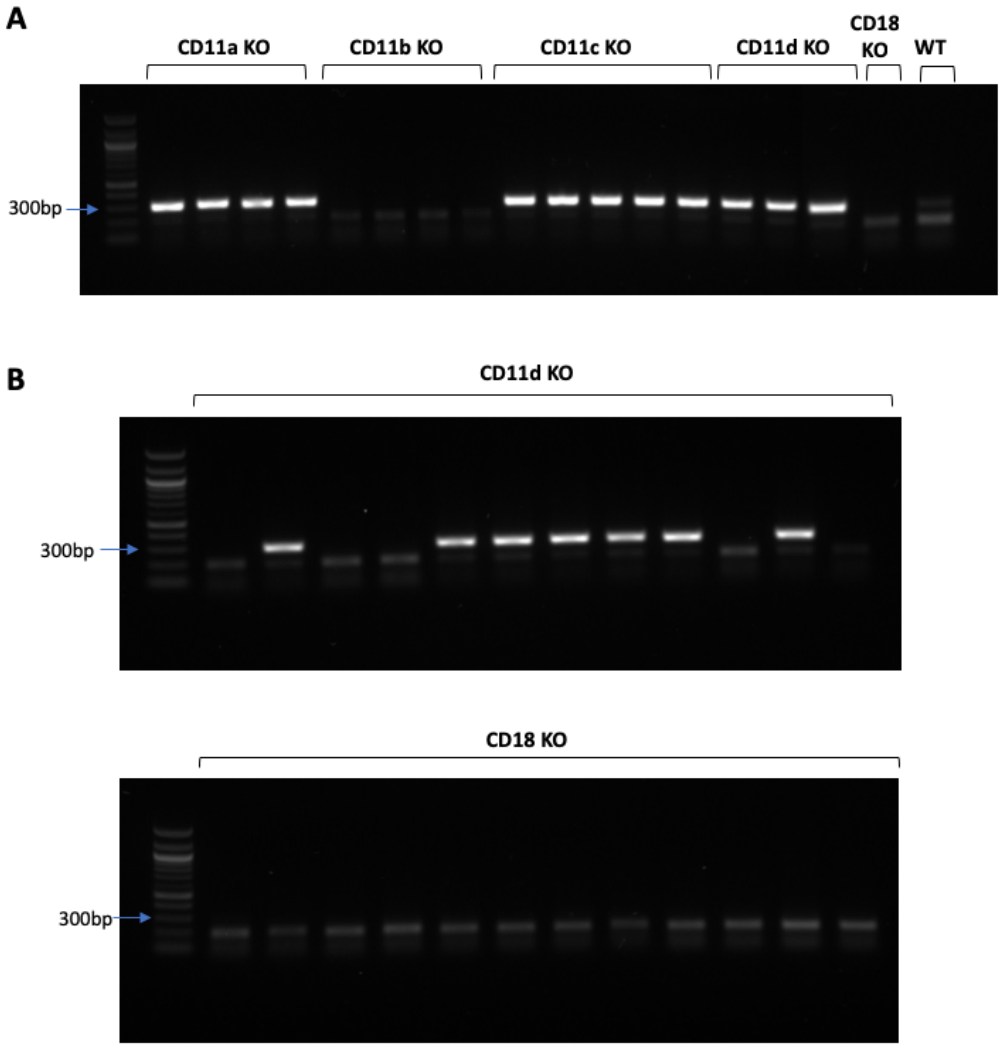

Figure 3-12 Mutated form of *Dock2* (*Dock2*^{Hsd}) is expressed in CD11a, CD11c and CD11d KO mouse strains.
DNA from digested ear clips of CD11a KO, CD11b KO, CD11c KO, CD18 KO or C57BL/6 (WT) mice. *Dock2*^{Hsd} was amplified by PCR and the resulting fragments were separated by gel electrophoresis on a 1% agarose gel (1X TAE 1X SYBR safe). The expected band size of the mutated form of *Dock2* is 305bp. **(A)** Analysis of DNA from CD11a KO and CD11b KO line original breeders, CD11c KO line first litter, CD11d KO and CD18 KO lines original mice obtained from embryos. **(B)** Analysis of DNA from CD11d KO and CD18 KO line breeders.

3.2.6 Determination of immune phenotypes in CD11a and CD11c KO mice due to loss of Dock2 or integrin expression

Dock2 is a guanine nucleotide exchange factor that regulates membrane polarisation and cytoskeletal dynamics. It is expressed exclusively in haematopoietic cells, therefore the mutation in this gene is particularly relevant for mice used to study immune cells. The main immune phenotypes that have been characterised due to *Dock2*^{Hsd} are an increase in memory CD8 T cells, loss of marginal zone B cells, iNKT cells and pDCs[309].

Both Dock2 and β_2 integrins play important roles in leukocyte migration. It was, therefore, imperative to determine which effects described earlier in this chapter were due to the β_2 integrin deficiency and which phenotypes were due to the mutation in *Dock2*. In order to answer these questions, mice from the CD11a and CD11c KO lines (where the most significant differences were seen in DCs and CD4 T cells) were crossed to create CD11a/CD11c hets (Figure 3-13). These mice were heterozygous for the expression of both CD11a and CD11c but retained homozygous expression of *Dock2*^{Hsd}. The phenotype of cells from these integrin hets was compared with the homozygous integrin KO *Dock2*^{Hsd} mice (CD11a KO & CD11c KO), mice that express WT integrins/Dock2 (WT) and mice with WT integrins/*Dock2*^{Hsd} (Dock2).

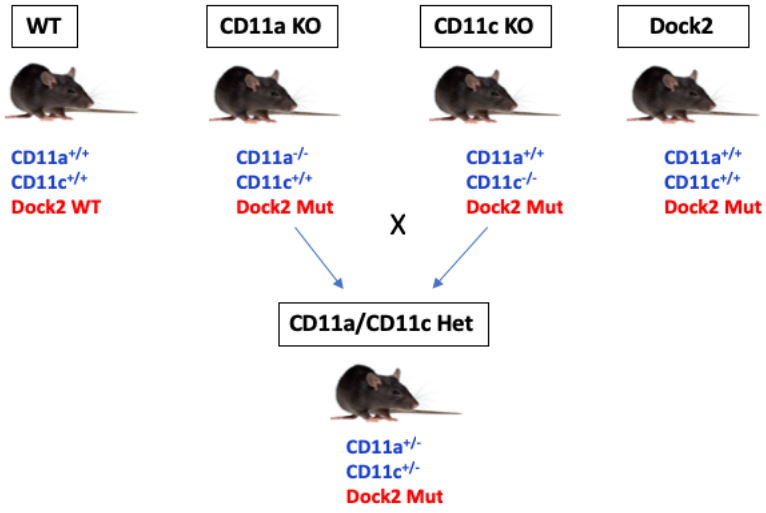

Figure 3-13 Mice used to determine effects downstream of β2 integrin-deficiency or *Dock2*^{Hsd}.
C57BL/6 (WT) mice were purchased from Harlan, CD11a KO mice were purchased from Jackson Labs, and CD11c KO mice gifted from Christie Ballantyne (Baylor College of Medicine, Texas, USA). CD11a/CD11c het mice were bred in-house by crossing CD11a and CD11c KO strains. Dock2 mice were bred in-house from the offspring of crossed F1 generation from CD11c KO mice crossed with WT C57BL/6 (see Figure 1-21). The integrin and Dock2 genotypes are shown.

Before assessing the immune phenotypes, β_2 integrin expression was first assessed on total splenocytes from WT, CD11a KO, CD11c KO and CD11a/CD11c het mice. The aim was to assess the altered integrin expression in CD11a/CD11c het mice to determine if they had the expected intermediate level of integrin expression. The expression of CD18 was significantly lower on cells from CD11a KO mice compared to WT, CD11c KO and CD11a/CD11c hets (Figure 3-14). CD11a was expressed to a similar extent on cells from WT and CD11c KO mice, but was absent in CD11a KO mice, as expected. The expression of CD11a on cells from CD11a/CD11c hets was higher than the CD11a KO mice but lower than the CD11c KOs, indicative of heterozygous expression. The Dock2 mice showed lower expression of CD11a than the CD11c KO mice but no difference compared to WT. The expression of CD11c was significantly increased on cells from CD11a and CD11a/CD11c hets than WT, CD11c KO or Dock2 mice. There was no significant difference in the mean expression of CD11c between WT and CD11c KO mice, suggesting that there was a high level of background staining or due to a low number of $CD11c^+$ cells present in the spleen.

As expected, the expression of both CD11a and CD11c in the CD11a/CD11c het mice was higher than their respective homozygous KO mice. The expression of CD18, CD11a and CD11c was similar on cells from WT mice regardless of their Dock2 expression (WT and Dock2 groups).

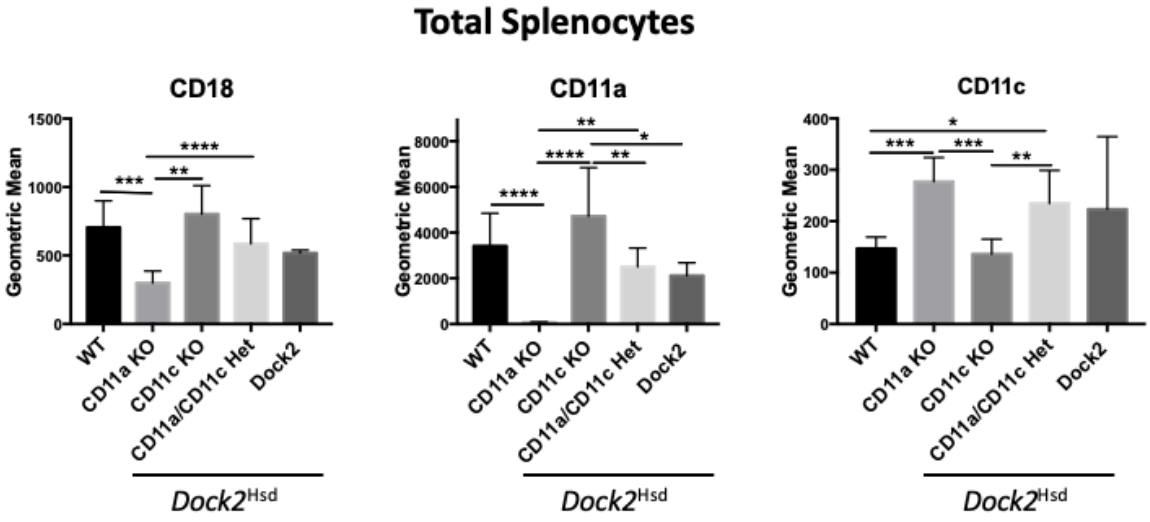

Figure 3-14 Expression of β2 integrins in integrin-deficient dock2 mutant mice.
Splenocytes from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice were analysed by flow cytometry for expression of β_2 integrins and ICAM-1. Data shown is geometric mean from total live single cells. WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, data from 3 independent experiments. Dock2 n=3 from 1 experiment. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

The CD11a/CD11c deficient *Dock2*^{Hsd} mice displayed higher integrin expression than the KO mice but, in some cases, reduced compared to WT. There are two possible functional consequences of this altered integrin expression, either the intermediate level of CD11a/CD11c expression will be: 1) sufficient for cell function so the het mice will appear as WT or 2) insufficient to restore integrin function so the het mice will display the same phenotype as KO.

Next, I wanted to confirm the mutation in *Dock2* resulted in the expected altered immune phenotype that has been reported. One of the major immune cell phenotypes that occurs in mice with *Dock2*^{Hsd} is the loss of marginal zone B cells (MZ B cells)[309,317]. To confirm that *Dock2*^{Hsd} resulted in a loss of MZ B cells in the integrin-deficient mice, splenic B cells were analysed from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice.

The total B cell population in the spleen can be identified by gating on CD19^{+} cells, then MZ B cells are identified as CD21/35^{hi} CD23^{lo} [318] (Figure 3-15A). The Dock2 mice (which have WT integrin expression but express *Dock2*^{Hsd}; see Figure 3-13) were obtained from in-house breeding of the CD11a/CD11c KO lines to remove expression of *Dock2*^{Hsd}. This was done by crossing the CD11a or CD11c KO mice with WT C57BL/6, then the F1 generation were crossed, and this resulted in the generation of some mice that had WT integrin expression but expressed *Dock2*^{Hsd}.

However, the PCR to test for *Dock2*^{Hsd} does not discriminate between mice that are heterozygous or homozygous, therefore it was known that the mice in the Dock2 group expressed *Dock2*^{Hsd}, but they may have 1 or 2 copies of the mutated gene. Analysis of MZ B cells from the Dock2 mice revealed that 2 out of the 3 mice had a population of MZ B cells but the other mouse was deficient (Figure 3-15B). This suggests that mouse number 3 was homozygous for *Dock2*^{Hsd} whereas mice 1 and 2 were heterozygous. This *Dock2*^{Hsd} homozygote mouse appears to have a population of CD23^{hi} B cells that is absent in the WT and het mice, though this was not investigated further. In the WT spleen, MZ B cells represented around 7% of the total B cell population (Figure 3-15C). Whereas in CD11a KO, CD11c KO and CD11a/CD11c het mice the MZ B cells were completely absent. Both the percentage and numbers of MZ B cells were significantly decreased in all of the integrin-deficient mice compared to WT.

Analysis of the MZ B cell populations confirmed that, as expected, the homozygous mutation in *Dock2* found in the CD11a KO, CD11c KO and CD11a/CD11c het mice resulted in a complete loss of MZ B cells in the spleen. The data also revealed that in the Dock2 group (mice with *Dock2*^{Hsd} but WT integrin expression), 2 out of 3 mice were likely heterozygous for *Dock2*^{Hsd}. This must be taken into consideration when interpreting the results from experiments using the Dock2 group of mice (Figures 3-16 to 20).

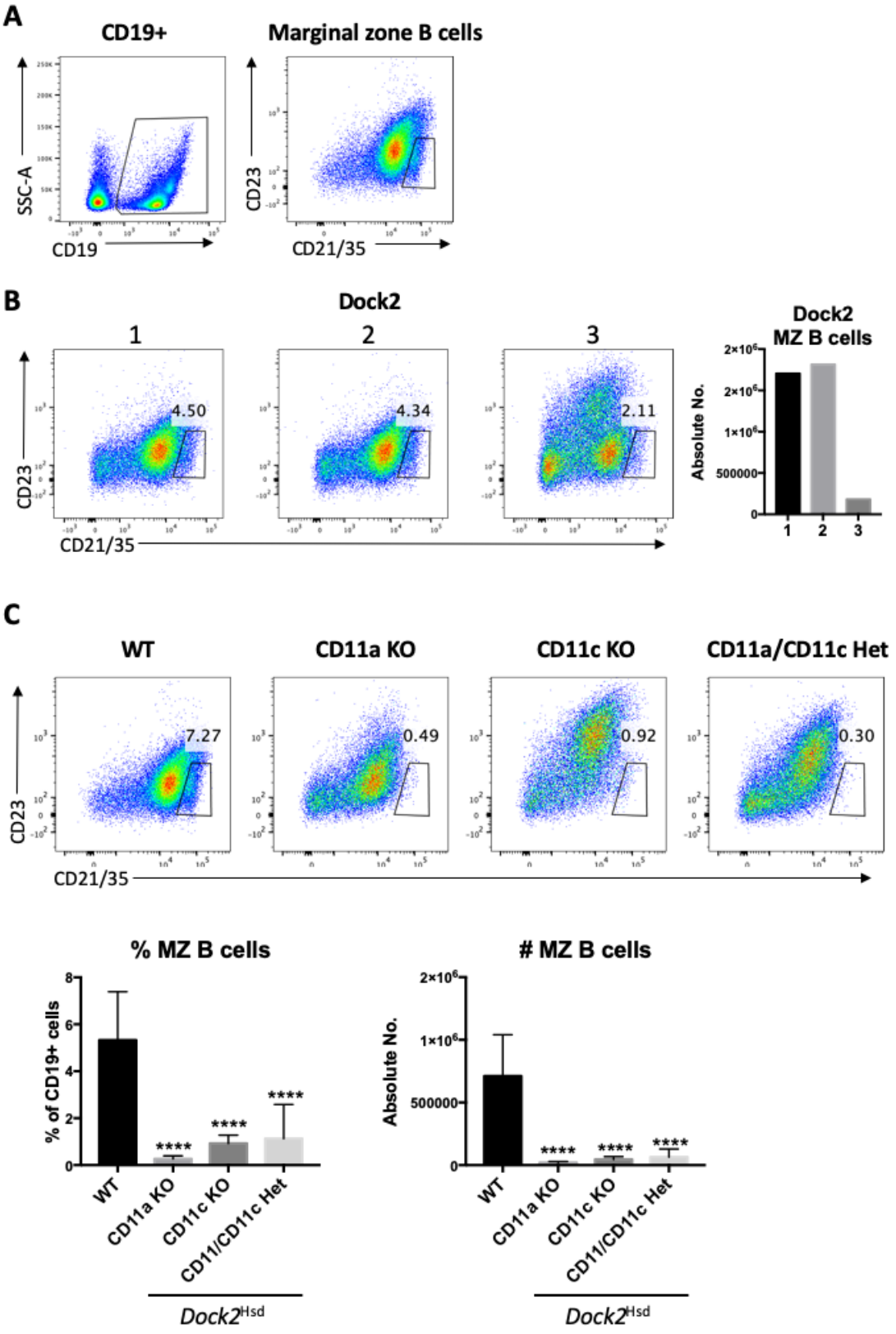

Figure 3-15 Loss of marginal zone B cells in mice with dock2 mutation.
Splenocytes from WT, CD11a KO, CD11c KO or CD11a/CD11c het mice were analysed by flow cytometry. **(A)** Cells were gated on CD19+ (total B cells) then CD21/35^{hi} CD23^{lo} (marginal zone B cells). **(B)** Plots and the absolute numbers of marginal zone B cells from replicates of Dock2 mice. **(C)** Representative plots showing marginal zone B cells, percentages and numbers of CD19^{+} cells and marginal zone B cells. n=9, data pooled from 3 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

Having confirmed CD11a and CD11c KO mice possess the *Dock2*^{Hsd} mutation at the genomic level and display the expected immune phenotype of marginal zone B cell loss, I next analysed their DC phenotype. In the initial experiments in this chapter, DCs were generated *in vitro* to determine which β_2 integrin subunits are responsible for regulating the activation state of BMDCs. First, β_2 integrins and ICAM-1 expression were assessed on the BMDCs and it was found that BMDCs lacking CD11a or CD11c expressed reduced levels of CD18 and CD11b but expressed higher levels of ICAM-1 than WT (Figure 3-1). This same analysis was carried out using BMDCs generated from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice to determine whether these effects were due to the loss of β_2 integrin expression or due to Dock2-deficiency.

The expression of both CD18 and CD11b was significantly lower on BMDCs from β_2 integrin KO mice compared to WT, but expression on BMDCs generated from Dock2 mice was equivalent to WT (Figure 3-16). This was true regardless of the maturation status of the BMDCs. This indicates that, as expected, the downregulation of CD18 and CD11b on CD11a/CD11c-deficient BMDCs is due to the loss of integrin expression and not the absence of functional Dock2. As expected, CD11a KO and CD11c KO BMDCs lacked expression of CD11a and CD11c respectively. The CD11a/CD11c het BMDCs expressed CD11a and CD11c, but at significantly lower levels than WT. There was no difference in the expression of either CD11a or CD11c on Dock2 BMDCs compared to WT. The expression of ICAM-1 was higher on immature CD11a KO and CD11a/CD11c het BMDCs compared to WT. However, following maturation, all integrin-deficient and Dock2 BMDCs expressed increased ICAM-1 compared to WT.

This data confirms the expected loss/reduced expression of CD11a and CD11c on BMDCs based on their genotype. The mutation in the *Dock2* gene did not impact on β_2 integrin expression but did contribute to the upregulation of ICAM-1 on mature BMDCs.

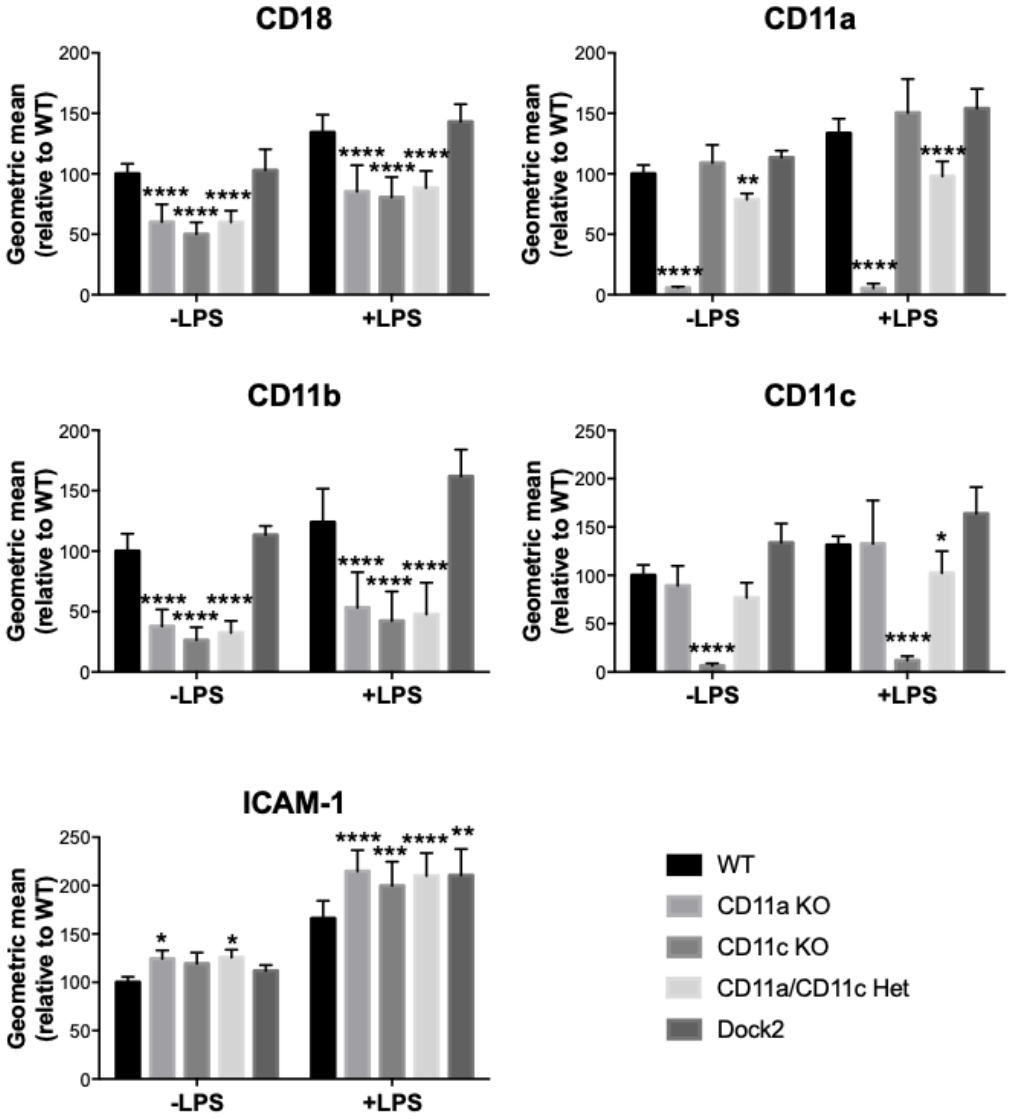

Figure 3-16 Altered expression of β₂ integrins on BMDCs is independent of Dock2. BMDCs generated from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice were cultured with or without 50ng/ml LPS overnight then cell surface marker expression was measured by flow cytometry. Values shown are relative to WT -LPS from individual experiments. WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, data pooled from 3 independent experiments. Dock2 n=3, data from 1 experiment. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

After confirming the reduced β_2 integrin expression on BMDCs was independent of Dock2, the expression of co-stimulatory molecules and the chemokine receptor CCR7 were assessed. The previous results (Figure 3-2) demonstrated that in the absence of CD11a or CD11c, BMDCs express higher levels of CD40, CD80 and CD86. The expression of CCR7 was increased only on mature CD11a KO BMDCs.

All of the immature BMDCs expressed similar levels of the markers analysed (Figure 3-17). However, mature BMDCs from CD11a KO, CD11c KO and CD11a/CD11c het mice expressed significantly higher levels of CD40, CD80 and CD86 than WT

mice. The expression of these co-stimulatory molecules was equivalent in WT and Dock2 BMDCs. In contrast to the previous experiments, there was no significant difference in CCR7 expression across any of the groups.

The aim with comparing the phenotype of cells from CD11a/CD11c het mice with CD11a and CD11c KO, was that intermediate levels of integrin expression may be sufficient to rescue any effects that occur due to integrin loss. Based on this idea, the expectation was that CD11a/CD11c hets would display a similar phenotype to the Dock2 and WT groups. In contrast to this, the enhanced expression of co-stimulatory molecules on CD11a and CD11c KO BMDCs was maintained in the CD11a/CD11c hets. This data indicates that reduced β2 integrin expression is sufficient to induce the enhanced expression of co-stimulatory molecules on BMDCs and importantly, this effect is due to the loss of integrin expression and independent of the mutation in Dock2.

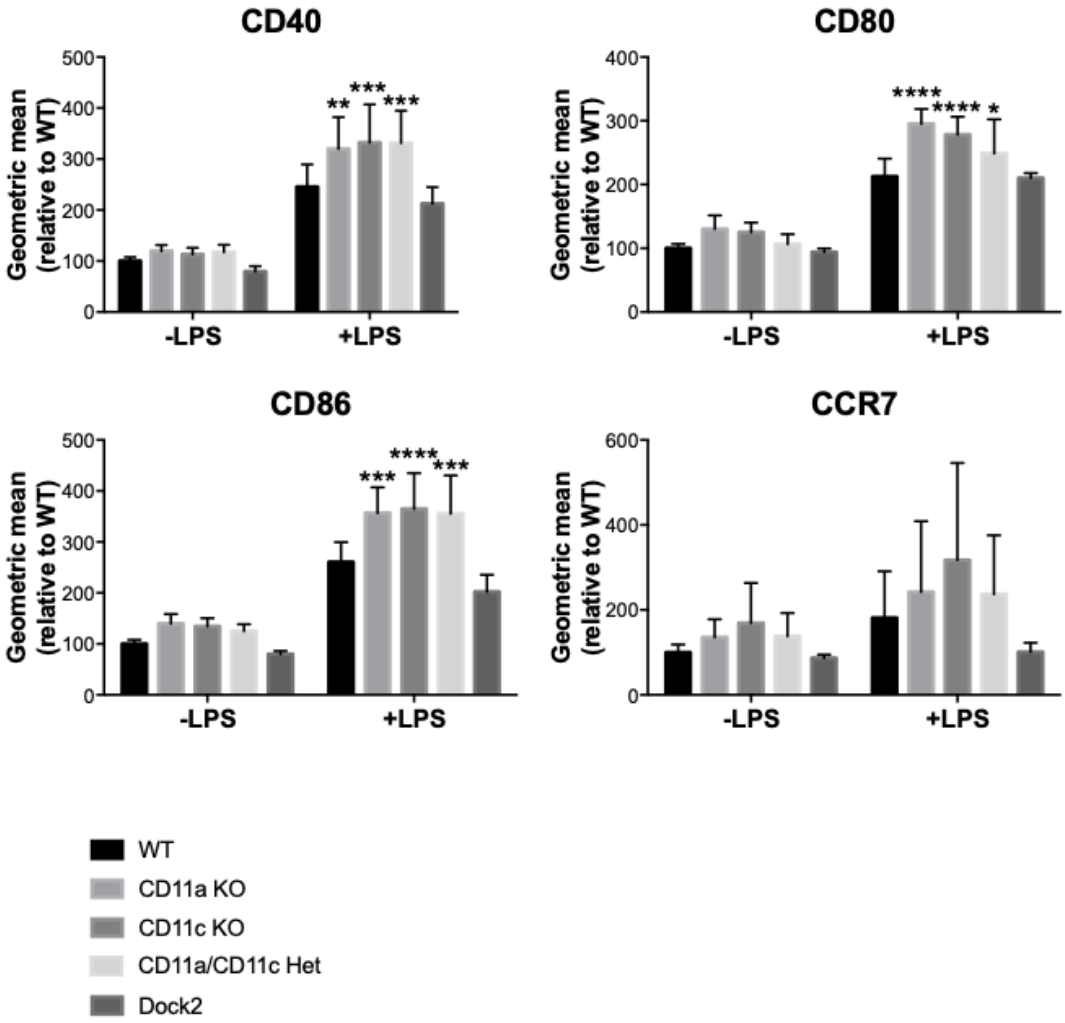

Figure 3-17 Elevated expression of co-stimulatory molecules on CD11a and CD11c KO BMDCs is independent of *Dock2*^{Hsd}.
BMDCs generated from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice were cultured with or without 50ng/ml LPS overnight then cell surface marker expression was measured by flow cytometry. Values shown are relative to WT from individual experiments. WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, data pooled from 3 independent experiments. Dock2 n=3, data from 1 experiment. Statistical differences were determined using a two-way ANOVA. Data represents mean + SD. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

The enhanced activation of β_2 integrin-deficient DCs generated *in vitro* was found to be an integrin-dependent effect. The next question was whether the increased proportions of DCs in secondary lymphoid tissues of CD11a and CD11c KO mice (Figure 3-7) is also an integrin-dependent effect. To test this, DCs were identified by *ex vivo* analysis in the CD11a/c het mice.

An increased percentage of DCs was identified in the spleen of CD11c KO mice and CD11a/CD11c het compared to WT, but there was no difference in the CD11a KO or Dock2 mice. There was variability in the data from CD11a KO mice (indicated by the large error bar), which may explain why the percentage of DCs was not significantly different in this group. No significant differences were seen between

any groups in the mLN or pLN analysis. Calculation of the absolute numbers of DCs showed a greater number of DCs in the spleen of CD11a KO, CD11c KO and CD11a/CD11c het mice compared to WT, but no difference in the Dock2 mice. Similar to the percentage data, the numbers of DCs did not differ in the mLN and pLN.

As found with the BMDC data, the CD11a/CD11c het group displayed the same phenotype as the CD11a and CD11c KO mice, but not the WT or Dock2. This indicates that intermediate expression of CD11a/CD11c is sufficient to cause an increase in DCs. As the increase in splenic DCs in CD11a, CD11c KO mice and CD11a/CD11c hets was not found in WT or Dock2 mice it can be concluded that the expression of *Dock2*^{Hsd} does not influence DC numbers *in vivo*.

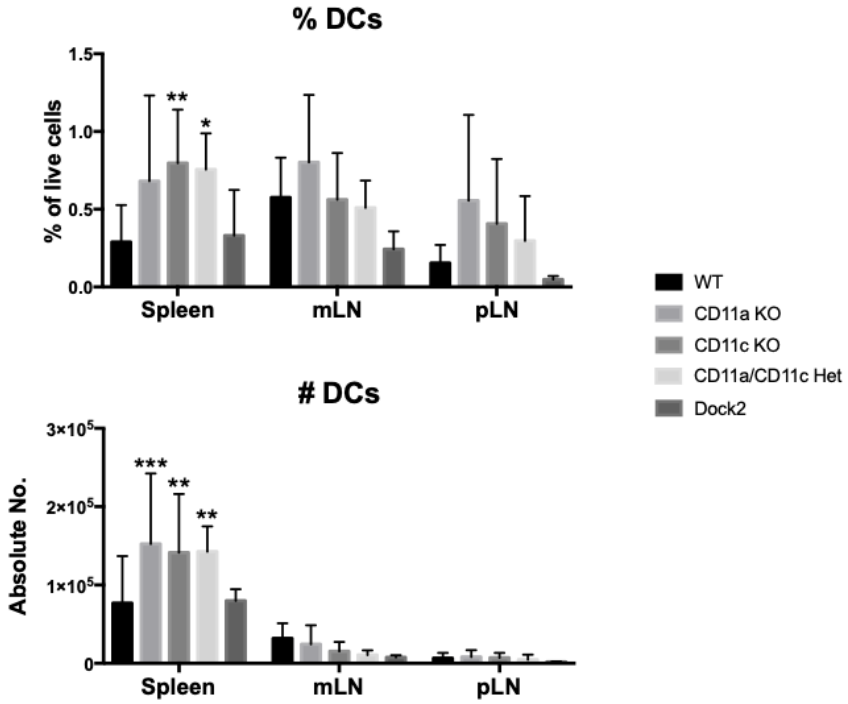

Figure 3-18 Increased proportions and numbers of DCs *in vivo* is due to the β_2 integrin defect.
Cells were isolated from WT, CD11a KO, CD11c KO, CD11a/CD11c het or Dock2 mutant mice then DCs analysed by flow cytometry. As per the gating strategy in (Figure 3-7A). WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, data pooled from 3 independent experiments. Dock2 n=3, data from 1 experiment. Data represents mean + SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

The enhanced activation of BMDCs and increased proportion of DCs *ex vivo* from CD11a and CD11c KO mice have now been confirmed as integrin-dependent effects, and not due to the expression of *Dock2*^{Hsd}. To determine the potential effect the increased number of DCs may have on the subsequent T cells response, the

phenotype of CD4 T cells was assessed in β_2 integrin deficient mice earlier in this chapter (Figure 3-10). Before looking at T cell activation, the percentages and numbers of CD4 T cells were first analysed. It is known that CD11a plays a major role in αβ T cell extravasation into LNs[68] and Dock2 also plays a role in regulating this process[319]. Therefore, the deficiency in both integrin and Dock2 may contribute to altered T cell populations in lymphoid organs. The percentages and numbers of CD4 T cells were assessed in the WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice to determine the effect of integrin deficiency vs loss of Dock2 on the overall CD4 T cell population present in the spleen and LNs.

The percentage of CD4 T cells in the spleen was decreased in CD11a KO mice compared to WT but remained equivalent in CD11c KO, CD11a/CD11c het and Dock2 mice (Figure 3-19). In the mLN, all of the CD11a/CD11c deficient mice displayed a lower percentage of CD4 T cells but the deficiency of Dock2 alone had no effect. In the peripheral LNs, the proportion of CD4 T cells was significantly reduced in CD11a KO and to a lesser extent in CD11c KO mice. However, this effect was lost in the CD11a/CD11c het LNs and, again, mice with the Dock2 mutation displayed a similar percentage of CD4 T cells to WT mice.

Despite a decreased percentage in the spleen of CD11a KO mice, the number of CD4 T cells was similar across all of the groups. On the contrary, in the mLN and pLNs there was a β_2 integrin-dependent defect in the number of CD4 T cells. Mice with the mutated form of *Dock2* and reduced expression of CD11a/CD11c showed a significantly lower number of CD4 T cells, whereas mice with *Dock2*^{Hsd} and WT integrin expression had numbers equivalent to WT.

In summary, the loss of CD11a and/or CD11c expression leads to a reduced number of CD4 T cells in the mLN and peripheral LNs of mice but the absence of functional Dock2 has no effect on CD4 T cell distribution.

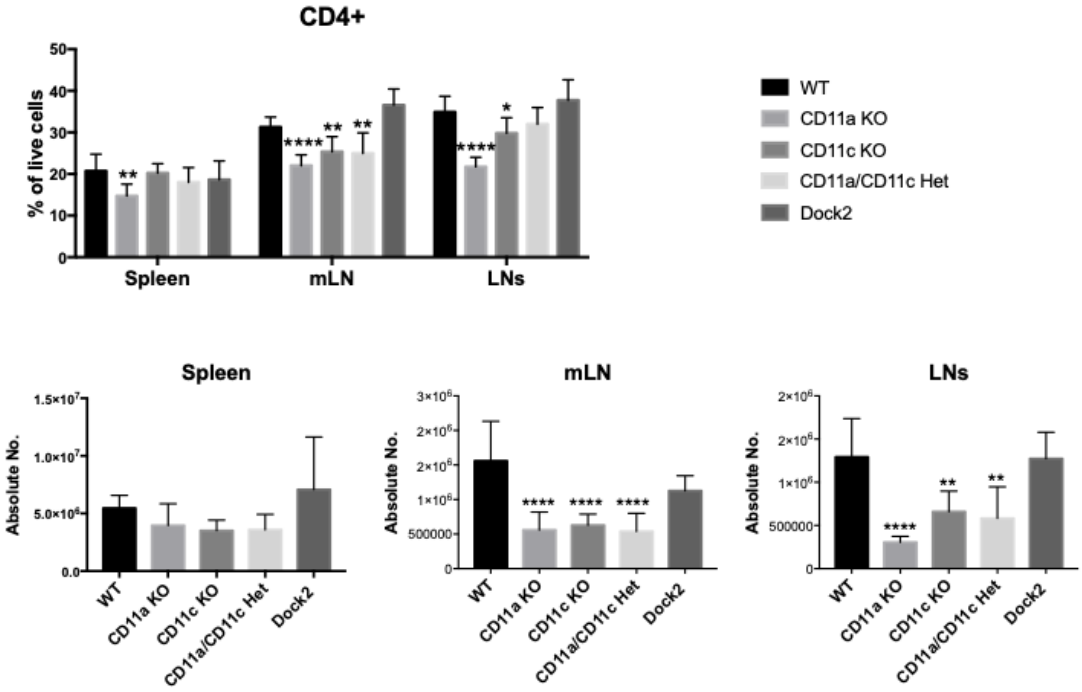

Figure 3-19 Reduced numbers of T cells in LNs is due to the β2 integrin defect.
Cells were isolated from the spleen, mesenteric lymph nodes (mLN) and peripheral lymph nodes (LNs) of WT, CD11a KO, CD11c KO, CD11a/CD11c het or Dock2 mutant mice. Cells were gated on CD3+CD4+ then T cell numbers and percentages calculated. WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, 3 expt; Dock2 mutant n=3, 1 expt. Data represents mean + SD. Statistical differences were determined using a two-way or one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

After identifying that *Dock2*^{Hsd} does not impact the number of CD4 T cells in spleen and LNs, the phenotype of these cells was then analysed. Previous experiments showed an increase in the expression of activation markers on CD4 T cells in mice lacking CD11a or CD11c (Figure 3-10).

In the spleen, CD4 T cells from CD11a KO, CD11c KO and CD11a/CD11c het mice displayed increased expression of CD44 compared to WT (Figure 3-20A), as was found previously in the CD11a and CD11c KO mice (Figure 3-10). Expression of CD44 on T cells from the Dock2 mice was variable but not significantly different from WT. In contrast to the previous findings, there was no difference in the expression of CD62L on CD4 T cells from CD11a or CD11c deficient mice. Similarly, the expression of CD25 and CD69 was equivalent on CD4 T cells regardless of their genotype.

In the mLN, there were no differences in the expression of CD62L, CD25 or CD69 (Figure 3-20B). The percentage of CD44^{+} CD4 T cells was greater only in the CD11c KO mice, as found previously (Figure 3-10). However, unlike the initial experiments,

CD11a KO T cells expressed CD44 at the same levels as WT. In pLNs, the data also showed inconsistencies with the previous experiments, where an increase in CD44 expression was identified in the absence of CD11a and CD11c, and CD11c KO mice showed higher expression of CD25 and reduced CD62L. CD4 T cells from all mice with the Dock2 mutation, regardless of β_2 integrin expression, expressed similar levels of CD44 and CD25 to those from WT mice (Figure 3-20C). The percentage of $CD62L^+$ T cells was similar between WT and β_2 integrin-deficient CD4 T cells but higher on T cells from Dock2 mice. In contrast to the initial experiments, there was also a significant increase in the percentage of $CD69^+$ CD4 T cells from CD11a KO mice compared to WT. No difference was seen in CD69 expression on the other groups.

It is unclear why the activation state of CD4 T cells from CD11a and CD11c KO mice was not consistent with the previous experiments. The altered expression of CD44 in the spleen appears to be an integrin-dependent effect, and this is potentially true in the mLN as well. However, this was not accompanied by the expected decrease in CD62L expression, and levels of CD44 were unaltered in the pLN. It is therefore difficult to make firm conclusions about the effect of β_2 integrin deficiency versus loss of Dock2 on the phenotype of CD4 T cells *in vivo*. Further analysis, including cytokine profiling, would be required to confirm the specific roles for integrins and Dock2 in CD4 T cell activation *in vivo*.

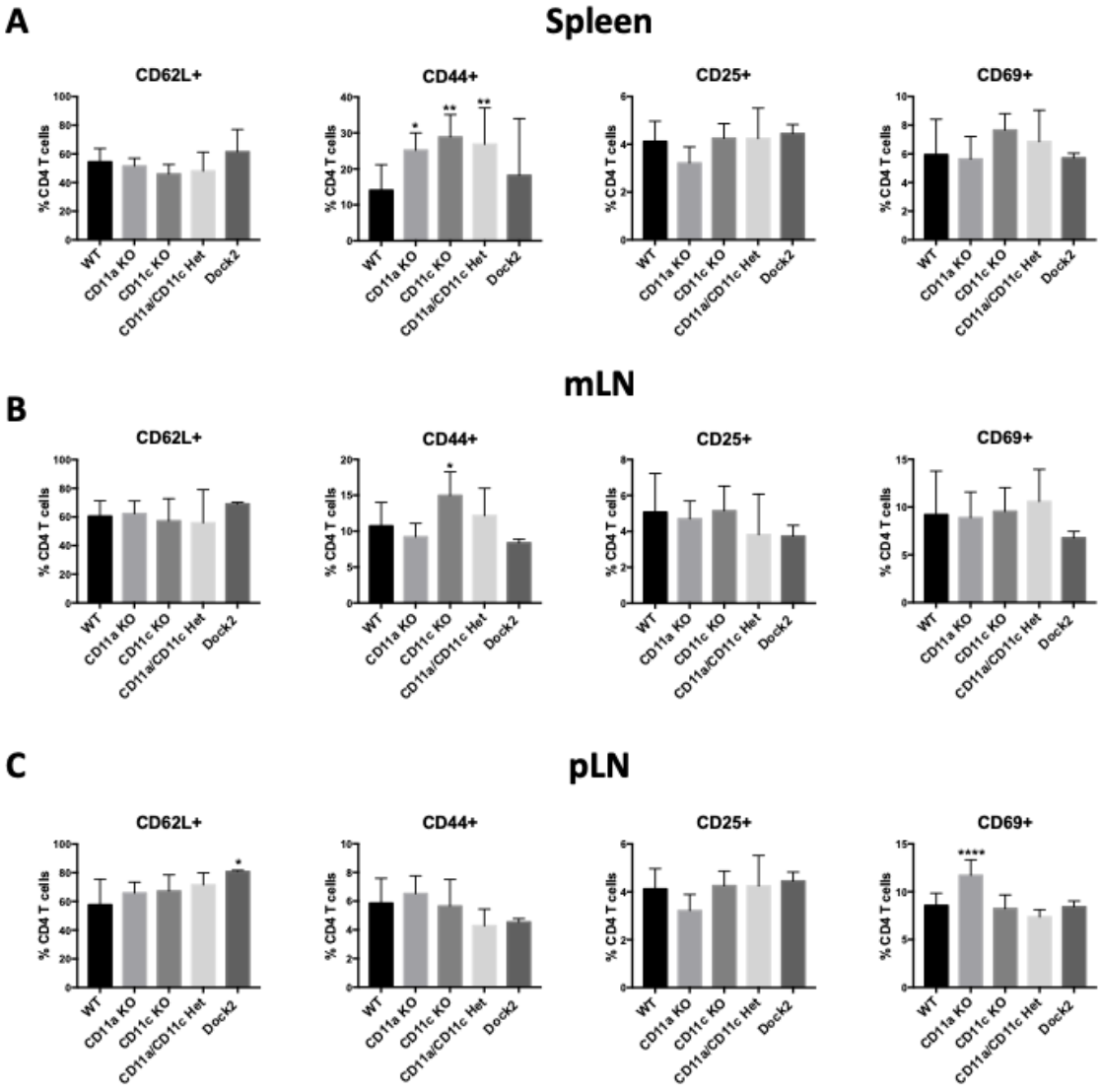

Figure 3-20 Loss of β_2 integrins and Dock2 have inconsistent effects on CD4 T cell phenotype.
Cells were isolated from WT, CD11a KO, CD11c KO, CD11a/CD11c het and Dock2 mice and analysed by flow cytometry. Cells were gated on CD3+CD4+ then activation marker expression was assessed on CD4 T cells from the spleen **(A)**, mesenteric LN (mLN) **(B)** and peripheral LN (pLN) **(C)**. WT, CD11a KO, CD11c KO, CD11a/CD11c het n=9, data pooled from 3 independent experiments; Dock2 n=3, data from 1 experiment. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

In summary, it was identified that CD11a KO, CD11c KO and CD11d KO mouse strains possessed an additional mutation in the *Dock2* gene. The CD11a and CD11c KO mice displayed a loss of MZ B cells due to the expression of *Dock2*^{Hsd}, whereas, the β_2 integrin-deficiency was responsible for the enhanced co-stimulatory molecule expression on BMDCs, increased population of DCs *in vivo* and reduced number of CD4 T cells in LNs.

