Regulation of T cell migration by the guanidine exchange factor Vav1

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Well we know where we're going
But we don't know where we've been
And we know what we're knowing
But we can't say what we've seen
And we're not little children
And we know what we want
And the future is certain
Give us time to work it out

We're on a road to nowhere
Come on inside
Taking that ride to nowhere
We'll take that ride
We're on a road to nowhere

(Talking Heads)

Declaration of originality

I hereby declare that this thesis is of my own composition, describes my own work except where specifically stated and has not been submitted in any other application for a higher degree.

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Abstract

T cell migration to sites of inflammation is an essential step of an effective immune response. T cell recruitment is enhanced by the recognition of their cognate antigen presented by the endothelium and their retention in the tissue is controlled by resident antigen presenting cells. Vav1 is a guanidine exchange factor for RhoGTPases that is activated through TCR signalling and is important for the cytoskeletal re-arrangements occurring during T cell activation and migration. In this study we have investigated the role of Vav1 in the recruitment and retention of antigen-specific T cells to sites of inflammation.

HY-specific A^b-restricted WT and Vav1-/- T cells were generated and fully characterised for specificity and expression of migration-related markers. Vav1-/- T cells showed defects in adhesion, migration in response to ICAM-1, CXCL12 and CXCL10 and increased migratory speed in *in vitro* assays. Despite displaying defective motility *in vitro*, both constitutive migration and recruitment of Vav1-/- T cells to antigenic sites occurred normally. However, retention of Vav-1-/- T cells into antigenic tissue was profoundly impaired. This may be due to the inability by Vav-1-/- T cells to respond to 'stop' signals delivered by co-engagement of TCR and CD28. In contrast to recent reports on wild type T cells, Vav1-/- T cell migration and retention to sites of inflammation was not affected by engagement of the co-stimulatory molecule CD28, suggesting that Vav-1 recruitment during CD28-mediated signalling is instrumental for Vav-1-mediated regulation of T cell traffic

Abbreviations

Antigen presenting cells	APCs
Allophycocyanin	APC
Bone marrow	BM
Bovine serum albumin	BSA
Calponin Homology	CH
Carboxyfluorescein diacetate succinimyl ester	CFSE
Casitas-B-lineage lymphoma protein	Cbl
Central nervous system	CNS
Common variable immunodeficiency	CVID
Cutaneous lymphocyte antigen	CLA
Cystein rich domain	CD
Cytotoxic T lymphocyte associated protein	CTLA-4
Dbl homology domain	DH
Dendritic cells	DCs
Diaglycerol	DAG
4,6-diamidino-2-phenylindole	DAPI
Dulbecco's modified eagle media	DMEM
Endothelial cells	ECs
Ethylenediaminetetraacetic acid	EDTA
Extracellular signal regulated kinase	ERK
Experimental autoimmune encephalomyelitis	EAE
Experimental autoimmune uveitis	EAU
ezrin-moesin-radoxin	ERM
Fluorescein isothiocyanate	FITC
Fluoerescence activated cell sorter	FA
Foetal calf serum	FCS
Forkhead box O	Foxo
Glutamic acid decarboxylase	GAD
Glycosylation-dependent cell adhesion molecule-1	GlyCAM
Granulocyte macrophage colony stimulating factor	GMCSF
Grb2-related adaptor downstream of Shc	Gads
Guanidine exchange factors	GEFs
Guanidine triphosphate	GTP
High endothelial venules	HEV
Histocompatibility	H
Human immunodeficiency virus	HIV
Human leukocyte antigen	HLA

N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid	HEPES
I kappa B kinase	IKK
Immunoglobulin	Ig
Immunoreceptor tyrosine based activation motifs	ITAMs
Inducible co-stimulatory molecule	ICOS
Inositol triphosphate	IP3
Interferon	IFN
Interleukin	IL
Intracellular adhesion molecules	ICAMs
Intraperitoneally	Ip
Intravenously	Iv
Junction adhesion molecules	JAMs
Linker for activation of T cells	LAT
Leukocyte associated function antigen	LFA
Leukocyte specific protein tyrosine kinase	Lck
Lipopolysaccharide	LPS
Lymphocytic choriomeningitis virus	LCMV
Macrophage inflammatory protein	MIP
Macrophage receptor 1	Mac1
Major Histocompatibility Complex	MHC
mitogen activated protein	MAP
Monocyte chemottactic protein	MCP
Mucosal addressin cell adhesion molecule	MadCAM
N-formyl-methionylleucyl-phenylalanine	fLMP
Nuclear factor of activated T cells	NFAT
Nuclear factor kappa B	NFkb
Optimal cutting temperature	OCT
Phorbol myristate acetate	PMA
Phosphate buffer saline	PBS
Phosphoinositide 3 Kinase	PI3K
Phosphoinositol 4.5 bisphosphate	PIP2
Phospholipase C	PLCγ
Phospholipids phosphoinositol-3-phosphotase	PIP3
Phycoerythrin	PE
Pleckstrin homology	PH
Programmed cell death	PD
Protein kinase C	PKC
P-selectin glycoprotein ligand-1	PSGL-1

Src homology 2 domain	SH2
Src homology 2 domain-containing leukocyte protein of 76 kDa	Slp-76
T cell receptor	TCR
Thymus and activation-related chemokine	TARC
Thymus-expressed chemokine	TECK
Transporter for antigen presentation	TAP
Tumour necrosis factor	TNF
Vascular adhesion protein	VAP
Vascular cell adhesion molecule	VCAM
Very late antigen	VLA
WASp-interacting protein	WIP
Wiskott-Aldrich syndrome protein	WASp
Wild type	WT
Zeta chain associated protein 70	ZAP70

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Introduction

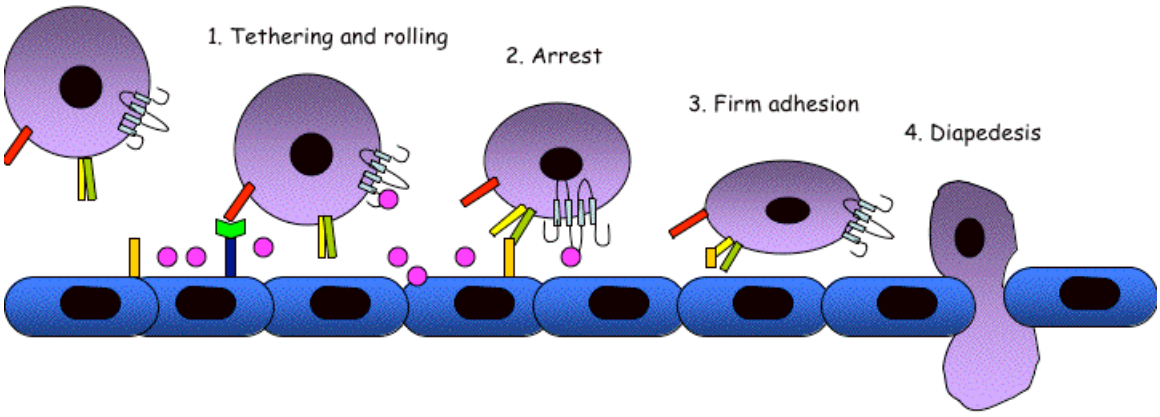
1. T cell migration

1.1. The multi-step paradigm of lymphocyte migration

From the moment they complete their development and leave the thymus, T cells constantly traffic in the bloodstream. Naïve T cells circulate in the blood and enter lymphoid organs such as lymph nodes or spleen, until they encounter antigen and get activated. Activated effector T cells must then exit the blood and enter peripheral tissues during the inflammatory response, while memory T cells continue re-circulating between peripheral tissues and secondary lymphoid organs (reviewed in Choi *et al*., 2004; reviewed in Cines *et al*., 1998; reviewed in Janeway *et al*., 2001).

The process of exiting the bloodstream through the vascular endothelium is regulated by several steps, which have been summarised as the "multi-step paradigm" of lymphocyte migration *(***Figure 1***)*. More specifically, lymphocytes tether and roll on the surface of the vascular bed, a process primarily mediated by selectins and some integrins. Chemokines present on the surface on the endothelium subsequently increase integrin affinity leading to arrest and tight adhesion to integrins. Finally, T cells cross the endothelium, in a process known as diapedesis. (reviewed in Cines *et al*., 1998; (reviewed in Ebert *et al*., 2005; reviewed in Garrood *et al*., 2006).

Figure 1: The multi-step paradigm of lymphocyte migration



Circulating T cells tether and roll on the surface of the vascular bed in a process mediated by selectins presented on the endothelium, and some integrins on the T cell (1). Chemokines presented on the apical surface of the endothelium in conjunction with shear force mediate integrin conformational changes leading to arrest (2) and firm adhesion (3). The cell undergoes morphological changes and transmigrates through the endothelium (4).

1.1.1 Rolling: selectins and their ligands

While lymphocytes are circulating in the bloodstream, they may tether and roll on the surface of the endothelium, in a process mediated by selectins. This mechanism slows the migrating lymphocytes down and allows them to initiate contact with the endothelium (reviewed in Garrood *et al*., 2006).

Selectins are type I transmembrane glycoproteins that bind to sialytated carbohydrate moieties (reviewed in Garrood *et al*., 2006; reviewed in Patel *et al*., 2002). They are primarily expressed on microvillus structures on the endothelial cell (EC) (Bruehl *et al*., 1996) (Stein *et al*., 1999), which promotes contact between the EC and the lymphocyte, thus mediating rolling (Stein *et al*., 1999).

Three selectins have been described, L-selectin, P-selectin and E-selectin (**Table** 1). L-selectin is expressed in most leukocytes and can bind to glycosylation-dependent cell

adhesion molecule (GlyCAM), mucosal addressin cell adhesion molecule (MadCAM), E-selectin, CD34 and Sgp200 in the high endothelial venules (HEV) of lymph nodes (reviewed in Garrood *et al*., 2006; reviewed in Patel *et al*., 2002). L-selectin has been shown to have an important role in lymphocyte migration, particularly in homing to lymphoid organs (reviewed in Garrood *et al*., 2006). T cells from L-selectin-deficient mice show defects in rolling (Arbones *et al*., 1994), while T cells from mice expressing low levels of L-selectin also display a similar phenotype, including increased rolling velocity *in vivo* (Galkina *et al*., 2007). In addition, L-selectin is important in the recruitment of T cells to inflammatory sites (reviewed in Cines *et al*., 1998), as L-selectin deficient mice show reduced T cell infiltration to sites of inflammation (Arbones *et al*., 1994)

P-selectin is a 140KDa protein expressed in platelets and ECs of inflamed tissues, where it is stored in granules which allow its effective expression during inflammation (reviewed in Cines *et al*., 1998; reviewed in Patel *et al*., 2002). Its major ligand is P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed on both myeloid and lymphoid cells (reviewed in Garrood *et al*., 2006).

E-selectin is expressed on inflamed endothelium, but unlike P-selectin, it is transcriptionally activated during the inflammatory response by inflammatory mediators such as tumour necrosis factor (TNF)-alpha and interleukin (IL)-1 (Bevilacqua *et al*., 1987; reviewed in Garrood *et al*., 2006; reviewed in Patel *et al*., 2002). Its major ligand is cutaneous lymphocyte antigen (CLA) in humans and PSGL-1 in mouse, while it can also bind L-selectin (reviewed in Garrood *et al*., 2006).

Table 1: Selectins and their ligands

Selectin	**Ligands**
L-selectin (CD62L)	GlyCAM, MadCAM, E-selectin, CD34 and Sgp200
P-selectin	PSGL-1
E-selectin	CLA, L-selectin

Other than selectins, activated T cells have also been shown utilise CD44 for entry into sites of inflammation. The latter can bind to hyaluronate and this interaction has been found to induce rolling and adhesion (DeGrendele *et al*., 1997). In addition, vascular adhesion protein (VAP)-1 has been shown to be important in rolling as well as transmigration to inflammatory sites and lymphoid tissues (Stolen *et al*., 2005). Integrins, which will be discussed in detail later on, are also thought to be involved in rolling on the vascular endothelium (Janeway *et al*., 2001).

1.1.2. Arrest: chemokines

Once lymphocytes start rolling on the endothelium, they may succumb to shear forces of the blood flow and become detached, or they may arrest. This event is controlled by chemokines (reviewed in Ebert *et al*., 2005).

Chemokines are small proteins with four conserved cysteins joined by a disulphide bond. They can be distinguished as CC or CXC chemokines, depending on whether the cysteins are adjacent or separated by one amino acid (reviewed in Baggiolini, 1998; reviewed in Ebert *et al*., 2005). They can also be distinguished functionally to homeostatic or inflammatory chemokines. The former are constitutively produced independently of inflammation and are thought to regulate homing to different tissues as will be explained later on, while the latter are only produced under inflammatory conditions and are thought to recruit effector T cells during inflammation (reviewed in Ebert *et al*., 2005).

Chemokines can be soluble or be presented on the apical surface of the EC, anchored on glucosaminoglycans (reviewed in Garrood *et al*., 2006). Once they have stimulated the T cells by binding to the transmembrane G protein coupled chemokine receptors, they can induce actin polymerisation and lamellipodia formation which are important for migration (reviewed in Ebert *et al*., 2005). In addition, they may increase integrin avidity by mediating conformational changes, which are necessary for the next step of transendothelial migration, firm adhesion (reviewed in Baggiolini, 1998).

1.1.3. Firm adhesion: integrins

Integrins are heterodimeric transmembrane proteins which belong to the integrin superfamily and can bind to ligands on the surface of cells or to the extracellular matrix. They consist of a large α chain that pairs non-covalently with a smaller β chain. There are several sub-families of integrins which are defined based on the β chain. β1 integrins include very late antigen 1 (VLA-1, $\alpha_1\beta_1$) and VLA-4 ($\alpha_4\beta_1$), while β2 integrins include leukocyte associated function antigen (LFA-1, $\alpha_L\beta_2$) and macrophage receptor 1 (Mac-1 $\alpha_M\beta_2$) (reviewed in Janeway *et al*., 2001). In lymphocytes, they may exist in high and low affinity binding states, the latter being induced by chemokine-mediated conformational changes, thus allowing efficient binding to their ligands (reviewed in Garrood *et al*., 2006).

Integrins may bind to adhesion molecules on the surface of ECs, such as intracellular adhesion molecules (ICAMs) ICAM-1, ICAM-2 and ICAM-3, all of which are important in lymphocyte activation and migration and can bind to LFA-1 on the surface of T cells (reviewed in Janeway *et al*., 2001) (reviewed in Ebert *et al*., 2005). The differential distribution of adhesion molecules that bind distinct integrins on T cells is thought to regulate their migration to different sites, as will be discussed later on; thus, adhesion molecules are also sometimes termed vascular addressins as they can direct T cells to different sites of the body (reviewed in Ebert *et al*., 2005).

Integrins are activated via "inside-out" signalling. More specifically, upon binding of the chemokine receptor on the lymphocyte to immobilised ligand, a signalling cascade is initiated which results in increased integrin avidity and activation (reviewed in Muller, 2002). The latter involves conformational changes or integrin clustering and in turn leads to binding of the adhesion molecule on the surface on the EC, resulting in firm adhesion (reviewed in Muller, 2002). Integrins can also initiate "outside-in" signalling pathways, i.e. internal signalling in response to external stimuli (reviewed in Garrood *et al*., 2006).

Of the various possible heterodimers, two integrins have been shown to be most important in T cell migration, namely LFA-1 and VLA-4. The ligands for LFA-1 are ICAM-1 (CD54), 2 (CD102) and 3 (CD50) (de Fougerolles *et al*., 1991)(reviewed in

Janeway *et al*., 2001), which are cell surface adhesion molecules of the immunoglobulin superfamily (Hopkins *et al*., 2004). ICAM-1 is expressed on vascular epithelial cells and ECs (Dustin *et al*., 1986), and its expression is upregulated by inflammatory cytokines such as IL-1 and TNF-a (Henninger *et al*., 1997) (Hopkins *et al*., 2004). ICAM-2 is primarily expressed on the resting endothelium (Lehmann *et al*., 2003) and is thought to facilitate lymphocyte transmigration and have a role in angiogenesis, as demonstrated in studies using ICAM-2 deficient mice (Huang *et al*., 2005).

ICAM-1 and ICAM-2 are thought to have a redundant role in lymphocyte re-circulation to lymphoid tissues, as antibody blocking of either ICAM-1 or ICAM-2 did not affect lymphocyte recirculation, while blocking of both molecules dramatically diminished entry to the lymph nodes. However, ICAM-1, is thought to be more important in migration to sites of inflammation, as antibody blocking of ICAM-1 alone resulted in dramatic decrease in migration to inflamed skin *in vivo*, which was not observed with antibody blocking of ICAM-2 (Lehmann *et al*., 2003).

Furthermore, the ligand for VLA-4 is vascular cell adhesion molecule 1 (VCAM-1, CD106). It is expressed at lower levels than ICAM-1 but follows similar activation kinetics in inflammation (Henninger *et al*., 1997) and has been involved in several inflammatory diseases such as inflammatory bowel disease, atherosclerosis and asthma (reviewed in Cook-Mills, 2002). Other than VLA-4, it can also bind to other adhesion molecules such as $\alpha_4\beta_7$, whose importance in T cell homing will be discussed in detail in chapter 1.3.2 (reviewed in Garrood *et al*., 2006).

The role of both LFA-1 and VLA-4 in transendothelial migration has been extensively demonstrated. Antibody blocking of LFA-1 led to 80% inhibition of transmigration (Schreiber *et al*., 2007) while antibody blocking of VLA-4 also significantly reduced transmigration (Pryce *et al*., 1997). In addition, ICAM-1 and VLA-4 presented on the apical surface of the endothelium have been shown to cluster with actin-rich structures and form a major docking structure *in vitro*, thus facilitating lymphocyte transmigration (Barreiro *et al*., 2002).

Integrin activation and firm adhesion to the endothelium brings the re-circulating lymphocyte to a stop and allows for diapedesis to occur.

1.1.4. Cell polarisation

An essential part of lymphocyte transendothelial migration is polarisation of the migrating cell. The latter is the result of a change in the distribution of filamentous actin which alters the normally spherical shape of the cell into a polarised one and is mediated by chemokines. Polarisation may occur during a variety of cell-to-cell interactions, such as antigen presentation as well as interaction with the endothelium (reviewed in Sanchez-Madrid and del Pozo, 1999).

As the cell polarises, two distinct parts are established, the leading edge and the uropod/trailing edge (del Pozo *et al*., 1995). The former is characterised by the presence of chemokine receptors and integrins in the active conformation, while the latter is primarily enriched with adhesion receptors, suggesting that the leading edge is involved in chemotaxis and the uropod with adhesion and de-adhesion of the cell, as well as recruitment of other lymphocytes (reviewed in Sanchez-Madrid and del Pozo, 1999). Lipid rafts, which are cholesterol–rich structures that facilitate signalling (reviewed in Katzav, 2004) are thought to be fundamental in establishing cell polarity, as cholesterol depletion abolished the formation of both the leading edge and the uropod (Gomez-Mouton *et al*., 2001).

In migrating lymphocytes, actin is re-distributed throughout the cell, and is mostly concentrated at the leading edge. An abundance of receptors can also be found at this part of the cell, such as integrins, urokinase plasminogen activator receptor and chemokine receptors (reviewed in Sanchez-Madrid and del Pozo, 1999), as well as the ganglioside lipid-raft associated protein GM3 (Gomez-Mouton *et al*., 2001). It is thus thought that the leading edge, as the name suggest, directs migration of the cell, primarily mediated by the chemokine receptors (Fais and Malorni, 2003).

The uropod is characterised by the presence of intracellular adhesion molecules such as CD2, CD44, CD43, as well as PSGL-1 and L-selectin (del Pozo *et al*., 1995; reviewed in Sanchez-Madrid and del Pozo, 1999). The ganglioside lipid raft associated protein GM1 has also been found to localise the uropod (Gomez-Mouton *et al*., 2001). Uropod formation is induced through triggering by adhesion molecules, such as ICAM-1, 3 and VCAM-1, as well as chemokines, such as CCL5 and monocyte chemottactic

protein (MCP)-1. In fact, distinct chemokines can stimulate uropod formation in different T cell subsets, for example macrophage inflammatory protein (MIP-)1α in CD4+ T cells and MIP-1β in CD8+ T cells (del Pozo *et al*., 1995).

In terms of function, the uropod is thought to mediate contact with the ECs and the extracellular matrix via the adhesion molecules that accumulate in this part of the cell (reviewed in Sanchez-Madrid and del Pozo, 1999). In addition, the uropod has also been found to be important for the contact between polarised T cells, in an interaction mediated by ICAM-1,3 and LFA-1 (del Pozo *et al*., 1997).

Cell polarisation requires cytoskeletal re-organisation, which is mediated by intracellular pathways such as phosphoinositide (PI) 3 kinase and Rho guanosine triphosphate (GTP)ases (reviewed in del Pozo *et al*., 1995; Sanchez-Madrid and del Pozo, 1999). The importance of these pathways will be discussed in detail later on.

1.1.5. Diapedesis

Diapedesis is possibly the least understood step of lymphocyte migration. Although traditionally lymphocytes have been thought to transmigrate between the EC junctions, termed paracellular route, recent evidence has shown that they can also migrate through the EC itself, known as transcellular route (reviewed in Engelhardt and Wolburg, 2004).

In the paracellular route, lymphocytes are thought to pass between the ECs, transiently replacing the molecules in the tight junctions. Molecules that are thought to be important in this process include CD31, CD99, VE-cadherin, ICAM-1, and junction adhesion molecules (JAMs) (reviewed in Engelhardt and Wolburg, 2004; reviewed in Garrood *et al*., 2006; reviewed in Muller, 2002). Of these molecules, CD31 has been studied in great detail. It is a member of the immunoglobulin superfamily, and is expressed in both ECs and haemopoetic cells. It is thought to form homophilic interactions between the T cell and the EC, thus facilitating migration. Its importance in diapedesis has been demonstrated *in vitro* as antibody blocking prevented the migration of monocytes and neutrophils (Muller *et al*., 1993). This effect has also been observed *in vivo,* as injection of a blocking antibody prevented leukocyte accumulation on to the sites of inflammation (Bogen *et al*., 1994; Muller, 2003). However, leukocytes can still

transmigrate to some extent in CD31-deficient mice, suggesting that other mechanisms compensate for the loss of CD31 (Duncan *et al*., 1999).

CD99 is expressed on the surface of most leukocytes and is found concentrated at the borders between confluent ECs. It is thought to be a co-stimulatory molecule, and recently has been shown to have a role in migration. As with CD31, it forms homophilic interactions between the migrating leukocyte and the EC (Muller, 2003). Antibody blocking experiments resulted in dramatic decrease in transendothelial migration of monocytes *in vitro* (Schenkel *et al*., 2002). Interestingly, blocking of both CD99 and CD31 had an additive effect in inhibition of migration, suggesting that the two molecules control different aspects of diapedesis (Schenkel *et al*., 2002).

VE-cadherin is located at the junctions between ECs. It is a member of the cadherin superfamily and is thought to be important for permeability as blocking antibodies result in increased permeability of ECs (Muller, 2003).

The JAMs (JAM A, B, C) belong to the immunoglobulin superfamily and are expressed on leukocytes and ECs. As with CD99 and CD31, JAMs are capable of homophilic interactions, although recent evidence suggests that they also interact with integrins on the surface of the migrating leukocyte (Muller, 2003). JAM A has been found to engage LFA-1 and thus control T cell transendothelial migration through the tight junctions. JAM B can interact with JAM C and VLA-4 while JAM C can interact with Mac –1 (reviewed in Engelhardt and Wolburg, 2004). Binding of the JAM proteins to lymphocyte integrins suggests a promising role in lymphocyte migration.

The importance of ICAM-1 in lymphocyte migration has already been highlighted. Briefly, ICAM-1, the ligand for LFA-1, is thought to form a docking structure with VCAM-1 on the surface of the EC, thus facilitating migration (Barreiro *et al*., 2002). In addition, a recent study using human umbilical cord ECs and Chinese hamster ovary cells expressing ICAM-1 showed that engagement of ICAM-1 by LFA-1 resulted in the formation of actin-rich projections. The latter surrounded the leukocytes and based on the time-scale they occurred, were thought to have a role in diapedesis (Carman *et al*., 2003). Millan *et al*. (2006) also observed that translocation of ICAM-1 to F-actin and caveaolin-1- rich areas of the endothelium resulted in transendothelial migration of human T cells, further confirming the importance of this molecule in diapedesis.

Other than the paracellular route of migration, research has also extensively focused on the transcellular route, i.e. migration through the ECs. The transcellular route was initially discovered in migration through the blood brain barrier and into the central nervous system (CNS) in mouse models of experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU) (Engelhardt and Wolburg, 2004; Greenwood *et al*., 1994; McMenamin *et al*., 1992; Raine *et al*., 1990). However, this phenomenon is not isolated to the CNS; neutrophils were found to cross the endothelial monolayer of the skin through the transcellular route as well in guinea pigs injected with their natural ligand, the chemoattractant *N*-formyl-methionylleucyl-phenylalanine (fLMP) (Feng *et al*., 1998).

It is thus thought that, although the paracellular route is probably the main route of transendothelial migration, under certain circumstances and possibly limited to certain tissues, the transcellular route is also possible (reviewed in Engelhardt and Wolburg, 2004).

1.2. Shear force in lymphocyte migration

Under physiological conditions, lymphocyte migration does not happen under static conditions; shear force is constantly applied by the blood to the migrating lymphocyte. Although traditionally a largely ignored subject, it has become clear that shear force directly influences the different aspects of lymphocyte transendothelial migration.

As mentioned earlier, chemokines mediate integrin conformational changes, allowing the lymphocyte to tightly adhere to the endothelium and subsequently transmigrate (Baggiolini, 1998). *In vitro* studies have shown that chemokines presented on the apical surface of the cytokine-treated human endothelium trigger rapid transendothelial migration of human lymphocytes in the presence of shear flow (Cinamon *et al*., 2001; Schreiber *et al*., 2007). This effect was not observed under static conditions, suggesting that flow directly affects this interaction (Schreiber *et al*., 2007).