3.2.7 Generation and Characterisation of β_2 integrin KO mice with WT expression of Dock2

Upon the discovery that CD11a KO, CD11c KO and CD11d KO mouse strains had a mutation in the *Dock2* gene in addition to the intended β_2 integrin deficiency, the mice were then bred to restore WT expression of Dock2. For the CD11d KO line, only some of the mice expressed *Dock2*^{Hsd} and were likely heterozygous, whereas others expressed *Dock2*^{WT}. Breeders were selected that did not express *Dock2*^{Hsd} so that all future offspring would not possess this mutation. For the CD11a and CD11c KO lines all of the mice were affected by the *Dock2* mutation, therefore it was assumed that the mice were homozygous for *Dock2*^{Hsd}. To restore WT expression of *Dock2*, CD11a and CD11c KO mice were crossed with WT mice (C57BL/6 mice purchased from Envigo) (Figure 3-21). This produced an F1 generation of mice that were heterozygous for both their respective β_2 integrin and *Dock2*^{Hsd}. The F1 generation were then crossed and breeders from their offspring were selected that had WT expression of *Dock2*, and either heterozygous or homozygous expression of the relevant integrin subunit. From the F3 generation breeders were then selected that lacked expression of *Dock2*^{Hsd} and were homozygous KO for the β_2 integrin receptor to continue the line.

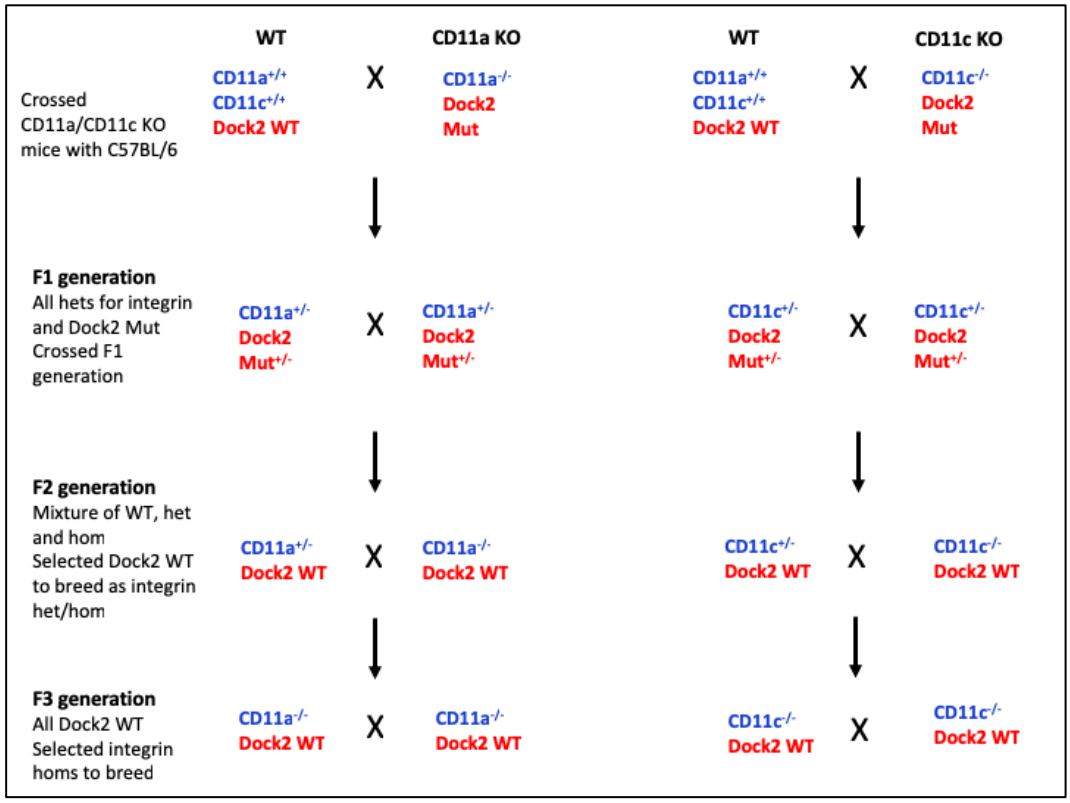

Figure 3-21 Breeding strategy for CD11a and CD11c KO mouse strains to remove *Dock2*^{Hsd}.

In order to confirm the findings that marginal zone B cell loss (Figure 3-15) and enhanced CD4 T cell activation (Figure 3-10, Figure 3-11) in CD11a and CD11c KO mice were due to *Dock2*^{Hsd}, the phenotype of β_2 integrin α-chain deficient mice, all with WT expression of *Dock2*, was then compared to WT and total β_2-deficient mice (CD18 KO).

First, the total cell numbers obtained from the spleen, mLN and pLN of these mice were calculated and compared with the mice affected by *Dock2*^{Hsd}. The total cell counts from the original strains affected by *Dock2*^{Hsd} showed a significant decrease in the number of cells in the spleen, mLN and pLN of CD11a and CD11c KO mice compared to WT (Figure 3-22A). Note, this data is the same as Figure 3-8, with the CD18 KO group added. Interestingly, in total β_2 integrin KO mice (CD18 KO) the cell number was also lower in the mLN and pLN, but numbers were greatly increased in the spleen compared to WT mice. Despite some of the CD11d KO mice expressing *Dock2*^{Hsd} (probably heterozygous), there was no difference in cell numbers from these mice or in the CD11b KOs. However, in the integrin-deficient mice with WT expression of *Dock2* there was no difference in the number of splenic cells in any of the α-chain KO mice compared to WT, and in the mLN and pLN the decreased number was only seen in the CD11a KO and CD18 KO but not in the CD11c KO mice.

This suggests that the decreased cellularity of the spleen of CD11a and CD11c KO mice was due to *Dock2*^{Hsd}. This reduction in cellularity may therefore be attributed to the loss of MZ B cells. Similarly, the reduced cellularity of CD11c KO LNs was also due to *Dock2*^{Hsd}, though the reason for this remains unknown. However, the decreased cell number in the mLN and pLN of CD11a KO mice was due to the loss of CD11a expression. This is to be expected due to the essential role for CD11a in leukocyte extravasation into the LNs.

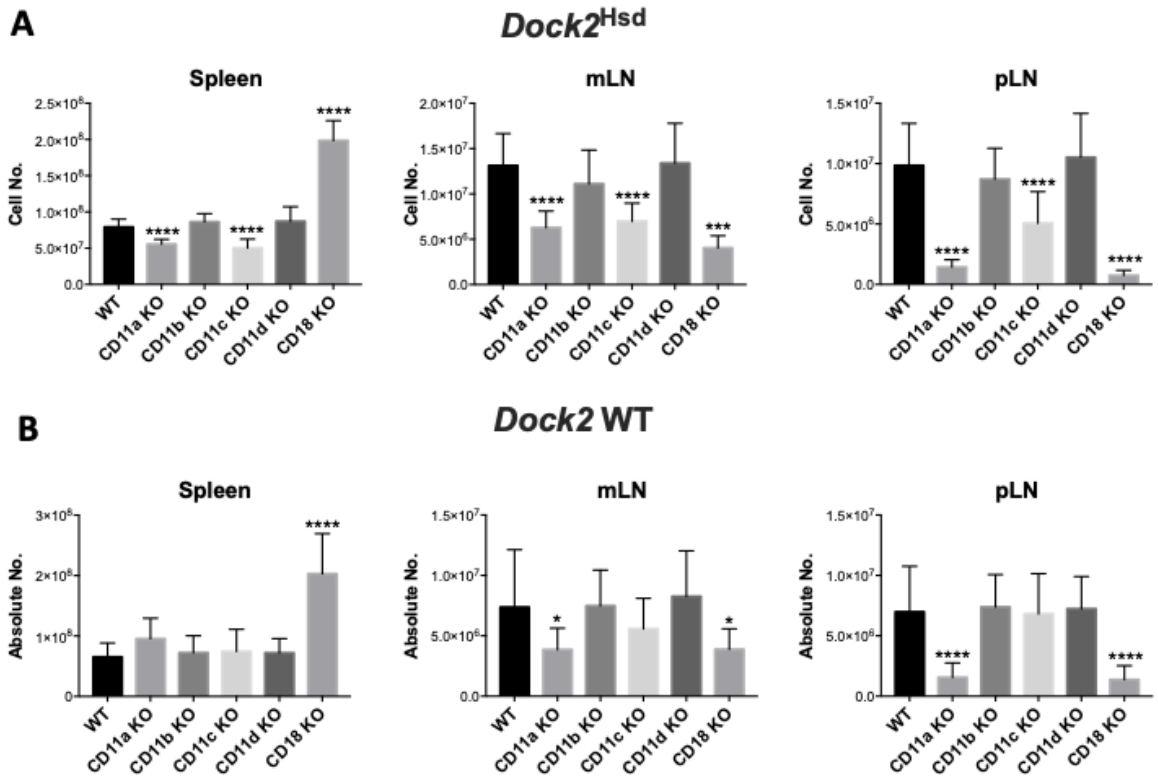

Figure 3-22 Reduced total cell numbers in the spleen of CD11a KO, spleen and LNs of CD11c KO mice were due to *Dock2*^{Hsd}.
Cells were isolated from the spleen, mesenteric LN (mLN) and peripheral LN (pLN) of WT, CD11a KO, CD11b KO, CD11c KO, CD11d KO, and CD18 KO mice and total cell numbers calculated. Counts from mice affected by *Dock2*^{Hsd} **(A)** and with WT *Dock2* expression **(B)**. n=16, data pooled from 6 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

I previously showed that CD11a KO and CD11c KO mice displayed one of the most striking immune phenotypes associated with *Dock2*^{Hsd}, the loss of MZ B cells[309,317]. To confirm the expression of *Dock2*^{WT} in integrin-deficient mice rectified this defect, MZ B cells were analysed from α-chain and β_2 KO mice that expressed *Dock2*^{WT}. The population of MZ B cells in the spleen was restored in *Dock2* WT integrin-deficient mice (Figure 3-23A). In the CD11c KO mice, the MZ B cell population was in fact increased, both by percentage and number, compared to the WT spleen (Figure 3-23B). Similar numbers of MZ B cells were seen in WT, CD11a KO, CD11b KO, CD11d KO and CD18 KO mice.

The presence of MZ B cells in CD11a and CD11c KO mice confirms the impairment was due to *Dock2*^{Hsd} and the breeding strategy (outlined in Figure 3-21) successfully restored Dock2 function in the integrin-deficient strains. Further to this, the loss of CD11c expression in the absence of *Dock2*^{Hsd} resulted in an increased number of MZ B cells, suggesting a potential role for CD11c in regulating MZ B cells.

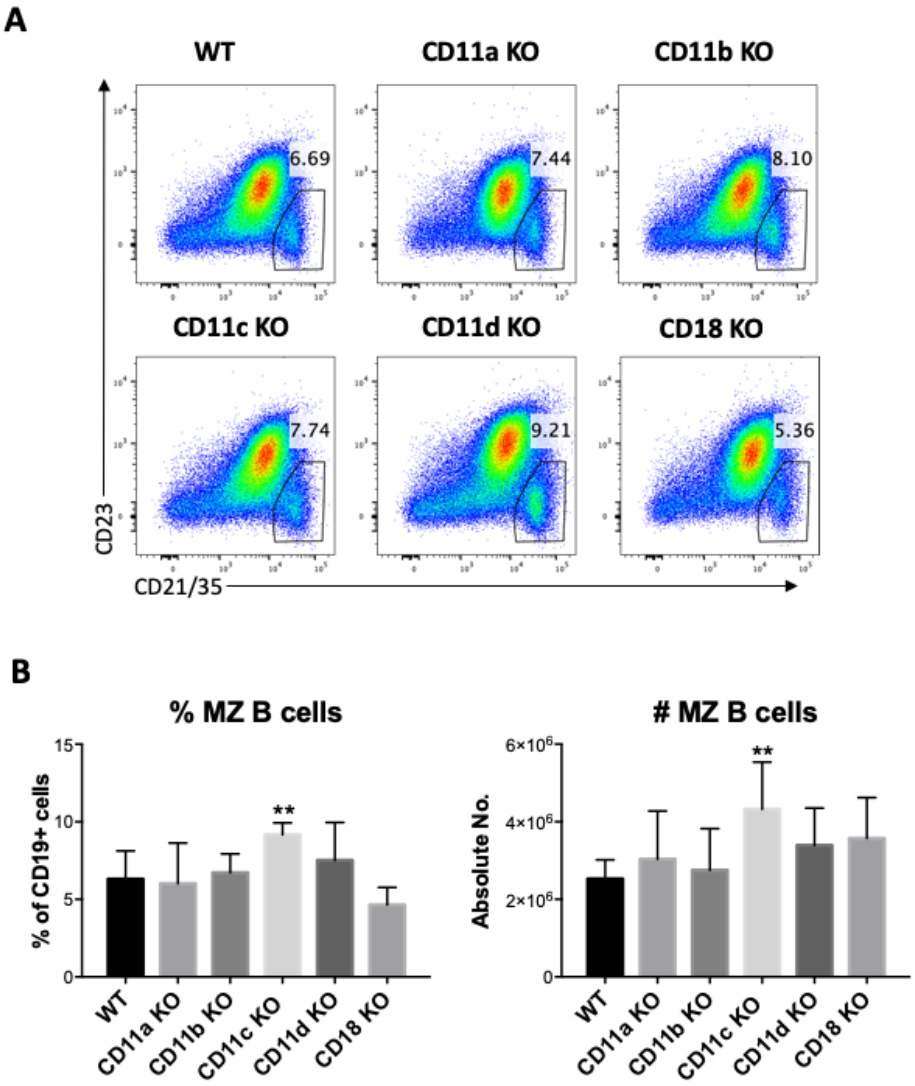

Figure 3-23 Confirmation that loss of marginal zone B cells in CD11a KO and CD11c KO mice was due to the mutation in Dock2.
Cells were isolated from the spleen of WT, CD11a KO, CD11b KO, CD11c KO, CD11d KO and CD18 KO mice (all WT for Dock2) then cells were analysed by flow cytometry. MZ B cells were gated on $CD19^{+}$ $CD21/35^{hi}$ $CD23^{lo}$. **(A)** Representative plots of marginal zone B cells as a percentage of $CD19^{+}$ cells. **(B)** Percentages and numbers of marginal zone B cells. n=9, data pooled from 3 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001.

Following confirmation that *Dock2*^{Hsd} removal from CD11a and CD11c KO mice strains resulted in the expected return of MZ B cells, the CD4 T cell population was then analysed. Data obtained from experiments using the CD11a/CD11c het mice suggested that the loss of CD11a and/or CD11c expression, but not Dock2, caused a reduction in the number of CD4 T cells in LNs (Figure 3-19). To confirm these findings, CD4 T cells were analysed from β_2 integrin-deficient mice with *Dock2*^{WT} expression. There was no difference in the number of CD4 T cells in the spleen of CD11a KO, CD11b KO, CD11c KO or CD11d KO mice compared to WT, but numbers were increased in CD18 KO mice (Figure 3-24A). In both the mLN and

pLN, there was a significant decrease in CD4 T cell numbers in CD11a KO and CD18 KO mice compared to WT. Equivalent numbers of CD4 T cells were found in the mLN and pLN of WT, CD11b KO and CD11d KO mice.

There were inconsistencies in activation marker expression on CD4 T cells between the initial experiments carried out in integrin-deficient mice with *Dock2*^{Hsd} (Figure 3-10) and experiments using CD11a/CD11c het mice (Figure 3-20). The initial data showed that CD4 T cells from the tissues of CD11a and CD11c KO mice displayed a more activated phenotype, but this was not apparent in the repeat experiments. To clarify this contradictory data, the expression of activation markers was assessed on CD4 T cells from the spleen of β_2 integrin-deficient mice with *Dock2*^{WT} expression. There was a reduced expression of CD25 on CD4 T cells from CD11a and CD18 KO mice compared to WT, whereas higher expression was seen on cells from CD11d KO mice (Figure 3-24B). CD11a KO CD4 T cells also displayed lower levels of CD69, whereas CD69 expression was upregulated on cells from CD11c and CD11d KO mice compared to WT. Fewer CD4 T cells expressed CD62L in the CD18 KO spleen, but no difference was found on T cells from any other integrin-deficient mice. CD44 expression was similar across all genotypes.

In the initial experiments using integrin-deficient mice expressing *Dock2*^{Hsd}, CD4 T cells from the spleen of CD11a and CD11c KO mice produced significantly higher levels (~2-fold) of IFNγ and IL-17 following *in vitro* re-stimulation (Figure 3-11). To determine whether the mice with *Dock2*^{WT} retained this phenotype, the levels of IFNγ and IL-17 were measured in response to PMA/ionomycin in CD4 T cells from integrin-deficient mice expressing *Dock2*^{WT}. The percentage of CD4 T cells producing IFNγ was similar across the β_2 integrin-deficient mice, except for the CD11d KO cells that showed a small but significant increase compared to WT (Figure 3-24C). In contrast to this, there was increased production of IL-17 in CD4 T cells from CD11a and CD18 KO mice, but the CD11b, CD11c and CD11d KO T cells produced similar levels to WT. The increase in IL-17 production was similar in magnitude (~2-fold) to that seen in CD11a KO mice expressing *Dock2*^{Hsd}.

This suggests that the absence of CD11a but not CD11c leads to a decreased number of CD4 T cells in LNs and enhanced production of IL-17 by T cells in the spleen. Loss of β_2 integrin expression does not have consistent effects on CD4 T

cell activation marker expression, but additional experiments are required to examine this further. Finally, the enhanced IFNγ production by CD4 T cells in CD11a KO mice was due to the *Dock2*^{Hsd} mutation.

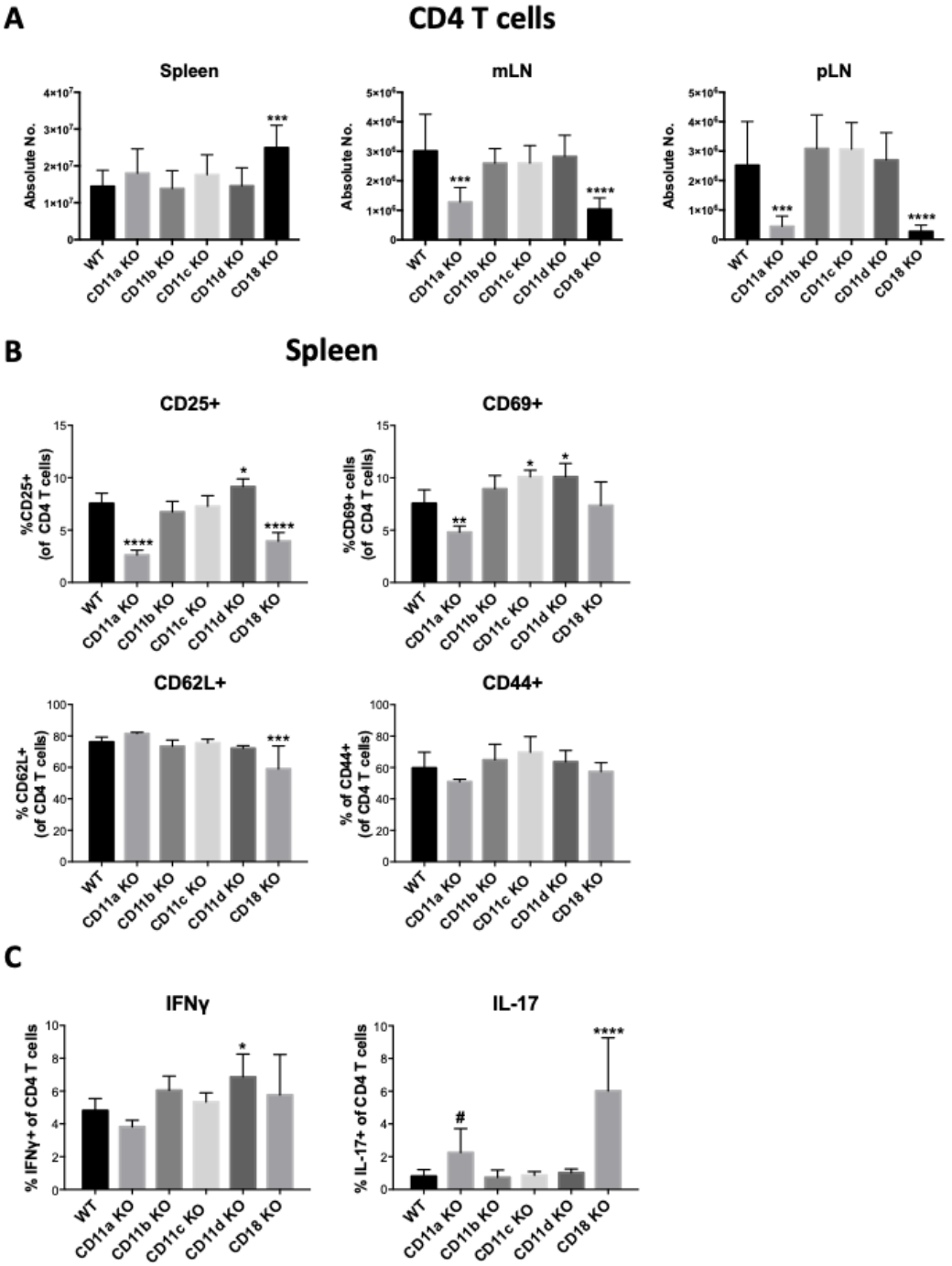

Figure 3-24 Reduced numbers of CD4$^+$ T cells in CD11a/CD18 KO mice and enhanced IL-17 production in the spleen due to the integrin-deficiency.
Cells were isolated from the spleen, mesenteric LN (mLN) and peripheral LN (pLN) of WT, CD11a KO, CD11b KO, CD11c KO, CD11d KO and CD18 KO mice (all WT for Dock2). **(A)** Cells were gated on CD3$^+$CD4$^+$ then T cell numbers calculated. **(B)** Percentage of splenic CD4 T cells expressing activation markers. **(C)** Production of IFNγ and IL-17 from splenic CD4 T in cells following *in vitro* re-stimulation with PMA/ionomycin. n=6, data pooled from 2 independent experiments. Data represents mean + SD. Statistical differences were determined using a one-way ANOVA. *p<0.05, ** p<0.01, ***p<0.005, ****p<0.0001, #p<0.01 when CD18 KO group excluded.

3.3 Discussion

In summary, I set out with the aim to determine which β_2 integrin family members regulate DCs and how this impacts the subsequent T cell response. However, this was confounded by the discovery that the integrin-deficient mouse strains used contained an additional mutation in a gene called *Dock2*. I have now identified the major phenotypes due to *Dock2*^{Hsd} are a loss of MZ B cells and reduced number of CD4 T cells in the LNs of CD11c KO mice. The loss of both of these cell types is likely to have contributed to the reduced total cell number in the spleen of CD11a and CD11c KO mice, and in the LNs of CD11c KO mice affected by *Dock2*^{Hsd}. Importantly, the increased activation of *in vitro* generated BMDCs and increased proportion of DCs found *in vivo* in CD11a KO and CD11c KO mice*,* were due to the loss of integrin expression. Further work is ongoing in the lab to follow up on the mechanism(s) by which CD11a and CD11c integrin subunits regulate the various aspects of DC phenotype and function.

3.3.1 Implications of the *Dock2* mutation identified in several strains of β_2 integrin-deficient mice

DCs are antigen presenting cells that play an essential role in mediating the induction of an immune response or tolerance of immune cells to specific antigens. It is essential to better understand the mechanisms regulating DCs and their ability to induce immune activation versus tolerance, to identify potential therapeutic targets for diseases where there is a loss of tolerance, such as autoimmunity. In recent years it has become apparent that β_2 integrins are important regulators of immune activation, particularly in DCs. CD18 KO BMDCs showed enhanced cytokine production in response to TLR stimulation[182] and the loss of β_2 integrin receptor function increased co-stimulatory molecule expression on DCs under steady state conditions[73]. DCs lacking functional β_2 integrins induced greater Th1 responses[73] whereas DCs expressing a constitutively active form of CD11a inhibited the generation of Th1 cells shown by reduced T-bet expression and IFNγ production[88]. These studies demonstrate that β_2 integrins are important for the negative regulation of DC function and limiting the priming of T cell responses. However, it has not yet been determined which of the subunits and associated signalling pathways are responsible for this regulation.

Chapter 3 Characterisation of β2 integrin-mediated regulation of dendritic cells and the impact on interactions with T cells

The aim of this chapter was to determine which β_2 integrin subunit(s) regulate DCs *in vitro* and *in vivo*, and to assess the impact that integrin loss on DCs has on their ability to prime T cells. These questions were answered using β_2 integrin-deficient mice that lack a specific subunit: CD11a, CD11b, CD11c or CD11d. My initial findings indicated that CD11a and CD11c KO mice have significant differences in DC and T cell activation compared to WT: CD11a and CD11c KO BMDCs expressed higher levels of co-stimulatory molecules and displayed reduced adhesion to ICAM-1. *Ex vivo* analysis of spleen and LNs found an increased percentage of DCs in CD11a and CD11c KO mice, and increased activation and cytokine production in CD4 T cells. Together, these initial findings suggested that integrin subunits CD11a and CD11c mediate regulation of DC phenotype that, in turn, keeps T cell activation in check.

While this work was underway, a publication highlighted that an additional mutation in a gene called *Dock2* was present in several genetically modified mouse strains including Siae and Cmah KO mice[309]. They proposed this mutation originated from the C57BL/6Hsd colony at Harlan in the USA. As these mice were used for backcrossing of newly generated genetically modified strains, other mouse colonies may express the mutated form of *Dock2,* termed *Dock2*^{Hsd}. Using PCR to test for the *Dock2*^{Hsd} gene it was found that CD11a, CD11c and CD11d KO mice expressed the mutated form of *Dock2* but CD11b KO and CD18 KO mice did not. The *Dock2*^{Hsd} allele is a functional mutation that results in a failure of protein translation, therefore mice possessing the Dock2^{Hsd} mutation pheno-copy Dock2 KO mice. The presence of *Dock2*^{Hsd} in addition to the intended β_2 integrin defect called into question whether the immune phenotypes described in the early sections of this chapter (Figures 3-1 – 3-11) were due to the loss of integrin subunits or Dock2.

Both Dock2 and integrins play important roles in cytoskeletal rearrangement, cell adhesion and migration. Given that the functions of β_2 integrins and Dock2 in immune cells are similar, it was imperative that any effects resulting from *Dock2*^{Hsd} were identified, and that the findings describing CD11a and CD11c as mediating the negative regulation of DC and T cell phenotypes were confirmed.

3.3.1.1 Uncovering Dock2-related phenotypes using CD11a/CD11c hets

The initial approach taken to uncover which altered immune phenotypes were caused by *Dock2*^{Hsd} and which were due to the β_2 integrin loss, was to cross the CD11a and CD11c KO mice to create CD11a/c hets. These mice still expressed homozygous *Dock2*^{Hsd} but were heterozygous for both CD11a and CD11c. The CD11a/CD11c het mice expressed intermediate levels of both of these integrin receptors and it was hypothesised that this level of expression would be sufficient to restore integrin function. If this hypothesis was correct then BMDCs generated from CD11a/CD11c hets would express co-stimulatory molecules at the same levels as WT, and the increased expression of T cell activation markers seen in CD11a and CD11c KO mice would be lost. Comparison of immune phenotypes in mice that were CD11a or CD11c homozygous KO or heterozygous for CD11a/CD11c expression with WT mice that either expressed WT *Dock2* or *Dock2*^{Hsd} allowed the separation of effects that were integrin-dependent or Dock2-dependent.

However, it was identified in the Dock2 group (WT integrin expression but non-functional Dock2), only one of the three mice was homozygous for *Dock2*^{Hsd} and the other two were likely heterozygous based on the presence of MZ B cells in the spleen. As mice that were heterozygous for *Dock2*^{Hsd} did not display an absence of MZ B cells it is likely that this trait is recessive. In the analysis of DCs, both *in vitro* and *ex vivo*, the hom and het *Dock2*^{Hsd} mice clustered closely together and appeared similar to the WT mice. The fact that the increased co-stimulatory molecule expression on BMDCs and increased number of DCs found in the spleen were not seen in the Dock2 mice, despite variability in the expression of *Dock2*^{Hsd}, further confirms the increased activation of DCs as an integrin-dependent and not a Dock2-dependent effect.

3.3.1.2 Reduced cell numbers in CD11a and CD11c KO mice due to *Dock2*^{Hsd}

To confirm the findings from experiments using CD11a/CD11c het and integrin WT *Dock2*^{Hsd} mice, cells from CD11a and CD11c KO mice bred to restore *Dock2*^{WT} were analysed to confirm which effects were due to the integrin deficiency (figures 21-23). There was a decreased total cell number in the spleen of CD11a and CD11c KO mice with *Dock2*^{Hsd} however, in the same strains with *Dock2*^{WT} expression the cell counts were equivalent to WT. A potential explanation for this is that the reduced

total cell number in the spleen was due to the loss of MZ B cells. MZ B cells absent from the spleen in CD11a and CD11c KO *Dock2*^{Hsd} mice were restored upon the removal of the Dock2 mutation. There was also no defect found in the numbers of any other leukocyte population examined from the spleen of CD11a and CD11c KO mice with restored Dock2 function (CD4 T cells, CD8 T cells, γδ T cells, NK cells, neutrophils, macrophages, DCs, eosinophils; McIntyre & Morrison unpublished data). Taken together, this data suggests that *Dock2*^{Hsd} caused a decreased total cell number in the spleen of CD11a and CD11c KO mice due to the loss of MZ B cells. In CD11c KO mice expressing *Dock2*^{WT}*,* the numbers of MZ B cells were in fact increased compared to WT mice. This finding was surprising as it has previously been shown that B cell development is unaffected by the loss of functional β_2 integrin receptors[320]. The equivalent number of MZ B cells in CD18 KO mice compared to WT is in agreement with this finding, but further work is required to determine why the loss of CD11c alone results in an increased number of MZ B cells.

In addition to changes in B cell populations there were also differences found in CD4 T cells. The number of CD4 T cells was reduced in the LNs of mice deficient in CD11a and CD11c that expressed *Dock2*^{Hsd}. When the mice were bred to remove *Dock2*^{Hsd}, this defect in CD4 T cells was lost from the CD11c KO but remained in the CD11a KO mice. Dock2 is known to play a role in T cell migration, as in Dock2 KO mice the homing of CD4 T cells to the mLN and inguinal LNs was impaired[319]. The reduced number of CD4 T cells in CD11a and CD11c KO mice with *Dock2*^{Hsd} is in agreement with these findings. The equivalent number of CD4 T cells found in the LNs of WT and WT *Dock2*^{Hsd} mice is likely due to the fact that 2/3 of the Dock2 mice were heterozygous for *Dock2*^{Hsd}. This would mean that one WT copy of *Dock2* is sufficient to mediate CD4 T cell migration into secondary lymphoid tissues. A reduced number of CD4 T cells was found in the mLN and pLN of CD11a KO mice regardless of their Dock2 expression. It has previously been established that CD11a is involved in the migration of T cells into LNs by mediating firm adhesion of lymphocytes to endothelial cells[68], therefore it is unsurprising that the decrease in CD4 T cells in CD11a KO mice is integrin-dependent. When comparing the average number of CD4 T cells from *Dock2*^{Hsd} CD11a KO mice (mLN $5.5x10^5$, pLN $3.1x10^5$) with the *Dock2*^{WT} CD11a KO (mLN $1.3x10^6$, pLN $4.5x10^5$), the number of LN CD4 T cells increased with the restoration of Dock2 function. This indicates that the

absence of *Dock2* expression had an additive effect on the impairment in CD4 T cell migration seen in CD11a KO mice.

3.3.1.3 Loss of *Dock2* and β_2 integrins contributed to changes in CD4 T cells

The phenotype of CD4 T cells was also characterised in β_2 integrin-deficient mice to determine whether the increased activation of T cells by CD11a KO DCs *in vitro* may also occur *in vivo*. Initial experiments showed increased activation of CD4 T cells in the absence of CD11a or CD11c expression, although these findings were not consistent when these same mice were compared to CD11a/CD11c hets, or when WT mice were compared to integrin-deficient mice with restored *Dock2*^{WT} expression. There is evidence linking both Dock2 and β_2 integrins to CD4 T cell activation. Dock2 is induced downstream of CD28 co-stimulatory signal to activate Rac, which is required for effective T cell activation[321] and *in vivo* transfer of antigen-specific Dock2 KO CD4 T cells show impaired activation in response to DC immunisation, based on CD25 and CD69 expression[322]. In β_2 integrin signalling-deficient mice, T cell activation was reduced following immunisation but unchanged during homeostasis[320].

In the experiment using mice expressing *Dock2*^{Hsd} there was a consistent increase in the percentage of CD44$^+$ CD4 T cells in the spleen of CD11a and CD11c KO mice, but this difference was lost when *Dock2*^{WT} expression was restored. This suggests that enhanced activation of CD4 T cells was due to Dock2. However, as the absence of Dock2 is linked to reduced T cell activation[322] and the *Dock2*^{Hsd} mice had a global loss of Dock2 function, the mechanism could be mediated by another cell population as opposed to a cell-intrinsic effect on T cells. There was a significant decrease in CD25 expression on CD4 T cells from both CD11a and CD18 KO mice. Expression of CD25 (IL-2Rα) is upregulated during activation of conventional T cells but it is also expressed highly on regulatory T cells (Tregs)[323,324]. It has been shown that CD18-deficient mice have a reduced number of CD4$^+$CD25$^+$ Tregs[325,326], therefore the reduced expression of CD25 may be due to the Treg deficiency in these mice, although a defect in activation of conventional CD4 T cells cannot be ruled out.

In contrast to this, CD4 T cells from CD11d KO mice expressed higher levels of CD25 and CD69 than WT. To my knowledge, the loss of CD11d expression has not

previously been linked to an increased activation of CD4 T cells. CD11d is generally expressed on myeloid cells and not T cells, therefore this effect may be due to the loss of CD11d altering another cell population that ultimately increases CD4 T cell activation. It has been shown that CD11d-deficient T cells have an impaired proliferative response to staphylococcal enterotoxins[58], suggesting that CD11d is required for an optimal T cell response. However, the CD11d KO mice used in the published study were the original strain donated to Jackson Labs and, therefore, the altered T cell response may be due to expression of *Dock2*^{Hsd}.

In the CD11d KO colony only some mice expressed *Dock2*^{Hsd}. This indicates that of the mice that tested positive, the expression was likely heterozygous. In the initial experiments comparing DC and T cell phenotypes in β_2 integrin-deficient mice, there were no significant findings identified in the CD11d KO mice. Given that the absence of splenic MZ B cells and reduced number of CD4 T cells in LNs were not seen in WT *Dock2*^{Hsd} mice with heterozygous expression, it is probable that there were no apparent changes in immune cell phenotype in the CD11d KO mice that were due to *Dock2*^{Hsd} because of the presence of at least one WT allele.

3.3.1.4 Altered DC phenotypes were independent of *Dock2*^{Hsd} and due to defects in β_2 integrin expression

It is clear the loss of both Dock2 and integrins contributed to changes in B and T cell populations in the β_2 integrin α-chain deficient mice. Yet, the main aim of this chapter was to explore the regulatory function of β_2 integrins in DCs. This was based on the previous report that the loss of β_2 integrin-mediated signalling results in enhanced activation of BMDCs and an increase in mature migratory DCs *in vivo*[73]. The generation of BMDCs from *Dock2*^{Hsd} mice with differing expression of CD11a and/or CD11c showed an upregulation of CD40, CD80 and CD86 in cells deficient in CD11a and/or CD11c but not those with WT integrin expression. Similarly, the increased number of DCs in the spleen of CD11a- and CD11c-deficient mice was also found to be β_2 integrin-dependent. The study that demonstrated β_2 integrin loss alters DCs used beta2$^{TTT/AAA}$ knock-in mice that have a mutated kindlin-3 binding site downstream of CD18, causing a loss of receptor function[73]. Importantly, these mice express *Dock2*^{WT}. Therefore, the increased co-stimulatory molecule expression by BMDCs is due to the loss of β_2 integrin expression. Taken together, this data shows that in the absence of CD11a or CD11c expression DCs show an

increased level of activation *in vitro,* suggesting that CD11a and CD11c are involved in the negative regulation of DCs.

I did not find any evidence to suggest that the DC phenotypes I described in β_2 integrin-deficient mice were related to the mutation in Dock2. However, Dock2 has previously been reported to influence the development and function of DC populations. A reduced number of plasmacytoid DCs (pDCs) has been described in the spleen and LNs of Dock2-deficient mice[309,327]. Dock2 is essential for pDC migration, in Dock2 KO mice pDCs are reduced in the spleen and LNs despite normal development in the bone marrow, and in *in vitro* assays were unable to migrate towards chemokine stimuli[327]. However, this is not true for cDCs as they express Dock180 in addition to Dock2 which have redundant functions, and the defects in the pDC population in Dock2 KO mice were not present in cDCs[327]. The majority of pDCs express B220[328], and in the *ex vivo* DCs analysis $B220^+$ cells were removed within the dump gate. The DC data from β_2 integrin-deficient mice only included cDCs and was therefore not impacted by the reduction in pDCs caused by *Dock2*^{Hsd}.

Finally, it is worth noting that the identification of *Dock2*^{Hsd} in CD11a KO, CD11c KO and CD11d KO mice is an important finding that must be taken into consideration when interpreting any results published previously using these mice. In addition to the lack of MZ B cells and reduced numbers of pDCs, mice expressing *Dock2*^{Hsd} have increased numbers of splenic CD8 T cells with a memory phenotype[309]. Indeed, a higher number of central memory CD8 T cells was also described in CD11a KO mice that were obtained from Jackson, so will express *Dock2*^{Hsd} [329]. This alteration in CD8 T cells may not be a CD11a-dependent effect, as interpreted by the authors, but instead due to *Dock2*^{Hsd}. Using Dock2 KO mice it was shown that Dock2-deficient T cells have impaired TCR clustering[330]. This decreased TCR clustering was also described in CD8 T cells from CD11a KO mice, where they attributed this effect to the absence of CD11a expression, however it is likely that these mice expressed *Dock2*^{Hsd} [331]. My data shows the increased expression of CD44 on CD4 T cells in the spleen of CD11a and CD11c KO mice and loss of MZ B cells was due to *Dock2*^{Hsd} and not the integrin-defect. Importantly, the altered phenotype of BMDCs and DCs *ex vivo* were β_2 integrin-dependent. The loss of MZ B cells and pDCs, increase in memory CD8 T cells in mice affected by *Dock2*^{Hsd} will

likely also have an indirect effect on other leukocyte populations. For example, pDCs can drive T cell responses, activate NK cells and recruit other immune cells through chemokine production[332]. Therefore, there will be knock-on effects on the phenotype and function of other leukocyte subsets. These findings highlight the importance of using multiple approaches to validate findings and not relying completely on genetically-deficient mouse models.

3.3.2 Regulation of DCs by β_2 integrins

My data demonstrates that, regardless of the mutation in *Dock2*, CD11a and CD11c act as negative regulators of DC activation. This conclusion can be made based on the following evidence: (i) BMDCs with reduced or absent CD11a and CD11c expressed higher levels of co-stimulatory molecules, whereas in integrin-sufficient BMDCs expression was unchanged whether from *Dock2*^{WT} or *Dock2*^{Hsd} mice. (ii) These findings are in line with previously published data, where mice with signalling-deficient β_2 integrin receptors showed an increased percentage of DCs in the spleen and BMDCs generated from these mice expressed higher levels of co-stimulatory molecules[73].

Initial experiments carried out used β_2 integrin-deficient *in vitro* generated BMDCs. First, integrin expression was assessed on the BMDCs to determine whether the loss of one β_2 integrin subunit affects the expression of the others. β_2 integrin family members have overlapping ligand repertoires and as a result there is a level of redundancy of function between the receptors. It is therefore possible that in the absence of one β_2 integrin, another may be upregulated to help compensate for the loss. Other than the loss of integrin expression expected based on the genetic KO, the only difference seen was in the levels of CD11b. CD11b expression was downregulated in CD11a, CD11c and CD11d KO BMDCs. However, as there were no significant differences in the activation or priming of T cells by CD11b KO BMDCs it is unlikely that the reduced CD11b expression in the other genotypes is contributing to the effects seen.

It was previously shown that in BMDCs expressing non-functional β_2 integrin receptors there was an enhanced expression of MHC II, CD40, CD80 and CD86[73]. From the results in the β_2 α-chain KO mice, the increased co-stimulatory molecule expression can be attributed specifically to the loss of CD11a and CD11c,

suggesting that these integrins are involved in the regulation of signalling pathways associated with co-stimulatory molecule gene expression. The transcription factor PU.1 induces the transcription of CD80 and CD86 in BMDCs[333] and can be activated by GM-CSF[334] and Akt[335]. It was previously shown that GM-CSF-induced activation of the MAPK pathway, including pAkt, was enhanced in β_2 signalling-deficient BMDCs[73]. Therefore, enhanced signalling through pAkt in the absence of CD11a and CD11c may activate PU.1 to induce greater transcription of CD80/CD86. There was no difference in MHC II expression in the absence of any of the individual CD11 subunits. This could be explained by the differential signalling pathways that control the expression of MHC II compared to CD80/CD86. The transcription of MHC II is activated by the transactivator protein CIITA, which can be induced by STAT5[336]. In the absence of β_2 integrin signalling, BMDCs showed comparable levels of pSTAT5[73], indicating that β_2 integrin-mediated regulation occurs independently of STAT5. There was no difference in the activation of BMDCs from CD11b mice, in agreement with the findings of Ling *et al.*, who reported similar expression of MHC II, CD40, CD80 and CD86 in WT and CD11b KO immature BMDCs [185]. However, in contrast to my findings the authors also demonstrated reduced expression of MHC II, CD80 and CD86 after LPS-stimulation in CD11b KO BMDCs. The concentration of LPS they used was 20 times greater than I used in this study (1μg/ml compared to 50ng/ml) and the duration of stimulation was longer at 24h, which may explain the inconsistency in results.

Following identification of increased co-stimulatory molecule expression on CD11a and CD11c KO BMDCs, the effect on the subsequent T cell response was then tested. In co-cultures of BMDCs with CD4 T cells there was increased proliferation and activation of T cells when cultured with CD11a KO but not CD11c KO BMDCs compared to WT, despite both CD11a and CD11c KO BMDCs displaying increased expression of co-stimulatory molecules. Although there were differences in the levels of co-stimulatory molecules between CD11a and CD11c KO BMDCs. The expression of CD40 was not significantly upregulated on CD11c KO BMDCs compared to WT, and in the absence of LPS the levels of CD86 were similar in CD11c KO and WT BMDCs. This indicates that there are subtle differences in co-stimulatory molecule expression on CD11c KO BMDCs, potentially explaining the enhanced T cell response with CD11a KO but not CD11c KO BMDCs.

The interactions between CD11a KO BMDCs and CD4 T cells *in vivo* resulted in an increase in the number of T cells in the draining LN, but these T cells displayed lower levels of activation marker expression. This is in contrast to the *in vitro* data where the absence of CD11a on BMDCs caused an increase in T cell activation. The higher number of OT-II T cells in the LN of mice that received CD11a KO BMDCs suggests that these cells induced greater recruitment or proliferation of the transferred CD4 T cells. CFSE staining of the transferred OT-II T cells could be used to investigate this in the future. A potential explanation for the reduced expression of CD25, CD69 and CD44 is that the CD11a KO BMDCs induced an earlier activation of the T cells, so after 3 days these receptors may already be downregulated. Whereas the WT BMDCs may activate the T cells at a slower rate, therefore at the 3 day timepoint the expression of activation markers may be at their peak. A time-course of analysis would be required to address this issue. Alternatively, the phenotype of the BMDCs may be changed when they are transferred *in vivo,* thereby losing their capacity to induce enhanced T cell responses. Recovering enough BMDCs from the draining LN after transfer makes this difficult to study.