Using *in vitro* video miscoscopy, a recent study using human peripheral blood lymphocytes has shown that lymph node chemokines, such as CCL21, induce integrin adhesiveness in conditions of shear force alone. Interestingly, in the absence of shear

force, CCL21 failed to trigger the activation of LFA-1 in T cells (Woolf *et al*., 2007). Therefore, it has been suggested that shear forces facilitate integrin signalling induced by chemokines, which allow for integrin conformational changes that result in adhesion to their ligands and lymphocyte transmigration (reviewed in Alon and Dustin, 2007).

Finally, some studies suggest that diapedesis can occur in the presence of continuous shear force alone; human lymphocytes were able to transmigrate *in vitro* following antibody blocking of LFA-1 and VLA-4 (Cinamon *et al*., 2001).

1.3. The concept of T cell homing

T cells do not migrate randomly; the expression of tissue-selective receptors (termed homing receptors), including integrins and chemokine receptors direct them to specific peripheral tissues. In addition, ECs express distinct adhesion molecules in different tissues (termed vascular addressins), which can also allow T cells to home to specific peripheral tissues.

Studies have demonstrated different homing potentials for different T cell subsets. Naïve T cells preferentially migrate from the blood to lymphoid organs via HEV where they may encounter antigen and become activated (see **Figure 1**) (Mackay *et al*., 1990; reviewed in Mora and von Andrian, 2006). In contrast, memory T cells have been shown to preferentially migrate from the blood to peripheral non-lymphoid tissues and then to lymphoid organs via the afferent lymphatic system (Mackay *et al*., 1990).

Memory T cells can be further subdivided based on their homing potential to central memory and effector memory T cells. The former express molecules such as CCR7 and preferential migrate to lymphoid organs such as peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches, as well as spleen, where they can activate antigen presenting cells (APCs) such as B cells and dendritic cells (DCs) (Sallusto *et al*., 1999; Weninger *et al*., 2001). In addition, unlike naïve T cells, central memory T cells have been found to utilise a second peripheral lymph node homing pathway in the absence of the CCR7 ligands CCL19 and CCL21, which is dependant on CXCL12

(Scimone *et al*., 2004). Central memory T cells have also been found to migrate more efficiently to the bone marrow compared to the other subsets (Mazo *et al*., 2005).

Effector memory T cells on the other hand do not express CCR7, and thus cannot respond to CCL21 and CCL19, which are primarily expressed on stromal cells of lymphoid tissues. They have in fact been shown to preferentially migrate to sites of inflammation, where they can mediate further inflammation and cytotoxicity (Sallusto *et al*., 1999; Weninger *et al*., 2001).

In terms of cytolytic function, IFNγ production was initially thought to be restricted in CD8+ effector memory T cells in response to DCs pulsed with bacterial superantigen (Sallusto *et al*., 1999). However, this has recently been contested, as both $CCR7^+$ and $CCR7^-$ T cells were found to be able to display cytotoxic activity in response to viral infection (Unsoeld *et al*., 2002).

As mentioned earlier, ECs can also regulate migration. This is due to the expression of distinctive adhesion molecules by ECs from different tissues, such as MadCAM in HEV of Peyer's patches (reviewed in Cines *et al*., 1998; Hillyer *et al*., 2003). In addition, ECs have been shown to produce different chemokines in different tissues, thus directing lymphocyte trafficking (reviewed in Baggiolini, 1998; Hillyer *et al*., 2003). They also contribute to lymphocyte compartmentalisation within lymphoid tissues by secreting chemokines that direct T cells to different anatomical sites, for example CXCR5 which directs T cells to the germinal centre of lymph nodes (reviewed in Baggiolini, 1998).

The homing potential of T cells based on the expression of adhesion molecules and chemokines receptors will be discussed in detail and are summarised in **Table** 2.

1.3.1 Homing to the lymph nodes

Naïve T cells trafficking in the blood can enter the lymph nodes via the HEV. This process follows the afore mentioned "multi-step paradigm" of lymphocyte migration and is tightly controlled by chemokines. The two chemokines that have been shown to be essential for entry to the lymph nodes are CCL19 and CCL21, both of which can bind to the chemokine receptor CCR7 (reviewed in Ebert *et al*., 2005)

CCR7 is primarily expressed by naïve T cells as well as central memory T cells, which retain the ability to migrate to the lymph nodes (reviewed in Ebert *et al*., 2005). The importance of CCR7 for homing to the lymph nodes was demonstrated in experiments using CCR7-deficient mice, which showed severe morphological alterations to their secondary lymphoid organs, primarily due to the lack of T cells (Forster *et al*., 1999). In addition, in a study using *plt* mice, which lack expression of the chemokines CCL19 and CCL21, homing to the mesenteric lymph nodes by adoptively transferred T cells was abolished (Okada *et al*., 2002).

Furthermore, the study by Okada *et al*. (2002) also demonstrated a role for CXCR4 in the migration of T cells to the lymph nodes. Using the *plt* mice, they showed that in the absence of CCR7 ligation by CCL19 or CCL21, some cells still migrate to the lymph nodes, and this is abolished in CXCR4-deficient mice (Okada *et al*., 2002). A more recent study suggested that CXCL12, the ligand for CXCR4 might contribute to the migration of central memory T cells, as antibody blockade of the former reduced central memory T cell homing to peripheral lymph nodes (Scimone *et al*., 2004).

L-selectin has also been shown to be involved in T cell homing to the lymph nodes. L-selectin is expressed by naïve T cells, and is though to be downregulated during the transition to memory phenotype (reviewed in Picker *et al*., 1993). Its importance in homing to the lymph nodes was demonstrated in L-selectin-deficient mice, which showed reduction in migration to all lymphoid organs as well as spleen (Arbones *et al*., 1994). In addition, a study by Galkina *et al*. (2007) showed that reduced expression of L-selectin correlated with reduced homing to the lymph nodes (Galkina *et al*., 2007).

Due to the lack of expression of other chemokine receptors, naïve T cells are naïve in migration potential, as homing is restricted to lymphoid tissue. It is thus thought that T cell priming allows activated T cells to gain homing receptors to different peripheral tissues, depending on the lymph node they get activated in, as will be explained later on (reviewed in Ebert *et al*., 2005).

1.3.2 Homing to the gut

Memory T cells can acquire homing receptors that direct them to the intestinal immune system when activated at the mesenteric lymph nodes or Peyer's patches (Stenstad *et al*., 2006). A distinctive feature of gut-tropic T cells is the acquisition of the chemokine receptor CCR9. The ligand for the latter is CCL25/ thymus-expressed chemokine (TECK), which is expressed in the small intestine, thus suggesting its importance in migration to gut (reviewed in Ebert *et al*., 2005; Kunkel *et al*., 2000; Zabel *et al*., 1999). Studies using CCR9-deficient T cells show that these T cells are significantly disadvantaged at localising to the intestine (Stenstad *et al*., 2006). In addition, a study by Svensson *et al*. (2002) directly showed that $CCR9^+$ T cells localise to the small intestine, and antibody blocking of TECK prevents their homing(Svensson *et al*., 2002). However, Stenstad *et al*. (2002) have suggested that there are CCR9-independent mechanisms for entering the lamina propria of the gut, as they still find some T cells in this part of the body in CCR9-deficient mice, which also display a distinct array of chemokine receptors to the lamina propria homing $CCR9^+$ T cells.

In addition to CCR9, $\alpha_4\beta_7$ integrin has been found to be important in homing to mucosal tissues (Abitorabi *et al*., 1996; Campbell and Butcher, 2002; Hamann *et al*., 1994; Schweighoffer *et al*., 1993). $\alpha_4\beta_7$ integrin preferentially binds to MadCAM1, which is a tissue-specific vascular addressin for mucosal ECs (Nakache *et al*., 1989; Streeter *et al*., 1988), and VCAM-1, but the latter with less affinity (Berlin *et al*., 1993; Pachynski *et al*., 1998; Rott *et al*., 1996).

Studies with α_4 or β_7 deficient mice have demonstrated their importance in T cell gut homing; α_4–deficient mice have no Peyer's patches (Arroyo *et al*., 2000), while β_7 -deficient mice show hypoplasia in their gut-assosiated lymphoid tissue, particularly the Peyer's patches, due to reduced lymphocyte adhesion on the HEV, but with surprisingly normal architecture (Wagner *et al*., 1996). In addition, antibodies to $\alpha_4\beta_7$ integrin have been shown to improve chronic colitis in tamarins (Hesterberg *et al*., 1996), suggesting that they block T cell migration to the intestine. The complete $\alpha_4\beta_7$ integrin heterodimer is thought to be required for homing to the gut, as $\alpha_4\beta_1$ preferentially binds to VCAM-1 (Berlin *et al*., 1993; Rott *et al*., 1996). $\alpha_4\beta_7$ integrin-positive T cells have been shown to

be generated in the intestinal lymphoid tissues (Campbell and Butcher, 2002) and express CCR9 (Svensson *et al*., 2002).

Finally, ICAM-1 and P-selectin have both been found to be important in the development of the intestinal immune system. ICAM-1 and P-selectin-deficient mice have fewer T cells in the intraepithelial lymphocyte compartment, lamina propria and the Payer's patches (Huleatt and Lefrancois, 1996; Steinhoff *et al*., 1998), implying a role for the two molecules in T cell migration to the gut.

1.3.3 Homing to cutaneous tissues

T cells can acquire skin homing receptors when activated in cutaneous lymphoid tissue (Picker *et al*., 1993). One of the most extensively studied molecules is CLA, which is the ligand for E-selectin (reviewed in Berg *et al*., 1991; Garrood *et al*., 2006) and is generated by post-translational carbohydrate modification of PSGL-1 in humans (Fuhlbrigge *et al*., 1997). Its importance in skin homing was first described by Picker (1990), who observed that human lymphocytes in inflammatory skin lesion specimens preferentially expressed an epitope (later named CLA) that reacted to the HECA-451 antibody (Picker *et al*., 1990). Furthermore, in studies examining patients with psoriatic arthitis, Pitzalis *et al*. (1996) observed that despite the presence of inflammatory lesions in both skin and joint, CLA^+ T cells preferentially migrated to the skin, thus confirming its importance in homing to cutaneous tissues (Pitzalis *et al*., 1996).

Most CLA^+ skin-homing T cells have been shown to express the chemokine receptor CCR4. The ligand to the latter is thymus and activation-related chemokine (TARC)/CCL17, which preferentially induces adhesion to the dermal endothelium (Campbell *et al*., 1999). $CD4^+$ T cells in skin lesions of patients with psoriasis have been found to express high levels of CCR4, and patients with cutaneous subtypes of system lupus erythromatosus express higher levels of TARC/CCL17 in the skin that healthy controls (Wenzel *et al*., 2005). However, a study examining the effects of different routes of immunisation in T cell homing in mice observed that CCR4 was not solely expressed by T cells generated through intracutaneous immunisation, and was in fact transiently expressed in activated T cells independently of the immunisation route. This study

suggests that CCR4 is probably not an exclusive marker for skin homing (Dudda *et al*., 2004).

Finally, CCR10 has also been shown to be expressed by most skin infiltrating T cells (reviewed in Garrood *et al*., 2006; Homey *et al*., 2002). The ligand for CCR10 is CCL27, whose intracutaneous injections in mice has been shown to attract T cells to the skin, while, more importantly, it is constitutively produced by epidermal keratinocytes (Homey *et al*., 2002).

1.3.4 Homing to sites of inflammation

Although evidence of the existence of homing receptors to other bodily sites is poorly understood, other molecules have been suggested which may play a role in the migration of T cells to distinct peripheral tissues. One example is VAP-1 which was first described by Salmi *et al*. (1992) as an EC marker, and whose expression in HEV is upregulated in inflammatory conditions (Salmi and Jalkanen, 1992) (Salmi *et al*., 1993). Interestingly, a blocking antibody to VAP-1 was shown to affect T cell adhesion to the hepatic endothelium, suggesting that it may play a role in T cell recruitment to the liver (McNab *et al*., 1996). A circulating form of VAP-1 was also found to be higher in inflammatory liver diseases and alcohol liver disease in particular, compared to colitis or rheumatoid arthritis (Kurkijarvi *et al*., 1998). In addition to VAP-1, recruitment to the liver is thought to require LFA-1, CCR5, which is involved in recruitment to portal tracts and CXCR3, which is important in recruitment to the hepatic parenchyma (Grant *et al*., 2002).

Several chemokine receptors have been implicated in the migration of T cells to the inflamed CNS. More specifically, in mouse models of EAE, T cells crossing the blood/brain barrier have been shown to express CCR7 and CXCR3 (Alt *et al*., 2002; Columba-Cabezas *et al*., 2003). As mentioned earlier, the ligands for CCR7 are CCL21 and CCL19, while CXCR3 can act as an alternative receptor. The two chemokines have been shown to be produced at high levels at the blood brain barrier in EAE, suggesting some involvement in T cell migration to the inflamed CNS (Alt *et al*., 2002). Moreover,

T cells from patients with inflammatory CNS conditions such as multiple sclerosis also express CCR7 and CXCR3, along with CCR5 (Giunti *et al*., 2003).

In addition to inflammatory CNS conditions, CXCR3 has been shown to be involved in the recruitment of T cells to sites of inflammation. T cells expressing CXCR3 have high homing potential to inflammatory sites (Qin *et al*., 1998), while it has also been involved in allergic rhinitis (Francis *et al*., 2007). Furthermore, CXCR3-deficient mice showed reduced cardiac allograft rejection, which a CXCR3 blocking antibody prolonged cardiac allograft survival in wild type mice, suggesting that this chemokine receptor is important in transplantation (Hancock *et al*., 2000).

Table 2: Summary of homing receptors guiding T cells to different tissues

Tissue	Receptor	Ligand	Comments	Reference
Lymph nodes	CCR7	CCL19/CCL21	Expressed by naïve and central memory T cells	Forster, 1999; Okada, 2002, Scimone, 2004
	CXCR4	CXCL12	Utilised by central memory T cells	
	L-selectin	GlyCAM, MadCAM,E-selectin, CD34 and Sgp200	Expressed by naïve T cells	Picker, 1993; Arbone, 1994; Galkina, 2007
Gut	CCR9	CCL25	CCR9 independent mechanisms for entry to lamina propria	(reviewed in Ebert., 2005; Kunkel 2000; Zabel 1999)
	$\alpha_4\beta_7$	MadCAM1	Complete heterodimer required for homing to the gut	Schweighoffer, 1993; Hamann., 1994; Abitorabi, 1996; Wagner, 1996; Arroyo, 2000
	ICAM-1	LFA-1	Deficient mice have fewer cells in the gut	Huleatt and Lefrancois, 1996
	P-selectin	PSGL-1	Deficient mice have fewer cells in the gut	Steinhoff., 1998
Skin	CLA	E-selectin	Glycosylation variant of PGSL-1	Picker, 1990; Berg., 1991; Pitzalis, 1996;
	CCR4	CCL17	Probably not an exclusive skin homing marker	Campbell, 1999;
	CCR10	CCL27	Intracutaneous injection of ligand attracts T cells	Homey, 2002
Sites of inflammation	VAP-1	unknown	Probably involved in migration to the inflamed liver	Salmi, 1993; McNab, 1996; Kurkijarvi, 1998
	CXCR3	CXCL10	Involved in migration during inflammatory liver disease, inflammatory CNS disease, allergic rhinitis, transplantation	Qin, 1998; Hancock, 2000; Alt, 2002;, Guinti, 2003; Francis, 2007

1.4. A role for dendritic cells in T cell homing

So how do T cells acquire homing receptors that direct them to peripheral tissues? It has already been hinted that the location of the lymphoid tissue where antigen stimulation occurred has an essential role in imprinting T cells with an "area code" that directs them to different parts of the body. T cells stimulated in the cutaneous-associated lymphoid tissue express higher levels of CLA (Picker *et al*., 1993), while those encountering antigen in intestinal lymphoid tissue acquire $\alpha_4\beta_7$ (Campbell and Butcher, 2002). It is thus reasonable to suggest that the lymphoid microenvironment, including resident APCs such as DCs, will have a role in the acquisition of homing receptors.

Interestingly, a study by Dudda *et al*. (2004) demonstrated that the immunisation route defines the homing potential of T cells. They observed that intracutaneous immunisation of DCs elicited a strong ear-swelling response, with the T cells from the auricular lymph node expressing high levels of CCR4 and E-selectin. In contrast, intraperitoneal immunisation resulted in the generation of $\alpha_4\beta_7$-expressing gut-homing T cells. It is interesting to note that intravenous immunisation did not elicit any particular response, probably because it did not target a specific lymphoid tissue.

Indeed DCs have been found to contribute to gut-specific imprinting of T cells, i.e. the acquisition of homing receptors allowing the T cells to migrate to the gut. Co-culture of murine T cells and DCs from the gut-associated lymphoid tissue, including mesenteric lymph nodes and Peyer's patches, have been shown elicit higher T cell expression of $\alpha_4\beta_7$ and CCR9 *in vitro* (Johansson-Lindbom *et al*., 2003; Mora *et al*., 2003; Mora *et al*., 2005). In addition, murine T cells stimulated by gut-associated lymphoid tissue DCs migrated better towards TECK and downregulated the expression of L-selectin (Johansson-Lindbom *et al*., 2003; Mora *et al*., 2003). Interestingly, once these T cells were adoptively transferred they migrated to the lamina propria and the intraepithelial lymphoid compartment of the gut, but not the colon, suggesting that different homing receptors exist for distinct parts of the gut (Mora *et al*., 2003)

DCs have also been shown to contribute to skin-specific imprinting of T cells. Studies have shown that murine T cells stimulated by DCs from peripheral lymph nodes expressed higher levels of P and E-selectin and had higher levels of mRNA of the

enzyme required for post-translational modification of PSGL-1. In addition, they expressed higher levels of CCR4 and migrated more efficiently to the inflamed skin when adoptively transferred (Mora *et al*., 2005).

The exact mechanism of imprinting is unclear, although Iwata *et al* (2004) observed an interesting effect when analysing vitamin A-deficient mice. Retinoic acid, a vitamin A metabolite, was found to induce $\alpha_4\beta_7$ and CCR9 expression *in vitro*, while at the same time downregulating CCR4 expression. In addition, vitamin A-deficient mice showed a reduction of $\alpha_4\beta_7{}^+$ T cells as well as depletion of T cells from the lamina propria of the gut. DCs from the gut associated lymphoid tissue seem to express high levels of retinoic acid, and consequently may be able to induce a gut homing phenotype in T cells (Iwata *et al*., 2004; Mora *et al*., 2005).

In addition to this, Sigmundsdottir *et al*. (2007) recently observed that vitamin D3 has a similar effect on the imprinting of skin homing T cells. More specifically, the metabolite of vitamin D3, $1,25(OH)_2D_3$ was found to signal T cells to express the skin-homing CCR10 and suppress the gut-homing $a_4\beta_7$ and CCR9. More excitingly, they observed that DCs isolated from skin-draining lymph nodes were able to convert vitamin D3 to $1,25(OH)_2D_3$ *in vitro*, suggesting that DCs from skin-associated lymphoid tissue can induce a skin-homing phenotype in T cells (Sigmundsdottir *et al*., 2007)

Finally, it is important to note that the T cell homing phenotype was found to be reversible by Mora *et al*. (2005). When Peyer's patches-activated murine T cells were re-stimulated with peripheral lymph node DCs, they gained selectin ligand expression, while peripheral lymph node-activated T cells re-stimulated with Peyer's patches DCs can acquire $\alpha_4\beta_7$ expression. In addition, adoptive transfer of vesicular stomatis virus-specific, non-gut tropic, non-β_7-expressing T cells into mice infected with the virus induced up-regulation of β_7 integrin expression as well as migration to mucosal tissues, suggesting that T cells can be "re-educated" to migrate to different parts of the body depending on the challenge (Masopust *et al*., 2004; reviewed in Mora and von Andrian, 2006).

1.5. Exit from peripheral tissues

Although extensive studies have been done to identify how T cells enter peripheral tissues, their exit has been largely ignored. Traditionally, exit from peripheral tissues and entry to the afferent lymphatics was not thought to utilise specific molecular mechanisms, however recent evidence suggests that the chemokine receptor CCR7 is involved. More specifically, CCR7 has been implicated in the T cell exit from the skin, as T cells from CCR7-deficient mice, which were adoptively transferred to wild type mice by footpad injection were unable to reach the draining lymph nodes. In addition, cannulation of the skin-draining afferant lymphatics of sheep indicated that present T cells expressed functional CCR7 (Debes *et al*., 2005) .

CCR7 has also been implicated in T cell exit from the lung. CCR7-deficient T cells were found to accumulate in the lung after challenge with aerosolised antigen. In addition, adoptively transferred transgenic T cells expressing CCR7 in higher levels than the wild type were found in lower numbers in the lung after re-challenge with aerosolised antigen and were primarily accumulating in the draining mediastinal lymph nodes (Bromley *et al*., 2005).

2. Regulation of T cell migration by the TCR and co-stimulatory molecules

2.1. TCR-dependent control of T cell migration

Homing receptors, such as $\alpha_4\beta_7$, confer some level of specificity to T cell migration to tissues such as the gut. However, patrolling the latter, which can be up to 6 meters long in adult humans, would be a very inefficient way of finding their cognate antigen. It has therefore been hypothesised that additional mechanisms operate, which allow the T cell to localise its cognate antigen in the tissue. Indeed, evidence suggests that the endothelium may present antigen to T cells, therefore regulating antigen-specific T cell migration.

2.1.1 Functional consequences of antigen presentation

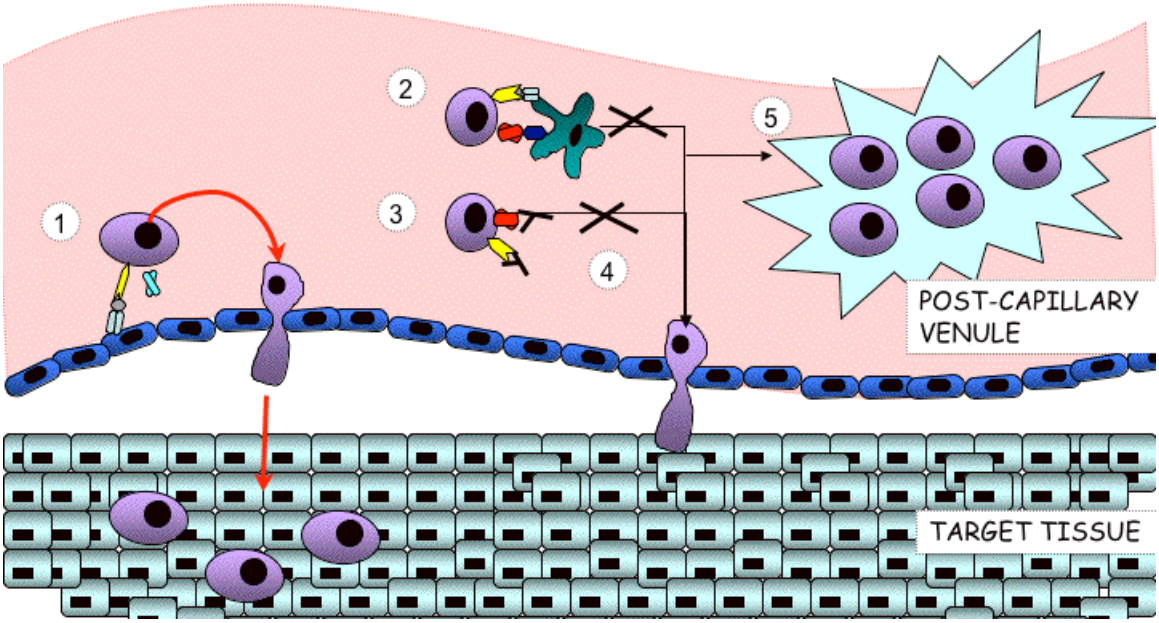
ECs have been shown to express Major Histocompatibility Complex (MHC) molecules, but expression differs between species; human ECs express both MHC class I and II while murine one only express class I, but can be induced to express class II upon interferon (IFN) gamma activation (reviewed in Choi *et al*., 2004; Haraldsen *et al*., 1998; Savage *et al*., 1995). Expression has been shown to differ between tissues as well, with non-lymphoid vascular ECs having the highest expression (reviewed in Choi *et al*., 2004). In addition to expressing MHC molecules, ECs have in fact been found to be able to present antigen to T cells (Bagai *et al*., 2005; Greening *et al*., 2003; Haraldsen *et al*., 1998; Murray *et al*., 1994; Savage *et al*., 1995), and this is thought to be in a proteasome dependent, transporter for antigen presentation (TAP) –dependent manner (Bagai *et al*., 2005).

Other than T cell receptor (TCR) stimulation in the context of MHC, T cells require an additional signal in order to become activated and not become anergic. This is normally delivered by the co-stimulatory molecules CD80 and CD86, which can activate CD28 on the surface of the T cell (reviewed in Choi *et al*., 2004; reviewed in Janeway *et al*., 2001). In addition to CD80/CD86 and CD28 mediated co-stimulation, other molecules such as inducible co-stimulatory molecule (ICOS) and OX40, which bind to

the ICOS and OX40 ligands respectively, have also been shown to provide co-stimulatory signals for T cells (reviewed in Lane, 2000; reviewed in Rudd and Schneider, 2003). Expression of co-stimulatory molecules by ECs has been shown to differ between species, with human ECs lacking expression of both CD80 and CD86 and murine ones having low expression of CD80 (Marelli-Berg *et al*., 2001a; reviewed in Marelli-Berg and Jarmin, 2004). Other co-stimulatory molecules, such as ICOS-L (Khayyamian *et al*., 2002) and programmed cell death (PD)-1 (Eppihimer *et al*., 2002) have been shown to be constitutively expressed in human ECs, and in conjuction with adhesion molecules are thought to partially compensate for the lack of CD80/CD86 and prevent anergy induction upon antigen presentation by the endothelium (reviewed in Marelli-Berg and Jarmin, 2004).