When integrin-deficient DCs were stained *ex vivo* for activation marker expression the altered co-stimulatory molecule expression found in the BMDCs was not replicated, with the exception of higher CD86 expression on splenic DCs from CD11c KO mice. There was a high variability in the data from DCs stained *ex* vivo, demonstrated by large error bars, therefore it was not possible to detect any subtle effects. This discrepancy between *in vitro* and *in vivo* DCs, may be due to factors within the microenvironment *in vivo* that affect expression of these markers. In particular for CD40 the difference in expression was only seen when DCs were activated with LPS, suggesting an inflammatory insult may be required to detect any differences in this marker. In addition, the generation of BMDCs produces a mixed population of DCs and macrophages[337] compared to the analysis of DCs *ex vivo*, where monocyte-derived cells were excluded. In the *in vivo* setting DCs were analysed by gating out other leukocytes then gating on MHC II^+ cells, therefore the purity of DCs analysed may have impacted the results, discussed in detail below. There is also evidence that BMDCs display a transcriptional signature related to subsets of migratory DCs found *in vivo*[337], so the enhanced expression of co-

stimulatory molecules may be subset-specific and consequently not detected by analysis of total DCs *in vivo*.

It was previously found that when β_2 integrin receptors are functionally impaired, DCs in the spleen expressed higher levels of CD86[73]. An increase in CD86 expression was also identified in splenic DCs from CD11c KO mice, indicating that the effect seen in the signalling-deficient mice may be due to the specific loss of CD11c. However, it must be taken into consideration that the markers used to identify the DCs were different. In the beta2$^{TTT/AAA}$ KI mice, the integrin receptors have impaired signalling but are still expressed on the cell surface, therefore CD11c was used as marker to identify DCs. The use of CD11c KO mice meant that an alternative gating strategy had to be used to identify DCs from the β_2 integrin α-chain KO mice. This was done by using 'dump' markers to exclude monocytes/macrophages (CD64), pDCs (B220), B cells (CD19/B220), NK/NK T cells (NK1.1), neutrophils (Gr1) and T cells (TCRβ). Cells were then gated on MHC II$^+$ and these were considered DCs. Using this method, the DCs were of 80-90% purity based on the percentage of CD11c$^+$ identified in WT, CD11a KO, CD11b KO and CD11d KO mice. This means that 10-20% of the cells are not DCs as they did not express CD11c. Potential sources of contamination would be cell types that were not removed by the dump gate and are known to express MHC II. This includes: ILCs[338], stromal cells[339] and γδ T cells[262]. The addition of CD45 to the panel would remove any stromal cells and replacement of TCRβ with CD3 would allow the exclusion of γδ T cells in addition to αβ T cells. Further optimisation of the gating strategy for DCs in β_2 integrin-deficient mice will clarify whether the findings from BMDCs generated *in vitro* are not replicated with *in vivo* DCs, or if the effects were lost due contamination of other cell populations.

One of the main unanswered questions is why the loss of CD11a and CD11c results in enhanced DC activation but not CD11b or CD11d. This could potentially be explained by the differential expression of the receptors on DCs, distinct ligand binding or downstream signalling pathways. BMDCs expressed CD11a, CD11b and CD11c (Figure 3-1), but CD11d was not measured due to the lack of a commercially available murine CD11d antibody. It could be hypothesised that higher expression of CD11a and CD11c than CD11b would indicate the functional significance of these subunits in BMDCs. Contrary to this, CD11b was expressed highest by BMDCs

(95%), followed by CD11a (70-80%) and then CD11c (65%) (relative expression shown in Figure 3-1). This suggests that the negative regulation of DCs by CD11a and CD11c is not governed by cellular expression levels of the receptors.

An alternative explanation is that CD11a and CD11c share a ligand specificity that differs from CD11b and CD11d. CD11a has the most restricted β_2 integrin ligand repertoire, only recognising the ICAM family (ICAM-1-5) and CD11c has also been shown to bind ICAM-1[103]. CD11b and CD11d have a high degree of structural homology and can recognise a similar diverse ligand repertoire including ICAM-3, VCAM-1, fibronectin, fibrinogen and other matrix proteins[22–25]. But unlike CD11b, CD11d does not display affinity for ICAM-1[24]. CD11b and CD11c also share a high degree of structural homology and the ability to bind ICAM-1[25], meaning there is not an obvious ligand that conveys specificity for CD11a and CD11c. However, ICAM-1 can exist in different isoforms, which consist of a combination of immunoglobulin (Ig) domains numbered 1-5[340]. The ability of ICAM-1 to bind CD11a is isoform-dependent[341] and CD11a binds to Ig domain 1 whereas CD11b binds to Ig domain 3[342]. The CD11c-binding Ig domain has not been characterised, but there may be a specific isoform/s of ICAM-1 that demonstrate specificity for CD11a and CD11c over CD11b. In my adhesion assay data, CD11a and CD11c were the major mediators of BMDC binding to ICAM-1, suggesting it may be specifically integrin binding to ICAM-1 that regulates DCs.

Downstream of β_2 integrin receptor binding signalling pathways are activated by the intracellular cytoplasmic tails. The β_2 integrin α chains differ in length and sequence, with different phosphorylation residues on their cytoplasmic regions[343]. The specific signalling molecules that bind individual α chains are not well characterised, unlike the CD18 chain, but shared downstream signalling molecules may explain the negative regulation of DCs by CD11a and CD11c, with different pathways induced compared to CD11b and CD11d.

In addition to the reason for DC regulation being specific to CD11a and CD11c receptors, the mechanism of this regulation is also unknown. BMDC cultures contained GM-CSF and IL-4, therefore it is likely that the β_2 integrin-mediated regulation of DCs occurs downstream of signalling from the receptors for these cytokines. It was previously shown that in the absence of β_2 integrin signalling, GM-

CSF treatment of BMDCs enhanced phosphorylation of Syk, p38, Erk and Akt but not STAT5[73]. This suggests that under homeostatic conditions, β_2 integrin receptor signalling may induce inhibitors of the MAPK and PI3K pathways. This could occur through the targeting of GM-CSF-induced signalling molecules for degradation via phosphorylation that enables degradation by ubiquitination, as was demonstrated for the signalling adapters TRIF and MyD88 in macrophages downstream of CD11b[183]. Thus, in the absence of β_2 integrins, signalling molecules downstream of GM-CSF are unconstrained.

3.4 Conclusions

In conclusion, I have identified that the loss of CD11a and CD11c leads to enhanced co-stimulatory molecule expression on BMDCs generated *in vitro*. These findings demonstrate that CD11a and CD11c function as negative regulators of DC activation. The presence of an additional mutation in the *Dock2* gene in CD11a, CD11c and CD11d KO mouse strains contributed to altered immune phenotypes in addition to the β_2 integrin-deficiency. Loss of *Dock2* expression caused a loss of MZ B cells and in CD11c KO mice reduced CD4 T cell numbers in LNs. Whereas the defect in CD4 T cells in CD11a KO mice was due to the loss of both *Dock2* and CD11a. The enhanced expression of co-stimulatory molecules on DCs *in vitro* and increased percentage of DCs *in vivo* in CD11a and CD11c KO mice was independent of *Dock2.* These findings have provided new information on how integrins regulate DCs, with the identification of the specific β_2 integrin receptor subunits, CD11a and CD11c as negative regulators of DCs. Further studies can now focus on the potential ligands for these receptors and the mechanism of this regulation.

Chapter 4 Characterisation of γδ T cells in β_2 integrin-deficient mice

4.1 Introduction

β_2 integrin receptors play an essential role in αβ T cell migration and function. CD11a is the β_2 integrin most highly expressed on T cells and plays a major role in cell adhesion of CD4 and CD8 T cells. The binding of CD11a to ICAM-1 expressed on antigen presenting cells mediates the formation of the immune synapse, an important interaction for the differentiation and activation of T cells [75,304]. In a similar interaction, CD8 T cells adhere to target cells via CD11a and this interaction is necessary for effective cell killing through release of cytotoxic granules [77,87]. CD11a-ICAM-1 contacts are also required for αβ T cell migration, both extravasation from the bloodstream into tissues and for entry into lymph nodes via the high endothelial venule [64,68].

β_2 integrins are also expressed on γδ T cells however, their role is much less understood than on αβ T cells. Previous studies suggest that unlike αβ T cells, the migration of γδ T cells to sites of inflammation is not dependent on β_2 integrins. Models of experimental autoimmune encephalitis (EAE) and psoriasis carried out in β_2 integrin-deficient mice demonstrated that γδ T cells were able to effectively migrate to the primary site of inflammation, central nervous system and skin respectively, despite lacking the adhesion receptor [295,296]. There has also been work demonstrating that mice deficient in β_2 integrins can develop inflammation associated with an increase in γδ T cells. In a model of reduced CD18 expression (CD18^{hypo}PL/J) mice spontaneously develop skin inflammation, with IL-17-producing γδ T cells found in the skin lesions and draining lymph nodes [296]. CD11a KO mice can develop periodontal disease as they age, which is also associated with an increased number of IL-17-producing γδ T cells in the oral mucosa [302]. There is additional evidence of expanded γδ T cells in integrin-deficient mice without signs of inflammation. An increased number of γδ T cells has been described in the cervical lymph nodes and spleen of CD18 KO mice [297,303].

Despite several studies demonstrating an expansion of γδ T cells in the absence of β_2 integrins, the mechanism of this expansion has not been identified. Furthermore, the number of γδ T cells in other mucosal tissues, where they perform essential

functions such as the lungs and intestines, were not investigated. To address this, I used total β_2 integrin deficient mice, CD18 KO, to assess γδ T cells more globally under steady-state conditions. It is worth noting that some strains of CD18 KO mice have been reported to develop a spontaneous dermatitis[5], however the colony housed at our mouse facility are on a C57BL/6 background which are resistant to the development of skin inflammation[344].

4.1.1 Hypothesis and Aims

Given that several mouse models indicate the loss of β_2 integrin expression leads to an increased number of γδ T cells, my hypothesis is that β_2 integrins are a novel regulator of γδ T cells.

The overall aim of this chapter was to examine how the absence of β_2 integrins alters the phenotype and localisation of γδ T cells under steady state conditions. This is further divided into 4 sub-aims:

1. At which lymphoid/mucosal sites are γδ T cell numbers increased in CD18 KO mice?

2. Is the increased number of γδ T cells due to a global expansion or specific subset/s?

3. Which β_2 integrin family member/s (CD11a/b/c/d) is responsible for the phenotype?

4. Does β_2 integrin expression differ on subsets of γδ T cells?

4.3 Results

4.3.1 CD18 KO mice have a tissue-restricted increase in γδ T cells

The first aim was to determine which lymphoid and mucosal sites display an increase in γδ T cell numbers in CD18 KO mice compared to WT. Cells were isolated from the spleen, blood, lungs, uterus, skin (ears), small intestine lamina propria (LP), small intestine epithelium (Ep), colon, Peyer's patches, mesenteric lymph nodes (mLN), peripheral lymph nodes (pLN; inguinal, brachial and axillary pooled), gingiva (oral mucosa), thymus and bone marrow. These were analysed by flow cytometry to determine the percentage and number of γδ T cells present in each tissue. Evaluation of αβ T cells was provided as a comparator population. A defined gating strategy was used to determine cell populations (Figure 4-1).

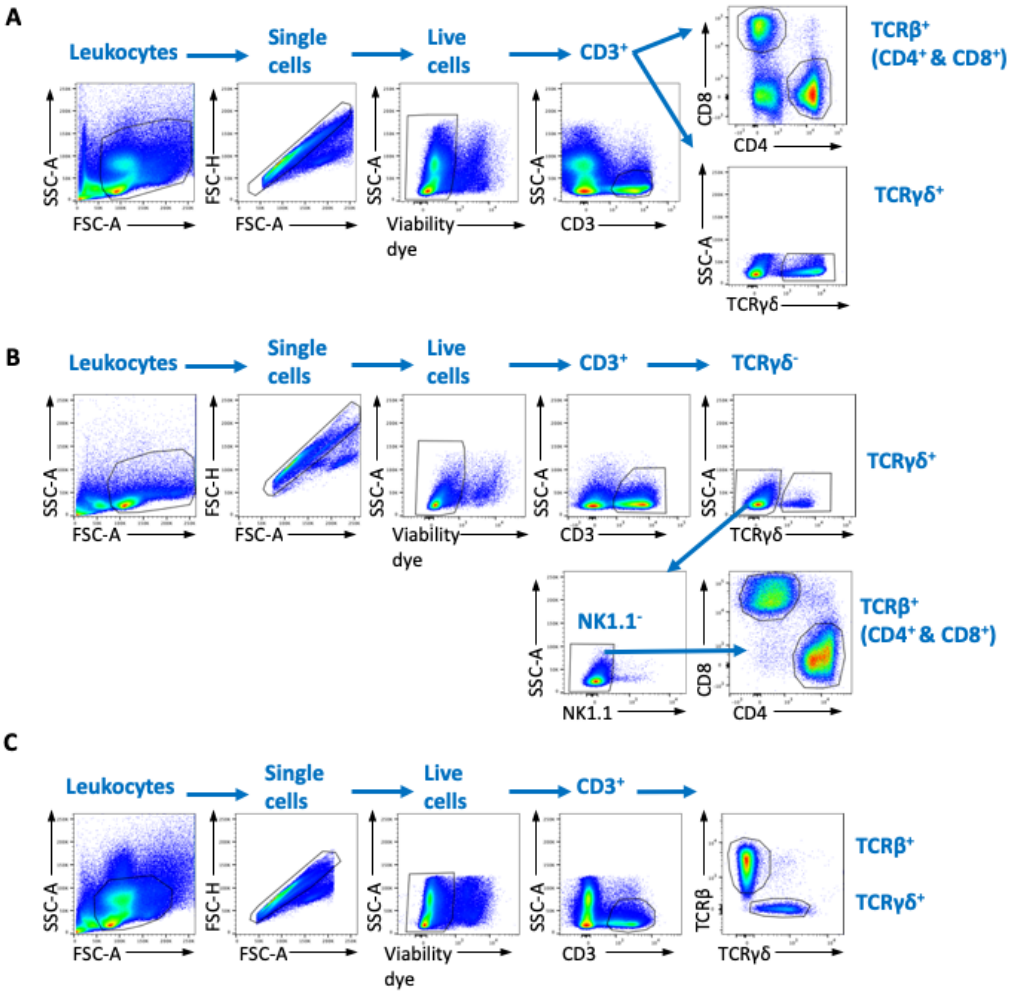

Figure 4-1 Flow cytometry gating strategy to identify αβ and γδ T cells.
(A) Gating strategy used to identify αβ and γδ T cells for data shown in figures 4-2 and 4-3 with the exception of small intestine, colon, mLN and Peyer's patches, which were gated as shown in **(B)**. **(C)** Gating strategy used to identify αβ and γδ T cells for figures 4-4 onwards. Examples shown in **(A & C)** from CD18 KO spleen are representative of WT and of other tissues. Example shown in **(B)** from WT mLN is representative of CD18 KO and other intestinal tissues.

The comparison between WT and CD18 KO mice revealed that with the exception of the skin, colon and thymus, there was a substantial increase in the percentage of $CD3^+$ γδ T cells in CD18 KO mice (Figure 4-2). This was accompanied by the expected decrease in proportion of αβ T cells. Enumeration of the absolute cell numbers (Figure 4-3) revealed that the actual differences in γδ and αβ T cells were site-specific. Notably, the absolute number of γδ T cells were greater only in the spleen, blood, lungs and uterus (Figure 4-3). In the skin, small intestine, colon, Peyer's patches, lymph nodes, gingiva, thymus and bone marrow there was no difference in the number of γδ T cells. In addition, the number of αβ T cells was decreased in the colon, Peyer's patches, mLN, pLN, thymus and bone marrow. It has already been established that αβ T cell migration is dependent on β_2 integrins, in particular they require these integrin receptors to enter lymph nodes via high endothelial venules[68]. It is therefore unsurprising that CD18 KO mice show a decreased number of αβ T cells in several lymphoid tissues including the mLN, pLN and Peyer's patches.

This comprehensive tissue analysis indicates that in the absence of CD18 there is an increased number of γδ T cells in a restricted number of sites, namely the spleen, blood, lungs and uterus.

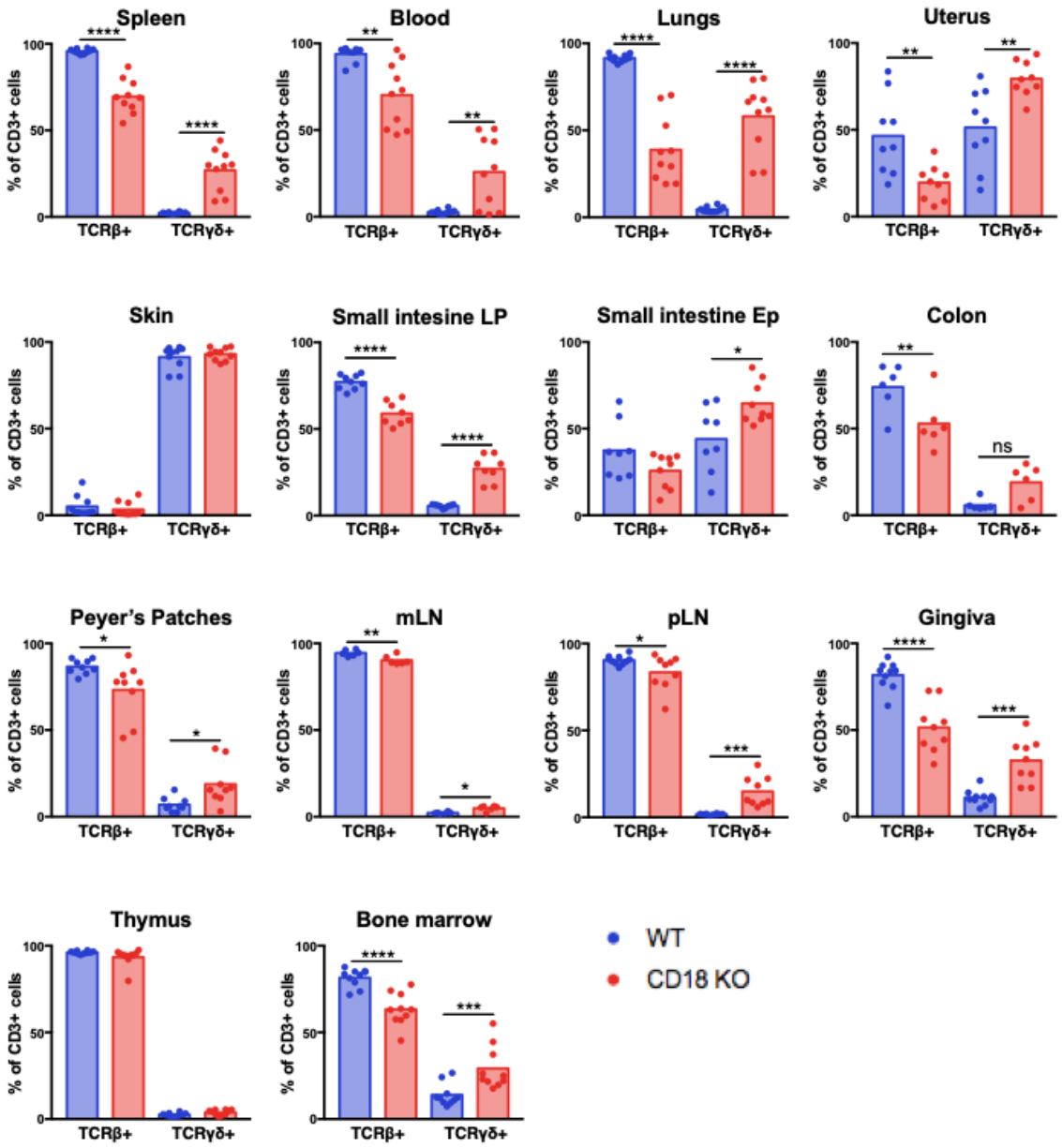

Figure 4-2 Increased proportion of γδ T cells in multiple tissues of CD18 KO mice. Cells were isolated from various tissues of WT and CD18 KO mice then analysed by flow cytometry. Percentage of αβ and γδ T cells as a proportion of $CD3^+$ are shown, as per the gating strategy in Fig 1-1A. Spleen, blood, lungs, skin, pLN, gingiva, thymus, bone marrow n=10. Uterus, Peyer's patches n=9. Small intestine LP WT n=9, CD18 KO n=8. Small intestine Ep WT n=9, CD18 KO n=8. Colon and mLN n=6. Male and female mice were used, aged 6-14 weeks. Data are pooled from a minimum of 3 independent experiments; each symbol representing an individual mouse. Statistical differences were determined using a two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

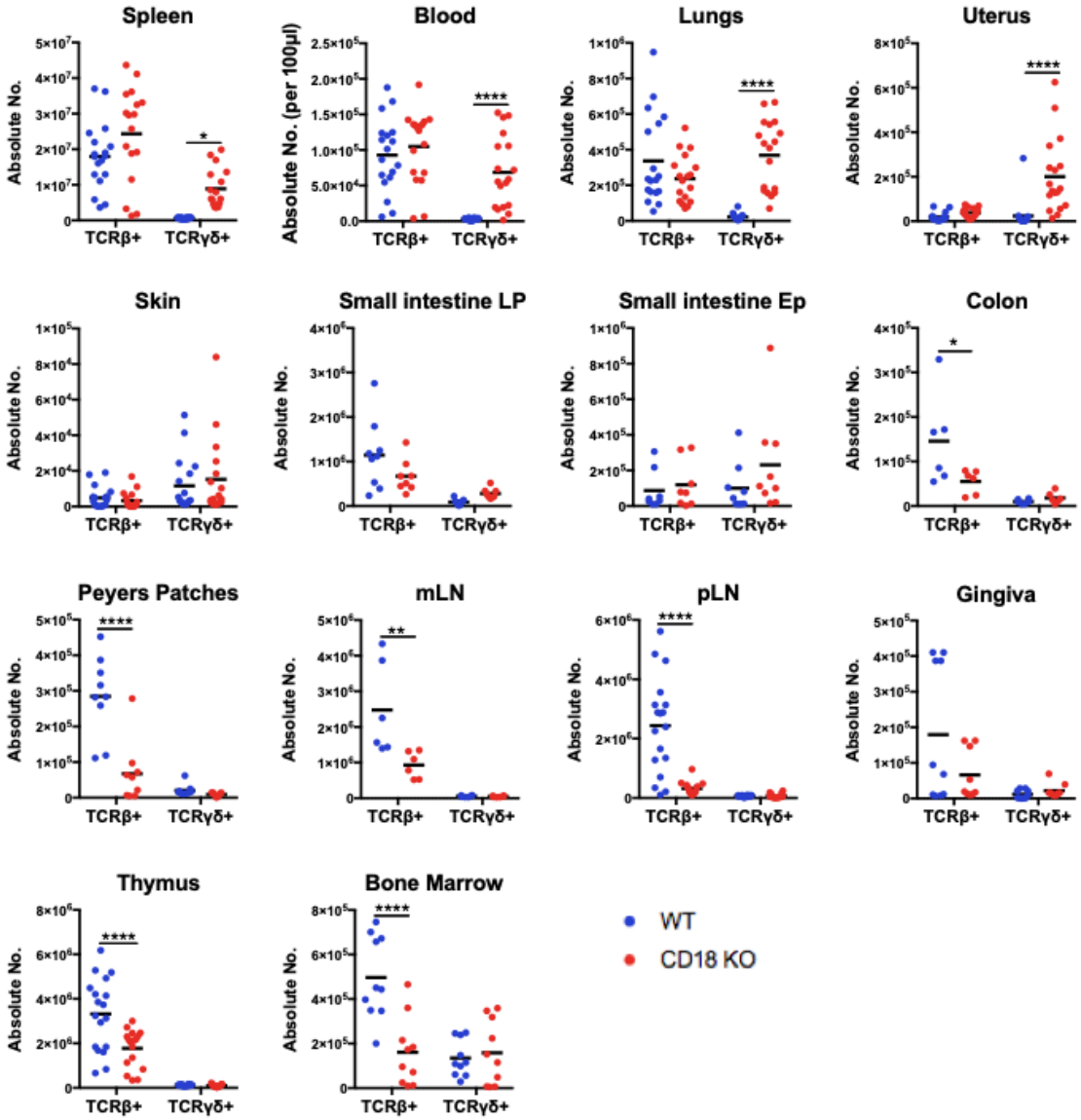

Figure 4-3 Increase in the number of γδ T cells in the spleen, blood, lungs and uterus of CD18 KO mice.
Cells were isolated from various tissues of WT and CD18 KO mice then analysed by flow cytometry. Absolute number of αβ and γδ T cells per tissue are shown. For blood numbers were calculated per 100µl. Spleen, blood, lungs, uterus, skin n=18. Gingiva and bone marrow n=10. Small intestine LP WT n=9, CD18 KO n=8. Small intestine Ep WT n=8, CD18 KO n=9. Colon and mLN n=6. Peyer's patches n=9. pLN WT n=18 CD18 KO n=16. Thymus n=20. Male and female mice were used, aged 6-14 weeks. Data from a minimum of 3 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Given that there was an increase in the number of γδ T cells found in the blood and also lungs, a highly vascularised organ, there could be contaminating cells in the lung samples that were circulating in the blood. To minimise contamination by leukocytes present in the vasculature, the lungs were perfused prior to removal. This will not completely remove all circulating cells present in the lung vasculature but does provide an appropriate comparator. Thus, to confirm that the cells analysed were present in lung tissue, γδ T cells recovered from perfused lung tissue were compared to unperfused. Both the percentage and number of γδ T cells isolated from unperfused or perfused lung tissue were similar (Figure 4-4). The same significant increase in γδ T cells can be seen when comparing CD18 KO to WT. There was no significant difference between the number of γδ T cells from unperfused and perfused lungs, regardless of the genotype, suggesting that the γδ T cells analysed are likely to reside in the lung tissue. Ideally, immunofluorescent microscopy could provide information on the location of γδ T cells within the lungs and visualise their position in relation to blood vessels and lung tissue. Unfortunately, although staining of lung sections for TCRγδ was attempted, due to technical issues it was unsuccessful. Alternatively, intravenous administration of labelled antibody (e.g. anti-CD45) prior to analysis of the lungs would confirm whether or not the γδ T cells are in the circulation (staining positive) or the tissue (staining negative).

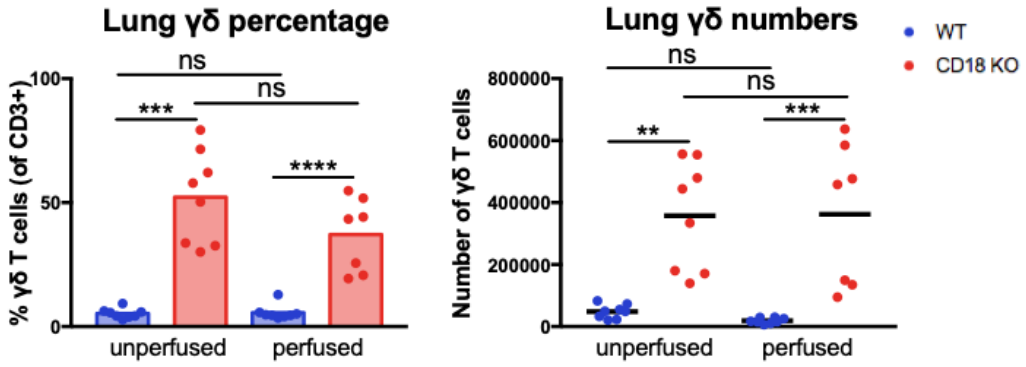

Figure 4-4 Perfusion of lungs does not affect the percentage/number of γδ T cells analysed. Cells were isolated from the lungs of WT and CD18 KO mice either immediately, or following perfusion with 2mM EDTA. WT/CD18 KO unperfused and WT perfused n=8, CD18 KO perfused n=7. 3 independent experiments; each symbol represents and individual mouse. Statistical differences were determined using a two-way ANOVA. ns not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

As noted above, analysis of individual tissues from CD18 KO mice identified that γδ T cell numbers are increased in the spleen, blood, lungs and uterus compared to WT. In order to assess the impact this has on the overall number of γδ T cells from

WT and integrin-deficient mice, the total number of γδ T cells was calculated by plotting the average number from all organs analysed. It was found that overall CD18 KO mice had a 6.5 times greater number of γδ T cells at $1.1x10^7$, compared to $1.7x10^6$ in WT mice (Figure 4-5). This was predominantly due to heightened numbers within the spleen, with CD18 KO mice displaying a 50-fold increase in γδ T cells in this organ. Similarly, in the lungs there was also a 50-fold increase in γδ T cells, whereas the increase was lower in the blood and uterus (8- and 4-fold respectively).

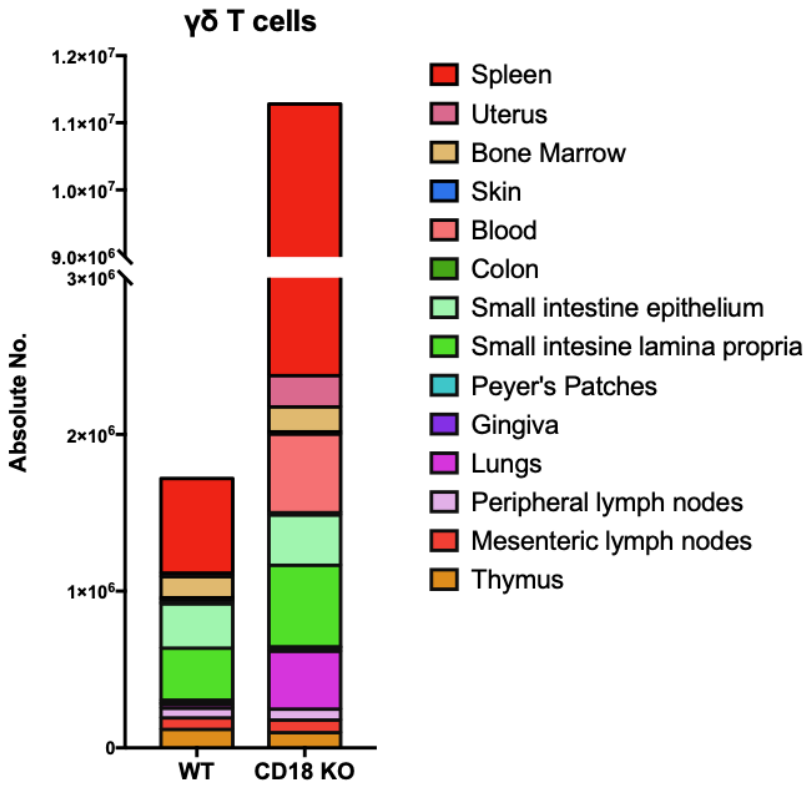

Figure 4-5 CD18 KO mice have 6.5 times more γδ T cells than WT.
Data from Figure 1-3 represented as the average number of γδ T cells per tissue, used to calculate the total number in WT and CD18 KO mice. For the blood the total number was calculated based on mouse blood volume 58.5ml/kg and an average weight of 25g[345].

In summary, the loss of CD18 expression leads to a 6.5-fold increase in the number of γδ T cells. This was due to higher numbers in a restricted number of sites; the spleen, blood, lungs and uterus. There were no differences in γδ T cell numbers found in the 10 other tissues examined.

4.3.2 Loss of β_2 integrin expression causes a specific increase in Vγ6Vδ1$^+$ IL-17-producing γδ T cells

After establishing that the increase in γδ T cells found in β_2 integrin-deficient mice is not restricted to the spleen and cervical LNs as previously reported [297,303], the next question was whether the expanded γδ T cells in CD18 KO mice were phenotypically different from those in WT mice. There are a number of cell surface markers, cytokines and transcription factors that are commonly used to characterise γδ T cells. The ability to produce either IFNγ or IL-17 broadly divides γδ T cells into two subsets. These subsets have different functions in response to infectious challenge and in diseases such as cancer can either promote or protect against tumour progression. Previous studies have identified that IFNγ-producing γδ T cells are CD62L^{hi} CD44^{lo} CD45RB$^+$ CD27$^+$ and express T-bet, whereas IL-17-producing γδ T cells are CD62L^{lo} CD44^{hi} CD45RB$^-$ CD27$^-$ and express RORγt [199,206,207].

Initial experiments evaluated the expression of surface markers. The majority of γδ T cells from the spleen of CD18 KO mice expressed CD44 but lacked expression of CD62L, CD27 and CD45RB (Figure 4-6A). Whereas those from a WT spleen mainly expressed CD62L, CD27 and CD45RB but lacked CD44. Both expression of the TCR and its co-receptor CD3 were higher on γδ T cells from CD18 KO compared to WT. High expression of these markers on γδ T cells has previously been shown to identify a population of IL-17-producing cells that express Vγ6 [346]. Combined, this implies that there may be an increase in Vγ6$^+$ γδ T cells in the CD18 KO spleen. This was further supported by the analysis of transcription factors T-bet and RORγt (associated with IFNγ and IL-17 production respectively), which showed an increased proportion of CD18 KO γδ T cells expressed RORγt in comparison with WT. There was no difference in the expression of T-bet (Figure 4-6A).

Based on the expression of surface markers and transcription factors, CD18 KO γδ T cells display a phenotype associated with IL-17 production. To investigate if this was the case, splenocytes from WT and CD18 KO mice were stimulated with PMA/ionomycin and their cytokine production was analysed. γδ T cells from CD18 KO mice produced higher levels of IL-17 and less IFNγ (Figure 4-6C). Despite this percentage difference, calculation of the number in the whole spleen showed that IL-17$^+$ γδ T cells were increased but there was no difference in the IFNγ$^+$ γδ T cells.

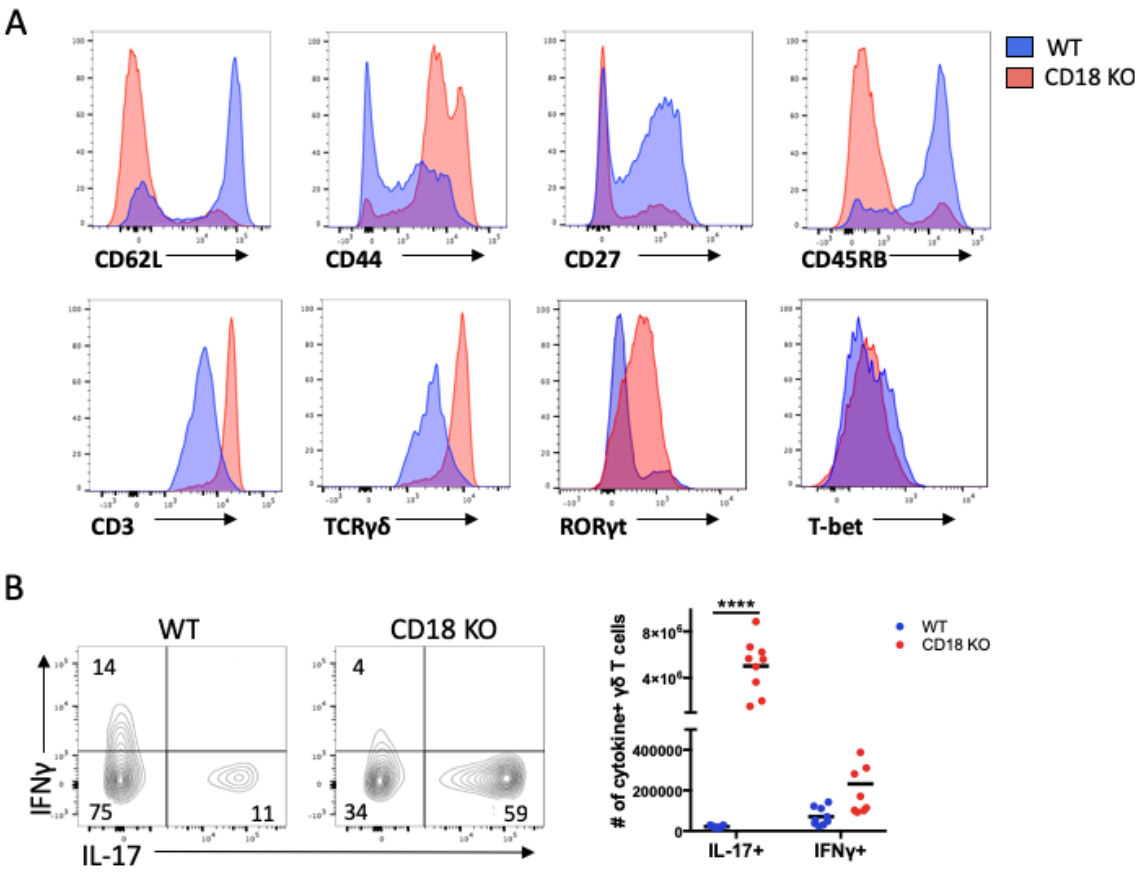

Figure 4-6 CD18 KO γδ T cells express high levels of CD44, CD3, TCRγδ, RORγt and IL-17. Analysis of γδ T cells from the spleen of WT and CD18 KO mice by flow cytometry. **(A)** Representative plots of cell surface markers and transcription factors. **(B)** Percentages and number of IL-17$^+$ and IFNγ$^+$ γδ T cells following *in vitro* stimulation with PMA/ionomycin for 4h. n=9. Data representative of 3 independent experiments. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

As γδ T cells from CD18 KO mice showed an increased ability to produce IL-17 following *in vitro* stimulation, the next question was whether CD18 KO mice display higher levels of IL-17 *in vivo*. The quantity of IL-17 was measured by ELISA in the serum and tissue supernatants (media from the whole tissue incubated for 4h) isolated from WT and CD18 KO mice. The levels of IL-17 were increased in the serum and spleen of CD18 KO mice compared to WT but there was no difference in the lungs or uterus. This suggests that circulating IL-17 is increased in CD18 KO mice, however, it is apparent that IL-17 production is not enhanced at all the sites where an increase in γδ T cell numbers was found.

The ELISA data showed that CD18 KO mice have increased levels of IL-17 in the serum and spleen, but this does not confirm that γδ T cells are the source of the IL-17. The ability of γδ T cells from the lungs and uterus (where an increased number of γδ T cells was found) to produce IL-17 had also not been assessed. To determine which cells produce IL-17 in the tissues of WT and CD18 KO mice, and therefore

assess the potential contribution of γδ T cell-derived IL-17 to the levels measured in supernatant samples, cells were re-stimulated *in vitro* with PMA/ionomycin and the phenotype of IL-17-producing cells was analysed. The gating strategy is shown in Figure 4-7B.

In the spleen, lungs and uterus of WT mice, the predominant IL-17 producers were a population of non-T cells ($CD3^-$) (Figure 4-7C). Conversely, in CD18 KO mice the main source of IL-17 was γδ T cells, with a significant increase found in the percentage of $IL\text{-}17^+$ cells that were $TCR\gamma\delta^+$ compared to WT. In all tissues this was accompanied by a significant decrease in the percentage of IL-17-producing non-T cells. This shows that γδ T cells in the spleen and lungs have an enhanced potential to produce IL-17 despite similar levels of IL-17 detected by ELISA. Interestingly, in the CD18 KO spleen there was also a decreased proportion of CD4 T cells and in the uterus a decrease in $CD45^-$ cells within the $IL\text{-}17^+$ population compared to WT.

When the total number of IL-17-producing cells were calculated and compared across tissues, a significant increase in the number of $IL\text{-}17^+$ cells was detected in the spleen but not lungs or uterus of CD18 KO mice compared to WT (Figure 4-7D). This shows that although there appears to be an increased number of IL-17-producing cells in the lungs and uterus, this is not significant when compared alongside the spleen, indicating that there is a greater increase in $IL\text{-}17^+$ cell numbers in the spleen. This may explain why there is an increase in IL-17 levels in the spleen but not lungs or uterus of CD18 KO mice.

Overall, this data indicates that in CD18 KO mice the predominant cell type capable of producing IL-17 is γδ T cells. Consequently, it is likely that the increased levels of IL-17 found *in vivo* are due to enhanced production by γδ T cells.

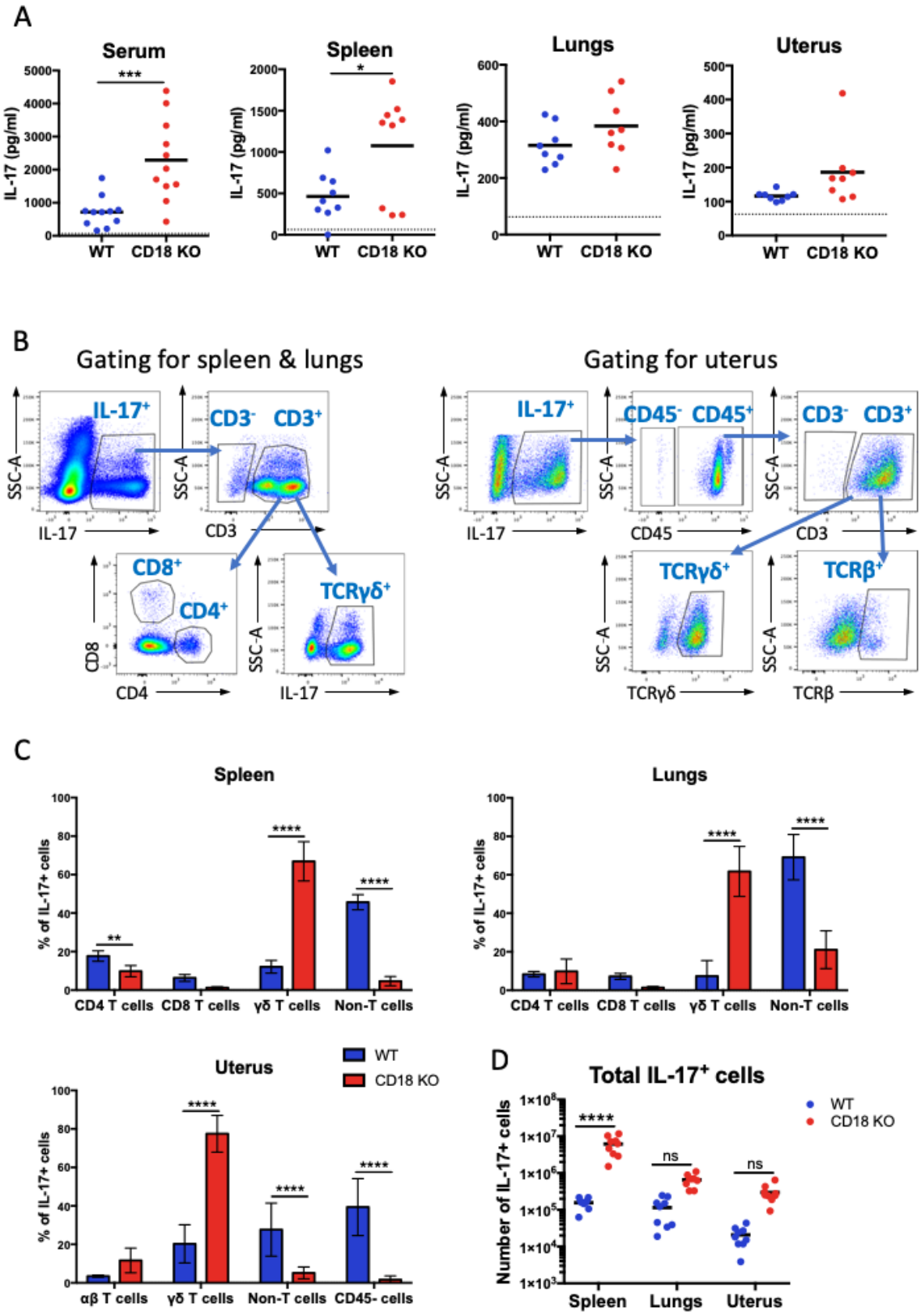

Figure 4-7 Increased levels of IL-17 in the serum and spleen of CD18 KO mice *in vivo* and in γδ T cells *in vitro*.
(A) Levels of IL-17 were measured by ELISA from serum isolated from the blood of WT and CD18 KO mice, and from tissue supernatants collected by incubating the whole tissue in complete media for 4h at 37°. Dotted line indicates the limit of detection (62.5pg/ml). Serum n=11, spleen n=9, uterus and lungs n=8. Each symbol represents an individual mouse, with mean shown. **(B&C)** The phenotype of IL-17$^+$ cells were analysed by flow cytometry, gating strategy **(B)** percentage of IL-17$^+$ cells of indicated phenotype. **(D)** Total number of IL-17$^+$ cells. Data represents mean ± SD. n=9. Data pooled from 3 independent experiments. Statistical differences were determined using a two-way ANOVA. ns not-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

I have identified that there is an increase in IL-17A-producing γδ T cells in β_2 integrin-deficient mice, specifically in the spleen, blood, lungs and uterus. The next question was whether the expanded population occurred due to the enhancement of one or more subsets of γδ T cells. Murine γδ T cells can be categorised according to the Vγ chain expressed as part of the TCR, each of which is associated with a specific wave of thymic development, tissue-homing properties and cytokine profile[196]. These are numbered Vγ1-7 (Heilig and Tonegawa nomenclature [224]), with IL-17 production predominantly by $V\gamma4^+$ and $V\gamma6^+$ cells (Figure 1-5, Table 1-1). In order to determine any Vγ chain bias in the expanded population, cells isolated from various tissues of WT and CD18 KO mice were analysed by flow cytometry for expression of Vγ chains that have commercially available antibodies (Vγ1, Vγ4 and Vγ5). Based on the data above (increased number of IL-17^+ γδ T cells in CD18 KO mice) it was hypothesised that the expanded population is likely to be either $V\gamma4^+$, $V\gamma6^+$, or a combination of both.