The functional consequences of antigen presentation to T cells differ significantly depending on the context it occurs (**Figure** 2, **Table** 3). Evidence suggests that engagement of the TCR, as occurs with DCs, results in loss of migratory ability, allowing the T cell to polarise to the APC and proliferate (Dustin *et al*., 1997). Antigen presentation by ECs does not result in proliferation in the absence of cytokines (Lodge and Haisch, 1993; Pardi and Bender, 1991) and in fact has been shown to increase migration (Marelli-Berg *et al*., 2000), while presentation by epithelial cells results in anergy induction and loss of migratory ability (Marelli-Berg *et al*., 2001a).

Figure 2: Effects of antigen presentation on T cell migration and activation



The effects of antigen presentation on T cell migration differ depending on the context it occurs. Low-avidity antigen presentation by ECs results in enhanced migration to target tissue (1). High-avidity antigen presentation by professional APCs such as DCs (2) or by antibody stimulation (3) delivers a stop signal to the migrating T cell (4), which allows it to proliferate (5).

Table 3: Functional consequences of antigen presentation

APC	Effect on T cell	Reference
DC	Stop signal Polarisation and proliferation	(Dustin *et al*., 1997)
EC	Increased migration	(Lodge and Haisch, 1993; Pardi and Bender, 1991) (Marelli-Berg *et al*., 2000)
Epithelial cell	Loss of migratory ability Anergy	(Marelli-Berg *et al*., 2001a).

Indeed, Dustin *et al*. (1997) showed that engagement of the TCR in murine T cells *in vitro* by peptide and IL-2 increased adhesion to ICAM-1 and delivered a stop signal to T cells, allowing them to re-polarise their microtubule organising centre from the uropod

to the contact areas (Dustin *et al*., 1997). Similar results were obtained in a study using murine $CD4^+$ T cells, where antibody engagement of the TCR and CD28 abrogated migration *in vitro* (Berg *et al*., 2002). Loading of external cognate antigen on the endothelium has also been shown to result in loss of migratory ability of $CD8^+$ T cells *in vitro*, suggesting that the avidity of the signal is important in determining the effect on T cell motility (Marelli-Berg *et al*., 2001b).

2.2. ECs can recruit T cells to sites of inflammation

The ability of ECs to influence T cell recruitment has been observed *in vitro* in a study using $CD4^+$ memory T cells. Recognition of antigen presented by the ECs resulted in two-fold increase of migration through human ECs (Marelli-Berg *et al*., 1999). In addition to this, $CD8^+$ T cell clones were found to migrate more through murine IFNγ-treated ECs, i.e. those induced to upregulate MHC class I expression, than resting ones *in vitro* (Marelli-Berg *et al*., 2000).

In line with these observations came a study by Greening *et al*. (2003) using human T cells and ECs. IFNγ treated glutamic acid decarboxylase (GAD) -pulsed ECs were found to increase the migration of GAD-specific T cells *in vitro*. Antibody-blocking of MHC or LFA-1 resulted in inhibition of migration suggesting that the enhanced migration of T cells is dependant on peptide presented by the endothelium as well as adhesion.

The ability of ECs to influence T cell motility has also been observed *in vivo*. A study by Savinov *et al*. (2001) showed that injection of $CD8^+$ T cells in IFNγ-deficient mice resulted in arrest in the pancreatic islet entrance rather that entry into the pancreas itself. This effect was reversed by systemic application of IFNγ, which as mentioned earlier, increases MHC class I and induces MHC class II expression, thus allowing for antigen presentation.,

Moreover, Savinov *et al*. (2003) subsequently observed that homing of diabetogenic T cells is dependent on presentation of insulin peptides produced by β cells and presented by pancreatic ECs. This process was found to be dependent on MHC I

expression, as in a mouse model of diabetes lacking MHC class I, $CD8^+$ T cells did not localise to the islets (Savinov *et al*., 2003).

Antigen-dependent recruitment of T cells has also been directly visualised *in vivo*. In this case, injection of male minor histocompatibility antigen HY-specific $CD4^+$ T cells resulted in recruitment and retention to the inflamed peritoneum in male but not female mice. Interestingly, visualisation of the vascular bed using intra-vital microscopy revealed that cognate recognition of the endothelium resulted in enhanced diapedesis of T cells in the tissue, without affecting rolling and adhesion. This suggests that recognition of antigen facilitated the selective recruitment of antigen-specific T cells (Marelli-Berg *et al*., 2004).

Based on the previous observations, two purposes for the role of TCR-mediated control of T cell motility have been suggested; first, it facilitates the recruitment of antigen-specific T cells into sites of inflammation by antigen displayed on the endothelium; and second, during antigen presentation by professional APCs, it allows for T cells to stop and polarise on the APC (reviewed in Marelli-Berg and Jarmin, 2004). Given the importance of the cytoskeleton in T cell migration, it is likely that molecules linking the TCR with the cytoskeleton will have a fundamental role in TCR-mediated T cell migration.

2.3. Overview of CD28 co-stimulation

Along with TCR triggering by the peptide:MHC complex, co-stimulation is also necessary for sustaining an effective immune response. One of the best-characterised co-stimulatory molecules is CD28, which is expressed in both naïve and effector T cells. Upon triggering of the TCR, CD28 binds to CD80 or CD86 on the surface of professional APCs initiating various signalling cascades, which lead to increased cytokine production, IL-2 mRNA stability and expression of the anti-apoptotic protein Bcl-XL (Parry *et al*., 2003). Thus, CD28 signalling is essential for the sustained proliferation and survival of T cells; in the absence of this signal, T cells become anergic or apoptose. CD28 is also thought to lower the threshold of T cell activation by reducing the number of TCRs

required for activation (reviewed in Alegre *et al*., 2001; Brunner-Weinzierl *et al*., 2004; Salomon and Bluestone, 2001)

The regulation of migratory events is associated with the activation of signalling pathways that mediate cell adhesion and cytoskeleton rearrangements, which CD28 has recently been found to engage.

2.3.1 Regulation of adhesion and cytoskeletal re-organisations by CD28

CD28-mediated signals have been involved in the regulation of T cell adhesion. CD28 cross-linking increased adhesion of human $CD4^+$ T cells to fibronectin, ICAM-1 and VCAM-1, in a process involving protein kinase C (PKC) (Shimizu *et al*., 1992). Similarly, CD28 was shown to enhance LFA-1:ICAM-1-mediated interactions between human T cells and CD80-expressing melanoma cells via PKC-activation (Turcovski-Corrales *et al*., 1995). Further evidence for the contribution of CD28-mediated signals to integrin function came from the observation that β1 integrin-mediated adhesion of a myelomonocytic cell line was enhanced by CD28 activation (Zell *et al*., 1998). This event involves an interaction of its Src homology 2 domain (SH2) with the p85 catalytic subunit of PI3kinase, a process regulated by the adaptor molecule Casitas-B-lineage lymphoma protein (Cbl)-b (Zell *et al*., 1998).

CD28 has also been implicated in the regulation of cytoskeletal re-organization. CD28 triggering increases F-actin levels in mouse T cells, in a process involving the Ras homologue GTPases Rac1 and Cdc42 (Kaga *et al*., 1998a, b). Cdc42 activation upon CD28 engagement is thought to be mediated by Src kinases independently of zeta chain associated protein (ZAP)-70 activation (Salazar-Fontana *et al*., 2003). CD28 can also activate the guanidine exchange factor (GEF) for Rho GTPases Vav1 independently of the TCR (Michel *et al*., 2000).

In addition to activating Rho GTPases, CD28 engagement has also been found to induce actin polymerisation in a Wiskott-Aldrich syndrome protein (WASp)-dependent manner (Badour *et al*., 2007). The latter molecule plays a major role in coupling TCR signals with cytoskeletal re-arrangements, in a process mediated by Vav1 and Rho GTPases as will be explained in chapter 3. WASp interacts with the actin related protein

(Arp)2/3 complex, and together they induce actin filament nucleation (reviewed in Sechi and Wehland, 2004). CD28 is thought to interact with WASp via PI3kinase and sorting nexin 9 activation (Badour *et al*., 2007).

2.3.2 Regulation of migration by CD28

Experimental models of autoimmunity have highlighted a role for CD28 co-stimulation in the localisation of memory T cells to antigenic sites. Resistance to EAE in CD80/CD86- and CD28-deficient mice correlated with reduced development of parenchymal T cell infiltrates of the CNS following immunization with MOG_{35-55} peptide, despite the occurrence of efficient T cell priming (Chang *et al*., 1999). Selective deficiency of CD80 or CD86 molecule expression led to disease development comparable to wild type mice, suggesting a redundant role for CD28 ligands in the development of T cell-mediated inflammation (Chang *et al*., 1999).

Similarly, in an EAE model induced by PLP_{56-70} immunisation of female non-obese diabetic mice, antibody blockade of CD80/CD86 abrogated tissue infiltration, inflammation and demyelination. Notably, the complete lack of histological damage was only observed when CD80 and CD86 monoclonal antibodies were used in conjunction (Girvin *et al*., 2000).

A direct involvement of CD28 in the control of T cell migration has been suggested by recent studies. CD28-deficient murine T cells stimulated with APCs or peptide secrete significantly less CC chemokine MIP1α (Herold *et al*., 1997). In addition, CD28-dependent up-regulation of OX40 is instrumental to CXCR5 expression and helper T cell migration to germinal centres in mice (Walker *et al*., 1999).

We have recently reported that CD28 co-stimulatory signals are essential for primed T cells to reach their antigenic sites. $CD28^{Y170F}$ mice carry a mutation in the cytoplasmic tail of CD28 that abrogates PI3K recruitment without leading to defects in clonal expansion (Okkenhaug *et al*., 2001). OT-II (ovalbumin-specific, MHC class II restricted TCR-transgenic) and OT-II/$CD28^{Y170F}$ double transgenic mice were generated and their T cells were shown to proliferate equally following immunization with ovalbumin. However, only OT-II T cells were able to localise to ovalbumin-expressing

peritoneal tissue and cavity, suggesting that CD28-induced, PI3K-mediated signals are necessary for the recruitment of T cells to antigenic sites (Mirenda *et al*., 2006).

In addition, we observed that dominant CD28 signals were also able to overcome the requirement for TCR engagement to sustain T cell localization to non-lymphoid tissue. Optimal antibody activation of CD28 in HY-specific H2-D^b-restricted CD8+ T cells led to unregulated infiltration of kidney, liver, spleen, heart, and 'homing-privileged' gut tissue in syngeneic female mice. Enhanced trafficking was specifically induced in memory but not in naïve T cells and abrogation of PI3K activation in CD28-activated $CD28^{Y170F}$ HY-specific CD8+ T cells prevented this effect (Mirenda *et al*., 2006).

Aberrant migration of primed T cells into non lymphoid tissue due to dominant CD28 signalling may explain the multiple organ failure described in healthy human subjects recently treated with a CD28 superagonist (TGN1412, Tegenero). This compound was found in the pre-clinical trials to stimulate and expand T cells independently of the TCR-triggering, in particular Th2 and $CD4^+$ $CD25^+$ regulatory T cells (Rodriguez-Palmero *et al*., 1999). Despite the lack of pro-inflammatory effects in the pre-clinical trials, when administered in six healthy male individuals, the compound led to multi-organ failure, severe lymphopenia and a cytokine storm, with increased levels of the pro-inflammatory cytokines TNFα, IL-2, IL-10 and IFN–γ (Suntharalingam *et al*., 2006). Given that the human T cell repertoire comprises around 50% memory T lymphocytes, the CD28 superagonist may have induced uncontrolled extravasation of memory T cells into non-lymphoid tissue, and subsequent T cell-mediated tissue damage. The specific effect on memory T cells may have been overlooked in experimental models where the T cell repertoire is mainly composed by naïve T lymphocytes (reviewed in David and Marelli-Berg, 2007).

3. The Vav family of proteins

The Vav proteins are a family of GEFs, which in mammals consist of three known members, Vav1, 2 and 3. The first member to be described was Vav1, which was initially termed vav, where it was identified as a proto-oncogene present primarily in cells of haematopoetic origin (Coppola *et al*., 1991; Katzav *et al*., 1989). Vav1 was subsequently mapped to chromosone 19 (Martinerie *et al*., 1990). The isolation of the other two members of the Vav family, Vav2 and Vav3, followed, both of which were found to be present in cells of haematopoetic and non-haematopoetic origin (Movilla and Bustelo, 1999; Schuebel *et al*., 1998). Analysis of the structure of Vav proteins suggested that they are important in both signalling as well as cell architecture as will be explained in the next chapter.

The Vav family of proteins can be found in different species such as Drosophila *melanogaster* (termed DroVav) where their effector functions are conserved; DroVav acts as a GEF for Drosophila GTPases (Hornstein *et al*., 2003). In addition, DroVav is also thought to regulate the actin cytoskeleton, as a mutated version, DMVav was found to induce lamellipodia and membrane ruffling when injected in NIH3T3 fibroblasts, a phenotype similar to the one induce by the mammalian Vav (Couceiro *et al*., 2005).

3.1. Structure

As Vav1 is the focus of this study, its structure will be explained in detail (**Figure** 3). Vav1 is a 95KDa GEF for RhoGTPases which is composed of domain characteristic of signal transducers. More specifically, Vav1 has a dbl homology domain (DH), which is thought to be essential for all its biological activities (Zugaza *et al*., 2002). The DH domain is thought to have the GEF activity for RhoGTPases (reviewed in Tybulewicz *et al*., 2003) as it contains Tyr174 which is involved in GEF activity of Vav1. The DH domain is autoinhibited through an N-terminal autoinhibitory arm, and phosphorylation

of Tyr174 results in release of the DH domain, thus exposing the GEF site and allowing Vav1 to act as a GEF for RhoGTPases (Aghazadeh *et al*., 2000)

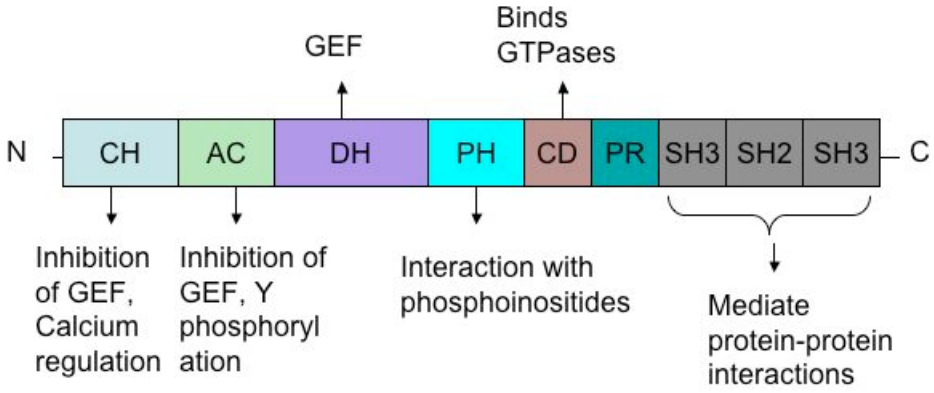
Furthermore, Vav1 has a calponin homology (CH) domain which is thought to negatively regulate the GEF activity of the DH domain and is involved in Ca^{2+} signalling (Cao *et al*., 2002; reviewed in Tybulewicz *et al*., 2003). In addition, the CH domain has also been found to be important in nuclear factor of activated T cells (NFAT)- activator protein-1 (AP-1) – mediated gene transcription and development of effector function in lymphocytes as indicated in mutagenesis studies (Billadeau *et al*., 2000; Zugaza *et al*., 2002)

Vav1 also has an SH2 and two SH3 domains, which are known to mediate protein-to-protein interactions and are generally present in signalling molecules. In Vav1 in particular, the SH domains are thought to mediate the interaction with Src homology 2 domain-containing leukocyte protein of 76 kDa (Slp-76) during TCR signalling (reviewed in Hornstein *et al*., 2004; reviewed in Tybulewicz *et al*., 2003) and to act as scaffolding ensuring proper phosphorylation and engagements of downstream effectors (Zugaza *et al*., 2002). Mutation of the SH2 and SH3 domains have been shown to impair the transforming activity of Vav1, suggesting a role in its the oncogenic properties (Groysman *et al*., 1998; Katzav, 1993).

Moreover, Vav1 has a pleckstrin homology (PH) domain which, as the DH domain, is thought to be essential for all biological activities (Zugaza *et al*., 2002). The PH domain has recently been shown to regulate Vav1 GEF activity *in vivo*, as well as selectively regulate proliferation of $CD4^+$ T cells in mice (Prisco *et al*., 2005).

Finally, Vav1 has cystein rich domain (CD), which contains two zinc ions that are throught to be required for the formation of the Vav1/Rac1 complex (Brooun *et al*., 2007; Heo *et al*., 2005).

Figure 3: The structure of Vav1



Structural studies have shown that Vav1 has a CH, DH, PH, CD and SH domains which are important in its function as a GEF for RhoGTPases as well as TCR signalling.

3.2. Vav1 in the development of T cells

Vav1 was initially described as being important for the development of cells of haematopoetic origin (Coppola *et al*., 1991) with Vav1 expression in the newborn mouse concentrated in haematopoetic tissues, and in the adult in the thymus, spleen and lymph nodes (Bustelo *et al*., 1993). Subsequent studies using antisense RNA or homologous recombination to abolish Vav1 function showed conflicting results in the relevance of Vav1 in murine embryonic stem cell haematopoetic development (Wulf *et al*., 1993; Zmuidzinas *et al*., 1995), with Zhang *et al*. *(*1995) discrediting previous studies as they observed little effect in haemotopoietic development in the absence of Vav1 when using gene targeting on embryonic stem cells (Zhang *et al*., 1995). In addition, Luger *et al*. (1996), suggested that Vav1 is important for erythroid cell development but dispensable for myeloid cell growth and differentiation. In this case, they diminished Vav1 function with anti-sense oligonucleotides in an adult human system, and observed no defects in myeloid cell proliferation and maturation.

In T cells, the importance of Vav1 has been demonstrated by the extensive studies using Vav1-/- mice, the latter generated through deletion of Vav1 in embryonic stem cells (Fischer *et al*., 1995) or disruptive mutation of Vav1 from the germline by homologous recombination (Tarakhovsky *et al*., 1995; Turner *et al*., 1997). One of the first studies on

Vav1-/- mice showed that Vav1-deficient embryonic stem cells generate a limited number of T and B cells, indicating that Vav1 is important in T and B cell development (Fischer *et al*., 1995).

Subsequent studies showed that Vav1-/- mice have altered thymic development, with reduced thymic cellularity resulting in decreased thymus size due to the decline of double positive thymocytes (Fischer *et al*., 1998; Tarakhovsky *et al*., 1995; Turner *et al*., 1997). A recent study using thymocytes expressing Vav1 with a mutation in the DH tyrosine 174 (Y174F) mimicked the phenotype of Vav1-/- mice, i.e. diminished thymic cellularity and decreased double positive population. This indicates that tyrosine 174 is a key residue in Vav1 regulating T cell development (Miletic *et al*., 2003).

Furthermore, Vav1-/- mice have altered T cell development with a partial block at the $CD4^+$ $CD8^+$ double positive to single positive $CD4^+$ or $CD8^+$ mature T cell, possibly due to impaired pre-TCR signalling and defective positive selection (Fischer *et al*., 1998; Turner *et al*., 1997). Tarakhovsky *et al*. (1995) also observed a reduction of single positive $CD4^+$ or $CD8^+$ mature T cells in the spleen and lymph nodes of Vav1-/- mice. In addition, this study showed a disproportionate ratio of CD4:CD8 mature T cells in the spleen but not the lymph node of Vav1-/- mice. Interestingly, Vav1-/- mice show normal development of mature γδ T cells, with comparable numbers of cells in the spleen and comparable levels of TCR expression (Swat *et al*., 2003).

Moreover, a study by Gomez *et al*. (2000) indicated an interesting link between Vav1 and Rac1 in development using Vav1-/- mice. More specifically, transgenic mice expressing a constitutively active form of Rac1 (L61Y40Rac-1) in Vav1-/- background displayed full reconstitution of thymic cellularity and prevented the skewed profile of depleted douple positive thymocytes. This study suggests that some of the defects in T cell development in Vav1-/- mice may be due to defective Rac1 activation.

A recent study has investigated the role of the other Vav proteins in T cell development. Fujukawa *et al*. (2003) examined Vav1/2/3-/- mice as well as different combinations of Vav proteins to investigate the function of each protein and identify potential redundancies. Their results show that Vav1 is the most important regulator of T cell development, as mice lacking Vav2 and 3 showed no T cell abnormalities. Vav3 did partly compensate for the loss of Vav1, and the triple Vav1/Vav2/Vav3 knockout had a

more severe developmental block than the Vav1/Vav3 knockout, with an almost complete absence of double positive thymocytes, suggesting that Vav2 also has a role in T cell development.

3.3. Vav1 in T cell function

The role of Vav1 in mature T cell function has also been extensively researched. Several studies have shown impaired TCR-induced proliferation in T cells from Vav1-/- mice, but normal proliferation with signals that bypass the antigen receptor, such as phorbol myristate acetate (PMA) or ionomycin (Costello *et al*., 1999; Fischer *et al*., 1998; Penninger *et al*., 1999; Zhang *et al*., 1995). Vav1-/- γδ T cells, which were shown to have normal development, also display reduced proliferation (Swat *et al*., 2003).

In addition, Vav1-/- T cells show reduced IL-2 production, which is the direct result of impaired CD3/CD28 signalling (Tarakhovsky *et al*., 1995). The importance of Vav1 in IL-2 production has also been demonstrated in a study examining the effects of methylation inhibitors in a mouse model of EAE. This study suggested a correlation between arginine methylation of Vav1 in autoreactive $CD4^+$ T cells and amelioration of disease due to reduced IL-2 production (Lawson *et al*., 2007). These findings have also been confirmed in studies using Vav1-/- Jurkat T cells generated through gene targeting, where reduced activity of the IL-2 promoter and reduced IL-2 production have been reported (Cao *et al*., 2002). Vav1-/- T cells also show reduced IL-4 production when examined by flow cytometry, and a skewed Th1 T cell profile (Tanaka *et al*., 2005).

Studies on T cells from Vav1-/- mice have also shown defective Ca^{2+} flux i.e. undetectable or at very low levels, resulting from defective signalling (Costello *et al*., 1999; Fischer *et al*., 1998). This has also been confirmed in studies examining Vav1-/- Jurkat T cells (Cao *et al*., 2002). Similar results have been obtained from cells from patients with common variable immunodeficiency (CVID). The latter, and more specifically T-CVID , is a primary dysfunction of the immune system, making patients more susceptible to bacterial infections, autoimmunity and cancer (Sneller, 2001). T-CVID is characterised by defective TCR-dependent early tyrosine phosphorylation

cascade in T cells. Close examination of T cells from these patients revealed that they have reduced Vav1 mRNA levels, which resulted, amongst other defects, in impaired Ca^{2+} flux (Paccani *et al*., 2005).

Vav1-/- mice are also thought to display impaired T cell help in the context of immunoglobulin class switching. Vav1-/- mice show defective immunoglobulin class switching and germinal centre formation, a phenotype which was reversed when using chimeras with normal T cells, suggesting that the defect is due to impaired T cell help (Gulbranson-Judge *et al*., 1999).

The role of Vav1 in effector T cell function has been examined in a study by Penninger *et al*. (1999), who investigated the role of Vav1 in cytotoxic T cell function. By challenging Vav1-/- mice with viral infections, they observed that Vav1-/- T cells had impaired primary cytotoxic responses *in vivo*, as Vav1-/- mice showed reduced footpad swelling after viral infection. The ability to respond was found to depend on viral challenge, with Vav1 being necessary for vesicular stomatitis virus clearance. However, once re-stimulated *in vitro*, Vav1-/- T cells were able to clear infection, suggesting that Vav1 is important for primary cytotoxic responses.

Korn *et al*. (2003) further examined Vav1-/- T cell effector function in a study of EAE. They observed that priming of Vav1-/- T cells with the EAE peptide MOG_{35-55} was defective despite effective antigen presentation, and Vav1-/- mice were not susceptible to EAE development. In addition, they examined cytokine secretion in Vav1-/- T cells through stimulation with MOG_{35-55} peptide, which did not result in IL-2, IL-4 or IFNγ induction *in vitro*.

Finally, in a transplantation study using Vav1-/- and Vav2-/- mice, Vav1-/- T cells showed reduced graft versus host disease reaction, as adoptively transferred carboxyfluorescein diacetate succinimyl ester (CFSE) labelled Vav1-/- T cells did not proliferate in the spleen of severe combined immunodeficiency mice. More importantly, both Vav1-/- and Vav2-/- mice showed increased heart allograft survival compared to the WT, with only mild cellular rejection observed upon histological examination of the transplant after 100 days (Weckbecker *et al*., 2007). This study indicates that Vav1 plays an important role in effector T cell function in the context of transplantation.

3.4. Vav1 in the cell cycle and apoptosis

In addition to its role in T cell development and function. Vav1 is also involved in the cell cycle and the prevention of apoptosis. Indeed Vav1 has been shown to be a caspase target, and to be cleaved into two products, Vav1p76 and Vav1p19 (Hofmann *et al*., 2000).

Vav1-/- T cells show defects in cycling, which is thought to be the result of reduced phosphorylation of forkhead box O (Foxo)1. The latter is a member of the forkhead family of transcription factors and plays a critical role in the regulation of the cell cycle and apoptosis (Coffer and Burgering, 2004). As Foxo1 is a target of PI3kinase, it is thought that Vav1 controls the TCR/CD28 dependent phosphorylation of Foxo1 in T cells via Akt (Charvet *et al*., 2006).