In line with prior studies, the majority of the γδ T cells in the WT spleen, blood and lungs were $V\gamma1^+$ or $V\gamma4^+$ (Figure 4-8A). Over 70% of the γδ T cells in these tissues were accounted for in WT mice using the available Vγ1, Vγ4 and Vγ5 antibodies. In contrast, CD18 KO mice showed a decrease in the proportion of $V\gamma1^+$ and $V\gamma4^+$ cells, with $V\gamma1^-V\gamma4^-V\gamma5^-$ cells representing over 85% of the γδ T cell repertoire in the spleen, blood and lungs. In the WT uterus 90% of γδ T cells were $V\gamma1^-V\gamma4^-V\gamma5^-$. This is expected as the predominant subset in the uterus expresses Vγ6 [198]. In the CD18 KO uterus the percentage of $V\gamma1^-V\gamma4^-V\gamma5^-$ γδ T cells was increased to 98%. In the small intestine over 50% of the repertoire was unidentified in WT mice. The likely explanation is that the majority of these cells are $V\gamma7^+$, the predominant subset located in the intestine [202,347]. Interestingly in CD18 KO mice the lamina propria displayed an increase in the $V\gamma1^-V\gamma4^-V\gamma5^-$ population similar to the spleen, blood, lungs and uterus whereas in the epithelium there was no difference in repertoire between the WT and CD18 KO mice. In the skin of both WT mice and CD18 KO mice, all γδ T cells could be accounted for and, as expected, 95% were $V\gamma5^+$.

Despite changes in the proportions of multiple γδ T cell subsets in CD18 KO mice, calculation of the absolute numbers of each subset revealed that only the $V\gamma1^-V\gamma4^-V\gamma5^-$ γδ T cells were significantly altered (Figure 4-8B). The number of this subset was increased in the same tissues that we found the total γδ numbers raised in

(spleen, blood, lungs and uterus; Figure 1-3) and surprisingly also in the small intestine lamina propria.

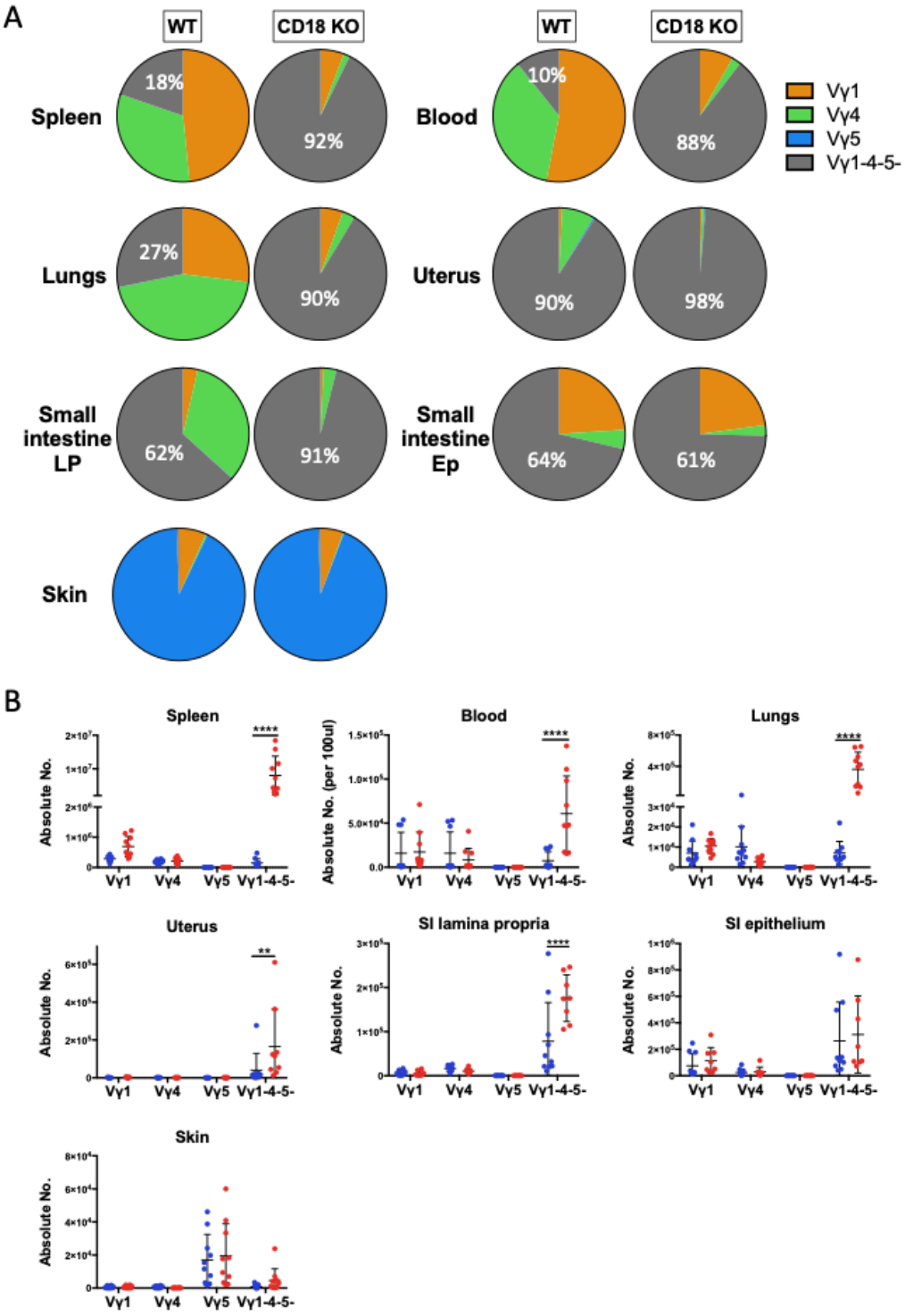

Figure 4-8 Increased number of Vγ1$^-$4$^-$5$^-$ γδ T cells in the spleen, blood, lungs, uterus and small intestine lamina propria of CD18 KO mice.
Cell were isolated from WT and CD18 KO mice lymphoid/mucosal tissues then analysed by flow cytometry. Samples were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$4$^-$5$^-$. **(A)** Percentage of total γδ T cells expressing each TCR chain. Number in white indicates percentage of Vγ1$^-$Vγ4$^-$ cells shown in the grey segment. Representative plots shown. **(B)** Absolute numbers of each γδ subset. n=10, 4 independent experiments. Each symbol represents an individual mouse, with mean shown. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The increased number of γδ T cells found in CD18 KO mice do not express Vγ1, Vγ4 or Vγ5. The remaining subsets are Vγ2, Vγ6 and Vγ7. Vγ2 is expressed at the transcript level in all γδ T cells, however the transcripts are either out of frame or do not correlate with expression on the cell surface[225,226]. $V\gamma7^+$ γδ T cells are the predominant subset in the intestines and tend to produce IFNγ [202,347]. The expanded $V\gamma1^-V\gamma4^-V\gamma5^-$ were therefore expected to express Vγ6. To test this, RNA was isolated from CD18 KO splenocytes sorted on $V\gamma1^-V\gamma4^-$ cells then RT-PCR was performed for Vγ and Vδ chains (Figure 4-9A&B).

The PCR results showed that CD18 KO $V\gamma1^-V\gamma4^-$ cells express the Vγ6 chain but not Vγ7 (Figure 4-9C), confirming my hypothesis. The flow cytometric analysis of Vγ chain expression was also confirmed, as expression of Vγ1, Vγ4 and Vγ5 could not be detected. All γδ T cell receptors exist as dimers of a γ and δ chain. Vγ6 is an invariant TCR that pairs with Vδ1[198]. PCR analysis for the six Vδ chains demonstrated that the $V\gamma1^-V\gamma4^-$ CD18 KO γδ T cells predominately express mRNA for Vδ1.

In summation, the current data supports the concept that the increased population of $V\gamma1^-V\gamma4^-$ γδ T cells in CD18 KO mice are $V\gamma6^+V\delta1^+$ cells. In the rest of the thesis, γδ T cells from the spleen, blood, lungs and uterus that were gated on $V\gamma1^-V\gamma4^-$ or from the thymus gated on $V\gamma1^-V\gamma4^-V\gamma5^-$ will be referred to as Vγ6.

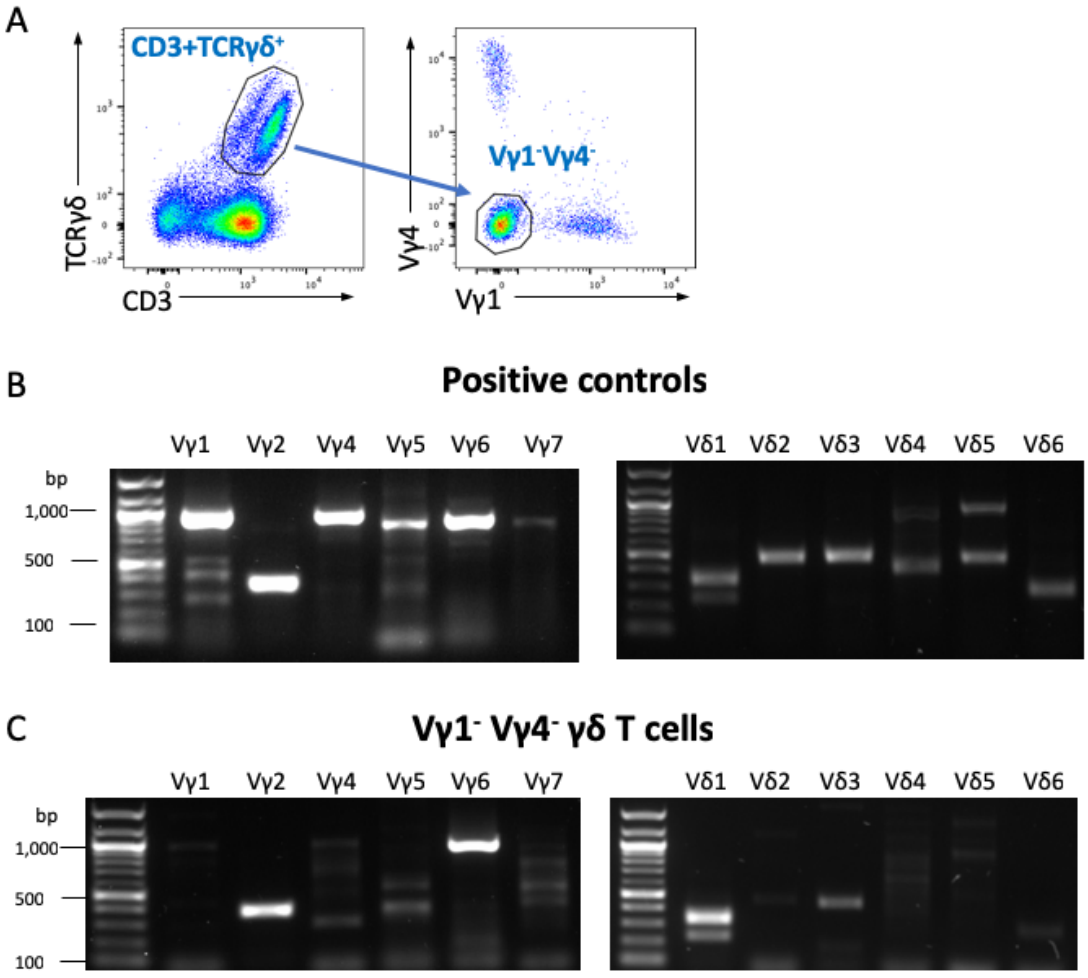

Figure 4-9 Expanded γδ T cell population in CD18 KO mice expresses Vγ6 and Vδ1. CD18 KO splenocytes were sorted by FACS for $CD3^{+}TCR\gamma\delta^{+}$, $V\gamma1^{-}V\gamma4^{-}$ cells then RNA isolated and RT-PCR performed. **(A)** Gating strategy used for sorting. Representative gels showing expression of Vγ and Vδ chain mRNA from positive controls **(B)** and $V\gamma1^{-}V\gamma4^{-}$ cells **(C)**. For positive controls for Vγ1, Vγ2, Vγ4, Vγ6, and all Vδ chains total spleen RNA was used, for Vγ5 total skin RNA, and for Vγ7 small intestine RNA. Results are representative of 4 mice.

CD18 KO mice have an increased number of Vγ6$^+$Vδ1$^+$ γδ T cells in the spleen, blood, lungs and uterus. Next, I wanted to confirm that the raised levels of IL-17 identified by flow cytometry in the total γδ population and serum/spleen cytokine levels by ELISA, were due specifically to enhanced IL-17 production by Vγ6$^+$ cells. To address this, splenocytes from WT and CD18 KO mice were stimulated *ex vivo* with PMA/ionomycin and the expression of IL-17A and IL-17F were assessed by flow cytometry.

As expected, both Vγ4$^+$ and Vγ6$^+$ cells from WT mice produced IL-17A but Vγ1$^+$ γδ T cells did not (Figure 4-10A&B). Surprisingly some production of IL-17A was detected by Vγ1$^+$ γδ T cells from CD18 KO mice, with 15% of Vγ1+ cells producing IL-17A. However, the majority of this cytokine was produced by the Vγ6$^+$ cells, with 80% of these cells staining positive for IL-17A. There was a significant increase in the percentage of Vγ6$^+$ γδ T cells that were IL-17A$^+$ in CD18 KO mice compared to WT. In the samples from CD18 KO mice 15% of Vγ1$^+$ cells produced IL-17A compared to 0.9% of WT Vγ1$^+$ cells, but this was not statistically significant (Figure 4-10B). The levels of IL-17F were very low, with less than 5% of cells expressing the cytokine, and no differences were found in expression between WT and CD18 KO γδ T cells. This data indicates that the increase in levels of IL-17 seen in the CD18 KO total γδ T cell population is predominantly due to production by Vγ6$^+$ γδ T cells.

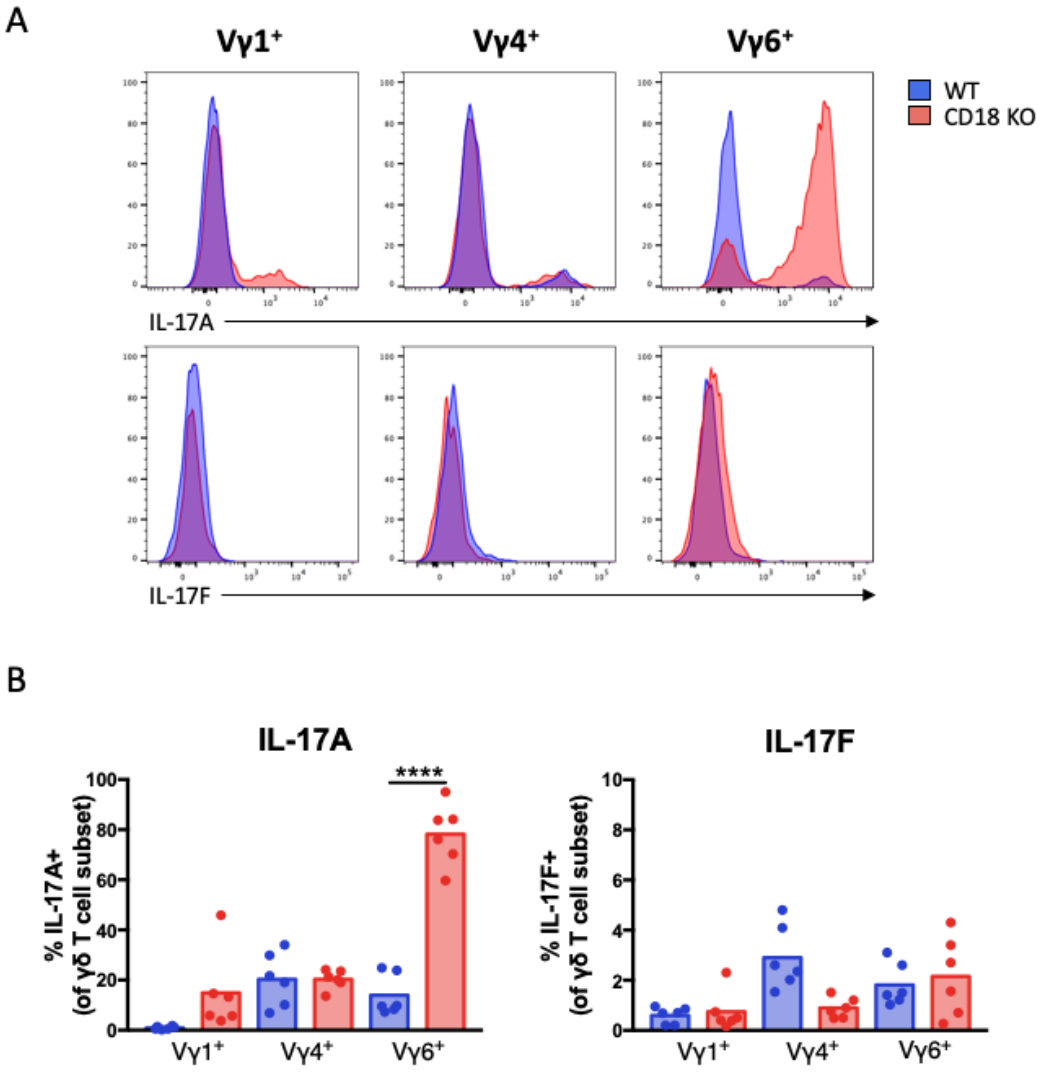

Figure 4-10 Increased IL-17 production by Vγ1$^-$Vγ4$^-$ γδ T cells in CD18 KO mice. Splenocytes were isolated from WT and CD18 KO mice and cytokine production by γδ T cells was assessed by flow cytometry, following *ex vivo* stimulation with PMA/ionomycin for 4h. Cells were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, or Vγ1$^-$4$^-$ (Vγ6$^+$). **(A)** Representative plots of Vγ subset IL-17A and IL-17F. **(B)** Percentage of each γδ subset expressing IL-17A or IL-17F. n=6, 2 independent experiments. Data represents mean; each symbol represents and individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In summary, the phenotype of γδ T cells from CD18 KO mice is different to WT as they are predominantly CD62L^{lo} CD44^{hi} CD45RB$^-$ CD27$^-$ RORγt$^+$. This is due to the specific increase in the Vγ6Vδ1-expressing subset of γδ T cells that show an enhanced production of IL-17A.

4.3.3 Increase in γδ T cells is due to the loss of CD11a expression

The β_2 integrin receptors are a family of four adhesion molecules. I have identified that mice lacking expression of the common β_2 chain have an increased number of IL-17-producing Vγ6$^+$ γδ T cells, therefore I next wanted to identify which of the β_2 integrin family member/s are responsible for this phenotype. To do this, γδ T cell numbers and cytokine production was analysed from mice that lack the individual α chains: CD11a KO, CD11b KO, CD11c KO and CD11d KO.

Flow cytometric analysis revealed that there was a greater number of γδ T cells in the spleen, blood and lungs of CD11a KO mice compared to WT (Figure 4-11A). Whereas there were no differences detected in CD11b KO, CD11c KO or CD11d KO mice (Figure 4-11A). Consistent with this, the percentage of γδ T cells that produced IL-17 was also only increased in the CD11a KO mice, with the proportion of IFNγ-producers in all of the α-chain KO mice remaining unchanged (Figure 4-11B&C). The absolute number of IL-17$^+$ γδ T cells was also higher in CD11a KO mice compared to WT, but in contrast to the findings in the CD18 KO, the number of IFNγ$^+$ γδ T cells also showed a significant increase in the CD11a KO mice. These data suggest that the expansion of IL-17-producing γδ T cells found in CD18 KO mice is due to the loss of CD11a expression.

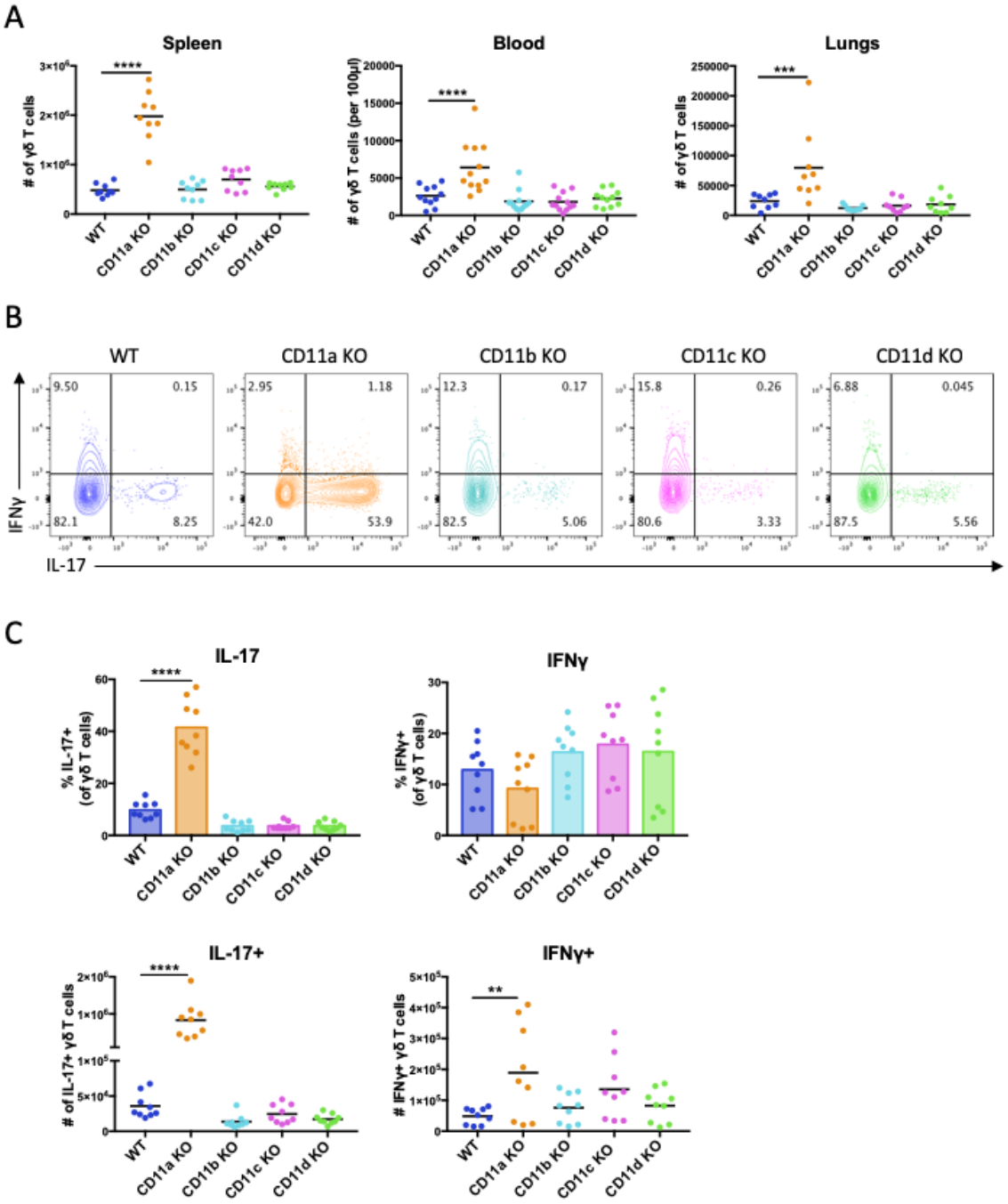

Figure 4-11 Increased number of IL-17-producing γδ T cells in CD18 KO mice is due to the loss of CD11a.
Cells were isolated from the spleen, blood and lungs of WT, CD11a KO, CD11b KO, CD11c KO and CD11d KO mice then analysed by flow cytometry. **(A)** Absolute numbers of γδ T cells. Levels of IL-17 and IFNγ from γδ T cells in the spleen were measured following *ex vivo* stimulation with PMA/ionomycin for 4h, representative plots **(B)**, percentages and absolute numbers **(C)**. n=9, 3 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3.4 The effect of location and subset on β_2 integrin and ICAM-1 expression on γδ T cells

After discovering the loss of CD11a expression results in increased γδ T cells, presumably Vγ6$^+$ cells as found in CD18 KO mice. I next assessed β_2 integrin expression on different subsets of γδ T cells in WT mice. The hypothesis was that CD11a expression may be higher on Vγ6$^+$ γδ T cells, suggesting this integrin may be of more functional importance than on the other subsets. The expression of CD11a, CD11b and CD11c was examined in the spleen, lungs and uterus (tissues where an increase in γδ T cell numbers was found) and in the pLN (site with no difference in γδ T cell numbers). CD11d expression could not be measured as there are currently no murine antibodies available.

Over 90% of γδ T cells expressed CD11a in all of the tissues examined (Figure 4-12A). In the spleen and pLN, all subsets expressed equivalent levels of CD11a. Conversely, in the lungs and uterus, Vγ6$^+$ γδ T cells expressed higher levels of CD11a than the Vγ1$^+$ and Vγ4$^+$ subsets. When comparing the mean expression of CD11a on Vγ6 γδ T cells across tissues there was a significant increase in both the lungs and uterus compared to spleen and pLN, as well as in the uterus compared to the lungs (Figure 4-12B).

In most tissues γδ T cells expressed very low levels of CD11b and CD11c, with the exception of the pLN where all subsets showed some expression of CD11b (Figure 4-12A&B). The majority of γδ T cells in the pLN were CD11b negative, but a small population of cells were CD11b$^+$. This population was greater in Vγ4$^+$ and Vγ6$^+$ than Vγ1$^+$ γδ T cells. The expression of CD11b was significantly higher on Vγ4$^+$ and Vγ6$^+$ cells in the pLN compared to spleen and lungs. The levels of CD11c were significantly increased on all subsets of γδ T cells from the spleen compared to pLN and lungs, although the percentage of CD11c$^+$ cells was less than 5. The functional relevance of low level CD11b and CD11c expression by γδ T cells in the pLN remains unknown.

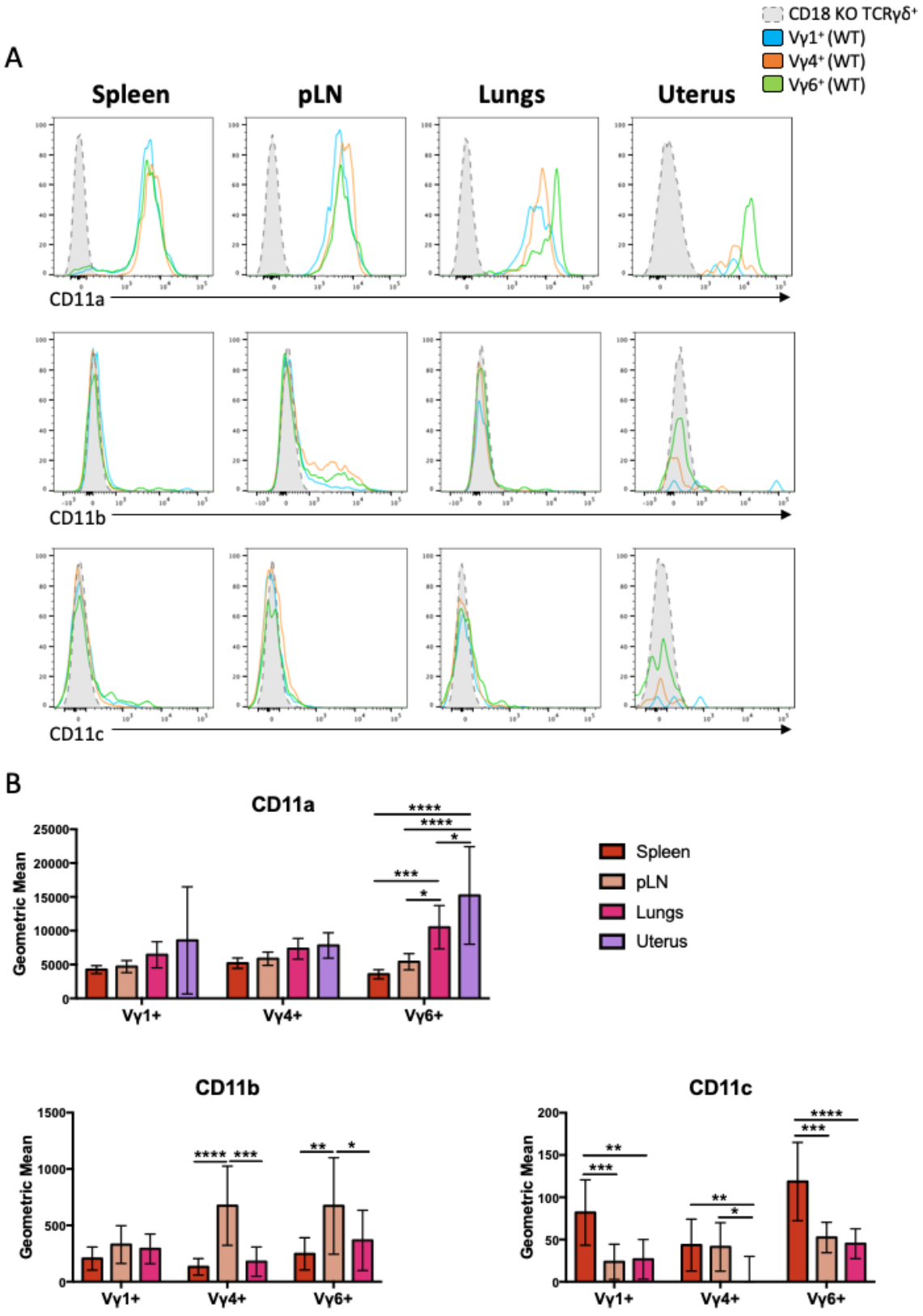

Figure 4-12 CD11a is expressed higher on Vγ1$^-$Vγ4$^-$ γδ T cells in the lungs and uterus. Expression of CD11a, CD11b and CD11c on γδ T cell subsets was measured by flow cytometry. Cells were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, or Vγ1$^-$4$^-$ (Vγ6$^+$). **(A)** Representative plots shown in. Grey with dotted line represents expression on CD18 KO γδ T cells. **(B)** Geometric mean of integrin expression. n=8, 3 independent experiments. Data represents mean ± SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

CD11a was expressed higher on Vγ6$^+$ γδ T cells that reside in the lungs and uterus, and loss of CD11a leads to Vγ6 expansion. The main ligand for CD11a is ICAM-1 (CD54), which is expressed on endothelial cells and leukocytes. Therefore, it is likely that the absence of interaction between CD11a and ICAM-1 is involved in mediating the expansion of γδ T cells in CD18 KO mice. It has been shown that γδ T cells in the spleen express ICAM-1[348], however comparison of ICAM-1 expression across tissues and subsets has not been done previously.

ICAM-1 expression was initially assessed on total γδ T cells from different tissues in WT mice (Figure 4-13A). Expression of ICAM-1 was readily detectable on γδ T cells in all tissues examined but expression was significantly lower on γδ T cells in the lungs compared to the spleen, pLN and uterus.

Expression of ICAM-1 was then analysed on γδ T cell subsets from WT and KO mice. In the spleen, where in WT mice Vγ1 and Vγ4 subsets predominate but in CD18 KO mice there is a large proportion of Vγ6$^+$ cells, there was a significant increase in Vγ1$^+$ and Vγ4$^+$ γδ T cell expression of ICAM-1 in CD18 KO compared to WT (Figure 4-13B). In the pLN, a site in which γδ T cells were not expanded in CD18 KO mice, there was an increased percentage of ICAM-1$^+$ Vγ4 and Vγ6 γδ T cells. The lungs and uterus are tissues in which Vγ6$^+$ cells preferentially localise and sites of expanded γδ T cell numbers in the absence of CD18. Vγ6$^+$ γδ T cells showed reduced expression of ICAM-1 in CD18 KO lungs compared to WT, whereas in the uterus there was no difference. Conversely for Vγ4$^+$ cells, there was an increase in ICAM-1 expression in the uterus but not lungs of CD18 KO mice.

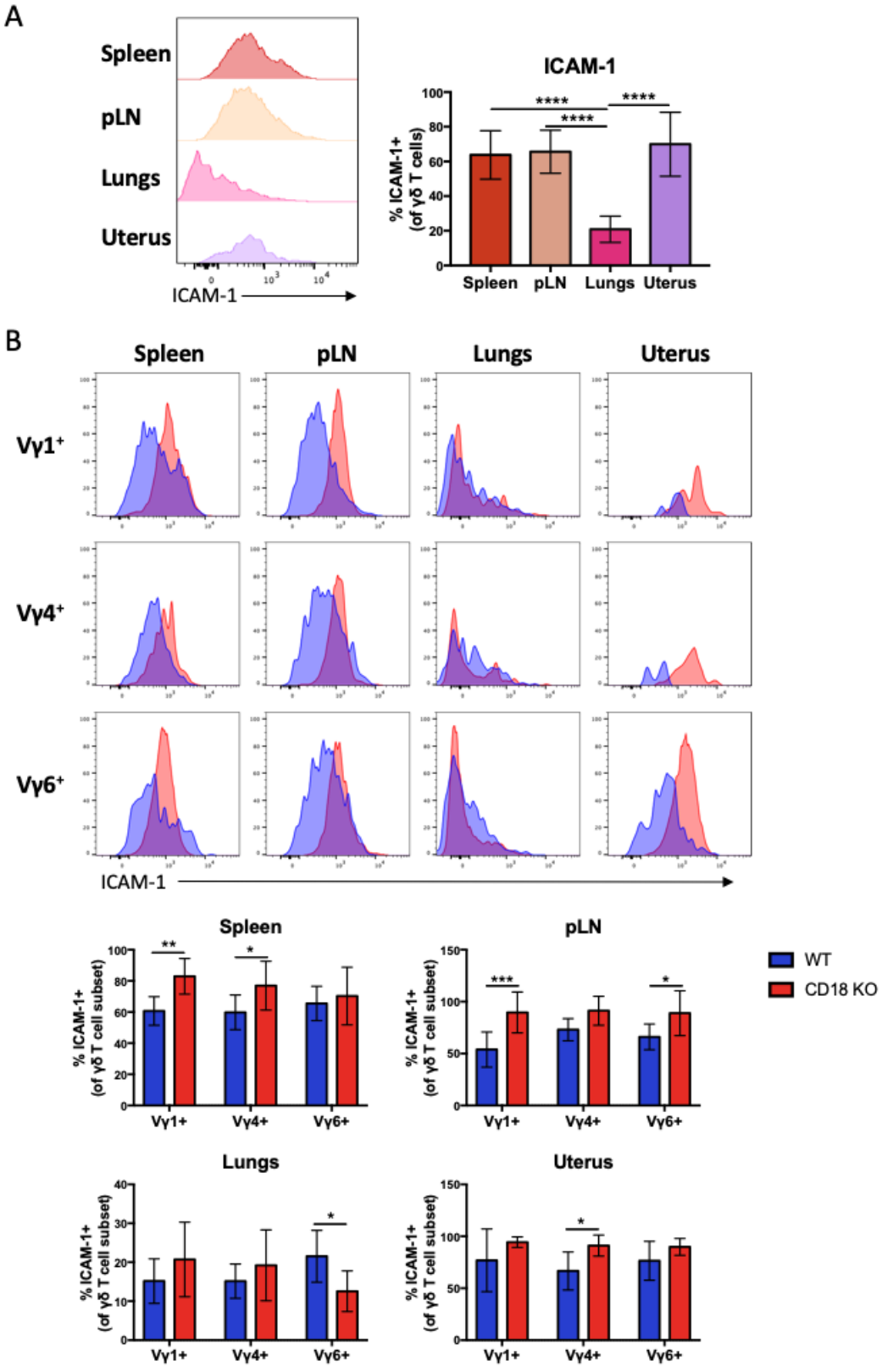

Figure 4-13 ICAM-1 expression is low on γδ T cells in the lungs and is expressed higher on γδ T cells from CD18 KO than WT spleen and LNs.
Levels of ICAM-1 on γδ T cells from WT and CD18 KO mice were analysed by flow cytometry. Cells were gated on $CD3^+TCR\gamma\delta^+$ then $V\gamma1^+$, $V\gamma4^+$, or $V\gamma1^-4^-$ ($V\gamma6^+$). **(A)** Expression of ICAM-1 on total γδ T cells from WT tissues. **(B)** Representative plots and percentages of $ICAM\text{-}1^+$ γδ T cell subsets from WT (Blue) and CD18 KO (red) mice. n=8, 3 independent experiments. Data represents mean ± SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The loss of CD11a expression, but not any of the other three β_2 integrin family members, is responsible for the increased number of γδ T cells identified in CD18 KO mice. Detailed analysis of β_2 integrin and ligand expression on γδ T cells across tissues identified that all γδ T cells in the tissues analysed express CD11a, but expression is higher on Vγ6$^+$ cells in the lungs and uterus than on other subsets. Together this data suggests that CD11a is important in the regulation of Vγ6$^+$ γδ T cells and that it's expression on Vγ6$^+$ cells may play a central role in their function within specific tissues.

4.4 Discussion

4.4.1 Tissue-restricted increase in Vγ6Vδ1$^+$ IL-17-producing γδ T cells in CD18 KO mice

In this chapter I have identified that in mice lacking β_2 integrins there is an expansion of γδ T cells specifically in the spleen, blood, lungs and uterus. It was previously reported that these mice have more γδ T cells in the spleen and cLNs but no difference in pLN (axillary and inguinal) [297,303]. This is in agreement with my findings that CD18 KO mice have increased γδ T cells in the spleen but not pLN. However, prior studies did not examine γδ T cells in other tissues, therefore this is the first data to identify an increase in γδ T cells in the lungs and uterus of CD18 KO mice. Another novel finding is that the expanded γδ T cells are Vγ6Vδ1$^+$, as previous studies did not investigate the subset of expanded γδ T cells in the absence of CD18. In a different mouse model where CD18 expression is reduced but not absent, mice develop psoriasis as they age, and increased numbers of γδ T cells were found in the psoriatic lesions of the skin and skin-draining lymph nodes [296]. The mice used in that study only develops spontaneous skin inflammation when the CD18-deficiency was on a PL/J background, whereas on a C57BL/6 background the mice are resistant[344]. The CD18 KO strain used in this thesis was on a C57BL/6 background, therefore it is unsurprising that the mice do not show any signs of skin inflammation. My results suggest that the enhanced γδ response in the absence of β_2 integrins is occurs in multiple, but restricted tissue sites.

One possible explanation for the increased number of γδ T cells in a restricted number of sites in CD18 KO mice is that there is a re-distribution due to migration into certain tissues being impaired. Calculation of the total γδ T cells across tissues found an overall 6.5 times increase in the number of γδ T cells in CD18 KO mice, with numbers increased in several tissues yet no sites with a defect in γδ T cells. It could be inferred that the increase in γδ T cell numbers is not due to a redistribution effect, but as not all tissues were examined there may be other sites with decreased γδ T cell numbers (e.g. peritoneum or liver). These data also suggest that migration of γδ T cells into tissues under homeostasis is not dependent on β_2 integrins. Although migration was not directly tested, this is in agreement with studies showing that in inflammatory disease models of EAE and psoriasis, γδ T cells can migrate effectively to tissue sites (CNS and skin)[295,296]. Further work is required to determine

whether β_2 integrins play any role in the migration of γδ T cells, but current evidence does not support their role in migrating into tissues.

An increase in both the percentage and number of γδ T cells was found in the spleen, blood, lungs and uterus of CD18 KO mice. In other lymphoid tissues (Peyer's patches, pLN, mLN, bone marrow) there was a higher percentage of γδ T cells as a proportion of total $CD3^+$ cells, but not in absolute number of γδ T cells – this was due to a reduced number of αβ T cells. This finding is expected, as αβ T cell migration is dependent on β_2 integrins hence CD18-deficient αβ T cells display reduced homing to lymphoid tissues, including pLN, mLN, Peyer's patches and bone marrow[64,68,186,297]. Interestingly there was also a decreased number of αβ T cells in the colon of CD18 KO mice. Leukocyte migration to the intestines is largely dependent on the integrin $\alpha_4\beta_7$[69], however this data suggests there may be a role for β_2 integrins in migration of αβ T cells to the colon. There was some discrepancy with the data obtained from the small intestine LP. Analysis of total γδ T cell numbers displayed no difference, however the number of $V\gamma1^-V\gamma4^-V\gamma5^-$ cells were significantly increased in this tissue. When quantified by imaging, there was no significant difference in the number of γδ T cells in the small intestine (data not shown). The predominant subset of γδ T cells found in the intestine express Vγ7[202,349], therefore it is likely that a large proportion of the cells within the $V\gamma1^-V\gamma4^-V\gamma5^-$ population will express Vγ7. This variability in the data may indicate subtle changes in the γδ T cell populations in the intestine, however, as the effects in this tissue were unclear compared to other tissues it was decided not to pursue this further.

Surface marker expression analysis revealed that β_2 integrin-deficient γδ T cells exhibited an altered profile consistent with a $V\gamma6^+$ subset phenotype. $V\gamma6^+$ γδ T cells are known to be $CD62L^{lo}$ $CD44^{hi}$, produce IL-17 and express high levels of CD3/γδ TCR but do not express CD27[199,206,346,350,351]. The $V\gamma6V\delta1^+$ phenotype of the expanded CD18 KO γδ T cells was then confirmed by PCR. In addition to the data from total γδ T cells, those expressing Vγ6 showed an increased production of IL-17A in CD18 KO compared to their WT counterparts. This suggests that the altered phenotype seen in the total γδ population in CD18 KO mice is not only due to there being an increased number of $V\gamma6^+$ cells, but also due to alterations in cytokine and surface marker expression on this population of γδ T cells.

In CD18 KO mice the levels of IL-17 were increased *in vivo* in the serum and spleen. Analysis of the total IL-17$^+$ cells showed that γδ T cells were the predominant source of IL-17 in CD18 KO mice following *in vitro* re-stimulation. In contrast to this, in WT mice the main population producing IL-17 in the spleen and lungs were non-T cells. A likely CD3$^-$ source of IL-17 are innate lymphoid cells (ILCs). This group of cells includes lymphoid-tissue transducer-like cells, natural killer cells, ILC2s and ILC3s, that are all known to produce IL-17[352–354]. In addition, the WT uterus also had a significant population of CD45$^-$IL-17$^+$ cells. This is in contrast to other papers that describe γδ T cells as the major IL-17-producing population in the uterus[355,356], but in those analyses only CD45$^+$ cells were examined. The CD45$^-$IL-17$^+$ cells are likely to be epithelial and stromal cells, which have previously been shown to express IL-17 in the mouse uterus[357].

Despite an increased number of IL-17-producing γδ T cells in the lungs and uterus, no difference was detected in the cytokine levels from these tissues. This could indicate that the Vγ6$^+$ cells have an increased potential to produce IL-17 but are not producing it in the lungs or uterus *in vivo*. This may be due to the absence of the appropriate stimuli required to induce IL-17 secretion, such as IL-1β and IL-23[200] that can be produced by activated macrophages and DCs[358–361]. Therefore, in an inflammatory setting when APCs come activated, Vγ6$^+$ cells from CD18 KO mice may produce higher levels of IL-17. This also highlights that the IL-17 data obtained from PMA/ionomycin stimulation may not reflect cytokine production *in vivo,* therefore cells other than γδ T cells may be contributing to the IL-17 measured from the serum. The levels of another isoform of IL-17, IL-17F, were also measured but could only be detected at very low levels (<5% of γδ T cells). Using an IL-17F reporter mouse it was shown that 12% of γδ T cells in the spleen express this cytokine[362]. This discrepancy indicates that IL-17F staining may have been suboptimal or that stimulation with reagents other than PMA/ionomycin may be required to induce IL-17F secretion.