In thymocytes, Vav1 activity has been found to be involved in peptide-specific apoptosis, which is in accord with its role in TCR-dependent positive and negative selection. In a study by Kong *et al*., (1998) Vav was found to regulate apoptosis in thymocytes through changes in the actin cytoskeleton and activation of PKC.

Moreover, a study by Tuosto *et al*. (2002) indicated that Vav1 activity prevents apoptosis, as in the absence of Vav1, the levels of pro-apoptotic Bax increased, leading to mitochondrial damage. The researchers have suggested that activation of the Src kinase leukocyte specific protein tyrosine kinase (Lck) activates T cells apoptotic pathways, an event counteracted by Vav1 through its co-operation with CD28 in boosting TCR signalling.

However, another study using a constitutively active form of Vav1 in haematopoetic progenitor cells showed increased apoptosis, including both early apoptotic (annexin positive) and late apoptotic (annexin and 7-AAD positive) cells. In addition, cells expressing the constitutively active form of Vav1 showed reduced expression of anti-apoptotic BCL-XL and BCL-2. (Gu *et al*., 2006).

3.5. Vav proteins and the actin cytoskeleton

Vav1 is a GEF for RhoGTPases whose role in cytoskeletal remodelling will be described later on. As a regulator of RhoGTPase function, it is reasonable to postulate that Vav1 activity is necessary for re-arrangements of the cytoskeleton. The evidence of Vav1's involvement in this process will be described in detail in this chapter.

3.5.1. Vav1 interacts with WASp

Vav1 has been shown to associate with WASp which is thought to couple TCR signals with the cytoskeleton. WASp encodes for a proline-rich protein, a mutated form of which causes Wiskott-Aldrich syndrome, an X-linked recessive immunodeficiency disorder characterised by eczema, thrombocytopenia and recurrent infections (Derry *et al*., 1994). T cells from WASp patients display cytoskeletal abnormalities (Gallego *et al*., 1997), while other WASp-deficient cells show reduced microvilli, impaired migration in response to chemoattractants and increased cell polarisation (Anton *et al*., 2002; Gallego *et al*., 1997; Remold-O'Donnell *et al*., 1997). The actin polymerisation function of WASP is regulated by a tyrosine phorphorylation of tyrosine 291 (Cory *et al*., 2002). A proline-rich WASp-interacting protein was isolated (WIP) which co-immunoprecipitated with WASP in lymphocytes and has actin binding motifs (Ramesh *et al*., 1997). WIP1-/- T cells fail to proliferate, secrete IL-2, polymerise F-actin, polarise and form conjugates with superantigen-presenting B cells (Badolato *et al*., 1998) (Anton *et al*., 2002). WIP co-operates with Vav1 for NFAT/ AP1 gene transcription via its 112-237 amino acid terminal domain; complex formation between WASp and WIP is necessary for this function (Savoy *et al*., 2000).

WASp has been shown to interact with the actin-related protein (Arp)2,3 complex to perform actin filament enucleation (reviewed in Sechi and Wehland, 2004; Welch *et al*., 1997). WASp could be recruited to the TCR through association of its C-terminal domain SH3 domain and Slp-76 (reviewed in Sechi and Wehland, 2004) , which would

place it in proximity to Vav1. WIP may also play a role in recruiting WASp to the TCR and bring it in proximity to Vav1 (reviewed in Sechi and Wehland, 2004).

3.5.2. Vav1 is involved in the formation of the immunological synapse

Vav1 has been shown to have an important role in the formation of the immunological synapse, also known as supramolecular activation cluster. The latter is the tight interphase between the T cell and the APC which is thought to facilitate the degradation of the TCR, and hence cessation of signalling (reviewed in Hornstein *et al*., 2004; Monks *et al*., 1998). Upon TCR triggering, molecules involved in T cell signalling become associated with specialised regions of the cell membrane known as lipid rafts, which are enriched with cholesterol, phospholipids and glycosphyncolipids (reviewed in Janeway *et al*., 2001). Examples of such molecules include Src family kinases such as Lck and Fyn, which phosphorylate downstream signalling molecules such as Vav1 (reviewed in Janeway *et al*., 2001). Upon phosphorylatuon, Vav1 translocates to the lipid rafts and is responsible for transducing signals necessary for the transcription of NF-AT and nuclear factor kappa B (NfkB) (Manicassamy *et al*., 2006). This facilitates downstream signalling cascades which lead to activation of transcription factors and gene expression (reviewed in Hornstein *et al*., 2004; Monks *et al*., 1998). The formation of the immunological synapse involves changes in the T cell cytoskeleton, allowing for the T cell to polarise towards the APC. These changes are thought to be mediated primarily by integrin receptors such as LFA-1 (reviewed in Hornstein *et al*., 2004; Monks *et al*., 1998).

A number of studies have implicated Vav1 in the formation of the immunological synapse, and in particular for the TCR– induced activation of LFA—1. More specifically, Vav1-/- thymocytes were defective in transducing LFA-1 "inside-out" signals for the formation of conjugates and cell polarisation (Ardouin *et al*., 2003). This effect was also observed using peripheral Vav1-/- T cells from TCR-transgenic mice, which showed reduced ability to form peptide-specific conjugates and reduced LFA-1 clustering (Krawczyk *et al*., 2002; reviewed in Cantrell, 2003).

In addition, Vav1 is necessary for the formation of lipid raft clusters, as lipid rafts in Vav1-/- T cells fail to translocate to the immunological synapse upon antigen

stimulation (Villalba *et al*., 2001). However, a different study using Vav1-/- Jurkat T cells observed no defects in lipid raft clustering or PKCθ translocation to lipid rafts (Cao *et al*., 2002).

Finally, Vav1-/- T cells have defective TCR/MHC class II accumulation in the centre of the immunological synapse (Wulfing *et al*., 2000), diminished actin cap formation and TCR clustering following TCR engagement, as well as abolished interaction of actin with CD3ζ (Fischer *et al*., 1998; Hornstein *et al*., 2004).

3.5.3. Vav proteins induce cytoskeletal re-arrangements in different cell types

Extensive research has shown that all Vav proteins can induce cytoskeletal re-arrangements, which are important for cell polarisation, adhesion and spreading. This has been directly shown by Fischer *et al*. (1998), who observed that loss of Vav1 in thymocytes results in severe defects of actin polymerisation.

Firstly, Vav1 is important in adhesion of thymocytes and T cells. In thymocytes, loss of Vav1 results in lack of adhesion to fibronectin, laminin, collagen type IV, vitronectin and ICAM-1, following TCR activation (Krawczyk *et al*., 2002). The same phenotype was observed with Vav1-/- T cells, suggesting the importance of Vav1 in T cell adhesion (Krawczyk *et al*., 2002).

The ability of Vav1 activity to mediate T cell adhesion has been confirmed in a study using Jurkat T cells. In this case, adhesion of Jurkat T cells to fibronectin was shown to result in rapid phosphorylation of Vav1 (Yron *et al*., 1999).

In addition, Vav1 is important in T cell spreading, the latter being a process intermediary to adhesion and motility which is important in determining whether a cell proliferates, becomes quiescent or dies post-adhesion and is characterised by adhesion and actin polymerisation (McGrath, 2007). T cells with a truncated form of Vav1 lacking the SH domain display defects in spreading on VCAM-1-coated plates when stimulated with CXCL12. Cells expressing lower levels of Vav1 were also shown to have increased levels of detachment from VCAM-1-coated plates in conditions of shear stress (Garcia-Bernal *et al*., 2006).

Vav1 has also been shown to inactivate ezrin-moesin-radoxin (ERM) proteins in T cells. The latter are regulated through threonine phosphorylation and are co-expressed and co-localised at locations where actin filaments are associated with the plasma membrane (eg membrane ruffles). They are thus thought to be regulators of cell rigidity (Roumier *et al*., 2001; Tsukita and Yonemura, 1999). Vav1-/- T cells show reduced ERM de-phosphorylation, suggesting that Vav1 may be responsible for ERM protein de-phosphorylation and indirectly for the regulation of cell rigidity (Faure *et al*., 2004).

Vav1 is also involved in cytoskeletal-dependent morphological changes in T cells. A study by Zaffran *et al*. (2001) showed that CD46 co-stimulation results in Vav1 phosphorylation and Rac1 activation, which in turn was shown to induce the formation of actin-rich lamellipodia and filopodia.

Vav proteins are also important in the cytoskeletal re-arrangements of other cell types. In neutrophils, all three Vav proteins have been shown to be expressed, with Vav1 and Vav3 being the most important. Vav1-/- and Vav3-/- neutrophils were shown to be defective in adhesion and spreading to their natural ligand C3bl, with the double knockout Vav1,3-/- having a more severe phenotype (Gakidis *et al*., 2004). In addition, neutrophils from Vav1-/- mice displayed reduced generation of F-actin in response to the chemoattractant fMLP (Kim *et al*., 2003).

Moreover, in platelets, Vav1 and Vav3 are redundant while Vav2 plays no role (Pearce *et al*., 2004). In terms of the cytoskeleton, Vav1,3-/- platelets adhere to fibronectin with the same efficiency as the wild type but show fewer filopodia and lamellipodia; Vav1-/- and Vav3-/- platelets show an intermediate phenotype (Pearce *et al*., 2007).

Furthermore, the role of the three Vav proteins in macrophages has not been studied extensively, but a study by Wells *et al*., (2005) showed reduced adhesive area in both Vav1-/- and Vav2-/- macrophages. However, Vav1-/- but not Vav2-/- macrophages showed reduced adhesion to fibronectin, suggesting a more important role for the former.

Vav proteins have also been shown to be involved in dendritic cell morphological changes. More specifically, the human immunodeficiency virus (HIV) protein Nef was found to trigger phosphorylation of Vav1 in infected DCs, which was followed by morphological changes such as formation of uropods, membrane ruffling, re-

arrangements of actin filaments and polarisation of vinculin. This study suggests that HIV infection of DCs results in activation of a Vav1-mediated signalling pathway which induces morphological changes (Quaranta *et al*., 2003). Indeed Vav1 has been found to be the binding partner for Nef during HIV infection, and to induce structural changes in the infected cell (Fackler *et al*., 1999) which may facilitate conjugate formation between DCs and T cells and enhance viral transmission (Quaranta *et al*., 2003).

Vav proteins have also been shown to play a role in osteoclast remodelling and spreading. More specifically, CSF treatment, which is known to affect osteogenesis, has been shown to translocate Vav3 to the plasma mebrane, leading to increased spreading of osteoclasts (Sakai *et al*., 2006).

The role of Vav proteins in remodelling of the cytoskeleton has also been examined through ectopic expression of the proteins on different cell types. One study used a human mammary epithelial cell line (MCF-10A) transformed with a constitutively active form of Vav1, resulting in morphological changes including membrane ruffling and lamellipodia formation. This study also identified the DH, PH or CD domains as being important for the morphological changes, as transformation of the human mammary epithelial cell line with a mutated form of Vav1 lacking the afore mentioned domains did not induce this phenotype (Wilsbacher *et al*., 2006).

Finally, extensive studies on the role of Vav protein in the regulation of the actin cytoskeleton have used fibroblasts. More specifically, Vav1 expression in REF-52 (rat embryo) fibroblasts induced filopodia formation (Han *et al*., 1997), while an oncogenic version of Vav1 expressed in NIH3T3 (mouse fibroblast) cell line resulted in both lamellipodia and filopodia formation (Lopez-Lago *et al*., 2000). Overexpression of Vav2 in a NIH3T3 cell line resulted in lamellipodia formation in a Src kinase dependent manner (Marignani and Carpenter, 2001). A study by Shuebel *et al*. (1998) observed different morphological changes induced by Vav1 and Vav2 when expressed in NIH3T3 cell line, suggesting that in this cell line, the two Vav proteins act using distinct signalling pathways .

3.6 Vav proteins and migration

As important regulators of the cytoskeleton, Vav proteins are likely candidates for the regulation of migration. Studies in T cells and other cell types have indeed indicated that Vav proteins are important regulators of migration and chemotaxis (summarised in **Table** 4).

Firstly, a study by Vicente-Manzanares *et al*. (2005) has shown that Vav1 has a role in T cell chemotaxis. More specifically, when using Vav1-/- murine T cells, they observed no effect on chemotaxis, lymphocyte migration and F-actin polymerisation in response to CXCL12. However, using human peripheral blood lymphocytes nucleofected with a dominant negative form of Vav1 resulted in impaired chemotaxis, actin polymerisation and cell polarisation. Redundancy between Vav proteins explains why no defect in migration to CXCL12 was observed in the murine Vav1-/- T cells. However, the dominant negative form of Vav1 would bind to the Vav interaction site on the CXCR4 signalosome, thus blocking access and preventing the other Vav proteins from compensating for the loss of Vav1.

Another study has also implicated Vav1 in CXCL12-mediated chemotaxis. In this case a dominant negative form of Vav1 abbrogated CXCL-12-mediated chemotaxis in Jurkat T cells (Ticchioni *et al*., 2002). This has been further examined by Garcia-Bernal *et al*., (2005) who showed that Vav1 and Rac1 are necessary for CXCL12-induced adhesion and transendothelial migration.