Vγ6-expressing γδ T cells preferentially localise to the lungs, uterus, tongue, dermis and peritoneal cavity[198–200]. As the expanded population of γδ T cells identified in CD18 KO mice were Vγ6$^+$, it was unsurprising that the lungs and uterus were sites with higher γδ T cell numbers. However, my data show that γδ T cell numbers were not increased in the skin or oral mucosa. For skin analysis the whole ear was digested, without separating the epidermis and dermis, with over 90% of the γδ T

cells identified expressing Vγ5. This subset is found exclusively in the epidermis[363], suggesting that cells from the dermis only represent a small fraction of those analysed and therefore any subtle changes to γδ T cells in the dermis may be lost. In the gingiva of adult mice Vγ6$^+$ cells represent only 14% of the total γδ T cell population[364], meaning the effect may be much less in this tissue or the phenotype in CD18 KO mice is truly specific to the lungs, uterus and spleen. The spleen is not a site where Vγ6$^+$ cells are described as preferentially homing to, although Vγ6-expressing γδ T cells have been shown to reside in the spleen[199,346,351]. This raises the question of why there is a higher number of Vγ6$^+$ γδ T cells in the spleen? A possible explanation is that the increasing number of Vγ6$^+$ cells exceeds the capacity within their resident tissues (lungs and uterus), so the γδ T cells are forced into the circulation and accumulate in the spleen. The enhanced number of γδ T cells in the blood fits with this theory. However further experiments to determine which tissues γδ T cells migrate into, such as adoptive transfer of Vγ6$^+$ γδ T cells into WT and CD18 KO hosts, are required to clarify this.

In a model of reduced CD18 expression, mice developed a spontaneous psoriasis associated with an increase in IL-17-producing γδ T cells[296]. In this model the expanded γδ population expressed Vγ4, not Vγ6. The fact that a reduction in CD18 expression is associated with increased Vγ4$^+$ cells, whereas total loss of CD18 results in Vγ6$^+$ cell expansion suggests that β_2 integrin regulation of γδ T cells is a finely tuned process. Alternatively, this may indicate a different mechanism during inflammation compared to homeostasis. Under homeostatic conditions, β_2 integrins may play an important role in the regulation of Vγ6$^+$ γδ T cells but during an inflammatory response, other stimuli may induce the specific expansion of Vγ4$^+$ γδ T cells. It should also be considered that the mice with reduced CD18 expression are on a different background (PL/J compared to C57BL/6) that renders them susceptible to the development of psoriasis[344,365]. It was suggested there is a single recessive allele in the PL/J mice that is responsible for disease susceptibility. The discrepancy between β_2 integrin loss causing expansion of Vγ4$^+$ versus Vγ6$^+$ γδ T cells could therefore be due to alterations in other genes that differ between mouse strains.

4.4.2 β_2 integrin expression depends on TCR expression and tissue localisation of γδ T cells

The expansion of IL-17-producing γδ T cells is due to the specific loss of CD11a. This β_2 integrin is expressed highly on T cells, both αβ and γδ, whereas other leukocyte populations express other β_2 integrins in combination with CD11a. I therefore hypothesise that the increase in Vγ6$^+$ γδ T cells is due to CD11a loss on T cells. This could be mediated in two different ways: either the direct loss of CD11a on γδ T cells themselves or through an indirect mechanism whereby loss of CD11a on αβ T cells affects other cell populations, that in turn drive the expansion of γδ T cells. The finding that CD11a is expressed at higher levels on Vγ6$^+$ γδ T cells in the lungs and uterus supports the idea that Vγ6$^+$ cells are more dependent on CD11a and that the effect may be intrinsic to γδ T cells.

In addition to CD11a expression, the CD11b subunit was also present on γδ T cells isolated from the pLN. This contrasts a previous report that γδ T cells in the spleen express low levels of CD11b whereas expression is absent in lymph node γδ T cells[26]. Another study found low level expression of both CD11b and CD11c on splenic γδ T cells [294]. Both of these studies quantified integrin expression by RNA-sequencing, therefore there may be discrepancies between expression at the RNA compared to protein level. It is clear that overall γδ T cell expression of CD11b and CD11c is very low or negligible. Therefore, the functional relevance of the expression of these integrin subunits in γδ T cells remains unclear.

I also characterised expression of ICAM-1, the main ligand for CD11a, on γδ T cells. More than half of the γδ T cells in the spleen, pLN and uterus expressed ICAM-1, whereas expression of ICAM-1 was much lower on γδ T cells isolated from the lungs. There was an increase in the percentage of γδ T cells expressing ICAM-1 in CD18 KO mice compared to WT. Although this effect was not limited to the Vγ6 subset but was found across Vγ1$^+$ and Vγ4$^+$ cells as well. This could indicate there is a regulatory loop between β_2 integrin expression (receptor) and ICAM-1 (ligand), whereby engagement of the receptor causes downregulation of its ligand. However, to my knowledge this has not been reported in the literature. The functional role of ICAM-1 expressed on γδ T cells has not previously been studied. In αβ T cells it has been shown that stimulation of ICAM-1 can promote T cell activation and cytokine production[366,367]. On CD8 T cells a correlation was found between ICAM-1

expression and cytotoxic activity[368]. ICAM-1 may play a similar role in mediating cell interactions, contributing to cytokine production and cytotoxic function in γδ T cells, however further work is required to clarify this. The β_2 integrin ligand ICAM-1 is expressed not only on leukocytes but also on endothelial, epithelial and stromal cells. In the lungs ICAM-1 is constitutively expressed on alveolar endothelial and epithelial cells[369,370]. In the uterus ICAM-1 expression is found on vascular, epithelial and stromal cells[371]. The levels of ICAM-1 on stromal cells were not assessed in WT and CD18 KO mice, but this may play a role in retaining γδ T cells in tissues or in regulating their function.

4.5 Conclusion

In this chapter I have provided a comprehensive analysis of γδ T cells in β_2 integrin-deficient mice. I have identified the specific expansion of γδ T cells in the spleen, blood, lungs and uterus of CD18 KO mice. These expanded γδ T cells are Vγ6Vδ1$^+$ and express high levels of CD44, IL-17, RORγt and CD3/γδ TCR. This expands the knowledge from previous studies of γδ T cells in CD18 KO mice, as two additional sites (lungs and uterus) as well as the subset (Vγ6$^+$) of expanded γδ T cells has been identified. In addition to this, analysis of γδ T cells from individual α-chain KO mice characterised that CD11a was the β_2 integrin family member responsible for this increase in γδ T cells. Expression of CD11a was higher on Vγ6$^+$ cells only in the lungs and uterus, potentially indicating an important functional role specific to the subset and tissue residency of the γδ T cells. Having characterised the specific subset of γδ T cells increased in CD18 KO mice, in the next chapter I will go on to investigate the potential mechanisms underlying their expansion.

Chapter 5 Defining the mechanism of γδ T cell increase in β_2 integrin-deficient mice

5.1 Introduction

I have identified that the loss of CD18 expression in mice leads to a tissue-restricted increase in Vγ6$^+$ IL-17 producing γδ T cells. The next step was to determine the underlying mechanism(s) of this expansion. I propose 3 possible mechanisms that could lead to the expansion of the Vγ6$^+$ subset in CD18 KO mice: (i) an increase in thymic output, (ii) enhanced proliferation in the periphery, or (iii) increased survival/reduced cell death. It should be noted however, that these scenarios are not mutually exclusive. It may be one or a combination of these proposed mechanisms that are responsible for the expanded Vγ6$^+$ population in mice lacking β_2 integrins.

5.1.1 Thymic output of γδ T cells

The initial thymic development of γδ T cells occurs primarily during embryonic/neonatal life. IL-17-producing γδ T cells in particular develop exclusively in the embryonic thymus and are then retained in the periphery through self-renewal[243]. Therefore, the loss of β_2 integrin-ligand interactions in CD18 KO mice may either alter the initial γδ T cell thymic development or their regulation in the periphery. Several studies have identified CD11a as an important factor for normal T cell development. CD11a expression was found to be critical for the development of common lymphoid progenitors in the bone marrow [89] and blocking CD11a in foetal thymic organ cultures (FTOCs) reduced the development of CD4$^+$CD8$^+$ T cells, whereas blocking ICAM-1 increased γδ T cell numbers[372]. Conversely, another study using CD11a KO mice described normal thymic T cell development [373] and in other FTOC experiments, blocking CD11a impaired T cell development from foetal liver progenitors but not foetal thymus progenitor cells[374]. Based on these studies it is unclear whether CD11a is required for normal T cell development, and importantly the impact of CD11a on γδ T cell subset development during different foetal stages was not examined. In adult CD18 KO mice, I found no difference in the number of thymic γδ T cells (Figure 4-3) but as Vγ6$^+$ cells are produced in the embryonic thymus, there may be an increased production in mice at this age.

5.1.2 Mechanisms of γδ T cell proliferation in the periphery

The second potential mechanism of Vγ6 expansion in CD18 KO mice is enhanced proliferation in the periphery. There are several cytokines that are known to induce proliferation of IL-17$^+$ γδ T cells including IL-7, IL-1β, IL-23 and TGF-β1, [235,303,375,376]. IL-7 can stimulate the proliferation of IL-17-producing γδ T cells [235], and enhanced expression of IL-7 on thymic stromal cells was shown to expand Vγ6$^+$ γδ T cells in the thymus of mice at E16.5 and at birth[377]. The production of IL-1β and IL-23 by myeloid cells can also stimulate the expansion of IL-17-producing γδ T cells[376]. This has led to the development of an *in vitro* protocol to expand IL-17-producing γδ T cells using a combination of IL-1β and IL-23[213]. It has been suggested that IL-1β is more important for inducing IL-17 production by Vγ4$^+$ compared to Vγ6$^+$ γδ T cells [200]. In agreement with this, Vγ6$^+$ cells were induced during pneumococcal infection in mice, but the number of these cells was significantly reduced when IL-23 KO mice were infected compared to WT or IL-1R KO mice [378]. This data indicates that IL-23 may preferentially induce Vγ6$^+$ over Vγ4$^+$ γδ T cells. TGF-β1 is required during thymic development of γδ T cells; in mice that lack TGF-β1 itself or the signalling adapter Smad3, there is a loss of IL-17$^+$ γδ T cells [375]. These studies highlight potential cytokines that may be elevated in the serum and/or tissues of CD18 KO mice and induce the expansion of Vγ6$^+$ γδ T cells. Some of these cytokines will be investigated in this chapter.

5.1.3 γδ T cell survival and apoptosis

The final proposed mechanism is that the increased number of Vγ6$^+$ cells in CD18 KO mice could be due to an accumulation resulting from increased survival and/or decreased apoptosis. In addition to driving proliferation, IL-7 has also been implicated in γδ T cell survival. There was a greater loss of BrdU-labelled γδ T cells in IL-7 KO mice compared to heterozygous controls 14 days post-BrdU administration, indicating that IL-7 is important for γδ T cell survival[246]. Another study suggested that IL-7 is required to induce proliferation but not survival of CD27$^-$ IL-17$^+$ γδ T cells. Following IL-7 stimulation *in vitro,* CD27$^-$ γδ T cells proliferated to a greater extent than their CD27$^+$ counterparts but expressed lower levels of *Bcl2*, a gene associated with cell survival [235]. However, *in vivo* administration of IL-7 was shown to upregulate expression of Bcl2 in IL-17$^+$ γδ T cells but not IFNγ$^+$ γδ T cells[247]. It is unclear the precise role that IL-7 plays in mediating the proliferation

versus survival of γδ T cells. This is a complex pathway that involves the coordination of many intracellular signalling molecules in addition to Bcl2. There may be a role for IL-7-mediated survival of Vγ6$^+$ γδ T cells in CD18 KO mice and interestingly this pathway is also linked to inhibition of apoptosis through up-regulation of anti-apoptotic proteins such as Bcl-xl and Mcl-1 [379].

Previous studies have demonstrated that β_2 integrins may be involved in mediating γδ T cell apoptosis: the co-stimulation of γδ T cells *in vitro* through the TCR and CD11a resulted in calcium-dependent apoptosis, however, this effect was lost when either receptor was activated alone [380,381]. This suggests that the engagement of both TCR and CD11a can trigger cell death in γδ T cells therefore, it is possible that in mice lacking CD11a/CD18 that a failure of apoptosis may occur.

5.1.4 Hypothesis and Aims

The overall aim of this chapter was to determine the mechanism of γδ T cell expansion/accumulation that occurs in CD18 KO mice. This was further divided into three main questions:

1. Is there an increase in the thymic development of Vγ6$^+$ γδ T cells in CD18 KO mice?

2. Are Vγ6$^+$ γδ T cells expanded in CD18 KO mice due to increased proliferation in the periphery?

3. Is there an accumulation of Vγ6$^+$ γδ T cells in CD18 KO mice due to an increase in survival and/or reduction in cell death?

5.2 Results

5.2.1 Thymic development of Vγ6$^+$ γδ T cells is unaffected by the loss of CD18, whilst Vγ4$^+$ development is impaired.

The first hypothesis for the increase in γδ T cells was that the thymic output of Vγ6$^+$ cells may be increased in CD18 KO mice compared to WT. The development of IL-17-producing γδ T cells occurs prior to birth[243] and for the Vγ6 subset is predominantly at embryonic days 16-18 (E16-18)[222]. To assess thymic development of Vγ6$^+$ γδ T cells, the populations of γδ T cells present in the thymus of WT and CD18 KO mice were analysed by flow cytometry at different ages: E18.5, 1 day old, 3 days old and 4 weeks old.

The total number of γδ T cells within the thymus was similar across ages and showed no difference between WT and CD18 KO (Figure 5-1A). Further analysis of γδ T cell subsets based on Vγ chain expression revealed that at E18.5 the WT thymus contained a population of γδ T cells that expressed either Vγ1, Vγ4, Vγ5 or Vγ6 (Figure 5-1B). The percentage of Vγ6$^+$ γδ T cells in WT mice was similar in E18.5, 1 day and 3 day old mice, then reduced in 4 week old mice. In the CD18 KO thymus there was an increased proportion of Vγ6$^+$ cells compared to WT, accompanied by a decrease in Vγ4$^+$, at E18.5, 1 day and 3 days but not in 4 week old mice. This could suggest a bias in γδ T cell development towards Vγ6 instead of Vγ4.

To determine if this was the case, the absolute numbers of Vγ subsets were calculated (Figure 5-1C). Surprisingly, there was no difference in the number of Vγ6$^+$ γδ T cells in the thymus at any age between WT and CD18 KO mice. However, the number of Vγ4$^+$ cells was significantly reduced in the neonatal but not in 4 week old mice.

Together, the data reveal that CD18 KO mice displayed no difference in the number of thymic Vγ6$^+$ γδ T cells in young mice. This suggests that the increased number of Vγ6$^+$ γδ T cells in CD18 KO mice is not due to enhanced thymic development. Unexpectedly however, the development of the other IL-17-producing subset, that express Vγ4, was impaired. This highlights that CD18 may be important for the development of Vγ4$^+$ γδ T cells.

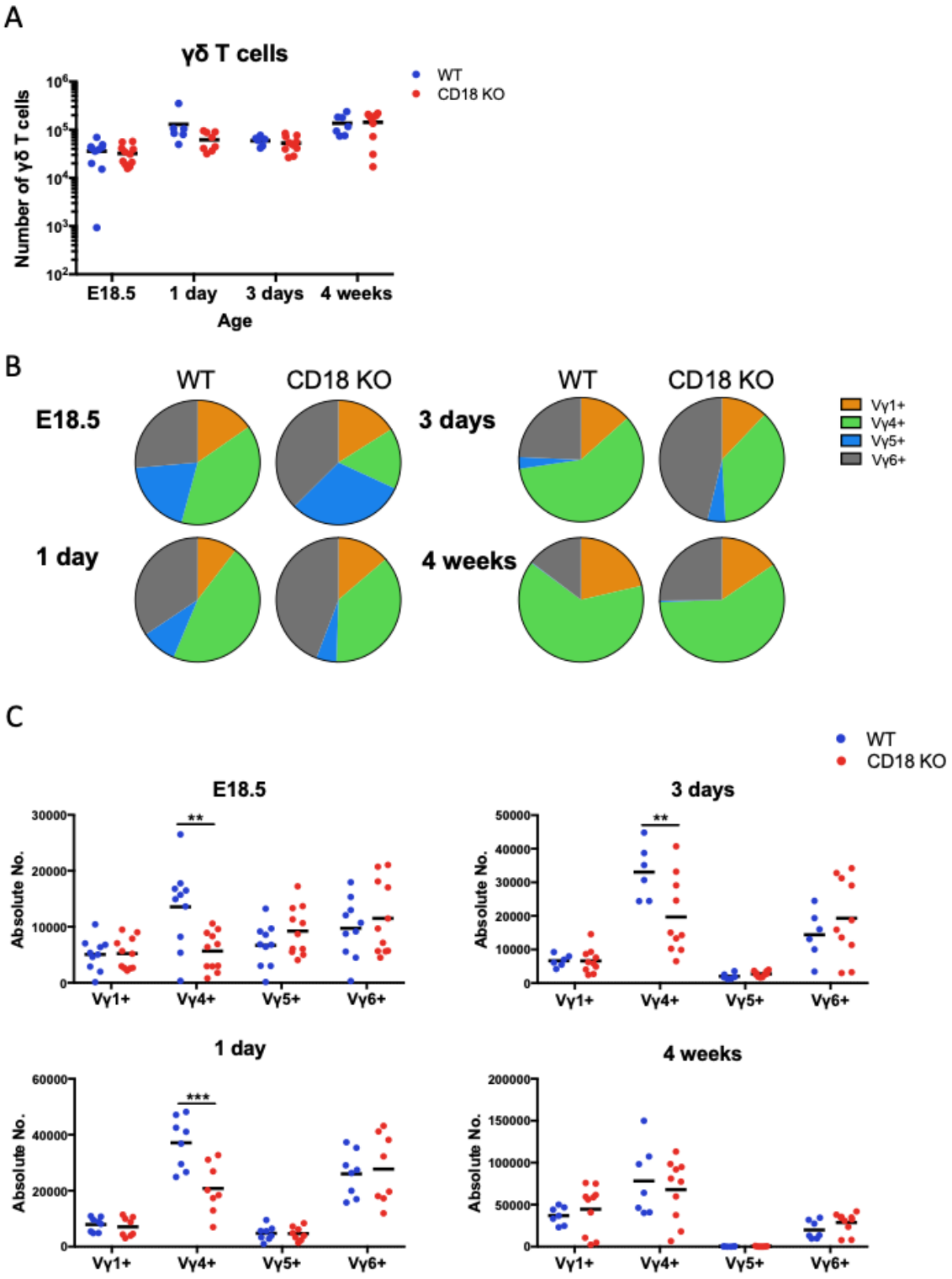

Figure 5-1 CD18 KO mice show no difference in Vγ6$^+$ thymic development but have impaired development of Vγ4$^+$ cells.
WT and CD18 KO mice were culled at E18.5, 1 day old, 3 days old or 4 weeks old and thymic cells were analysed by flow cytometry. Samples were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$4$^-$5$^-$ (i.e. Vγ6$^+$). **(A)** Total number of γδ T cells. **(B)** Representative pie charts of the percentage of γδ T cells expressing each Vγ chain. **(C)** Absolute number of each Vγ subset. WT 3 days n=6, WT 4 weeks n=7, WT & CD18 KO 1-day n=8, WT E18.5 & CD18 KO 3 days and 4 weeks n=10, CD18 KO E18.5 n=11. Data from a minimum of 3 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To further investigate the defect in Vγ4$^+$ cell development identified in the thymus of neonatal CD18 KO mice, the stages of thymic γδ T cell development were analysed based on maturation marker expression. During thymic development γδ T cells go through different stages of maturation. The surface marker CD24 is commonly used to differentiate between immature (CD24$^+$) and mature (CD24$^-$) populations because CD24 is downregulated on γδ T cells prior to exit from the thymus [382–384].

Within the immature population of thymic γδ T cells there was a significant decrease in the number of Vγ4$^+$ cells in CD18 KO mice compared to WT at E18.5 (Figure 5-2A). This was specific to the Vγ4$^+$ cells as the numbers of all other γδ T cell subsets were equivalent. In 1 day old mice there was a trend towards a decreased number of CD24$^+$ Vγ4$^+$ cells in the CD18 KO thymus, however, this was not significant. By 3 days old and 4 weeks old, the immature Vγ4$^+$ population was similar in number in WT and CD18 KO mice. This indicates that during embryonic development in CD18 KO mice there is a defect in the generation of immature Vγ4$^+$ γδ T cells.

During thymic development γδ T cells express different levels of the cell surface markers CD27, CD44 and CD45RB. CD27 is expressed on the earliest γδ-committed T cell progenitors and is downregulated upon maturation of IL-17-producing subsets [206,383]. CD44 and CD45RB are not expressed during early γδ T cell development but are upregulated on terminally differentiated γδ T cells [203]. Both IFNγ and IL-17-producers will express CD44, whereas CD45RB expression is only expressed by IFNγ-producers. Analysis of these surface markers showed a decreased percentage of CD44$^+$ and concurrent increase in CD44$^-$ immature embryonic Vγ4$^+$ cells from CD18 KO mice compared to WT (Figure 5-2B). Most of these cells (>90%) expressed CD27 and lacked CD45RB in both WT and CD18 KO, indicative of their expected immature phenotype. Conversely, the immature Vγ6$^+$ population in the CD18 KO thymus displayed a small but significant increase in the percentage of CD27$^-$ cells, potentially indicating a more mature phenotype. Although in contrast to this, there was also a greater proportion of CD44$^-$ Vγ6$^+$ cells, which may suggest a more immature phenotype. As the differences were not found in percentages of CD27$^+$ and CD44$^+$ Vγ6$^+$ cells, it is unclear whether the maturation of these cells is truly different in the CD18 KO thymus.

Together, examination of the immature CD24$^+$ γδ T cell populations in the thymus of WT and CD18 KO mice at E18.5 demonstrated a differential maturation status of the Vγ4-expressing γδ T cell subset. There was a reduced number of developing Vγ4$^+$ cells, and they displayed a more immature phenotype (based on CD44 expression). In contrast to this, the downregulation of CD27 and CD44 in Vγ6$^+$ cells from CD18 KO mice does not indicate a clear change in maturation status.

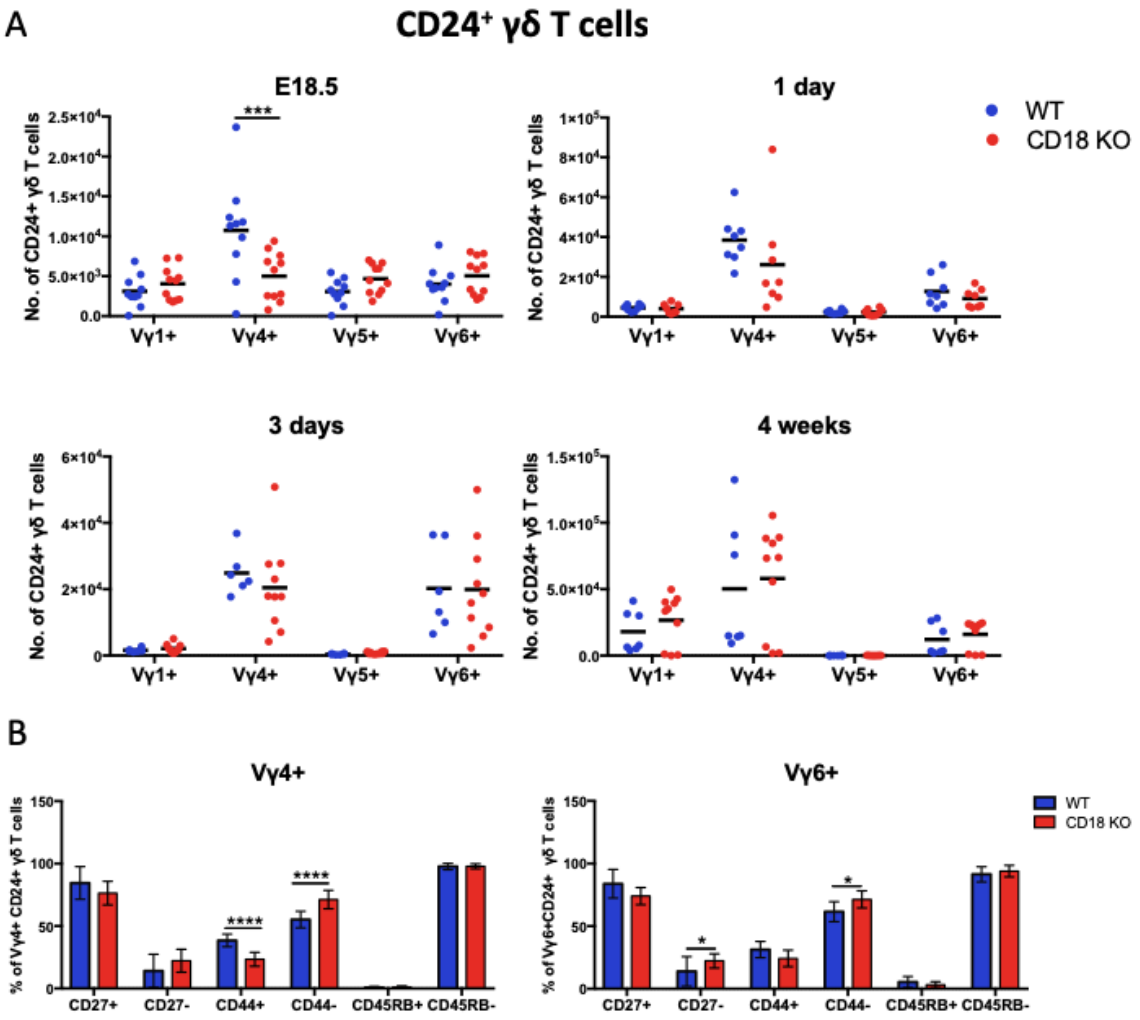

Figure 5-2 The embryonic CD18 KO thymus has a reduced number of Vγ4$^+$ γδ T cells with an immature phenotype.
Cells isolated from WT and CD18 KO thymus at E18.5 were analysed by flow cytometry. Cells were first gated on CD3$^+$TCRγδ$^+$CD24$^+$, then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$4$^-$5$^-$, followed by CD27, CD44 or CD45RB. **(A)** Percentage and number of CD24+ γδ T cells according to Vγ chain expression. **(B)** Percentage of Vγ4$^+$ or Vγ1$^-$4$^-$5$^-$ γδ T cells from E18.5 thymus that are positive or negative for CD27, CD44 and CD45RB. WT n=10, CD18 KO n=11. Data from 6 independent experiments. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Following the identification of reduced numbers and maturation of Vγ4$^+$ cells within the immature γδ population in CD18 KO mice, the mature (CD24$^-$) cells were then analysed. Due to the defect in Vγ4$^+$ cells occurring at the E18.5 immature stage of development, a subsequent reduction in mature Vγ4$^+$ cells was expected. The numbers of γδ T cell subsets within the CD24$^-$ thymic population were similar between WT and CD18 KO mice at the embryonic stage and immediately post-birth (Figure 5-3). However, by 3 days old the CD18 KO mice displayed a significantly reduced number of mature CD24$^-$ Vγ4$^+$ cells compared to their WT counterparts. This reduction was maintained into adulthood, as mice at 4 weeks old also showed a decrease in CD24$^-$ Vγ4$^+$ γδ T cells.

Within the mature population of γδ T cells, different stages of γδ T cell development can be further identified. A recent study by Sumaria *et al.* described a gating strategy to analyse the mature γδ T cells committed to either IFNγ or IL-17 production [203]. They showed that after gating on CD3$^+$TCRγδ$^+$CD24$^-$ cells, the expression of CD45RB and CD44 can segregate the cells into 4 discrete populations (Figure 5-4A). CD45RB$^-$CD44$^-$ cells have the potential to develop into either IL-17 or IFNγ producers. CD45RB$^+$CD44$^-$ are a population of developing IFNγ-producers that will then upregulate CD44 to become CD45RB$^+$CD44$^+$, a more terminally differentiated population. Whereas, in the IL-17 development pathway, the cells will upregulate CD44 to become CD45RB$^-$CD44$^+$.

Analysis of these 4 populations in the thymus of WT and CD18 KO mice showed that at E18.5 cells are present at all stages of the pathway (Figure 5-4B). At 1 and 3 days old, the mature IL-17-producers (CD45RB$^-$CD44$^+$) predominate, whereas at 4 weeks old most of the cells are of unknown commitment (CD45RB$^-$CD44$^-$). There was no difference in the number of cells at each stage of the development pathway at E18.5, 1 day and 3 day old mice between WT and CD18 KO. The only difference was seen at 4 weeks old, where the number of CD45RB$^-$CD44$^-$ cells were significantly decreased in CD18 KO mice. This suggests that the commitment of mature CD24$^-$ γδ T cells to the IL-17- and IFNγ-producing lineages is unaffected by the loss of CD18 expression. Interestingly, at 4 weeks old the total number of thymic cells was decreased in CD18 KO mice ($8.6x10^7 \pm 1.5x10^7$ cells) compared to WT ($1.04x10^8 \pm 3.4x10^7$ cells) (Figure 5-4C). Therefore, the decreased number of

uncommitted mature γδ T cells may contribute to the overall decrease in cellularity in the thymus.

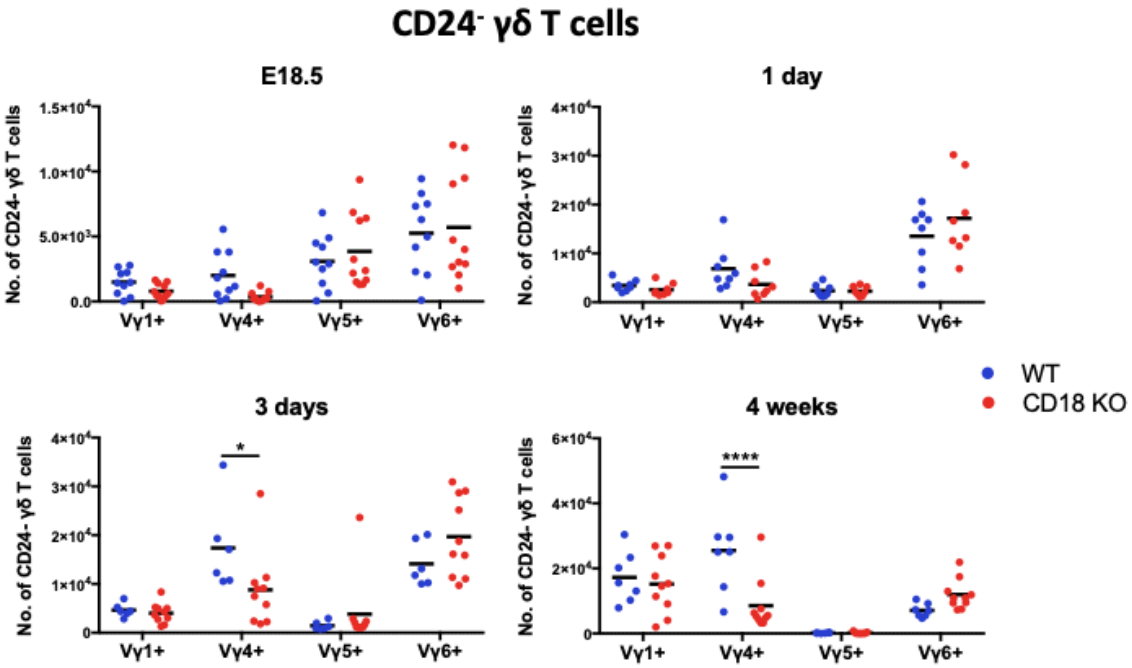

Figure 5-3 The defect in Vγ4$^+$ cell numbers in newborn CD18 KO mice is in the mature CD24$^-$ population of γδ T cells.
Cells isolated from WT and CD18 KO thymus at various ages were analysed by flow cytometry. Cells were first gated on CD3$^+$TCRγδ$^+$CD24$^-$, then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$4$^-$5$^-$ (Vγ6$^+$). Percentage of CD24- γδ T cells according to Vγ chain expression. WT n=10, CD18 KO n=11. Data from 6 independent experiments. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

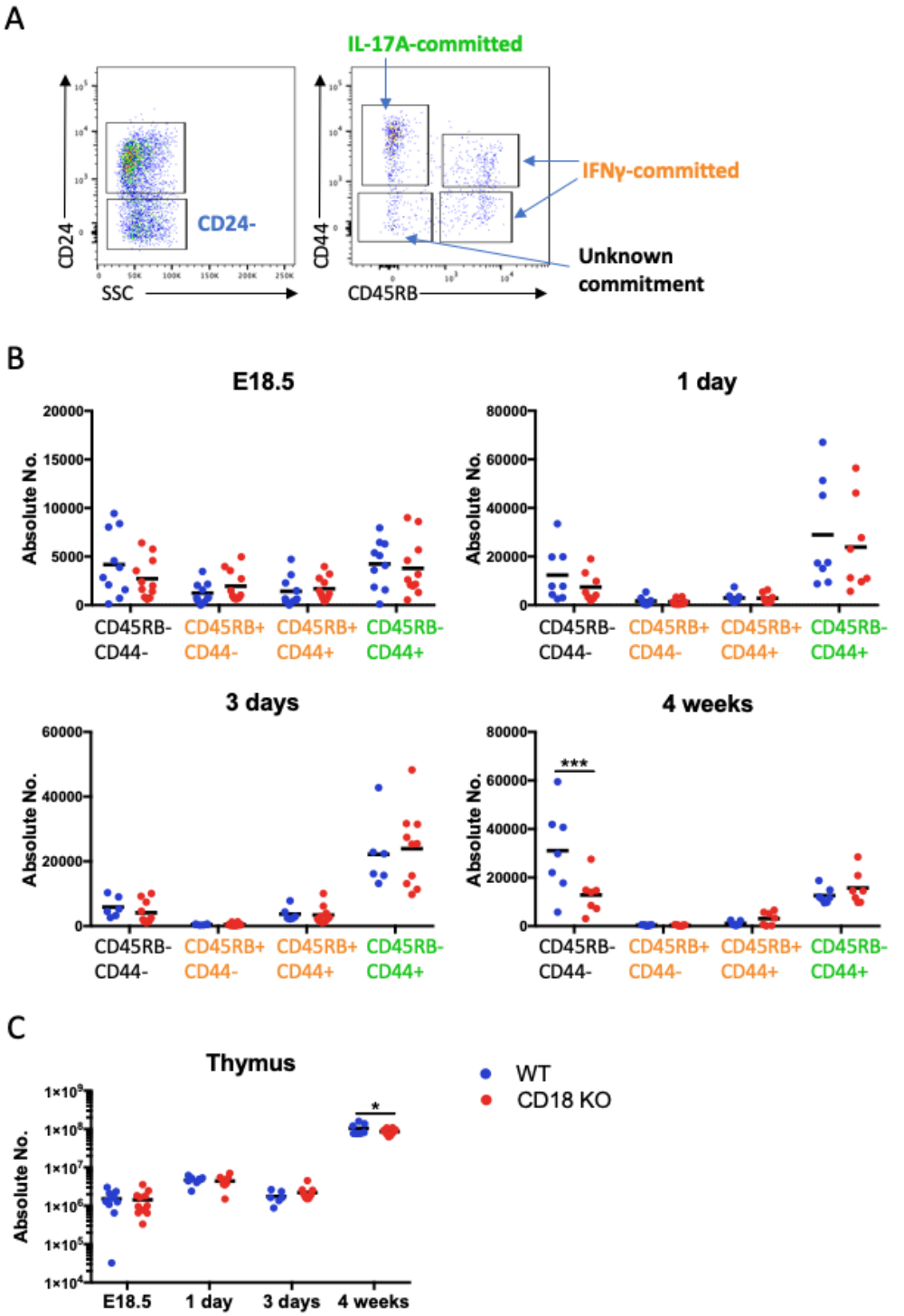

Figure 5-4 Commitment of mature (CD24$^-$) γδ T cells in the thymus to IL-17- and IFNγ-producing subsets is equivalent in WT and CD18 KO mice.
Cells isolated from WT and CD18 KO thymus at various ages were analysed by flow cytometry. Cells were first gated on CD3$^+$TCRγδ$^+$CD24$^-$ then separated into 4 populations based on expression of CD45RB and CD44. **(A)** Gating shown with representative plots from 1 day old thymus. **(B)** Percentages of γδ T cells expressing CD45RB/CD44. **(C)** Total number of thymocytes. WT 3 days n=6, WT & CD18 KO 4 weeks n=7, WT & CD18 KO 1 day n=8, WT E18.5 & CD18 KO 3 days n=10, CD18 KO E18.5 n=11. Data from a minimum of 3 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A defect in the thymic development of Vγ4$^+$ γδ T cells was identified in CD18 KO mice. This population is one of the predominant γδ T cell subsets present in lymphoid organs, along with Vγ1$^+$. I have already established that there is no difference in the number of Vγ4$^+$ cells in the spleen, blood or mucosal tissues of CD18 KO mice (Chapter 4, Figure 4-8). As this subset tends to reside within lymphoid tissues, the peripheral LNs (pLN), mediastinal LN (medLN), mesenteric LNs (mLN) and bone marrow were examined for the number of γδ T cell subsets. My hypothesis was that the defect in development of Vγ4 cells in CD18 KO mice would result in a peripheral defect in lymphoid tissues.

Calculation of Vγ4$^+$ cell numbers revealed a defect in the pLN of CD18 KO mice but no difference in the medLN, mLN or bone marrow (Figure 5-5). The number of Vγ1$^+$ and Vγ6$^+$ γδ T cells were equivalent between WT and CD18 KO at all the lymphoid sites analysed. This suggests that the impaired development of Vγ4$^+$ cells results in a peripheral defect specifically in the pLN.

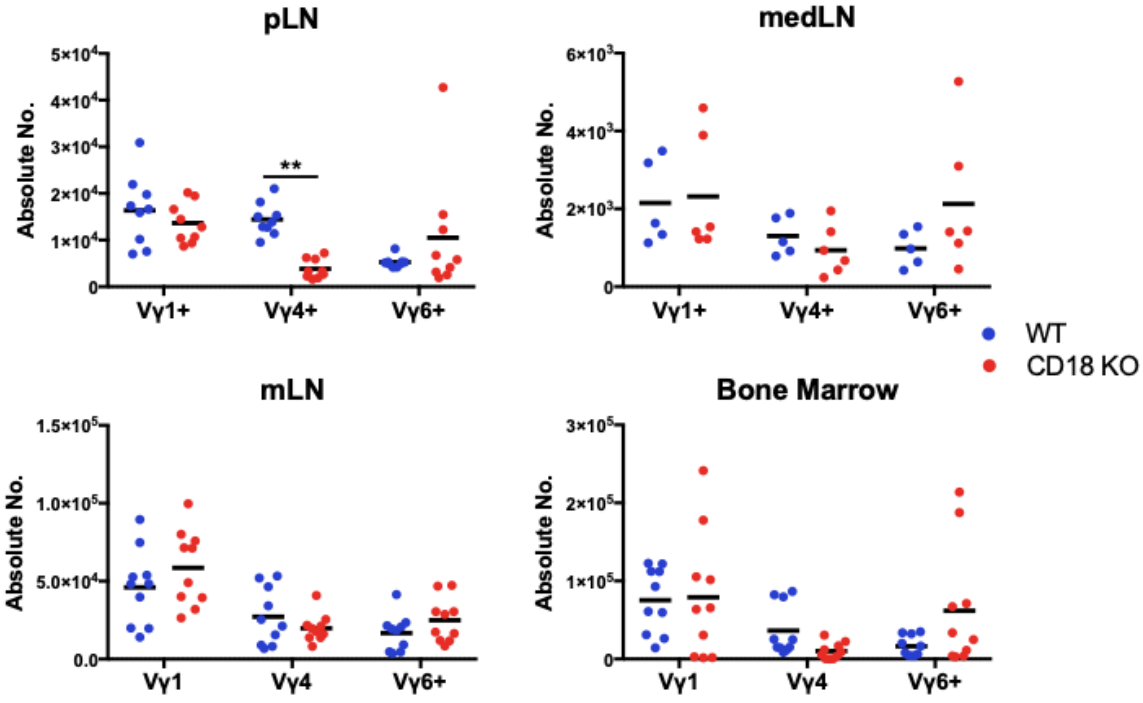

Figure 5-5 CD18 KO mice have a defect in numbers of Vg4+ γδ T cells only in the pLN. Cells isolated from the peripheral LN (pLN; axillary, brachial and inguinal pooled), mediastinal LN (medLN), mesenteric LN (mLN) and bone marrow of adult WT and CD18 KO mice were analysed by flow cytometry. Cells were gated on CD3+TCRγδ+ then Vγ1+, Vγ4+, Vγ6+ (Vγ1-4-5- for bone marrow and mLN; Vγ1-4- for pLN and medLN). Absolute cell numbers shown. pLN, mLN and bone marrow n=9 from 3 independent experiments. medLN n=6 from 2 independent experiments. Each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The results shown in figures 5-1 to 5-4 indicate that thymic development of Vγ6$^+$ cells was unaffected by the loss of β_2 integrin expression. Therefore, enhanced thymic output is not the reason for the increase in Vγ6$^+$ cell numbers in the CD18 KO adult mice. The next question was to determine when during the lifespan of the mouse this Vγ6$^+$ cell expansion occurs. To answer this, the Vγ repertoire was analysed from the spleen and lungs of WT and CD18 KO mice across different ages.

The Vγ repertoire in the WT lungs showed the presence of Vγ1, Vγ4, Vγ5 and Vγ6-expressing γδ T cells (Figure 5-6A). The percentage of Vγ6$^+$ cells increased from embryonic to newborn mice and was retained up to 4 weeks old. In contrast to this, the percentage of Vγ6$^+$ cells in CD18 KO lungs was higher than WT at 1 day old and further increased at 3 days and 4 weeks old. There was also a large proportion of Vγ5$^+$ cells in both genotypes at E18.5, that decreased by 1 day and was absent in the 4 week old mice. As Vγ5$^+$ cells are found exclusively in the epidermis of adult mice[363], it could be assumed that these are circulating cells that will become resident in the skin. The WT spleen showed a large proportion of Vγ1$^+$ and Vγ4$^+$ cells, with a smaller percentage of Vγ6$^+$ at all ages that were analysed. Note, for the spleen of E18.5 mice the percentage of γδ T cell subsets is not shown due to very low cell numbers, meaning that there were no representative plots due to high variability. The proportion of Vγ6$^+$ cells in the WT spleen was similar across all ages studied. In contrast to this, the CD18 KO spleen displayed a greater proportion of Vγ6$^+$ cells in 1 day old mice that was further increased at 3 days and again at 4 weeks old. In the lungs at E18.5 most of the cells were Vγ5$^+$, this population decreased with age and was absent by 4 weeks regardless of genotype, as these cells have homed to the skin.

There was a similar number of Vγ6$^+$ cells in the spleen and lungs of embryonic WT and CD18 KO mice. In the lungs, the number of Vγ6$^+$ cells was significantly increased in CD18 KO mice compared to WT from 1 day old onwards (Figure 5-6B). Whereas a higher number of Vγ6$^+$ cells in the CD18 KO spleen was only seen from 3 days old relative to WT mice. This data indicates that the increased number of Vγ6$^+$ γδ T cells in CD18 KO mice is present initially in the lungs from 1 day post-birth and by 3 days also in the spleen.

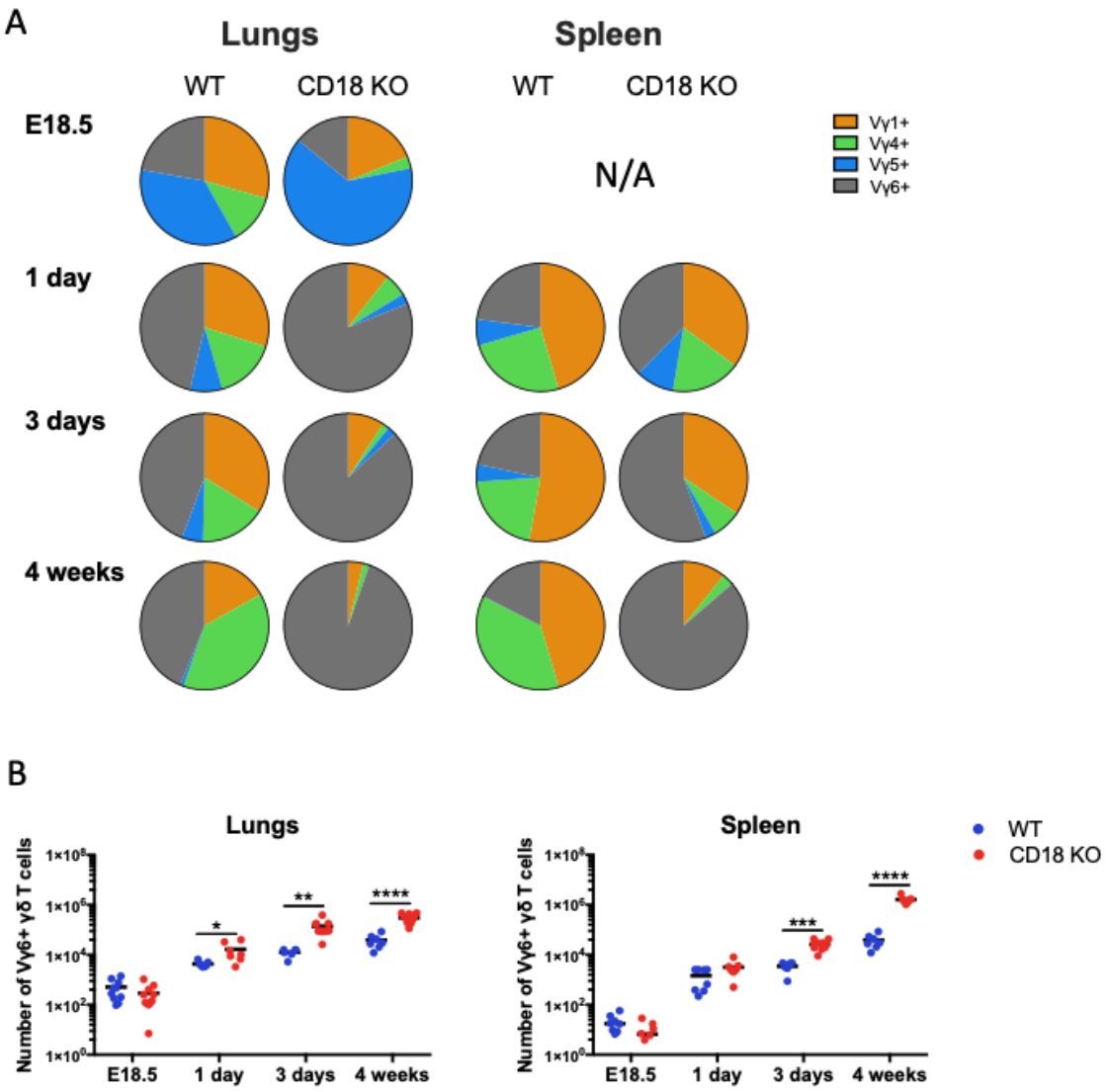

Figure 5-6 Increase in Vγ6+ γδ T cells in the spleen and lungs of young CD18 KO mice. Analysis of γδ T cells from the spleen and lungs of WT and CD18 KO mice at various ages. Cells were gated on CD3+TCRγδ+ then Vγ1+, Vγ4+, Vγ5+ or Vγ1-4-5-. **(A)** Representative pie charts of the percentage of γδ T cells expressing each Vγ chain. E18.5 spleen not shown due to very low cell number. **(B)** Absolute number of Vγ6+ (Vγ1-Vγ4-Vγ5-) γδ T cells. WT spleen & lungs 3 days n=6. CD18 KO spleen 1 day, WT/CD18 KO lungs 1 day, WT/CD18 KO spleen 4 weeks, WT lungs 4 weeks n=7. WT spleen 1-day n=8. CD18 KO spleen 3 days, CD18 KO lungs 3 days/4 weeks, WT spleen E18.5 n=10. CD18 KO spleen/lungs E18.5 n=11. E18.5 data from 6 litters, 1 day old from 6 litters, 3 day old from 4 litters, 4-week old from 5 litters; data from 4-6 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a Student's t test with false discovery rate approach two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Having determined that CD18 KO mice show an increased number of Vγ6$^+$ cells in the lungs and spleen by 1 and 3 days old, respectively, the γδ T cells were then analysed for the expression of markers associated with IL-17-producing γδ cells (CD45RB$^-$, CD44$^+$, CD27$^-$). The lungs of CD18 KO mice displayed a higher percentage of CD45RB$^-$, CD44$^+$ and CD27$^-$ γδ T cells than WT mice at 1 day, 3 days and 4 weeks old (Figure 5-7). There was also an increased percentage of CD45RB,

CD44$^+$ and CD27$^-$ γδ T cells in the spleen of 3 day and 4 week old CD18 KO mice In addition, there was a small increase in the proportion of CD27$^-$ γδ T cells in 1 day old mice. This data fits with the findings from Figure 5-6 that there is an increased number of Vγ6$^+$ cells with an IL-17-producing phenotype in CD18 KO mice from 1 day old in the lungs and from 3 days old in the spleen.

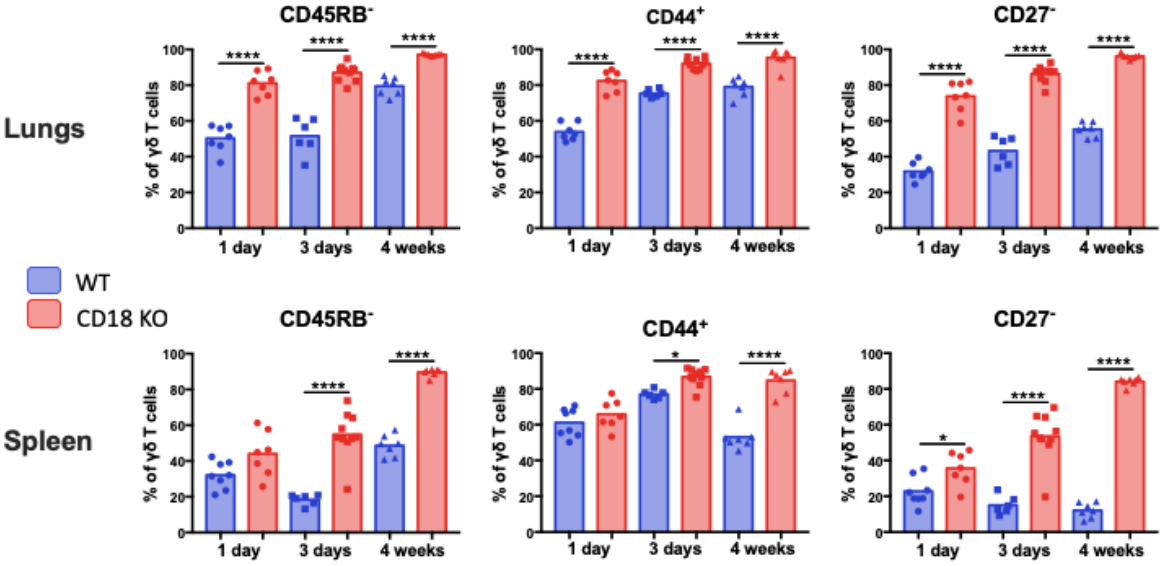

Figure 5-7 Increase in IL-17-producing (CD45RB$^-$CD44$^+$CD27$^-$) γδ T cells in the spleen and lungs of young CD18 KO mice
Analysis of γδ T cells from the spleen and lungs of WT and CD18 KO mice at various ages. Cells were gated on CD3$^+$TCRγδ$^+$. Data shown as percentage of γδ T cells that were CD45RB$^-$, CD44$^+$ or CD27$^-$. WT spleen & lungs 3 days n=6. CD18 KO spleen 1 day, WT/CD18 KO lungs, WT/CD18 KO spleen & lungs 4 weeks, n=7. WT spleen 1 day n=8. CD18 KO spleen & lungs 3 days, n=10. Data from 4-6 litters, with each litter run as an independent experiment; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In summary, the loss of β_2 integrin expression resulted in a reduced number of immature Vγ4$^+$ cells in the thymus and a delayed upregulation of maturation markers, revealing a novel role for β_2 integrins in promoting the development of Vγ4$^+$ γδ T cells. In contrast, thymic development of Vγ6$^+$ cells was unaffected but these cells were increased in CD18 KO lungs from 1 day old and in the spleen from 3 days old. This data indicates that the expanded population of Vγ6$^+$ cells in CD18 KO mice is not due to enhanced thymic output.

5.2.2 CD18 KO mice show no evidence of enhanced γδ T cell proliferation

The increased number of Vγ6$^+$ γδ T cells in CD18 KO mice was not due to enhanced thymic output. Next, I explored the second potential mechanism of Vγ6$^+$ cell expansion: that there may be increased proliferation of Vγ6$^+$ cells in the periphery of CD18 KO mice. The primary aim was to determine if the environment in mice lacking β_2 integrins preferentially induced proliferation compared to WT mice. To address this question, total T cells (CD3$^+$) were isolated from the spleen of CD18 KO mice, labelled with CFSE, and then transferred i.v. into either WT or CD18 KO hosts. The mice were culled 1-, 4- or 8-days post-transfer. Ideally, WT γδ T cells would be used as a control in this adoptive transfer experiment; however, due to low γδ T cell numbers and different subset ratios in WT mice, this was neither feasible nor appropriate.

Following the transfer of CD18 KO T cells, the thymus, spleen, blood, mLN, pLN and lungs of host mice were analysed by flow cytometry. The percentage of transferred cells that were recovered was calculated by dividing the sum of CFSE$^+$ cells from all tissues by the number that were initially transferred, then multiplying by 100. There was an average of 10% cell recovery from both WT and CD18 KO mice across all timepoints (Figure 5-8A). Prior to transfer the T cells were labelled with CFSE to allow assessment of cell proliferation. There was a loss of CFSE expression over the timecourse, however only a single peak was present, suggesting that the cells did not proliferate (Figure 5-8B). Although as there was no positive control to confirm this conclusion. Comparison of the mean CFSE expression showed no difference between WT and CD18 KO, indicating that the proliferative capacity of γδ T cells was unaltered between WT or CD18 KO hosts.

There was no evidence of γδ T cell proliferation over the 8 day timecourse, but the experimental set-up allowed the analysis of γδ T cell homing to different tissues in WT and CD18 KO mice. The number of transferred γδ T cells found in tissues would reflect the preferential migration and/or retention at this site. The ratio of γδ to αβ T cells was calculated in the initially transferred cells and in the CFSE$^+$ cells found in each tissue at 1-day post transfer. An increased ratio indicates a preferential migration/retention of γδ T cells to that tissue, whereas a decreased ratio indicates a preference for αβ T cells. A similar ratio of αβ:γδ T cells was found in the thymus

as in the transferred cells (shown as a grey dotted line) (Figure 5-8C), indicating equivalent migration of αβ and γδ T cells to this site. The ratio was increased in the spleen and blood, and to a greater extent in the lungs. Conversely, the ratio was decreased in the mLN and pLN. This suggests that there was a preferential recruitment and/or retention of γδ T cells in the spleen, blood and lungs, and for αβ T cells in the mLN and pLN. These findings were as expected because the majority of the transferred γδ T cells expressed Vγ6, and these cells are known to preferentially home to the lungs[199], whereas αβ T cells tend to home to LNs. The spleen, blood and lungs were the sites where most of the transferred γδ T cells were located. Therefore, the number of $CFSE^+$ γδ T cells was calculated in these tissues from WT and CD18 KO hosts. There was no difference in the number of transferred γδ T cells that were found in the spleen, blood or lungs of WT and CD18 KO hosts across all of the timepoints studied (Figure 5-8D), indicating that there was not a preferential migration or retention of γδ T cells in CD18 KO mice compared to WT.

Overall, this experiment demonstrated that, following adoptive transfer, CD18 KO γδ T cells do not proliferate within 8 days. Additionally, the CD18 KO γδ T cells preferentially migrate and/or are retained in the spleen, blood and lungs compared to αβ T cells. As there were no differences found in the γδ T cell numbers whether transferred into WT or CD18 KO mice, there is no evidence of an altered environment in the absence of CD18 that leads to enhanced proliferation or migration/retention.

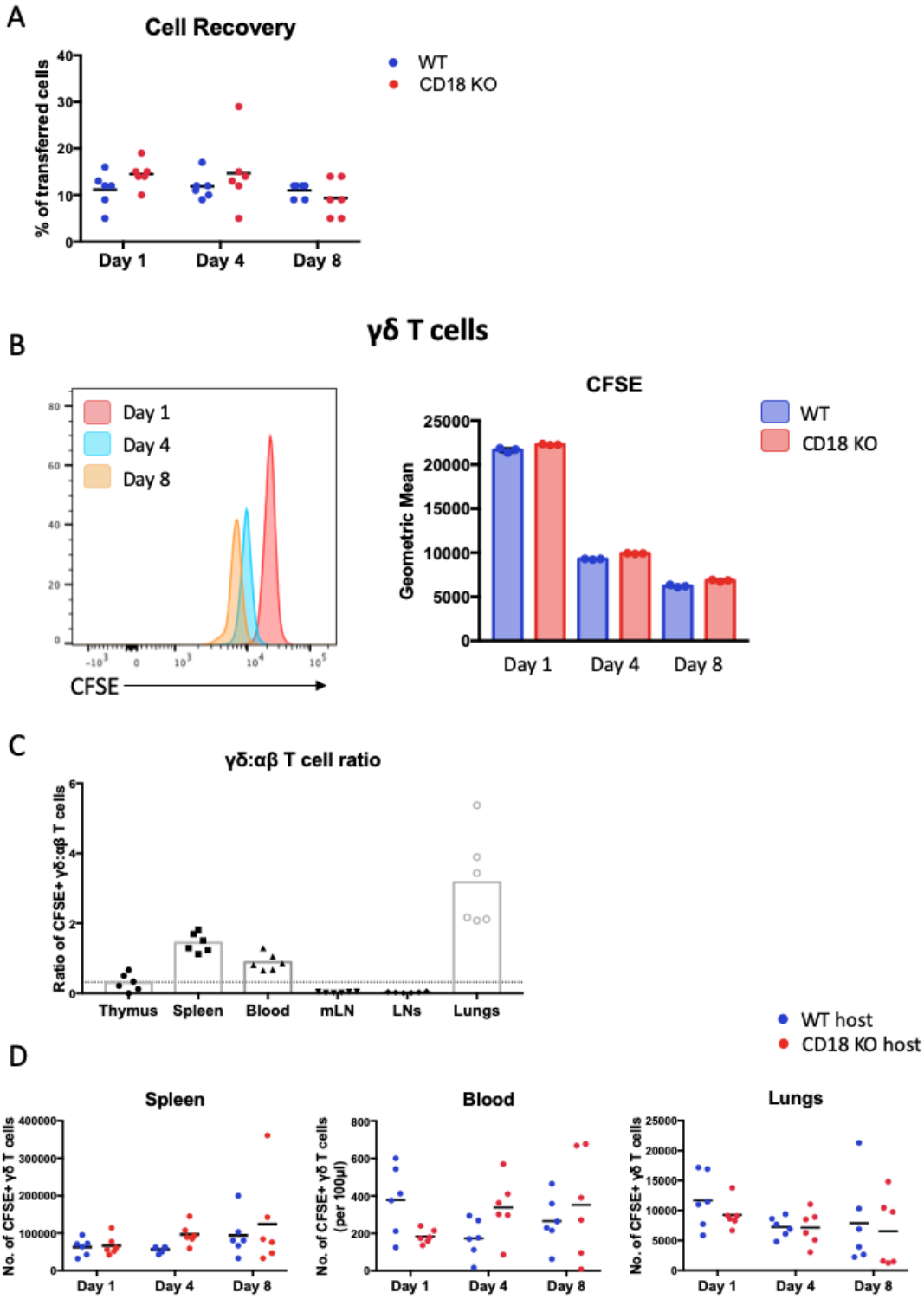

Figure 5-8 CD18 KO γδ T cells do not proliferate and display similar migration/retention when transferred into WT and CD18 KO hosts.
$CD3^+$ cells were purified from CD18 KO splenocytes, labelled with CFSE and then $2x10^6$ cells were transferred i.v. into WT or CD18 KO hosts. 1, 4 or 8 days after transfer, cells from the thymus, spleen, blood, mesenteric LNs (mLN), peripheral LNs (pLN) and lungs were analysed by flow cytometry. **(A)** Percentage of transferred cells calculated as the number of CFSE+ cells (total from all tissues harvested) divided by the number of CFSE+ cells transferred, multiplied by 100. **(B)** The mean expression of CFSE on γδ T cells. Representative plot shown from WT spleen. **(C)** The ratio of CFSE+ γδ to αβ T cells in different tissues of WT mice 1 day after transfer. Grey dotted line shows the ratio in the transferred cells. **(D)** The absolute number of CFSE+ γδ T cells from WT and CD18 KO hosts. n=6, data pooled from 2 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The data obtained from adoptive transfer of CD18 KO γδ T cells showed no evidence of proliferation in either WT or CD18 KO hosts. To further assess the proliferative capacity of γδ T cells *in vivo* within their native setting, WT and CD18 KO mice were administered with a single i.p. dose of BrdU, followed by 7 days in the drinking water. γδ T cells were then analysed by flow cytometry for BrdU incorporation as a measure of proliferation (BrdU labels cells undergoing DNA replication, i.e. in S-phase). Cells were also assessed for their expression of Ki67, a marker that is only expressed during active cell cycle (G1, S, G2 and M) [385]. The hypothesis was that Vγ6$^+$ cells, which increased in the spleen and lungs of CD18 KO mice, may display enhanced proliferation compared to WT. Vγ4$^+$ cells were used as controls as they display comparable numbers in the CD18 KO spleen and lungs to WT.

The levels of Ki67 were higher in γδ T cells from the lungs compared to the spleen, regardless of the subset (Figure 5-9A). When compared to WT, the expression of Ki67 was significantly increased in Vγ6$^+$ γδ T cells from both the spleen and lungs of CD18 KO mice. In Vγ4$^+$ cells, Ki67 levels were equivalent between genotypes. Similar to Ki67, the expression of BrdU was higher in the lungs than the spleen for both Vγ6$^+$ and Vγ4$^+$ γδ T cells (Figure 5-9B). This suggests that γδ T cells may proliferate preferentially in the lungs rather than the spleen. However, there was no difference in BrdU incorporation when comparing either the Vγ4$^+$ or Vγ6$^+$ γδ T cell subsets from WT and CD18 KO mice. This indicates that very few γδ T cells are actively undergoing DNA replication (S-phase) in either WT or CD18 KO mice within a 1 week period.

The levels of both Ki67 and BrdU detected in γδ T cells was very low, when compared with a positive control of cells from an inflamed LN. The increased expression of Ki67 on Vγ6$^+$ cells from CD18 KO mice could indicate that more cells are in active cell cycle. Based on this data it can be concluded that very few Vγ6$^+$ γδ T cells are proliferating within a 1 week period, and the levels of proliferation are similar between WT and CD18 KO mice.

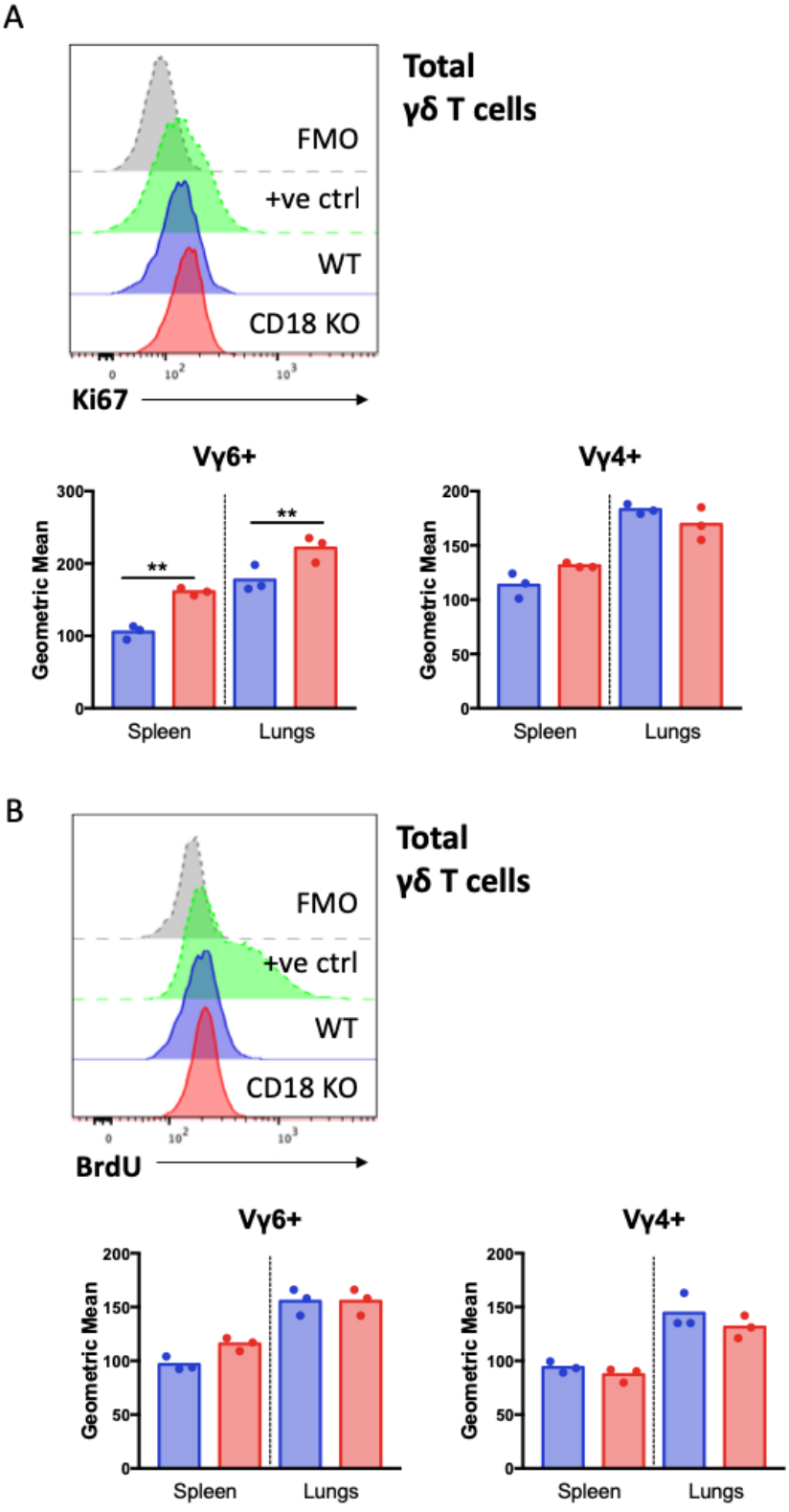

Figure 5-9 Increased expression of Ki67 in Vγ6⁺ CD18 KO γδ T cells but low levels of proliferation measured by 1 week of BrdU.
WT and CD18 KO mice were injected with 1mg BrdU i.p. then fed 0.8mg/ml BrdU in the drinking water for 7 days. Cells isolated from the spleen and lungs were analysed by flow cytometry for the expression of Ki67 **(A)** and BrdU incorporation **(B)**. Representative histograms shown from the spleen. Grey dotted line indicates the FMO, blue line is WT, red line is CD18 KO and green line is a positive control of cells from the inguinal LN of mice with tail lesions. Data is representative of 2 experiments; each symbol represents and individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

There was no difference found in the proliferation of Vγ6$^+$ γδ T cells in CD18 KO mice, measured by BrdU incorporation over 1 week. However, there was an enhanced expression of Ki67, which may indicate that the cells have a higher proliferation but at low levels, over a longer period of time. I next investigated the quantities of IL-1β and IL-23 in WT and CD18 KO mice, as these cytokines are known to induce the proliferation of IL-17-producing γδ T cells[200]. In addition to this, both IL-1β and IL-23 can induce IL-17 production by γδ T cells[244,386], therefore, higher levels of these cytokines could explain why CD18 KO Vγ6$^+$ cells produce more IL-17 (Figure 4-10). Previous studies using CD18 KO mice have also identified higher levels of IL-1β in the spleen[387] and increased IL-23 transcript was measured in the mLN of these mice[303]. However, the levels in the serum or other tissues where I have found increased Vγ6$^+$ cells (lungs and uterus) were not measured.

The levels of IL-1β and IL-23 in WT and CD18 KO mice were measured by ELISA, from the serum and tissues where γδ T cells were expanded. The serum cytokine results were inconclusive, as in at least half of the samples the cytokines were below the limit of detection (Figure 5-10A). Statistically, there was no significant difference in the serum levels of IL-1β or IL-23 from CD18 KO mice compared to WT, although it is possible that differences may be found with a more sensitive method of detection. Cytokine levels were detectable in tissues, where supernatant samples isolated from CD18 KO spleen cultures showed significantly higher levels of both IL-1β and IL-23 than WT. In contrast to this, the lungs and uterus displayed equivalent levels of both cytokines regardless of the genotype.

It is not only the levels of cytokine present that dictate the cellular response, but the expression of the corresponding receptor will also influence the strength of signal that the cell will receive. As the levels of IL-1β and IL-23 were increased in the spleen of CD18 KO mice, the levels of the receptors for these cytokines were measured on γδ T cells from the spleen and lungs of WT and CD18 KO mice.

WT and CD18 KO γδ T cells from the spleen and lungs expressed low levels of IL-1βR and IL-23R (Figure 5-10B). In the spleen, the expression of IL-1βR was absent on Vγ4$^+$ cells and very low on Vγ6$^+$ cells (Figure 5-10C). Although significantly higher expression of IL-1βR was detected on CD18 KO Vγ6$^+$ cells compared to WT, the levels were only slightly above the FMO. IL-23R was detected on both subsets

of γδ T cells in the spleen, with equivalent expression on $Vγ4^+$ cells, and a small, but significant increase in expression on CD18 KO $Vγ6^+$ cells compared to WT. The increased expression of IL-1βR and IL-23R on $Vγ6^+$ cells in the CD18 KO spleen is a very subtle effect therefore may not have any biological significance. Similar to those in the spleen, $Vγ4^+$ cells from the lungs did not express the IL-1βR regardless of genotype. Expression was found on $Vγ6^+$ cells from both WT and CD18 KO mice. γδ T cells from the lungs showed higher expression of IL-23R than in the spleen. However, as the data from the lungs was n=2, the experiment would have to be repeated to determine if there are any differences in the expression of IL-1βR or IL-23R on γδ T cells from WT and CD18 KO lungs.

In sum, no differences were detected in the serum levels of IL-1β or IL-23 as samples were largely below the limit of detection, but higher levels of IL-1β and IL-23 were found specifically in the spleen of $β_2$ integrin-deficient mice compared to WT. The expression of the corresponding receptors could be detected on both WT and CD18 KO γδ T cells, but only at low levels.

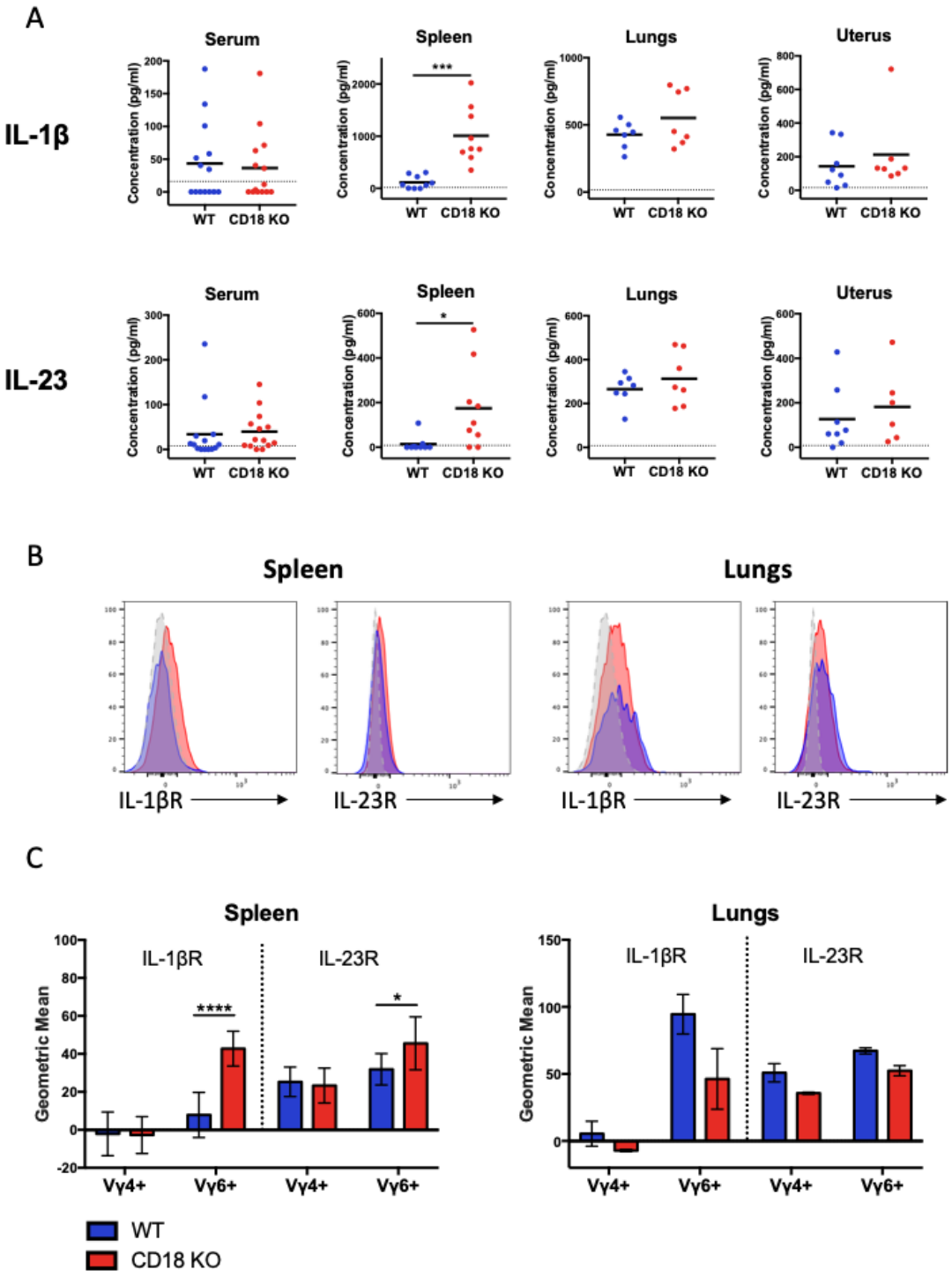

Figure 5-10 CD18 KO mice have increased levels of IL-1β and IL-23 in the spleen and higher expression of IL-1βR and IL-23R on Vγ6+ γδ T cells.
(A) Levels of IL-1β and IL-23 were measured by ELISA from serum and tissues isolated from WT and CD18 KO mice, tissue supernatants were collected by incubating the tissue in complete media for 4h at 37°C. Dotted line indicates the limit of detection: IL-23 8pg/ml, IL-1β 16pg/ml. Levels of the IL-1βR (CD121a) and IL-23R were measured on γδ T cells from the spleen and lungs of WT and CD18 KO adult mice by flow cytometry. **(B)** Representative plots of IL-1β and IL-23 expression on total γδ T cells from the spleen and lungs. Grey dotted line indicates the FMO, blue line is WT and red line is CD18 KO. **(C)** Mean expression of IL-1β and IL-23 on $V\gamma4^+$ and $V\gamma6^+$ cells. Serum n=14, spleen n=9, WT IL-1β/IL-23 uterus n=8, CD18 KO IL-1β uterus n=7, CD18 KO IL-23 uterus n=6. Each symbol represents an individual mouse. IL-1βR/IL-23R spleen n=9; data pooled from 3 independent experiments. IL-1βR/IL-23R lungs n=2; data from one experiment. Data represents mean ± SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

In summary, there was no evidence of enhanced proliferation of Vγ6$^+$ γδ T cells in CD18 KO mice after adoptive transfer or by BrdU incorporation. There were small but significantly increased levels of IL-1β and IL-23 identified in the spleen of CD18 KO mice, although the effect of this is unclear as the proliferation of γδ T cells in the spleen was equivalent in WT mice. Overall, this data indicates that either the mechanism of Vγ6$^+$ cell expansion in CD18 KO mice is not due to proliferation or that the cells proliferate in younger mice and are then retained in the adults.

5.2.3 Vγ6$^+$ γδ T cells from CD18 KO mice show no evidence of enhanced survival signalling downstream of the IL-7R but show reduced apoptosis in the lungs

Having found no differences in the thymic development or proliferation of Vγ6$^+$ γδ T cells in CD18 KO mice, I next explored my third hypothesis to explain the increased Vγ6$^+$ cell numbers in CD18 KO mice. I hypothesised that Vγ6$^+$ cells may accumulate due to an increased survival and/or failure of homeostatic cell death. IL-7 is an important pro-survival signal for γδ T cells[247,388], and in mice with reduced CD18 expression levels of the IL-7R were increased on CD27$^-$ γδ T cells (IL-17-producers) compared to WT[296]. Based on this knowledge, I further investigated IL-7 and the downstream survival signalling in Vγ6$^+$ cells from WT and CD18 KO mice.

First, the serum levels of IL-7 were measured by ELISA. The quantity of IL-7 was undetectable in the majority of serum samples from both WT and CD18 KO mice (Figure 5-11A). Suggesting that in general the circulating levels of IL-7 are very low. Next the expression of the IL-7Rα (CD127) was assessed on γδ T cells from the spleen and lungs. The IL-7R was expressed at higher levels on γδ T cells from the spleen of CD18 KO mice compared to WT whereas, expression on γδ T cells from the lungs was similar (Figure 5-11B). When compared on γδ T cell subsets, Vγ4$^+$ cells (which are equivalent in number) had similar levels of IL-7R whether from WT or CD18 KO mice (Figure 5-11C). Whereas, Vγ6$^+$ cells from CD18 KO mice (increased in number) had higher IL-7R expression than WT in the spleen but not the lungs. Interestingly, in the lungs almost 100% of γδ T cells stained positive for the IL-7R, and this was also true for those in the spleen of CD18 KO mice but not WT. This could indicate that there is an increased recirculation of Vγ6$^+$ cells from the lungs to the spleen in CD18 KO mice compared to WT. Alternatively, factors present in the CD18 KO spleen may induce upregulation of the IL-7R.

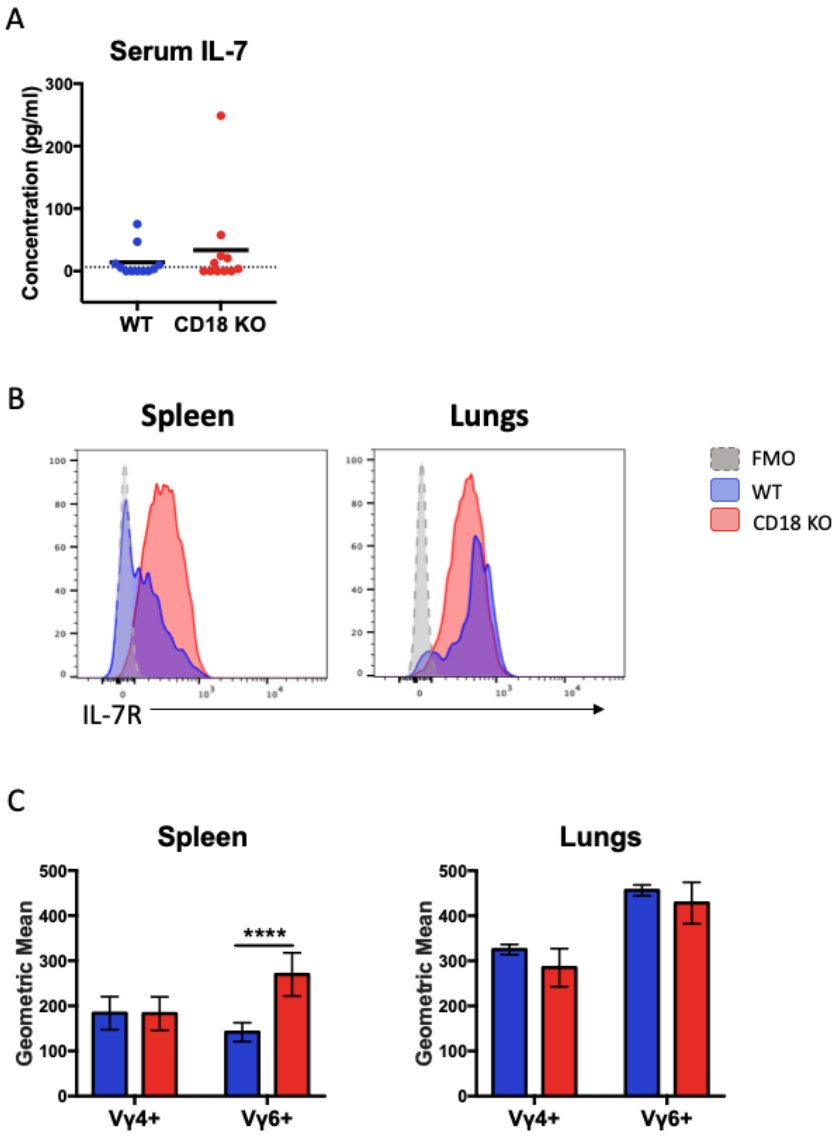

Figure 5-11 Vγ6⁺ cells from the CD18 KO spleen express higher levels of IL-7R and IL-1βR than WT.
(A) Levels of IL-7 were measured by ELISA in serum from WT and CD18 KO adult mice. Grey dotted line indicates the limit of detection, 6.3pg/ml. **(B&C)** Levels of the IL-7R (CD127) were measured on γδ T cells from the spleen and lungs of WT and CD18 KO adult mice by flow cytometry. **(B)** Representative plots of cytokine receptor expression on total γδ T cells. Grey dotted line indicates the FMO, blue line is WT and red line is CD18 KO. **(C)** Mean expression of cytokine receptors. n=8. Data pooled from 3 independent experiments. Data represents mean ± SD. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

It was found that Vγ6⁺ γδ T cells in the CD18 KO spleen expressed higher levels of IL-7R, this raised the question of whether this increased receptor expression could result in increased downstream survival signalling, which may contribute to the increased Vγ6⁺ cells in CD18 KO mice. One of the major anti-apoptotic molecules known to be induced by IL-7 and enhance cell survival is Bcl2 [389,390]. To assess if pro-survival signalling is altered in CD18 KO γδ T cells, Bcl2 expression was assessed by flow cytometry in γδ T cell subsets from WT and CD18 KO mice.

Initially, Bcl2 expression was examined in γδ T cells from the spleen of adult mice (Figure 5-12A&B). Vγ4-expressing cells from WT and CD18 KO mice expressed Bcl2 to a similar level. In contrast to this, the Vγ6$^+$ cells from WT mice expressed similar levels to Vγ4$^+$ cells whereas in CD18 KO mice Bcl2 was significantly decreased only in the Vγ6$^+$ population. This suggests that the increased expression of IL-7R on Vγ6+ cells in the spleen does not correlate with an enhanced expression of Bcl2, in fact the data shows the opposite. As the increased number of Vγ6$^+$ cells were present in young CD18 KO mice, meaning that the mechanism driving γδ T cell expansion is likely present prior to adulthood, Bcl2 expression was then analysed in 4-week old mice. γδ T cells from the spleen and lungs showed no significant difference in expression of Bcl2, regardless of subset, when comparing WT and CD18 KO mice (Figure 5-12C).

Despite expressing more IL-7R, Vγ6$^+$ γδ T cells in the spleen of CD18 KO mice do not express higher levels of the anti-apoptotic molecule Bcl2. This data does not support the hypothesis that Vγ6$^+$ cells in CD18 KO mice have enhanced survival signalling downstream of the IL-7R. On the contrary, Vγ6$^+$ cells in the spleen of adult mice display reduced Bcl2, but expression is unaltered in 4 week old mice. This suggests a potentially age-dependent effect on Bcl2 expression, but there was no evidence of increased survival downstream of the IL-7R mediated by Bcl2. Bcl2 is one of many anti-apoptotic molecules that enhance cell survival in a complex pathway, so it is possible that other pro-apoptotic or anti-apoptotic molecules, such as Bcl-xL, Mcl-1 or BIM, may be altered in CD18 KO Vγ6$^+$ γδ T cells.

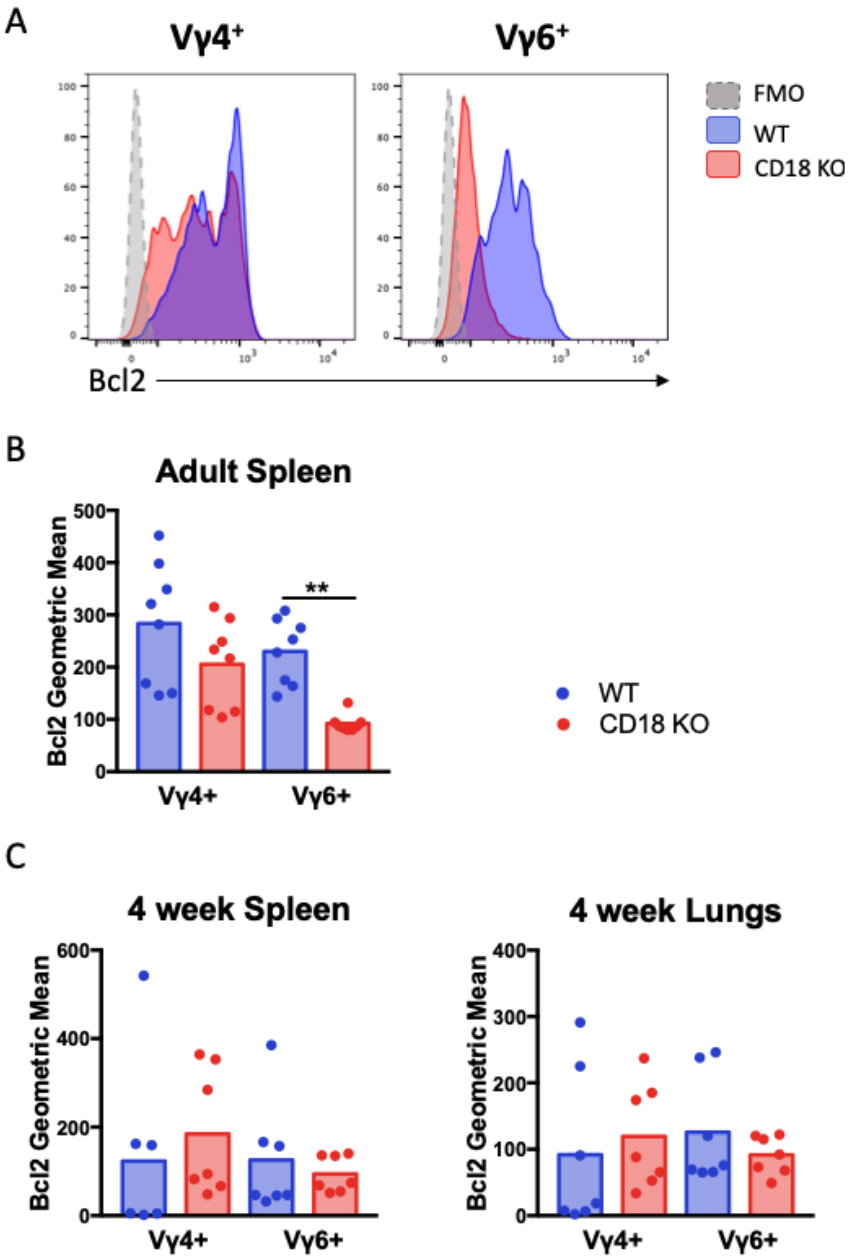

Figure 5-12 Bcl2 expression is decreased in splenic Vγ6⁺ cells from adult CD18 KO mice but not in 4-week old mice.
Bcl2 expression was analysed by flow cytometry in γδ T cells from WT and CD18 KO mice in the spleen of adults **(A&B)** and in the spleen and lungs of 4-week old mice **(C)**. Adult spleen n=8, 4-week old spleen/lungs n=7. Data pooled from a minimum of 3 independent experiments; each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Vγ6$^+$ γδ T cells in the spleen of CD18 KO mice express higher levels of IL-7R and reduced levels of Bcl2, whereas in the lungs the expression of IL-7R was equivalent to WT. This suggests that despite expressing higher levels of the receptor for IL-7 compared to WT, Vγ6$^+$ cells in the CD18 KO spleen do not have elevated downstream Bcl2. I next tested IL-7R signalling to determine if the reduced levels of Bcl2 could be explained by an impaired ability of CD18 KO Vγ6$^+$ cells to activate signalling pathways in response to IL-7. Cells isolated from the spleen and lungs of WT or CD18 KO mice were stimulated *in vitro* with IL-7 and the response of Vγ6$^+$ cells (higher IL-7R expression in the spleen) was compared with Vγ4$^+$ cells (equivalent IL-7R expression). The levels of the downstream phosphorylated signalling molecules pSTAT5 and pSTAT3 were measured 1, 10 and 30mins after stimulation.

Stimulation with IL-7 resulted in the upregulation of pSTAT5 in γδ T cells and to a lesser extent pSTAT3 (Figure 5-13A), as seen previously[235]. In both Vγ6$^+$ and Vγ4$^+$ γδ T cells from WT spleen there was a time-dependent increase in pSTAT5, with highest levels seen after 30mins (Figure 5-13B). When compared with CD18 KO γδ T cells, the levels of pSTAT5 were significantly higher in the WT cells after 10 and 30mins of IL-7 treatment. This was true for both Vγ4$^+$ and Vγ6$^+$ cells. This suggests that the induction of STAT5 phosphorylation in response to IL-7 is impaired in CD18 KO γδ T cells. The expression of pSTAT3 in splenic γδ T cells was not upregulated until 10mins after stimulation with IL-7. Unlike pSTAT5, the expression of pSTAT3 was induced to a similar extent in Vγ6$^+$ cells from WT and CD18 KO mice. While for Vγ4$^+$ cells, a defect in pSTAT3 levels in CD18 KO cells was seen after 10 and 30mins of IL-7, similar to the effect on pSTAT5. This shows that the loss of CD18 reduces the ability of Vγ4$^+$ cells to induce pSTAT3 downstream of the IL-7R, but not in Vγ6$^+$ cells. In the unstimulated samples, the expression of both pSTAT5 and pSTAT3 were slightly lower, although not significant, in the CD18 KO γδ T cells compared to WT.

The effect of IL-7 on signalling in γδ T cells from the lungs displayed different dynamics than those from the spleen (Figure 5-13C). For Vγ4$^+$ cells, the expression of pSTAT5 peaked after 10mins and only at this timepoint was there a significant decrease in expression in CD18 KO cells compared to WT. Unlike the Vγ4$^+$ cells from the spleen, where lower levels of pSTAT3 were seen, the dynamics of pSTAT3

induction in response to IL-7 were similar in cells from the lungs regardless of genotype. This shows that the reduced responsiveness of Vγ4$^+$ cells in the spleen is not mirrored in the lungs, indicating that the loss of β_2 integrin expression causes a tissue-specific rather than cell-intrinsic effect. pSTAT3 increased after 1min of IL-7 stimulation, peaked after 10mins then was slightly decreased after 30mins in Vγ4$^+$ cells from both WT and CD18 KO mice. This same effect was seen in Vγ6$^+$ cells, with no difference in the levels of pSTAT3 between WT and KO. This suggests that in Vγ6$^+$ γδ T cells, pSTAT3 signalling downstream of the IL-7R is unaffected by the loss of CD18. The induction of pSTAT5 differed in Vγ6$^+$ cells from the lungs: after exposure to IL-7 for 10mins, Vγ6$^+$ cells upregulated pSTAT5, however this high expression was retained after 30 mins. There was no significant difference pSTAT5 in Vγ6$^+$ cells from the lungs of WT and CD18 KO mice at any time following treatment with IL-7. This reiterates the point that the impaired IL-7R-mediated signalling in β_2 integrin-deficient γδ T cells is a tissue-specific effect.

Overall, Vγ4$^+$ and Vγ6$^+$ γδ T cells from CD18 KO spleen failed to upregulate pSTAT5 to the same extent as WT in response to IL-7. This effect was also seen for pSTAT3 in Vγ4$^+$ but not Vγ6$^+$ cells. In contrast, CD18 KO γδ T cells from the lungs upregulated pSTAT5 and pSTAT3 in a similar manner to WT, with the exception of Vγ4$^+$ cells that displayed reduced pSTAT5 at the peak of the response, although the difference was much smaller than those seen in the spleen. This indicates that the ability of γδ T cells to signal downstream of the IL-7R is altered in CD18 KO mice depending on their subset and location.

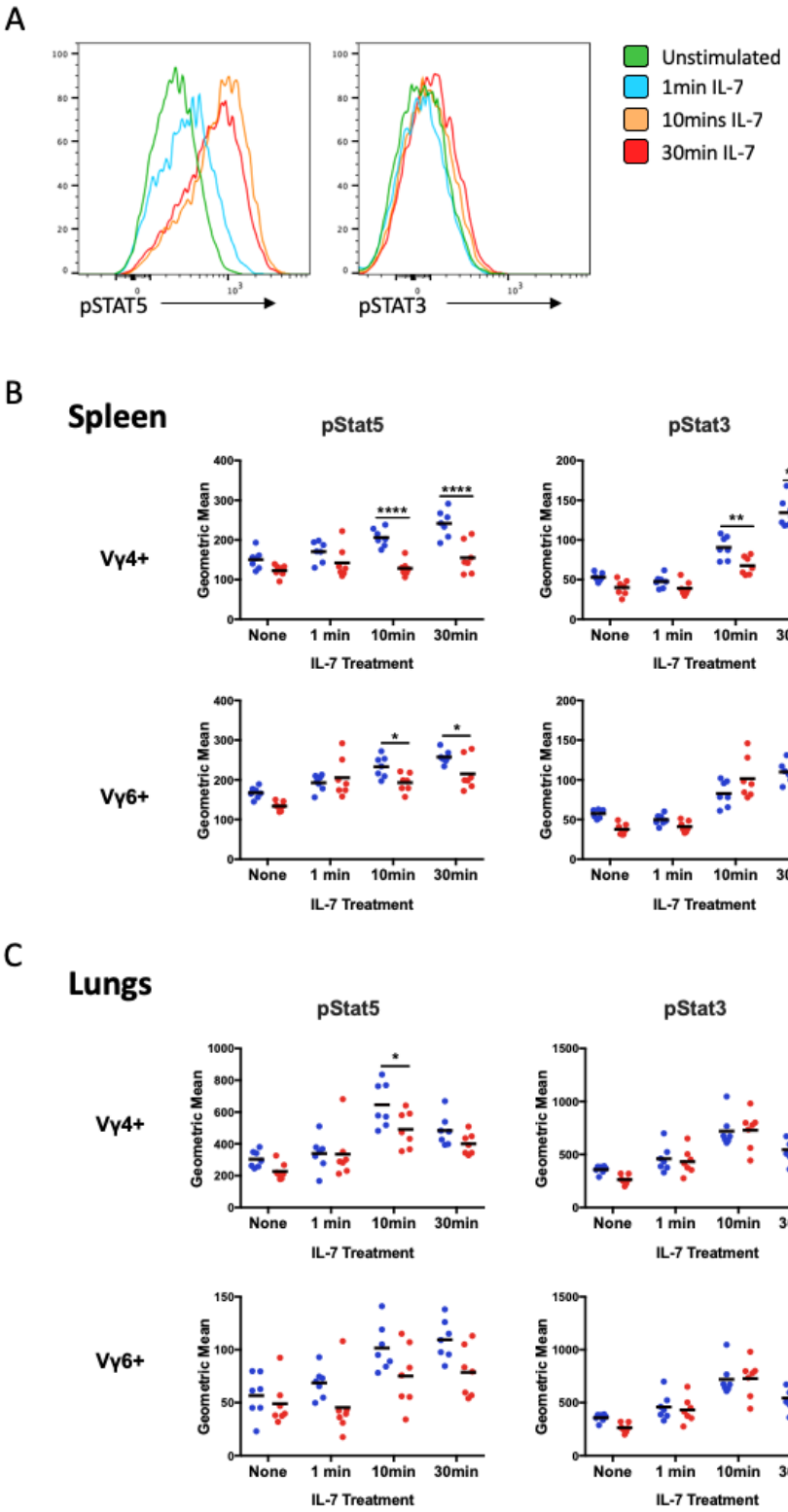

Figure 5-13 Vγ4+ and Vγ6+ γδ T cells from the spleen of CD18 KO mice display impaired STAT signalling in response to IL-7.
Cells from adult WT and CD18 KO spleen and lungs were stimulated *in vitro* with 100ng/ml IL-7 for 1, 10 or 30mins. Cells were then analysed by flow cytometry for the expression of phosphorylated STAT3 and STAT5. Samples were gated on $CD3^{+}TCR\gamma\delta^{+}$ then $V\gamma4^{+}$ or $V\gamma1^{-}4^{-}5^{-}$ ($V\gamma6^{+}$). **(A)** Representative plots from the total γδ T cells in CD18 KO lungs. Mean expression of pSTAT3 and pSTAT5 in cells from the Spleen **(B)** and Lungs (**C**). n=7. Data pooled from 2 independent experiments; each symbol represents an individual mouse, with mean shown. Statistical differences were determined using a two-way ANOVA. **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

I did not find any evidence to suggest that the increased Vγ6$^+$ cell numbers in CD18 KO mice corresponded to enhanced IL-7R-mediated signalling or downstream Bcl2 expression. I next explored the hypothesis that the cells may be accumulating due to decreased apoptosis. Apoptosis was measured in WT and CD18 KO γδ T cells by *ex vivo* staining with Annexin V and 7AAD, which stain cells undergoing early and late apoptosis. During the early stages of death Phosphatidylserine (PS), a phospholipid normally absent from the plasma membrane, translocates to the outer layer of the membrane. Annexin V is a protein that binds to PS and can therefore be used as a marker for early apoptosis [391]. As cell death progresses, the membrane integrity is lost, which allows the cell to passively take up dyes such as 7-amino-actinomycin-D (7AAD) [392,393].

Co-staining of cells with Annexin V and 7AAD allows segregation into three populations that indicates the state of apoptosis (Figure 5-14A). Cells that are alive will be Annexin V$^-$ 7AAD$^-$. Cells that are Annexin V$^+$ 7AAD$^-$ are undergoing early apoptosis. In the transition into late apoptosis, loss of membrane integrity will allow 7AAD to enter into the cell, resulting in a population of Annexin V$^+$ 7AAD$^+$ cells. Using this gating strategy, γδ T cell subsets were assessed for the different stages of apoptosis. The percentage of Vγ4$^+$ γδ T cells that were live, undergoing early or late apoptosis remained equivalent regardless of the tissue they were isolated from or the genotype of the mice (Figure 5-14B). In contrast, Vγ6$^+$ cells from the spleen of CD18 KO mice showed a significant increase in late apoptotic cells, accompanied by a decrease in those undergoing early apoptosis. In Vγ6$^+$ cells isolated from the lungs, there was a decreased proportion of late apoptotic cells, with an increase in live cells (Figure 5-14B&C).

The altered apoptotic state of Vγ6$^+$ cells in CD18 KO mice implies that, in the absence of β_2 integrins, more Vγ6$^+$ cells in the spleen are undergoing late stage apoptosis, as opposed to the early stage. Importantly, the most striking effect was seen in the lungs where there was a reduction in γδ T cell death in the CD18 KO mice specifically in the Vγ6$^+$ population. This decrease in apoptosis may contribute to the accumulation of Vγ6$^+$ cells in CD18 KO mice.

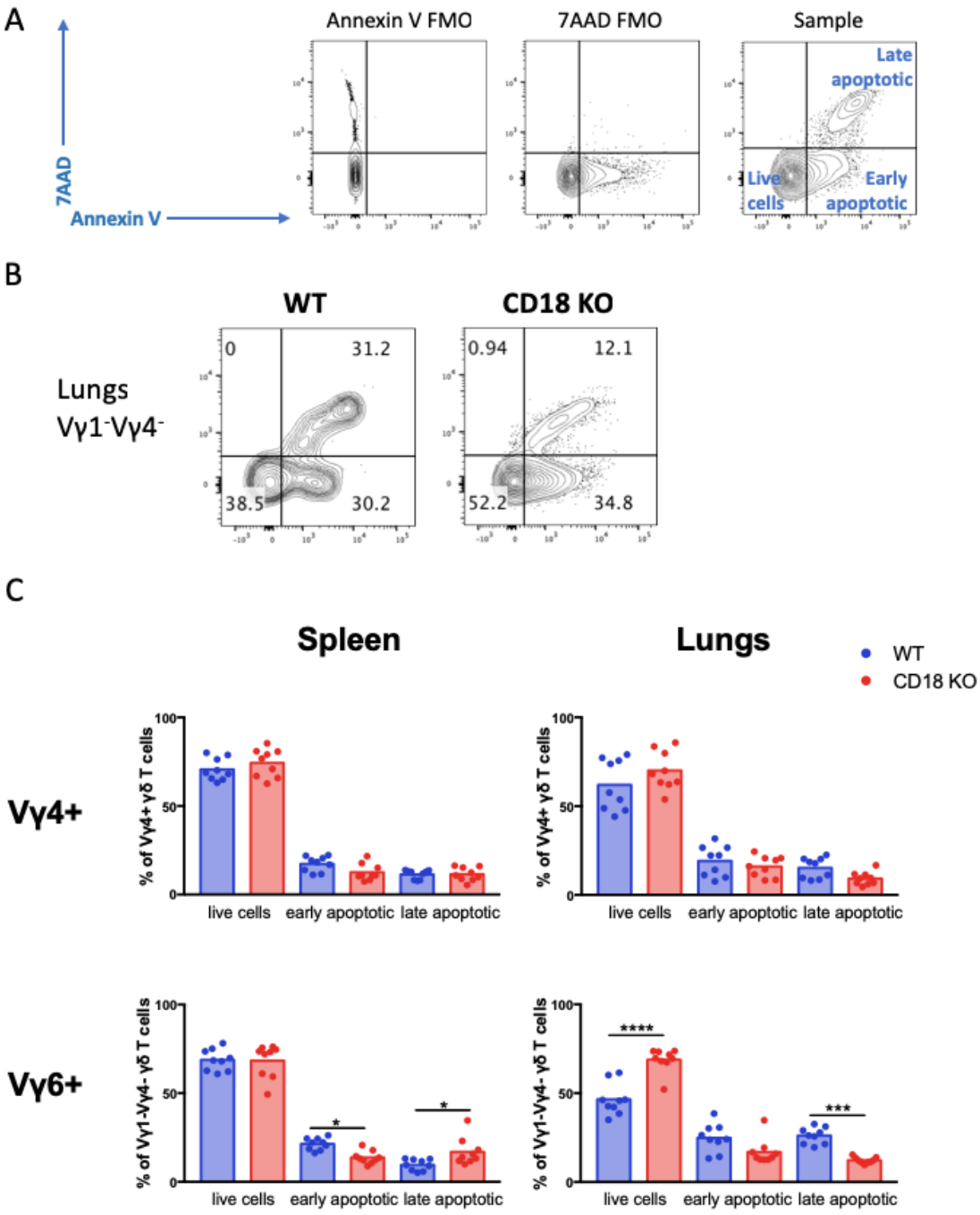

Figure 5-14 Reduced apoptosis of Vγ6⁺ γδ T cells in the lungs of CD18 KO mice. Cells were isolated from WT and CD18 KO spleen and lungs then analysed by flow cytometry. Samples were gated on $CD3^+TCR\gamma\delta^+$ followed by $V\gamma4^+$, or $V\gamma1^-4^-$ ($V\gamma6^+$), then Annexin V^- $7AAD^-$ (live cells), Annexin V^+ $7AAD^-$ (early apoptotic) or Annexin V^+ $7AAD^+$ (late apoptotic). **(A)** Gating strategy from WT spleen. **(B)** Representative plots of Vγ6+ cells from the lungs. **(C)** Percentages of γδ T cell subsets at each stage of apoptosis. n=9. Data pooled from 3 independent experiments; each symbol represents and individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

An increase was found in the apoptosis of Vγ6$^+$ γδ T cells in the lungs of WT mice compared to CD18 KO, however the expression of apoptosis markers indicates the current stage of death the cell is in, but not the upstream mechanism that triggered the apoptotic pathway within the cell. Therefore a potential upstream regulator of this altered cell death was explored. Fas (CD95) is a death receptor that when engaged on T cells triggers death by apoptosis[394]. Fas is known to be expressed on γδ T cells and it has been shown previously that in mice lacking Fas there is an expansion of γδ T cells[395]. I hypothesise that the reduced apoptosis of Vγ6$^+$ γδ T cells in the lungs of CD18 KO mice may be linked to reduced Fas expression.

The results show that Fas expression can be detected on γδ T cells (Figure 5-15A), and the levels of Fas were similar across Vγ4- and Vγ6-expressing γδ T cells in the spleen from both WT and CD18 KO mice (Figure 5-15B). Likewise, in the lungs there was no difference in the percentage of Fas$^+$ Vγ4$^+$ cells. Conversely, a significantly lower proportion of Vγ6$^+$ γδ T cells expressed Fas in the lungs of CD18 KO mice compared to WT (Figure 5-15A&B). This fits with my hypothesis that Vγ6$^+$ cells display reduced apoptosis, specifically in the lungs of CD18 KO mice due to reduced Fas expression.

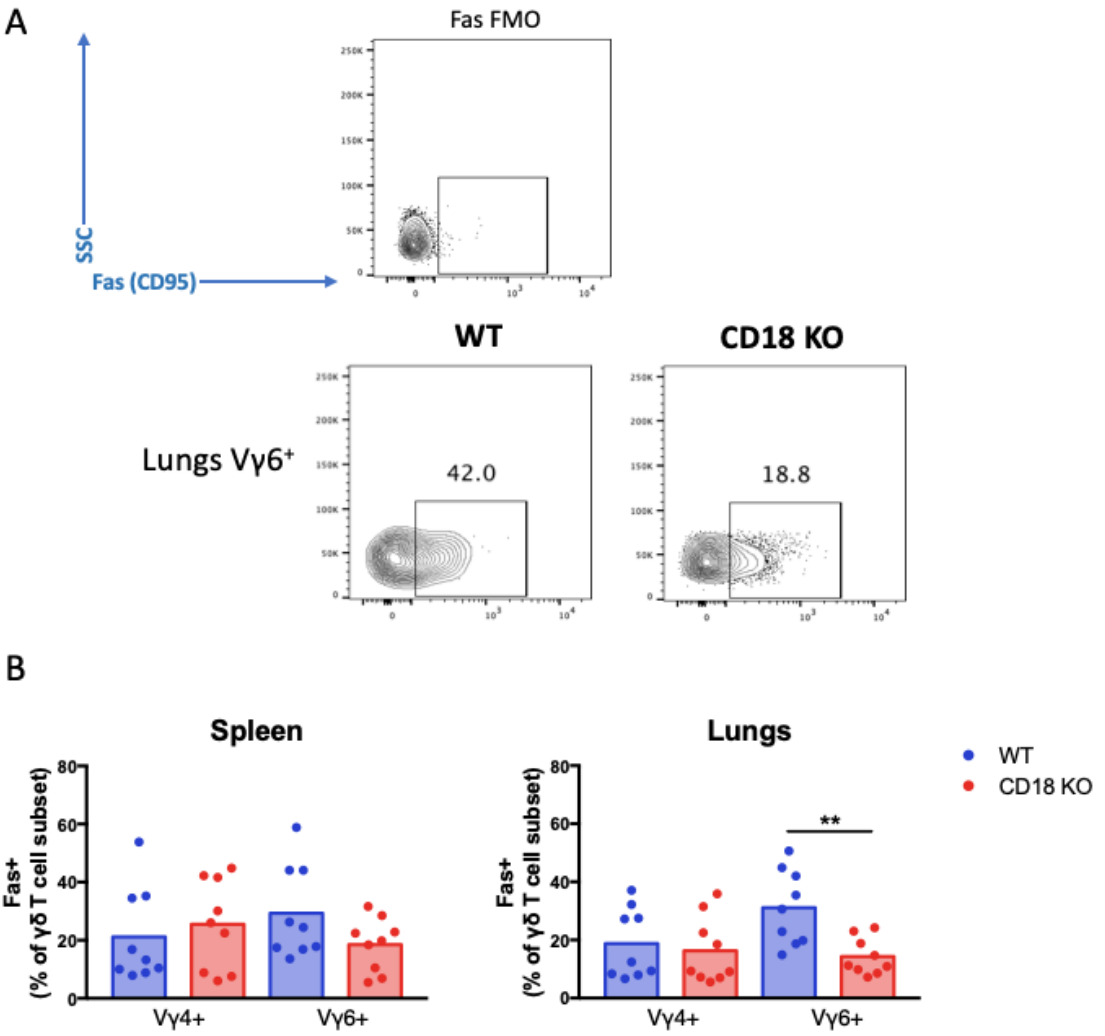

Figure 5-15 Expression of Fas is reduced on Vγ6$^+$ γδ T cells in the lungs of CD18 KO mice compared to WT.
Cells were isolated from adult WT and CD18 KO thymus, spleen and lungs then analysed by flow cytometry. Samples were gated on CD3$^+$TCRγδ$^+$ followed by Vγ1$^+$, Vγ4$^+$, or Vγ1$^-$4$^-$ (Vγ6$^+$), then Fas$^+$ (CD95). **(A)** Representative plots of the FMO and Vγ6 cells from the lungs. **(B)** Percentages of Fas$^+$ γδ T cell subsets. n=9. Data pooled from 3 independent experiments; each symbol represents and individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

There was a reduced expression of Fas on Vγ6$^+$ γδ T cells in the lungs of CD18 KO mice and these cells displayed a decreased apoptosis compared to WT mice. Based on these findings, I hypothesised that the increased number of Vγ6$^+$ γδ T cells in CD18 KO mice is due, at least partially, to a failure of homeostatic cell death which results in their accumulation. To examine if CD18 KO mice show an enhanced accumulation of Vγ6$^+$ cells as a result of reduced apoptosis (identified in adult mice), I determined the number of Vγ6$^+$ cells in the spleen and lungs of aged mice (20-24 weeks old) and plotted this alongside the numbers previously shown from embryonic, neonatal, 4 week old and adult mice (Figure 4-3 and Figure 5-6).

The number of Vγ6$^+$ γδ T cells in the lungs of WT mice steadily increased from E18.5 to 4 week old, were slightly reduced in adults then further increased in aged mice. In the CD18 KO lungs an increase in Vγ6 numbers was also seen between E18.5 and 4 weeks, but at a faster rate than in WT. The number of Vγ6$^+$ cells did not increase between 4 week old and adult mice, but did increase between adult and aged mice, at a similar rate to WT. In the spleen the difference in Vγ6 numbers between WT and CD18 KO mice then remained consistent in adult and aged mice.

Taken together, this data suggests that the number of Vγ6$^+$ cells in CD18 KO mice increases at a greater rate than in WT during early life. By 4 weeks old, the established difference in Vγ6 numbers between WT and CD18 KO mice does not further increase in adults or aged mice. Further work is therefore required to determine if the decreased apoptosis of Vγ6$^+$ γδ T cells in the lungs of CD18 KO results in an increased accumulation of these cells relative to WT mice.

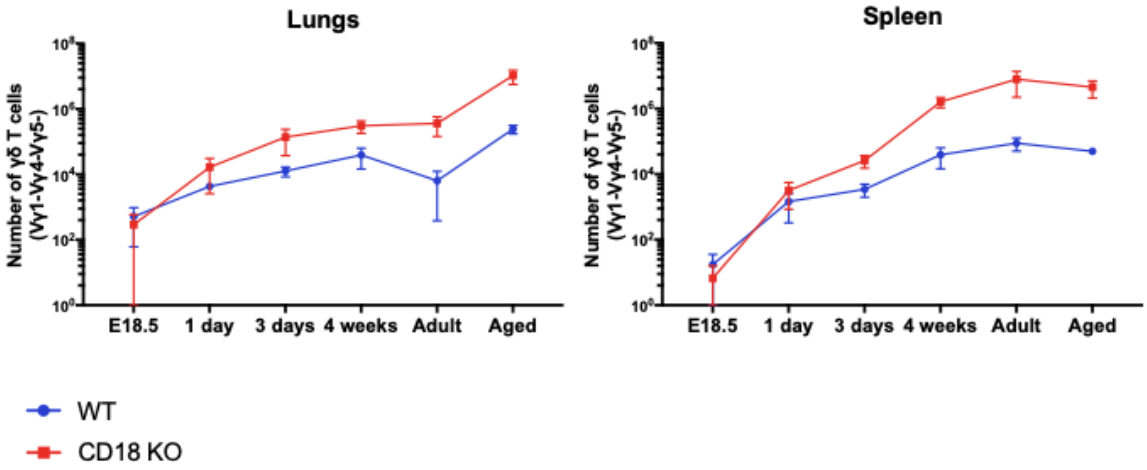

Figure 5-16 Differences in the number of Vγ6⁺ γδ T cells found in young CD18 KO mice compared to WT do not further increase from 4 weeks old onwards.
WT and CD18 KO mice were culled at E18.5, 1 day old, 3 days old, 4 weeks old, 8-12 weeks old (adult) and 20-24 weeks old (aged) and cells from the spleen and lungs were analysed by flow cytometry. Samples were gated on $CD3^+TCR\gamma\delta^+$ then $V\gamma1^-4^-5^-$ ($V\gamma6^+$). Absolute number of $V\gamma6^+$ cells in the spleen and lungs are shown. WT spleen & lungs 3 days n=6. CD18 KO spleen 1 day, WT/CD18 KO lungs 1 day, WT/CD18 KO spleen 4 weeks, WT lungs 4 weeks n=7. WT spleen 1 day n=8. CD18 KO spleen 3 days, CD18 KO lungs 3 days/4 weeks, WT spleen E18.5 n=10. CD18 KO spleen/lungs E18.5 n=11. Adult spleen/lungs n=10. Data pooled from 4-6 independent experiments. Aged spleen/lungs n=4 from 1 experiment. Data represents mean ± SD.

5.3 Discussion

The aim of this chapter was to explore potential mechanisms to explain the increased number of Vγ6$^+$ γδ T cells in CD18 KO mice. I found no evidence of enhanced thymic development or proliferation of these cells in the periphery, that would account for the elevated numbers of Vγ6$^+$ cells. In the spleen, Vγ6$^+$ cells expressed higher levels of the IL-7R accompanied by reduced downstream signalling and lower expression of Bcl2, whereas no differences were seen in the lungs. It can be concluded there is no evidence of enhanced IL-7-mediated survival of Vγ6$^+$ cells from CD18 KO mice. Reduced apoptosis was detected in Vγ6$^+$ cells from the lungs of CD18 KO mice, associated with a decreased expression of Fas. This data suggests that in β_2 integrin-deficient mice there is an accumulation of Vγ6$^+$ cells due, at least in part, to a reduced Fas-mediated apoptosis in the lungs.

5.3.1 Role for β_2 integrins in thymic development of Vγ4- but not Vγ6-expressing γδ T cells

The first hypothesis for the mechanism underlying Vγ6$^+$ γδ T cell expansion in CD18 KO mice explored, was an increase in thymic output. Surprisingly in embryonic and newborn mice the number of Vγ6$^+$ cells in the thymus was unaffected by β_2 integrin loss. As there was no difference in the thymic numbers of Vγ6$^+$ γδ T cells at any of the ages studied, it seems unlikely that the increased numbers in CD18 KO mice are due to altered development. In addition to this, greater numbers were seen in the tissues from 1-day post birth but at this age or during prior embryonic development the Vγ6$^+$ population in the thymus was normal. Unexpectedly, analysis of the embryonic/neonatal thymus identified a defect in the development of Vγ4$^+$ γδ T cells.

This defect was present in the immature Vγ4$^+$ cells in the embryonic thymus and in the mature population from 3 days old onwards. This suggests that CD18 is an important factor in promoting Vγ4$^+$ cell development but is not completely necessary. As CD18 loss initially affected the immature Vγ4$^+$ cells it is likely that the β_2 integrin-ligand interaction plays a role in the early stages of Vγ4$^+$ cell development. Within the immature population, the Vγ4$^+$ cells expressed lower levels of CD44, a marker that is upregulated during maturation, therefore in addition to the defect in Vγ4$^+$ cell selection there may be a delay in maturation. The impaired initial

thymic production also impacted the numbers of mature Vγ4$^+$ cells in the thymus and periphery. Interestingly, in adult mice the defect in Vγ4$^+$ cell numbers was only found in the peripheral LNs, but not at any other lymphoid site. In WT mice Vγ4$^+$ γδ T cells represented a significant proportion of the γδ T cells in the spleen, blood and lungs, yet in CD18 KO mice there was no difference in the number of Vγ4$^+$ cells despite their reduced development. This could indicate that the migration of Vγ4$^+$ cells into peripheral LNs is partially dependent on β_2 integrins. Alternatively, there may be factors present in the spleen/lungs but not pLNs that either expand or retain the Vγ4$^+$ cells. The expansion of Vγ4$^+$ cells in the spleen/lungs could overcome the thymic defect, or the absence of factors that retain Vγ4$^+$ cells in the pLNs would result in a preferential retention in other sites such as the spleen/lungs.

What remains unclear is how the loss of CD18 expression causes impaired development of Vγ4-expressing γδ T cells. To answer the question of which β_2 integrin subunit and ligand are required for normal Vγ4 development, *in vitro* foetal thymic cultures (FTOCs) using WT thymus and integrin/ligand blocking antibodies could be used. Blocking using an anti-CD18 would determine if β_2 integrin receptor-mediated interactions are required and using an anti-CD11a would confirm if this effect is CD11a-dependent, as was found for the peripheral effect on Vγ6$^+$ γδ T cells (Figure 4-11). Experiments using the α-chain KO mice to analyse thymic γδ T cell development in embryos/neonatal mice would also help to elucidate which β_2 integrin receptor(s) is involved in promoting Vγ4$^+$ cell development.

The main ligand for CD11a is ICAM-1, which is expressed on both thymic epithelial cells and thymocytes[372,396]. Therefore, the interaction between γδ T cell progenitors expressing CD18/CD11a and thymic epithelial cells expressing ICAM-1 may be required for optimal Vγ4$^+$ cell development. In particular, cortical thymic epithelial cells (cTECs) are known to play a role in IL-17-producing γδ T cell development: The loss of cTECs results in a defective production of Vγ4$^+$ cells, whereas the generation of Vγ6-expressing γδ T cells continues in greater numbers after birth[397]. This study highlights that epithelial cells in the thymus can play a differential role in Vγ4$^+$ versus Vγ6$^+$ cell development. Another factor known to be involved in thymic development of IL-17$^+$ γδ T cells is the transcription factor SRY-related HMG-box 13 (Sox13). The expression of Sox13 in Vγ4$^+$ cells is highest at the immature (CD24$^+$) stage, and in mice deficient in Sox13 there is a specific defect in the

development of these γδ T cells[218,219]. In CD18 KO mice it is also only Vγ4$^+$ cell thymic development that was impaired and at the earlier stage of development, not other γδ T cell subsets, therefore the loss of β_2 integrin expression may cause a defective upregulation of Sox13 that is required for optimal Vγ4$^+$ γδ T cell development. Examination of Sox13 expression in γδ$^+$ thymocytes from neonatal WT and CD18 KO mice would clarify whether Sox13 plays a role in the impaired development of Vγ4$^+$ γδ T cells in β_2 integrin-deficient mice. Another important consideration is which phenotype of Vγ4$^+$ cells show impaired development in CD18 KO mice. Vγ4$^+$ γδ T cells can produce either IL-17 or IFNγ, and the loss of Sox13 specifically affects the IL-17-producers[218,219]. Further analysis of Vγ4$^+$ cells during thymic development for the expression of CD27 and CD45RB (expressed only on IFNγ-producers) or for cytokine production following *in vitro* re-stimulation would identify which subset of Vγ4$^+$ are defective in CD18 KO mice.

5.3.2 No evidence for enhanced proliferation of Vγ6$^+$ γδ T cells in adult CD18 KO mice

There was no evidence of enhanced proliferation of γδ T cells from CD18 KO mice compared to WT within a 1-week timeframe in adult mice. This was concluded following measurement of proliferation by two methods: adoptive transfer of CFSE-labelled cells and *in vivo* labelling with BrdU. It is not entirely surprising that an enhanced proliferation of Vγ6$^+$ γδ T cells was not detected in adult CD18 KO mice compared to WT, because both Vγ4$^+$ and Vγ6$^+$ cells have a reduced rate of proliferation in adults compared to younger mice [200]. In addition to this, a study using a tamoxifen-induced γδ T cell-specific RFP labelling, the frequency of CD27$^-$CD44^{hi} RFP$^+$ γδ T cells (IL-17-producers, both Vγ4$^+$ and Vγ6$^+$) was the same at 7 weeks post tamoxifen treatment as after 2 weeks [245]. This suggests that the homeostatic turnover of these cells is slow, because they retained RFP expression.

There was a discrepancy in the data measuring proliferation of Vγ6$^+$ cells by Ki67 and BrdU (Figure 5-9), whereby expression of Ki67 was higher in CD18 KO cells but BrdU incorporation remained the same as WT. Ki67 is commonly referred to as a marker of proliferation[247,398], although it is more specifically associated with a cell being in active cell cycle[385]. A recent study described that Ki67 accumulates within cells during the S, G_2 and M phases and is degraded during the G_1/G_0 phases of the cell cycle [399]. They described that although Ki67 levels increase during cell cycle

progression, the initial expression depends on how long a cell has been quiescent for, i.e. the more time spent quiescent, the lower the initial level of Ki67. Whereas BrdU will be incorporated into a cell during DNA replication (i.e. S-phase), in the timeframe where BrdU is present. Taken together, the increased expression of Ki67, but not incorporation of BrdU, that I found in CD18 KO Vγ6$^+$ cells could indicate that they spend a longer time in active cell cycle whilst not progressing into proliferation. Alternatively, these cells may be proliferating at an increased rate, but slowly, i.e. longer than 1 week. Therefore, it can be concluded that there is no evidence to support enhanced proliferation of adult CD18 KO Vγ6$^+$ γδ T cells in a 1 week period, but a role for greater proliferation in these cells cannot be completely excluded.

The suggestion of potential changes in the cell cycle in β_2 integrin-deficient γδ T cells is interesting, as integrin receptors are known to be involved in cell cycle regulation. The progression of the cell cycle requires interactions between the cell and extracellular matrix (ECM) via integrins[400]. The cell cycle is tightly regulated by cyclin-dependent kinases (CDKs). Activation of the kinase activity of CDKs by cyclins stimulates the phosphorylation of substrate molecules, which drives progression into the next stage of the cell cycle. In human T cells the activation of the beta2 integrin ligand ICAM-1 has been shown to induce phosphorylation and subsequent inactivation of cdc2 Kinase[367]. In mice the homolog for cdc2 is CDK1, which is important for increasing the activation of integrins and their clustering at the site of cell adhesion (e.g. area of the cell interacting with the endothelium) in the G1 to S phase[401]. Following this, CDK1 inhibition triggers a loss of adhesion to enable entry into mitosis[401]. These studies highlight the potential interplay between integrin-mediated adhesion and cell cycle regulation, although further work is required to determine if these pathways are altered in β_2 integrin-deficient γδ T cells.

One of the remaining questions is where Vγ6$^+$ γδ T cells are expanding in CD18 KO mice. No difference was found in the proliferation of Vγ6$^+$ cells in adult mice, therefore there is not an obvious site of expansion. When γδ T cells from newborn mice were analysed, the Vγ6$^+$ cells were increased in number in the lungs of CD18 KO mice from 1 day old, but not in the spleen until 3 days old. I therefore hypothesise that the Vγ6$^+$ cells are expanding within the lungs; hence the increased numbers are first seen in this tissue, then due to recirculation the Vγ6$^+$ numbers are then higher in the spleen of CD18 KO mice. Although Vγ6$^+$ cells are thought of as a

tissue-resident population, a proportion of the cells have been shown to traffic into the blood and LNs under homeostasis, but migration to the spleen was not explored[402]. It is also possible that the loss of CD18 expression on Vγ6$^+$ cells impairs their retention within the lung tissue, thereby increasing their recirculation. The adoptive transfer of CD18 KO T cells did not show a difference in the number of γδ T cells that migrated to the lungs or spleen between WT and CD18 KO hosts. This suggests that the migration and/or retention of KO γδ T cells to the lungs is not due to a difference in factors present in the CD18 KO microenvironment but due to an intrinsic property of the KO γδ T cells. However, as this was not compared to the migration of transferred WT γδ T cells (due to issues with cell yield from WT) it cannot be concluded whether β_2 integrin deficiency affects γδ T cell retention in the lungs. The co-transfer of labelled WT and CD18 KO Vγ6$^+$ γδ T cells would allow a direct comparison to determine the effect on β_2 integrin loss on Vγ6 retention in the lungs, but this would require the *in vitro* expansion of the cells to obtain sufficient numbers for transfer.

Although there was no evidence for altered environmental factors in CD18 KO mice compared to WT causing altered expansion or migration of Vγ6$^+$ cells following adoptive transfer, increased levels of IL-1β and IL-23 were found in the spleen but not the lungs of CD18 KO mice. This is in agreement with a previous study describing increased IL-1β in the spleen of CD18 KO mice[387]. This could indicate that there is a higher number of cells producing these cytokines, or that production is increased by cells in the spleen. APCs are the predominant producers of both IL-23 and IL-1β. An increased number of MFs and DCs were found in the spleen of CD18 KO mice but not the lungs (McIntyre & Morrison; data not shown), both of which are known sources of IL-1β and IL-23. A previous study suggested that in CD18 KO mice the expansion of γδ T cells is due to enhanced levels of IL-23[303,403]. They describe a model whereby under homeostatic conditions MFs and DCs phagocytose apoptotic neutrophils, leading to the downregulation of IL-23. Whereas in CD18 KO mice the defective migration of neutrophils into tissues prevents IL-23 downregulation, leading to an increase in IL-17 production by γδ T cells. They showed that *in vitro* culture of BMDCs with apoptotic neutrophils decreased their IL-23 secretion (measured by the ability of conditioned media to induce IL-17 secretion from splenocytes) and *in vivo* transfer of WT neutrophils, but not CD18 KO, decreased the mRNA expression of the p40 subunit of IL-23 in the mLN, and serum

levels of IL-17. This suggests that neutrophil migration can affect the levels of IL-23 and IL-17, however the direct impact of IL-23 on the number of γδ T cells was not demonstrated. In agreement with this model, I found increased levels of IL-23 in the spleen of CD18 KO mice, although the study did not directly measure IL-23 levels. Furthermore, I have demonstrated that the increase in IL-17-producing γδ T cells in CD18 KO mice is due to a specific subset of Vγ6-expressing cells. It should also be noted that the neutrophilia described in the CD18 KO mice (caused by a defect in neutrophil migration) was found in both the CD11a and CD11c KO strains (McIntyre & Morrison; unpublished data), but the γδ T cell expansion was specific to the CD11a-deficient mice. This suggests that there is not an intrinsic link between the expanded neutrophils and γδ T cells, which was the mechanism suggested in the paper, therefore alternative or additional mechanisms may be involved. I have identified a novel mechanism for the increase in γδ T cells through a reduced apoptosis of Vγ6$^+$ cells in the lungs. This may occur in addition to the neutrophil-mediated effects, although further experiments are required to determine whether the defect in neutrophil migration affects the γδ T cell numbers through the proposed mechanism.

There could be altered levels of additional cytokines (other than IL-1β or IL-23) in CD18 KO mice that induce the proliferation of Vγ6$^+$ γδ T cells, which I did not look at in my study. IL-2 has been implicated in the maintenance or expansion of Vγ6$^+$ cells, as mice deficient in IL-2 or IL-25 (IL-2R) displayed reduced numbers in the periphery despite normal thymic development[350]. It has also been shown that Vγ6$^+$ cells bind IL-2 with a higher affinity than Vγ4$^+$ cells[248]. Overall, as there were no differences found in the proliferation of Vγ6$^+$ γδ T cells in adult CD18 KO mice, the levels of IL-23, IL-1β, IL-7, and IL-2 should be compared in WT and CD18 KO neonatal mice, where there is more likely to be an enhanced proliferation that is potentially cytokine-driven.

Environmental factors other than cytokines could also play a role in the increased number of Vγ6$^+$ cells in CD18 KO mice. In recent years there has been evidence suggesting a role for the microbiota in the regulation of Vγ6$^+$ γδ T cell numbers. Vγ6$^+$ CD3^{bright} γδ T cells in WT mice were decreased when they were housed in germ-free conditions or treated with antibiotics[346]. Another study found that, in the absence of the IL-17 receptor, Vγ6$^+$ IL-17-producing γδ T cells were increased and

the proposed mechanism was that microbiota-activated CD103$^+$ DCs were driving γδ T cell proliferation through cell-cell contacts[351]. It is therefore possible that the absence of CD18 results in alterations to the microbiota that modify the immune response, resulting in Vγ6 expansion. Although the role of the microbiota in regulating the increased number of Vγ6$^+$ cells in CD18 KO mice was not directly addressed, it can be speculated whether it is likely to play a role. Firstly, it should be noted that the CD18 KO mice were housed in individually vented cages, which limits environmental exposure. Secondly, I used littermate controls in some experiments to control for potential differences in the microbiota. The analysis of γδ T cells in embryonic/neonatal mice used WT littermates, where an increased number of Vγ6$^+$ cells was found in the spleen and lungs. Also, for the experiments in chapter 4 where Vγ chains were analysed by flow cytometry, a mixture of WT mice that were bought externally and WT littermates from the CD18 KO colony were used. Importantly, the increased number of Vγ6$^+$ cells in CD18 KO mice was consistent regardless of the source of the WT mice. It was also shown in a model of reduced CD18 expression that mice developed psoriasis at the same incidence and magnitude when under germ-free or specific-pathogen free conditions[365]. This suggests, though does not definitively prove, that the mechanism of γδ T cell expansion in CD18 KO mice is likely to be independent of the microbiome.

5.3.3 Vγ6$^+$ γδ T cells in β$_2$ integrin-deficient mice display no difference in IL-7-mediated survival

The increased expression of IL-7R on Vγ6$^+$ cells in the spleen but not the lungs of CD18 KO mice highlights that the cells may have a different capacity to respond to IL-7 or be exposed to different levels of IL-7, depending on the tissue microenvironment in which they reside. Interestingly, Vγ6$^+$ cells in the lungs of both WT and CD18 KO mice expressed high levels of the IL-7R. In the WT spleen IL-7R expression was reduced compared to the lungs, whereas in the CD18 KO spleen the Vγ6$^+$ cells expressed levels similar to those in the lungs. This could be explained by an increased recirculation of Vγ6$^+$ cells from the lungs to the spleen in CD18 KO mice, compared to WT where the cells remain more tissue-resident. If the increased numbers of Vγ6$^+$ cells originate in the lungs, then the numbers may exceed the level at which the cells can be effectively retained in the tissue, therefore the cells enter the circulation and accumulate in the spleen. If Vγ6$^+$ cells from the lungs retain their

high levels of the IL-7R when they migrate to the spleen, this would explain why in CD18 KO mice the Vγ6$^+$ cells in the spleen express higher levels of the IL-7R.

Given that differences were found in the IL-7R expression on γδ T cells, I further explored the downstream signalling. The *in vitro* stimulation of spleen and lung cells from WT and CD18 KO mice with IL-7 resulted in the upregulation of pSTAT5 and to a lesser extent pSTAT3, as seen previously[235]. In CD18-deficient cells the upregulation of pSTAT5 was impaired in both Vγ4$^+$ and Vγ6$^+$ cells whereas a pSTAT3 defect was only seen in the Vγ4-expressing γδ T cells. The phosphorylation of both STAT3 and STAT5 are known to be induced by IL-7R engagement[404] but pSTAT3 upregulation occurs to a greater extent in IL-17-producing γδ T cells than in IFNγ producers[235]. Again, the alterations in γδ T cells from the CD18 KO spleen were not found in those isolated from the lungs, re-enforcing the tissue specific differences seen between lungs and spleen. The IL-7 stimulation assay was performed using unsorted cells, so the number of Vγ6$^+$ cells per well from CD18 KO mice would have been higher than from WT. It could be argued that the quantity of IL-7 per cell would therefore be lower in the cultures from KO mice, however it has been estimated that using 0.01-0.1ng/ml IL-7 *in vitro* recapitulates the levels found *in vivo* [405] and maximal levels of pSTAT5 should be induced after exposure to 0.1ng/ml of IL-7 [406]. As the levels used here were far in excess of this (100ng/ml), the maximal signalling is likely to have been achieved. The defect in pSTAT5 signalling was also found in the Vγ4$^+$ population, for which numbers were equivalent between WT and CD18 KO mice, suggesting that cell numbers and IL-7 availability are not responsible for the defect in STAT phosphorylation.

Vγ6$^+$ cells in the spleen showed impaired signalling immediately downstream of the IL-7R (Figure 5-13) despite expressing higher levels of receptor (Figure 5-11). This higher expression of IL-7R was accompanied by a decreased expression of Bcl2 (Figure 5-12), which is in agreement with a previous study that identified higher expression of Bcl2 in IL-7R^{low} γδ T cells compared to IL-7R^{high} [407]. The reduced expression of Bcl2 in CD18 KO splenic Vγ6$^+$ cells suggests a reduced survival, which fits with the apoptosis data, where Vγ6$^+$ cells from the spleen of CD18 KO mice displayed an increase in apoptosis compared to WT. However, this reduced Bcl2 expression was only found in Vγ6$^+$ cells from the spleen of adult mice (8 weeks or older) but not in mice aged 4 weeks old (Figure 5-12). This may indicate that there

is an age-dependent effect, whereby the initial increase in Vγ6$^+$ cells occurs in the young mice following birth then during adulthood the cells accumulate. This accumulation is at least partially driven by a reduced apoptosis in the lungs, although in the spleen there may not be enough survival signals to sustain the expanded Vγ6$^+$ population, hence the increased apoptosis and decreased Bcl2 expression. Expression of the IL-7R is downregulated in response to IL-7 binding, so the increased expression on Vγ6$^+$ cells in the spleen may reflect lower levels of IL-7. I also found that Vγ6$^+$ cells in the spleen showed impaired signalling downstream of the IL-7R.

It was only the initial signalling immediately downstream if the IL-7R in response to IL-7 that was measured. Therefore, the impact this impaired signalling through pSTAT3 and pSTAT5 has on downstream pathways and on cell proliferation is unknown. *In vitro* IL-7 stimulation of cells with reduced CD18 expression for 5 days selectively increased the CD27$^-$ γδ T cells compared to cells from WT mice[408]. Therefore, a longer culture of γδ T cells from WT and CD18 KO mice using IL-7 would help to elucidate if there is a difference in their functional response to IL-7, however as the starting cell populations from the spleen/lungs of WT and CD18 KO mice are different, the γδ T cells would need to be sorted prior to culture. This adds the caveat of the low number of γδ T cells that can be obtained from WT mice. Overall, there was no evidence of enhanced IL-7-mediated survival of Vγ6$^+$ γδ T cells in CD18 KO mice, shown by short-term *in vitro* cultures and *ex vivo* staining for Bcl2.

5.3.4 Vγ6$^+$ γδ T cells in β$_2$ integrin-deficient mice display reduced Fas-mediated apoptosis in the lungs

There was a decreased apoptosis of Vγ6$^+$ cells in the lungs of CD18 KO mice compared to WT. This effect was found to be both γδ subset-dependent and tissue-specific as the apoptosis of Vγ4$^+$ cells was unaffected by β$_2$ integrin loss, as were the Vγ6$^+$ cells in the spleen. Analysis of the death receptor Fas on Vγ6$^+$ cells, showed that in the lungs of CD18 KO mice these cells express lower levels of Fas, in addition to their reduced apoptosis. It is therefore likely that the reduced apoptosis of Vγ6$^+$ cells in the lungs of CD18 KO mice is at least partially mediated by reduced Fas-mediated signalling compared to WT mice. A decrease in apoptosis indicates that there is an increase in cell survival, although the mechanism of this higher

survival is unclear as this was not an IL-7-dependent effect. Bcl2 expression in Vγ6$^+$ cells was either unchanged or significantly reduced in CD18 KO mice compared to WT. This suggests that the increase in Vγ6$^+$ γδ T cells in β_2 integrin-deficient mice is not due to an enhanced survival mediated by the upregulation of Bcl2. The anti-apoptotic molecule Bcl2 is part of the intrinsic death pathway, whereas the Fas receptor activates the extrinsic apoptosis pathway[409]. Given that reduced expression of Fas was identified on Vγ6$^+$ cells in the lungs of CD18 KO mice, it is likely that the increased survival was mediated by a reduction in signalling downstream of the extrinsic apoptosis pathway. Caspase-8 is activated downstream of Fas, which can cleave other downstream caspases such as caspase-3[410]. Assessment of these caspase molecules in Vγ6$^+$ cells from WT and CD18 KO mice would determine if there are differences in the activation of caspases downstream of the Fas receptor.

The lower levels of Vγ6$^+$ cell apoptosis in the lungs of CD18 KO mice were identified in adult mice, therefore I hypothesised that there may be an increased accumulation of Vγ6$^+$ cells compared to WT, due to a failure of homeostatic cell death. Analysis of aged mice showed that the difference between WT and CD18 KO Vγ6$^+$ cell numbers present in adults does not become greater as the mice age, indicating that there is not an enhanced accumulation of these cells in CD18 KO mice compared to WT. However, reduced apoptosis may contribute to the retention of Vγ6$^+$ cells in CD18 KO mice, while other mechanisms could retain the cells in WT mice. The comparison of Vγ6$^+$ γδ T cell numbers across all the ages analysed (Figure 5-16) highlights that the difference between WT and CD18 KO mice occurs prior to 4 weeks of age. Data from neonatal CD18-deficient mice, showed that Vγ6$^+$ cells are increased in the tissues from 1 day post birth. Taken together with the data showing that there is no difference in Vγ6$^+$ cell proliferation in adult mice, I propose that there is an expansion of Vγ6-expressing γδ T cells in young CD18 KO mice and these cells are then retained in the adults.

5.3.5 The loss of CD18 may affect the expression of receptors and ligands that are β_2 integrin-dependent or independent

One of the important questions that remains unanswered is whether the loss of β_2 integrin expression has a cell-intrinsic effect on the γδ T cells or affects another cell type that indirectly leads to the expansion of Vγ6$^+$ cells. The generation of mice with

a γδ T cell-specific loss of CD18 expression would be an effective way to test this, although this would first require a CD18-floxed mouse to be created. It could be argued that as the Vγ6$^+$ cell expansion is dependent on CD11a (Figure 4-11), and T cells rely on CD11a, whereas other leukocytes express multiple β_2 integrin receptors that may compensate for the loss of CD11a, the defect could be T cell-mediated. To rule out the potential involvement of αβ T cells, the CD18 KO mice could be crossed with an αβ-deficient line. Alternatively, CD18 KO γδ T cells could be used to reconstitute γδ T cell deficient mice by adoptive transfer. If the γδ T cell phenotype remained, then an indirect effect of αβ T cells would be excluded. Determining if the Vγ6$^+$ cell expansion effect is intrinsic to γδ T cells will be pivotal in the future plans for this study.

A second question is why the expansion effect is restricted to Vγ6-expressing γδ T cells. For some subsets of γδ T cells, molecules that drive their specific expansion and retention have been identified. Both the development and retention of Vγ5$^+$ cells is linked to their interaction with Skint1 [214,215]. The expression of Skint1 in thymic epithelial cells is involved in Vγ5$^+$ cell selection, after which they home to the epithelium where Skint1 is expressed in keratinocytes. Similarly the expression of Btnl1 in the intestines selectively drives the maturation and expansion of Vγ7$^+$ γδ T cells[216]. The expression of such a ligand for the Vγ6-expressing γδ T cells has not yet been identified. It is possible that a specific ligand exists that is important for the proliferation and retention of Vγ6$^+$ cells in sites such as the lungs, and that it is upregulated in CD18 KO mice. Alternatively, the γδ T cell interactions with the epithelium mediated by β_2 integrins themselves may be driving the enhanced population of Vγ6$^+$ γδ T cells, as discussed previously.

Unlike αβ T cells, γδ T cells are not restricted to recognising peptides presented in the context of MHC molecules. Several ligands for the γδ TCR have been identified including the algal protein phycoerythrin[250], phospholipid antigens in the context of CD1d[251] and more recently it was shown that the Vγ7 TCR has distinct sites to bind both antigen and the co-stimulatory molecule Btnl[217]. However, to date the ligand for Vγ6$^+$ γδ T cells has not been identified. This population of γδ T cells express a TCR with an identical sequence (invariant), but can be induced by a variety of stimuli including infection with Mycobacterium[411] or Listeria[412], ovarian cancer[283] and

CFA[199]. It is therefore likely that Vγ6$^+$ cells are not solely activated through a single TCR-restricted ligand.

Finally, it is possible that the increase in Vγ6$^+$ cells in CD18 KO mice may not be due directly to the loss of CD18, but instead to an indirect effect whereby CD18 loss results in the upregulation of another integrin receptor, or downregulation of something else within the cell. In CD18 KO mice the expression of VCAM-1 was found to be upregulated in the pulmonary arteries, accompanied by an 5-fold increase in the number of VLA-4$^+$ ($\alpha_4\beta_1$) leukocytes in the alveolar walls of these mice[413]. These VLA-4$^+$ leukocytes may represent the expanded population of γδ T cells. In humans, VLA-4-ICAM-1 interactions were shown to be involved in the interactions between γδ T cells and endothelial cells[298] and in mice Vγ1$^+$ and Vγ4$^+$ γδ T cells were described as expressing VLA-4[294]. Analysis of VLA-4 expression on Vγ6$^+$ γδ T cells from WT and CD18 KO mice may reveal an $\alpha_4\beta_1$ integrin-mediated migration or retention of these cells in the lungs. Although an increase in VLA-4 may cause the altered distribution of γδ T cells, it doesn't explain the increased numbers of Vγ6$^+$ cells found in CD18 KO mice, or the reduced apoptosis and expression of Fas that I have identified.

5.4 Conclusions

In conclusion, β_2 integrin receptors play an important role in promoting the development of Vγ4$^+$ γδ T cells and in the peripheral regulation of Vγ6$^+$ γδ T cells. The thymic production of Vγ4$^+$ cells was reduced in CD18 KO mice from the embryonic stage and into early life, which resulted in a defect in Vγ4$^+$ numbers, specifically in pLNs. The increased numbers of Vγ6$^+$ cells were present in the lungs of CD18 KO mice from 1 day post-birth and in the spleen from 3 day old mice. In adult mice there was no difference in IL-7R-mediated signalling or survival on Vγ6$^+$ cells, but they accumulated due to reduced apoptosis in the lungs of CD18 KO mice compared to WT. The decreased expression of Fas on CD18 KO Vγ6$^+$ cells suggests that the lower level of apoptosis was due to reduced Fas-mediated cell death. These findings highlight the novel and differential roles for β_2 integrins on IL-17-producing γδ T cell subsets. Further work is required to determine the precise mechanisms of these roles and establish the functional outcomes.

Chapter 6 Final Discussion

The β_2 integrins are a family of leukocyte-specific adhesion receptors that play key roles in cell migration, adhesion and interactions. Traditionally, the functions of β_2 integrins have been associated with immune activation. This is based on their involvement in the migration of αβ T cells and neutrophils to LNs and tissue sites during inflammation, immune synapse formation between αβ T cells and APCs, binding of cytotoxic T cells/NK cells to target cells, and phagocytosis of complement-coated particles by myeloid cells/granulocytes. More recently, there is accumulating evidence that β_2 integrins also play a regulatory role in the immune system. In both mice and humans, the loss of β_2 integrin expression is associated with inappropriate inflammation and autoimmunity. The overall aim of this thesis was to understand the immunoregulatory roles of β_2 integrins. This was explored in DCs and γδ T cells, based on preliminary evidence in the literature for integrin-mediated regulation of these cell types.

6.1 Summary of the role for β_2 integrins in DC regulation

In the absence of β_2 integrin expression, DCs were hyperresponsive to TLR-mediated signalling[182] and in the absence of TLR signals, DCs with functionally-deficient β_2 integrins displayed enhanced activation based on co-stimulatory molecule expression and IL-12 production[73]. These studies suggest that β_2 integrins act as a negative regulator of DC activation. In chapter 3, I showed the specific loss of CD11a or CD11c expression causes the enhanced co-stimulatory molecule expression on DCs generated. In addition, BMDCs lacking CD11a were capable of inducing greater T cell responses. This furthers our understanding of how β_2 integrins regulate DCs, through identification of the specific subunits responsible for this regulation, namely CD11a and CD11c.

6.2 Mechanisms of DC regulation by β_2 integrins

The primary aim in chapter 3 was to determine which β_2 integrin subunits are responsible for regulating DCs and what effect this has on the subsequent T cell response. Importantly, these studies were conducted using DCs deficient in each of the four α-subunits (CD11a, CD11b, CD11c or CD11d) to provide a complete and unbiased assessment of β_2 integrin-mediated regulation in DCs. This provides

additional knowledge compared to previous studies where DCs were examined from total β_2 integrin-deficient mice[73,97,182], CD11b KO alone[185] or CD11a compared with CD11b KO mice[182].

Whilst these studies were ongoing, it was discovered that the CD11a, CD11c and some CD11d KO mice expressed an additional mutation in the *Dock2* gene (*Dock2*^{Hsd}). The focus of chapter 3 then became to identify which effects described in the CD11a and CD11c KO mice were integrin-dependent and which were due to the unknown defect in Dock2 function. Crucially, I identified that the enhanced co-stimulatory molecule expression by CD11a and CD11c KO BMDCs in these KO mice was due to the β_2 integrin-deficiency and independent of *Dock2*^{Hsd}. Heterozygous expression of CD11a/CD11c was sufficient to induce these effects relative to WT or mice with hom/het expression of *Dock2*^{Hsd}. The alterations in DC phenotype should be confirmed in CD11a and CD11c KO mice with *Dock2*^{WT} expression to consolidate these findings.

The enhanced co-stimulatory molecule expression on CD11a and CD11c KO BMDCs generated *in vitro* may suggest that the loss of integrin expression has a cell-intrinsic effect on DCs, meaning that β_2 integrin receptors directly regulate DC activation. Alternatively, there may be changes in epigenetic programming in the mouse due to the altered environment. The enhanced expression of CCR7 on CD11a KO BMDCs suggests that integrin loss can also have indirect effects on DC function. To determine whether the increased levels of CCR7 enhance CD11a KO BMDC migration, transwell assays could be used to compare the migration of WT and CD11a KO BMDCs to the CCR7 ligands CCL19 and CCL21. In addition, the co-transfer of labelled WT and KO BMDCs would determine if there are differences in CD11a KO BMDC ability to migrate to LNs *in vivo.*

It is well established that CD11a expressed on CD4 T cells binds to ICAM-1 on the APC to mediate immune synapse formation, but what is less well characterised, is the importance of CD11a expressed on the DC binding to ICAM-1 on the T cell. I showed that the loss of CD11a on BMDCs reduced their ability to bind to ICAM-1, which is in agreement with previous findings where BMDCs expressing constitutively active CD11a displayed enhanced adherence to ICAM-1[88]. A decreased number of DC-T cell conjugates form when CD4 T cells are cultured with CD18 KO BMDCs compared to WT (Morrison, unpublished data), suggesting that

the loss of β_2 integrin expression on DCs does reduce their ability to form contacts with T cells. Despite this evidence for reduced adhesion to T cells, the co-culture of CD11a KO BMDCs with antigen-specific CD4 T cells demonstrated their ability to induce greater levels T cell activation, proliferation and differentiation than WT BMDCs. This may indicate that β_2 integrin expression on the T cell mediates binding that is sufficient for T cell activation. It should be noted; this data should first be confirmed using CD11a KO BMDCs with WT *Dock2* expression. Perhaps there is a threshold for the strength of interaction that is required to enable T cell activation, and despite reduced adherence by CD11a KO BMDCs, the binding is still above the required threshold. To investigate if this is the case, TCR signal strength could be measured using T cells from Nur77^{GFP} mice, which express GFP from the *Nr4a1* (Nur77) locus, so GFP is activated in response to TCR signalling and correlates with signal strength[414]. Co-culturing Nur77^{GFP} T cells with WT and CD11a KO BMDCs would determine if there are differences in the strength of signal provided to T cells and using anti-CD11a antibody to block the integrin on the T cell side of the interaction would determine if CD11a expressed on the T cell is required for sufficient TCR signalling. Further to this, the titration of an inhibitor of TCR signalling, e.g. Zap-70 inhibitor[415], would determine whether there is a threshold of TCR signalling required for T cell activation.

This reductionist approach of using β_2 integrin-deficient BMDCs generated *in vitro*, allowed the specific effects of integrin subunit loss in BMDCs to be assessed and enabled the generation of sufficient numbers of BMDCs to carry out functional assays. It will be important for future studies to validate these results *in vivo* to determine the physiological relevance and functional significance of these findings. When I analysed DCs *ex vivo* from β_2 integrin-deficient mice, I found no difference in their activation marker expression. There are several possible reasons for this discrepancy between integrin-deficient BMDCs *in vitro* and endogenous DCs *in vivo*. It is possible that the *in vitro* system is simply not representative of DCs *in vivo*. The GM-CSF/IL-4-cultured bone marrow cells that I refer to as BMDCs (based on expression of MHC II) are in fact derived from both common DC precursors (CDP) and common monocyte precursors[337]. This is in contrast to endogenous DCs that only develop from CDPs[128] therefore, BMDCs actually represent a mixed population of DCs and macrophages. However, a previous study using β_2 integrin signalling-deficient mice showed that *in vivo* splenic DCs, gated by CD11c$^+$ MHC II$^+$ cells, did express higher levels of CD86[73]. The β_2 α-chain KO mice have a global integrin

deficiency, therefore not only the DCs will be affected by integrin loss, and alterations in other immune cell populations may indirectly impact on the activation status of the DCs. The generation of a DC-specific β_2 integrin KO mouse, using $CD11c^{Cre}$ and $CD11a^{flox}$ mice, would determine any effects of integrin loss that are due to the absence on DCs. The analysis of DCs *ex vivo* encompassed a mixed population of DCs, as cells were gated on dump negative (to exclude T cells, B cells, NK/NKT cells, monocytes/MFs, pDCs and neutrophils) then MHC II^+. Therefore, the separation into specific subsets, e.g. cDC1 and cDC2, may reveal that β_2 integrin-mediated regulation of DCs is a subset-specific effect. Finally, the improvement of DC purity for analysis, as discussed in chapter 3, would also reduce variability in the data because the study using signalling-deficient mice[73] gated through CD11c which was not possible in my case, due to the use of CD11c KO mice.

6.3 The relevance of β_2 integrin-mediated regulation of DCs to disease

There is clear evidence that the loss of β_2 integrin expression is linked to the development of autoimmunity in both humans[119,120] and mice[122,124]. My data and previously published studies[73,88,97,182] suggest that loss of β_2 integrin function in DCs results in increased activation and enhances their ability to induce T cell responses. What remains to be determined is whether these two phenomena are linked. Does the loss of β_2 integrin expression/function in DCs contribute to the development of autoimmunity? The aberrant activation of DCs has been described in many autoimmune diseases[416]. It is possible that a reduction in β_2 integrin activation or expression results in a loss of negative regulation, thereby promoting DC-mediated priming of autoreactive T cells. This could be tested using mouse models of autoimmune disease but would require a DC-specific system. This could be achieved through the adoptive transfer of WT or integrin-deficient DCs, or the generation of DC-specific integrin KO mice. Work is also currently ongoing in the lab to assess β_2 integrin expression and activation on DCs from rheumatoid arthritis patients, which will help to determine the potential relationship between integrin-mediated regulation of DCs and autoimmune disease.

6.4 Summary of the mechanism of β_2 integrin-mediated regulation in γδ T cells

Mouse models of β_2 integrin-deficiency can develop inflammation in the skin or oral mucosa that is associated with an increase in IL-17-producing γδ T cells[296,302]. It was also shown under steady state conditions that CD18 KO mice have an increased number of γδ T cells in the cervical LNs[297] and IL-17$^+$ γδ T cells in the spleen[303]. Based on these findings my hypothesis was that β_2 integrins are a novel regulator of IL-17-producing γδ T cells. In chapter 4, I identified that in addition to the spleen, the loss of CD18 expression leads to increased numbers of γδ T cells in the lungs, uterus and blood. This increase was due to the specific expansion of Vγ6Vδ1$^+$ γδ T cells that display an enhanced potential for IL-17 production. This is the first evidence identifying β_2 integrins as a negative regulator of Vγ6$^+$ γδ T cells.

In chapter 5 I explored the potential mechanism(s) underlying the increase in Vγ6$^+$ γδ T cells in CD18 KO mice. Analysis of γδ T cells in the thymus and peripheral tissues of CD18 KO mice from foetal stage to adults revealed that β_2 integrins play different roles in Vγ4- and Vγ6-expressing γδ T cell subsets (summarised in Figure 6-1). The thymic development of Vγ6-expressing cells was unaffected by β_2 integrin-loss, whereas Vγ4$^+$ γδ T cell development was impaired (Figure 6-1A). This reduced thymic development resulted in a lower number of Vγ4$^+$ cells in peripheral LNs. On the contrary, an increased number of Vγ6$^+$ γδ T cells were found in the tissues (lungs & spleen) of neonatal mice (Figure 6-1B) and their numbers continued to increase in CD18 KO mice above their WT counterparts up to 4 weeks old. In the periphery of adult mice, the active proliferation of Vγ6$^+$ cells could not be detected, and I found no evidence to suggest any factors present in CD18 KO mice that were driving Vγ6$^+$ γδ T cell expansion. Vγ6$^+$ γδ T cells did display reduced apoptosis in the lungs and expressed lower levels of Fas, suggesting a reduced susceptibility to induced cell death via the Fas pathway (Figure 6-1C). Overall, these studies reveal a previously unknown role for β_2 integrins in promoting the thymic development of Vγ4$^+$ γδ T cells and regulating Vγ6$^+$ γδ T cell numbers in the periphery.

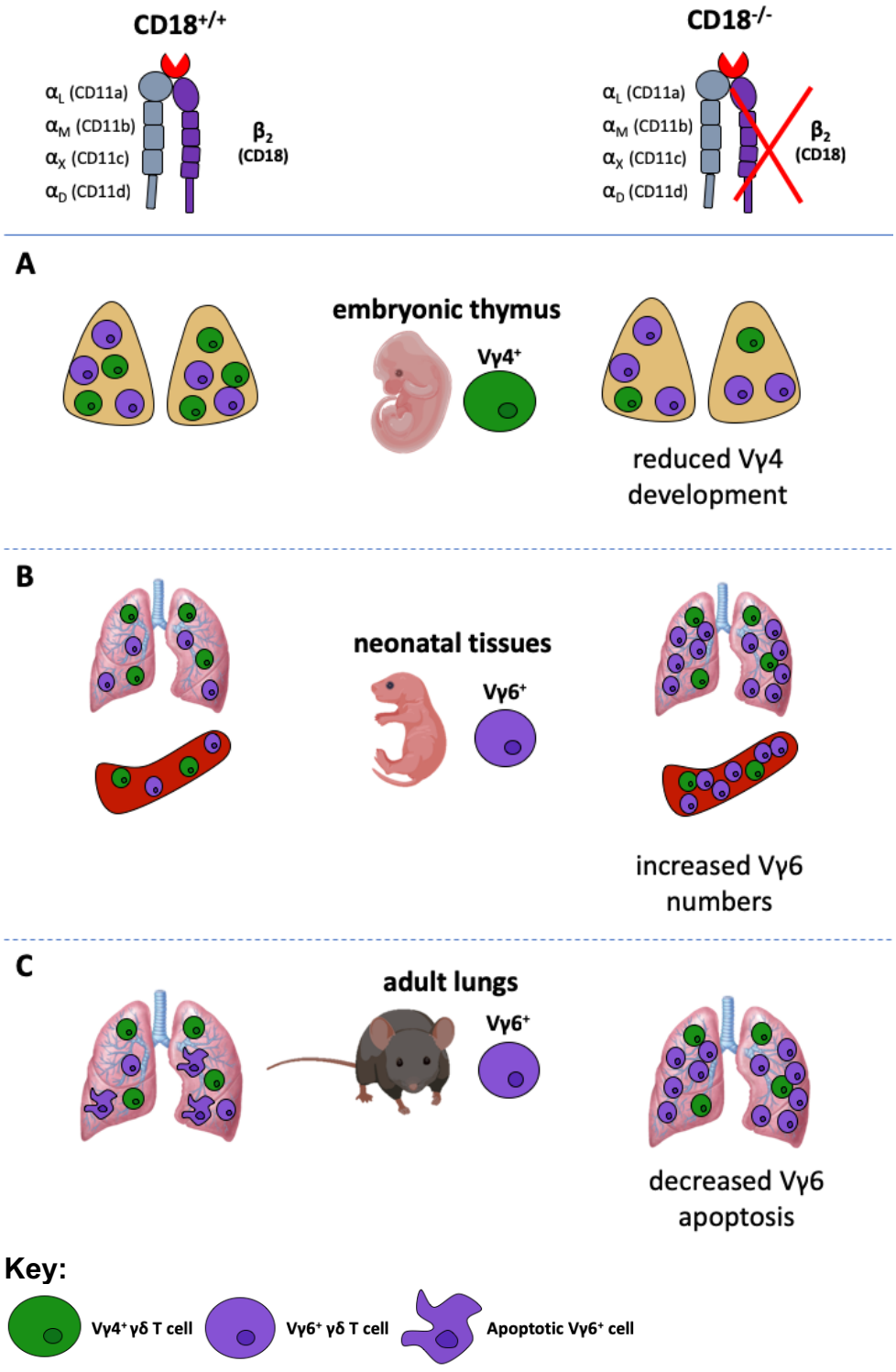

Figure 6-1 Differential effects of β_2 integrin loss of Vγ4$^+$ and Vγ6$^+$ γδ T cells

6.5 Potential mechanisms of γδ T cell expansion in β_2 integrin-deficient mice

The main unanswered question from this data is whether or not the increase in γδ T cells is due to a cell-intrinsic effect. β_2 integrins are expressed on most leukocytes but the levels of individual family members varies according to cell type. I identified the specific loss of CD11a causes an increase in γδ T cell numbers. CD11a is highly expressed on both γδ and αβ T cells, potentially indicating the increase in γδ T cells is a T cell-mediated effect. This raises the question of whether integrin loss on αβ T cells could indirectly increase γδ T cell numbers. A previous study demonstrated that αβ T cells inhibit the homeostatic expansion of γδ T cells[417]. As αβ T cell migration to LNs and tissues is impaired in CD18 KO mice this raises the possibility that the loss of β_2 integrin expression prevents αβ T cell-mediated regulation of γδ T cells due to altered localisation of αβ T cells. However, the mechanism of γδ T cell regulation identified was through competition with αβ T cells for IL-15[417], a cytokine required for the expansion of $CD27^+$ but not $CD27^-$ γδ T cells[235]. As the expanded population in CD18 KO mice are $CD27^-$ γδ T cells, this competition for IL-15 is unlikely to be the mechanism here. In addition to this, there is contradictory evidence that CD4 T cells in fact enhance IL-17^+ γδ T cells numbers and cytokine production[418]. However, my data showed increased numbers of γδ T cells present in mice from 1 day post-birth, an age at which very few αβ T cells are present, making it unlikely to be an αβ T cell-driven mechanism of γδ T cell expansion in the CD18 KO mice.

I propose that the loss of CD11a/CD18 expression on γδ T cells leads to a loss of regulation as a direct result of the absence of CD11a-mediated signals, thereby they increase in number. Analysis of CD11a expression on γδ T cells in WT mice showed higher expression on $Vγ6^+$ cells than other γδ T cell subsets in the lungs and uterus, the tissues where these cells usually reside. This may partially explain why the loss of CD18 expression only affects Vγ6-expressing cells in specific tissues. Under homeostasis, signals in the lungs/uterus tissues may induce CD11a upregulation to perform important functions in $Vγ6^+$ γδ T cells. In αβ T cells, particularly $CD8^+$, TCR-mediated signalling leads to the upregulation of CD11a[74,329]. Therefore, there may be a Vγ6 TCR ligand present in the lungs and uterus that drives enhanced CD11a expression. There is evidence that CD11a expression on γδ T cells is required for

their cytotoxic functions[79,80], but its functional role in other types of γδ T cell remains undetermined.

It cannot be ruled out that CD18 loss on a non-T cell population may be responsible for the increased number of γδ T cells in β_2 integrin-deficient mice. Expression of CD11a has been identified on neutrophils[66], dendritic cells[27] and macrophages[31], therefore the loss of CD11a is likely to also have a functional impact on these cells. A common approach to determine cell-intrinsic effects is to use bone marrow chimeras, however as Vγ6$^+$ γδ T cells are produced exclusively during embryonic/neonatal stages of thymic development, this would not provide any insight. It has previously been shown that following irradiation and bone marrow transplantation, Vγ6$^+$ cells remain absent whereas Vγ1- and Vγ4-expressing γδ T cells can develop[243]. The best approach to determine if β_2 integrin loss has a cell-intrinsic effect on γδ T cells would be to create a γδ-specific knockout of CD11a or CD18. This requires mice with a CD11a or CD18 lox-P flanked sequence (which do not currently exist) crossed with mice expressing TCRδ Cre recombinase.

The increased number of Vγ6$^+$ γδ T cells in CD18 KO mice occurs despite normal thymic output, indicating that factors in the periphery are contributing to the expansion of γδ T cells above the numbers found in WT mice. I was unable to detect proliferation of γδ T cells *in vivo* following BrdU labelling in adult mice or adoptive transfer of cells from adult mice, indicating that KO γδ T cells are not undergoing proliferation in adult mice. I identified that Vγ6$^+$ cells in the adult lungs express lower levels of Fas and display reduced apoptosis. This indicates a reduction in cell death, therefore is likely to contribute to the accumulation of Vγ6$^+$ cells in CD18 KO mice. Further experiments are required to determine if reduced Fas expression on CD18 KO Vγ6$^+$ cells renders them resistant to Fas-induced cell death. This could be determined by measuring the apoptosis of Vγ6$^+$ cells following incubation with Fas ligand and a cross-linking antibody in vitro. In addition to this, the reduction in Vγ6$^+$ γδ T cell apoptosis implies they have an increased survival. My data suggests that this survival is independent of IL-7R-mediated signalling and Bcl2, but further analysis of survival pathways may identify alterations in other survival-related signalling molecules.

Analysis of γδ T cell numbers in the tissues of WT and CD18 KO mice across different ages identified that the higher number of γδ T cells is present from 1 day

following birth. This indicates that the factor/s driving the increase in Vγ6$^+$ γδ T cells is present in CD18 KO mice from neonatal age. This raises the question of what mechanism is responsible for the increased γδ T cell numbers in neonatal CD18 KO mice. There are a number of factors that could be contributing to the increase in γδ T cells in CD18 KO newborn mice such as cytokines, loss of signalling downstream of β_2 integrin receptors or altered signalling via other receptors/ligands. RNA sequencing of Vγ6$^+$ cells from WT and CD18 KO neonatal mice would provide further information on the pathways that are altered in γδ T cells in the absence of β_2 integrins and may provide indications for the upstream regulators. For example, alterations in integrin-related pathways would indicate an intrinsic effect on the γδ T cells whereas changes in cytokine-related pathways would suggest the impact of factors in the microenvironment. It is possible that although no differences in the proliferation of Vγ6$^+$ cells are being detected in adult mice, there is an enhanced expansion of these cells in young CD18 KO mice. Analysis of γδ T cell proliferation in WT and CD18 KO neonatal mice would confirm if Vγ6$^+$ cells are proliferating earlier in life. Alternatively, the increased number of Vγ6$^+$ cells may be due to an enhanced survival, as suggested by the data obtained from adult mice. When Vγ6$^+$ cells leave the thymus, presumably only a proportion of those cells that migrate to the lungs will actually survive therefore there may be an increased survival of Vγ6$^+$ cells that initially seed the lungs in CD18 KO mice compared to WT. It has been hypothesised that Vγ5$^+$ γδ T cells initially colonise the epidermis to form small clusters that undergo homeostatic expansion to fill the niche[419]. Perhaps, if the same phenomenon occurs for Vγ6$^+$ cells, then either the initial colonisation or homeostatic expansion of Vγ6$^+$ γδ T cells is enhanced in the lungs of CD18 KO mice compared to WT.

6.6 Implications of the expanded Vγ6$^+$ γδ T cell population in CD18 KO mice

Having established there is an expansion/accumulation of Vγ6$^+$ γδ T cells in β_2 integrin-deficient mice, the next step would be to determine the functional consequences. There are many studies that have described protective or pathogenic effects of Vγ6$^+$ IL-17-producing γδ T cells in the setting of infection, autoimmunity or cancer. Mice deficient in γδ T cells showed impaired clearance of *Staphylococcus aureus* and efficient bacterial clearance was dependent on neutrophil recruitment via IL-17 production by γδ T cells[271,420,421]. In a similar

mechanism, fungal clearance following *Candida albicans* infection also required γδ T cell IL-17 production to induce neutrophil migration[274]. These studies show that Vγ6$^+$ γδ T cells are protective in response to certain infections. In an Aldara-induced model of psoriasis, Vγ6$^+$ γδ T cells were the main producers of IL-17 in the inflamed skin and exacerbated pathology[422]. In a model of autoimmune arthritis Vγ6$^+$ cells were also the predominant source of IL-17, and migration of these cells via CCL2 increased disease incidence[423]. In contrast to the examples of infection, Vγ6$^+$ cells play a pathogenic role in autoimmune disease. In the ID8 model of ovarian cancer, IL-17 production by Vγ6$^+$ cells enhanced tumour growth through recruitment of small peritoneal macrophages[283]. Similar to their role in autoimmunity, Vγ6$^+$ γδ T cells are also pathogenic in cancer.

Using these models in WT and CD18 KO mice, it would be expected that the increased population of Vγ6$^+$ γδ T cells would enhance the clearance of *S. aureus* or *C. albicans*, whereas exacerbate psoriasis, arthritis or cancer development. It was previously shown that CD11a KO mice display an increased susceptibility to tumour development using the B16 melanoma cancer model, despite NK cell cytotoxicity being unaffected[373]. As IL-17$^+$ γδ T cells have been shown to promote tumour growth in this model[424], the expanded population of Vγ6$^+$ cells may explain the increased susceptibility of CD11a KO mice to tumour development. However, the major caveat to infecting or inducing disease models in β_2 integrin-deficient mice is that the β_2 integrin defect is a global deficiency; therefore it would be difficult to attribute effects specifically to the expanded population of γδ T cells. In addition to this, the defective function of other immune cells may exacerbate or protect against disease. For example, the migration of αβ T cells is defective in CD18 KO mice and arthritis development is αβ T cell-dependent, meaning that CD18 KO mice will likely not develop disease to the same extent as WT. This was demonstrated in a study using the K/BxN model of arthritis, where CD11a KO mice were resistant to developing arthritis[124]. To circumvent this issue, models could be induced in WT mice or γδ T cell KO mice and then Vγ6$^+$ γδ T cells adoptively transferred. This would provide a more direct readout of γδ T cell function. Another question that remains is whether the CD18 KO γδ T cells are functionally different to WT. My data demonstrated that the Vγ6$^+$ cells have an enhanced capacity to produce IL-17 when re-stimulated *in vitro*, suggesting that they may produce higher levels of IL-17 than the WT counterparts. The transfer of WT and CD18 KO Vγ6$^+$ cells into WT mice during an infection or disease model would test this, however this would require *ex*

vivo expansion of the cells as only a small number of Vγ6$^+$ γδ T cells exist in WT mice, making it difficult to obtain sufficient numbers for transfer.

In addition to the question of what effect the increased number of Vγ6$^+$ γδ T cells in CD18 KO mice will have on their ability to respond to infection or disease, there are also a number of unanswered questions about the direct impact that integrin loss has on γδ T cell functional ability. An important function of β_2 integrins in several leukocyte populations is to enable migration[64,65,68], this raises the question of whether CD18 KO γδ T cells are able to efficiently migrate to target sites, e.g. to a tumour. My data showed that under steady state conditions, there was no defect in γδ T cell numbers in any of the tissues analysed, which suggests that γδ T cell migration is not dependent on β_2 integrins. However, this may not be the case during inflammation or disease and could be different depending on the subset of γδ T cell. The transfer of specific subsets of WT and CD18 KO γδ T cells into mice during an infection or cancer model would directly assess whether β_2 integrins are required to mediate γδ T cell migration to effector sites.

In addition to contributing to migration, β_2 integrins are also important for facilitating cell contacts. There is evidence from *in vitro* studies using human γδ T cells and integrin blocking antibodies to suggest that β_2 integrins are required for optimal killing of target cells by γδ T cells[79,80], although this has not yet been demonstrated *in vivo*. The cytotoxic capacity of γδ T cells also requires the delivery of signals mediated by receptors/ligands such as NKG2D[190] and FasL[425]. The ability of γδ T cells to receive signals via NKG2D and provide signals to target cells via FasL may be impacted by β_2 integrin loss, either through indirect effects on expression levels or by loss of cell interactions that are required to facilitate receptor-ligand binding. Analysis of NKG2D/FasL levels on γδ T cells from WT and CD18 KO mice, combined with culturing these γδ T cells with target cells to measure killing would help to clarify the effect of β_2 integrin loss on γδ T cell cytotoxic capability.

6.7 β_2 integrins promote Vγ4$^+$ γδ T cell development

My initial hypothesis was that β_2 integrins are a negative regulator of IL-17-producing γδ T cells. However, analysis of γδ T cell thymic development revealed an unexpected defect in Vγ4$^+$ γδ T cell development in the absence of CD18, suggesting that integrins play a role in promoting the development of this γδ T cell

subset. The mechanisms involved in the development of specific γδ T cell subsets are still not well understood. Vγ4$^+$ γδ T cells are capable of producing either IL-17 or IFNγ depending on their functional pre-programming. It has been suggested that IL-17-producers (that express Vγ6 or Vγ4) are generated in the thymus during embryonic and neonatal life, whereas Vγ4$^+$ cells that produce IFNγ are produced at the postnatal stage[426,427]. I did not characterise the phenotype of the Vγ4$^+$ cells that display impaired development in CD18 KO mice compared to WT. This could be done by staining cells from the neonatal thymus for IL-17/IFNγ and other markers that distinguish these subsets such as CD27, CD45RB and CD73[203,206,427]. The defect in Vγ4$^+$ γδ T cell development is present in the thymus of neonatal mice but not adults, which may suggest that the impaired development is likely to be the IL-17-producing Vγ4$^+$ cells. In the absence of Sox13, mice display a complete loss of Vγ4$^+$ γδ T cells but Vγ6$^+$ cells are present at normal frequency in the periphery[219], meaning the loss of β_2 integrin expression may reduce the levels of Sox13 and thereby impair Vγ4$^+$ γδ T cell development. Alternatively, the development of IFNγ-producing Vγ4$^+$ cells may be reduced in CD18 KO mice. There is evidence to suggest that strong TCR signals favour the development of IFNγ-producing γδ T cells[205,428], therefore cell adhesion via β_2 integrins could be required for optimal signalling, a function that has been demonstrated in αβ T cells[83,84]. The development of IFNγ-producing γδ T cells can also be promoted by co-stimulation via CD70-CD27[206], and again β_2 integrins may facilitate the cell interactions necessary to facilitate co-stimulatory molecule interactions. Further analysis of signalling pathways in thymic γδ T cells from WT and CD18 KO mice would elucidate the potential involvement of these molecules in promoting Vγ4 development downstream of β_2 integrin signalling.

The importance of β_2 integrin receptor interactions in promoting Vγ4$^+$ cell development in the thymus should also be further explored. Foetal thymic organ cultures (FTOCs) using a CD18 blocking antibody would confirm that β_2-mediated interactions are required for optimal Vγ4$^+$ γδ cell development. In addition, comparing Vγ4$^+$ cell development in FTOCs using CD18 KO donor thymocytes and WT stromal cells, with WT donor cells and CD18 KO stromal cells, would determine whether the defect in CD18 has an intrinsic effect on the γδ T cells.

Finally, the functional outcome of the reduced number of Vγ4$^+$ γδ T cells in CD18 KO mice should also be considered. In the B16 melanoma model, Vγ4$^+$ γδ T cells

play a protective role in decreasing the tumour burden through IFNγ and perforin production[279,429]. In CD18 KO mice the absence of anti-tumour Vγ4$^+$ cells in combination with the expanded pro-tumour Vγ6$^+$ cells would likely result in exacerbation of disease. Following infection with respiratory syncytial virus, Vγ4$^+$ cells producing IFNγ enhanced viral control and reduced disease severity[430] therefore, the reduced numbers of Vγ4$^+$ cells may enhance disease following viral infection. The protective role of Vγ4$^+$ cells has also been demonstrated in a model of sepsis, where IL-17 production by Vγ4-expressing γδ T cells delayed mortality in mice[431] whereas in a model of psoriasis IL-17-producing Vγ4$^+$ cells contributed to pathology[432,433]. The lower number of Vγ4$^+$ cells in CD18 KO mice could result in protection against pathology in psoriasis but exacerbated pathology in sepsis. Altogether these studies clearly demonstrate that the role of Vγ4$^+$ γδ T cells is model-dependent and differs according to their cytokine production. The determination of which type of Vγ4$^+$ cells (IFNγ- and/or IL-17-producers) show impaired development in β_2 integrin-deficient mice will elucidate the potential functional outcome.

6.8 Links to human γδ T cells

The data presented in this thesis demonstrated that in the absence of β_2 integrins, mice have an increased number of Vγ6$^+$ IL-17-producing γδ T cells. In future work the significance of these findings for human biology must be considered. In humans, β_2 integrin-deficiency exists in a disease called leukocyte adhesion deficiency (LAD). In type I LAD, patients have a mutation in the CD18 gene leading to reduced or absent β_2 integrin expression and in type III LAD, patients have mutations in kindlin-3 that prevent β_2 integrin activation[114]. It was shown previously that LAD I patients with periodontitis have an increase in IL-17-producing CD3$^+$ cells in their gingival tissue but not peripheral blood[302]. These cells were defined as TCRγδ$^-$ (although the data was not shown), and in the blood only the percentage of total T cells producing IL-17 was analysed. Further characterisation of the γδ T cell populations in LAD patients would provide more information on whether the loss of β_2 integrin expression results in an increase of γδ T cells in humans, as found in mice. Although a subset equivalent to the Vγ6$^+$ γδ T cells identified in mice does not exist in humans, analysis of the Vδ1- and Vδ2-expressing subsets, both of which have been shown to produce IL-17[236,237,434], may reveal a potential role for β_2 integrins in regulating specific subsets of human γδ T cells.

6.9 Potential therapeutic targeting of β_2 integrins

Pre-clinical and clinical studies have assessed the potential use of β_2 integrin blocking molecules, with mixed sucess[435]. In pre-clinical studies odulimomab, a monoclonal antibody against CD11a, reduced graft rejection in mice[436] and rats[437], and odulimomab protected against ischaemia and reperfusion injury following transplantation in primates[438]. This drug was not taken further into clinical trials. The blocking of CD11a or CD11b had a therapeutic effect in the mouse model of multiple sclerosis, EAE[439]. Whereas, rovelizumab, a humanised anti-CD11a, did not show any clinical benefit in a phase II trial using patients with acute exacerbations of MS[440]. Efalizumab, another humanised anti-CD11a, was shown to be an effective treatment for plaque psoriasis[441]. However, it was subsequently withdrawn due to 4 reported cases of multifocal leukoencephalopathy, a rare side effect thought to be caused by immunosuppression[442]. The CD11a antagonist BMS-587101 was shown to be protective in a mouse model of arthritis and inhibited T cell adhesion, proliferation and Th1 cytokine production[443]. It was designed for topical use in a phase II trial for psoriasis but was stopped after showing signs of hepatotoxicity[444]. A CD11a antagonist currently in clinical use is Lifitegrast, a small molecule inhibitor that acts as a competitive antagonist of CD11a binding to ICAM-1[445]. This drug is used in eye drops to decrease T cell-mediated inflammation and reduce eye dryness[446]. The success of Lifitegrast may be partially attributed to the topical application that limits potential side effects, such as broad immunosuppression.

These studies emphasise the potential issues with translating studies from animal models and the potential side effects of blocking β_2 integrins, such as immunosuppression. My data showed that in contrast to the functions previously described where β_2 integrins contribute to immune activation, these receptors also play a role in the negative regulation of DCs and γδ T cells. Taken together, this highlights the need for careful and effective drug design, considering the differential roles for β_2 integrins in the immune system.

6.10 Concluding Remarks

The data presented in this thesis has characterised the immuno-regulatory roles of β_2 integrins in DCs and γδ T cells. CD11a and CD11c act as negative regulators of DC activation and restrict the ability of DCs to induce T cell responses. Whereas in γδ T cells β_2 integrins play differential roles in γδ T cell subsets, promoting $V\gamma4^+$ γδ T cell development and regulating $V\gamma6^+$ γδ T cells in the periphery. These findings highlight the importance of β_2 integrins in regulating immune responses. Further work to understand the downstream pathways of this regulation will identify potential therapeutic targets for diseases where DC or γδ T cell responses are dysregulated.

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