Moreover, using an EAE model, Korn *et al*. (2003) showed that T cells from Vav1-/- mice show reduced T cell infiltration of the brain compared to the WT, which was partly reversed with administration of cytotoxic T lymphocyte associated protein (CTLA-4) Ig, suggesting a role for co-stimulatory signalling in the regulation of T cell migration by Vav1.

As with the regulation of the cytoskeleton, Vav proteins have been shown to affect motility in other cell types as well. In macrophages, Vav1-/- cells were selectively found to have reduced migratory speed, probably due to altered microtubule dynamics as demonstrated by the reduced levels of α-tubulin (Wells *et al*., 2005). In agreement with this was the study showing that loss of the Vav1 N terminal domain (i.e. oncoVav1)

resulted in increased spontaneous migration of macrophages four to five-fold, suggesting that Vav1 regulates migratory speed in macrophages. The same study also showed that loss of the Vav1 GEF activity abrogated chemotaxis (Vedham *et al*., 2005).

Furthermore, neutrophils from Vav1-/- mice were shown to have reduced migration to medium alone compared to WT cells, as well as reduced chemotaxis to high concentrations of chemoattractant FMLP both *in vitro* and *in vivo* (Kim *et al*., 2003). A different study using Vav1,3-/- neutrophils showed no defect in migration and chemotaxis in response to C3bl compared to wild type *in vitro*, but indicated higher migratory speed compared to the control (Gakidis *et al*., 2004).

Vav has also been shown to be involved in melanoma cell invasion, mediated by CXCL12. Melanoma cells express both Vav1 and 2, and blocking of the two Vav proteins resulted in impaired activation of Rac and inhibition of CXCL12-mediated invasion (Bartolome *et al*., 2006).

Finally, a study using human mammary epithelial cells transformed with a constitutively active form of Vav1 observed enhanced migration compared to the wild type control in response to epidermal growth factor which is necessary for the migration of these cells. This study also indicated a role for the the SH2 and CSH3 Vav1 domain in the migration of human mammary epithelial cells, as a mutant lacking this domain did not display this phenotype (Wilsbacher *et al*., 2006).

Table 4: Summary of experimental evidence implicating Vav proteins in migration

Cell type	Vav protein	Experimental system	Effect on migration	Comments	Reference
T cells	Vav1	Murine Vav1-/- cells *in vitro*	No defect in response to CXCL12	Dominant negative form blocked access to CXCR4, preventing compensation by other Vav proteins	Vicente-Manzanares, 2005
		Human T cells transfected with dominant negative form *in vitro*	Impaired chemotaxis, actin polymerisation and polarisation		
		Jurkat T cells trasfected with a dominant negative form of Vav1	Abrogated CXCL12 chemotaxis		Ticchioni, 2005
		Murine Vav1-/- T cells *in vitro*	Required for CXCL12-inducedtransendothelial migration	In conjunction with Rac1	Garcia-Bernal, 2006
		Adoptive transfer in EAE model	Reduced brain infiltration	Partly reversed by CTLA-Ig	Korn, 2003
Macrophages	Vav1	Murine Vav1-/- cells *in vitro*	Reduced migratory speed		Wells, 2005
		Murine Vav1-/- cells with no N-terminal domain *in vitro*	Increased spontaneous migratuon		Vedham, 2005
		Murine Vav1-/- cells with no GEF activity *in vitro*	Defect in chemotaxis		
Neutrophils	Vav1	Murine cells Vav1-/- *in vitro, in vivo*	Reduced chemotaxis		Kim, 2003
	Vav1, Vav3	Murine Vav1,3-/- cells *in vitro*	Increased migratory speed	No defect in chemotaxis	Gakidis, 2004
Melanoma cells	Vav1, Vav2	Murine cells with blocked Vav1,2 *In vitro*	Inhibition of CXCL12-mediated melanoma invasion		Bartolome, 2006
Mammary epithelial cells	Vav1	Constitutively active form *in vitro*	Increased migration		Wilsbacher, 2006

4. Vav1 and TCR/CD28 signalling

4.1. Overview of TCR signalling

Triggering of the TCR-CD3 complex by the MHC-peptide complex displayed by APCs leads to rapid activation of Src family kinases such as Lck, which is constitutively associated with the cytoplasmic domain of the co-receptors CD4 and CD8, and Fyn, which associated with the cytoplasmic domain of the ζ and CD3ε upon receptor clustering. Thus, both Lck and Fyn can phosphorylate immunoreceptor tyrosine based activation motifs (ITAMS) upon TCR trigerring (reviewed in Janeway *et al*., 2001).

Lck subsequently phosphorylates ZAP70 which in turn can phosphorylate linker for activation of T cells (LAT), Vav1 and Slp76. LAT is a cytosplasmic protein that can associate with the inside of the plasma membrane through palmitoylation of its cystein residues, which become associated with lipid rafts. The latter are cholesterol rich areas of the plasma membrane that are essential in the recruitment of signalling molecules and the propagation of signals from the membrane to the inside of the cell (reviewed in Janeway *et al*., 2001; reviewed in Katzav, 2004).

Once phosphorylated, LAT is thought to recruit Grb2-related adaptor downstream of Shc (Gads), which associates with Slp-76. In addition, upon phosphorylation of Slp76 by ZAP70, the former can bind to Vav1 through the latter's SH2 domain, thus recruiting Vav1 to the signalling complex (Aghazadeh *et al*., 2000; reviewed in Tybulewicz *et al*., 2003; Wu *et al*., 1996). This complex serves to propagate the signal from the plasma membrane to the inside of the cell through the activation of signalling pathways that ultimately result in gene transcription (reviewed in Janeway *et al*., 2001).

4.2. Upstream of Vav1

One of the first steps of TCR signalling post-triggering is the activation of the Src family kinases Lck and Fyn. Studies have focused on examining the effect the two kinases have on the activation of Vav1, and have shown that both can induce Vav1 phosphorylation. While Lopez-Lago *et al*. (2000) showed that Lck can indeed

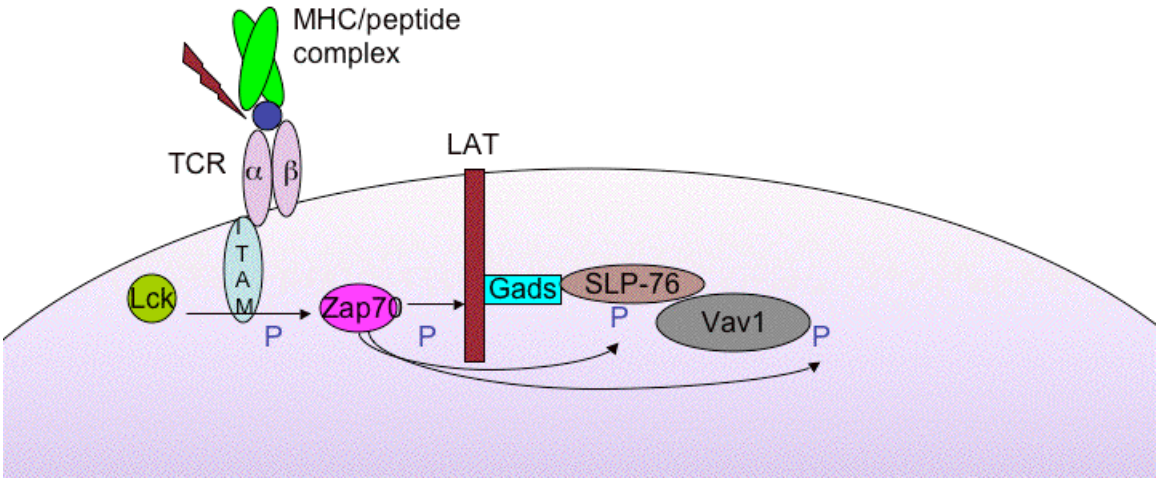
phosphorylate Vav1 at tyrosine residue 174, a study using JCaMI Jurkat T cells concluded that Vav1 is phosphorylated primarily by Fyn, with the later showing 40% higher phosphorylation than the unstimulated control (Huang *et al*., 2000).

Moreover, studies have shown that Vav1 can be activated by Syk and Zap70, two kinases that contain two SH2 domains and two regulatory regions (Bustelo, 2000). Syk is expressed primarily in immature T lymphocytes and is thought to interact with Vav1 through its SH2 domain, as a mutation in a conserved arginine residue of the latter abolished this interaction (Arudchandran *et al*., 2000; reviewed in Bustelo, 2000; Deckert *et al*., 1996). Once again, the Vav1 tyrosine 174 seems to be important, as the Y174F Vav1 mutant was not phosphorylated by Syk (Deckert *et al*., 1996).

Studies on ZAP70 have indicated that it has an important role in Vav1 activation. More specifically, ZAP70 has been shown associate with and tyrosine phosphorylate Vav1 upon TCR triggering (Gouy *et al*., 1995; Huby *et al*., 1995; Michel *et al*., 1998). The ZAP70 tyrosine 315 plays a critical role in the binding to the SH2 domain of Vav1, as a mutation in this residue impaired the function of ZAP70 and eliminated its interaction with Vav1 (Wu *et al*., 1997). In addition, ZAP70 has been shown to phosphorylate Slp76 in sites that allow for Vav1 binding, thus facilitating the downstream signalling pathway (Raab *et al*., 1997). ZAP70 also facilitates the translocation of Vav1 and Son of Sevenless to the lipid rafts upon TCR triggering, as demonstrated in studies using ZAP70-deficient Jurkat T cells (Salojin *et al*., 2000).

It is thought that under physiological conditions, the apparent requirement for Lck and Fyn for the phosphorylation of Vav1 is in fact to phosphorylate ZAP70 which can in turn phosphorylate Vav1. Syk kinase follows a similar mechanism of activation but does not require phosphorylation by Lck or Fyn for the final activation step. However, there is some evidence that Lck and Fyn can activate Vav1 directly, but it is thought that this would only occur under abnormal conditions (reviewed in Bustelo, 2000) A summary of the events occurring upstream of Vav1 post-TCR triggering is summarised in **Figure** 4.

Figure 4: TCR signalling upstream of Vav1



Upon TCR triggering by a peptide/MHC complex, activated Lck phosphorylates ZAP70, which phosphorylates LAT, Slp76 and Vav1. LAT recruits Gads, and along with phosphorylates Slp76 and Vav1, this forms the signalling complex.

Vav1 has also recently been shown to be activated by the co-stimulatory molecule CD46 (Zaffran *et al*., 2001). CD46 is expressed in human T cells and highly promotes T cell proliferation (Astier *et al*., 2000). It has also been shown to induce morphological changes mediated by Vav1 and Rac1 (Zaffran *et al*., 2001).

4.3. Adaptor molecules interacting with Vav1

Vav1 interacts with adaptor molecules which facilitate its downstream effects. Adaptors lack enzymatic activity, and primarily function as scaffolds by binding to other proteins. They can act both as positive and negative regulators of T cell function, with examples such as LAT, Slp76, Grb-2 and WASp (Wilkinson *et al*., 2004).

As mentioned earlier, LAT, a transmembrane adaptor, is phosphorylated upon TCR triggering, and can thus recruit Gads to the lipid rafts (reviewed in Janeway *et al*., 2001; Wilkinson *et al*., 2004). Studies have shown that LAT can be phosphorylated by ITK, which allows for its association with Vav1 via the LAT phosphorylated tyrosine 171 and

the Vav1 SH2 domain (Perez-Villar *et al*., 2002). This was further confirmed by studies using a Vav1 SH2 domain mutant, where association of Vav1 with LAT as well as its translocation to the plasma membrane were inhibited. (Arudchandran *et al*., 2000).

Slp76 is another adaptor protein which plays an integral role in the activation of downstream signalling pathways (Wilkinson *et al*., 2004). It associates with Vav1 via the latter's SH2 domain (Wu *et al*., 1996), and has been shown to be important in bringing together Vav1 and WASp, thus regulating the re-arrangement of the actin cytoskeleton (Zeng *et al*., 2003). In addition, Slp76 is involved in NFAT transcription and IL-2 production, with overexpression having a similar effect to that of Vav1 (Wu *et al*., 1996).

4.4. Negative regulators of Vav1

Research on the negative regulators of Vav1 has been far less extensive, but has identified one key regulator, Cbl-b. The latter is a ubiquitously expressed protein with a multidomain structure, which belongs to a well known family of E3 ubiquitin ligases that inhibit signalling cascades by downmodulating the activity of kinases, or ubiquitinating membrane tyrosine kinase receptors (Bustelo, 2000).

The observation that Cbl-b negatively regulates Vav1 came from a study that showed that Cbl-b overexpression in rodent fibroblasts resulted in inhibition of signalling pathways mediated by Vav1. The two molecules are thought to associate via the entire SH3-SH2-SH3 domain of Vav1 and proline-rich stretch in the central region of Cbl-b (Bustelo *et al*., 1997). In addition, T cells from cbl-b -/- mice stimulated with anti-CD3/CD28 were found to have higher GEF activity that the WT, suggesting that Cbl-b regulates the GEF activity of Vav1 (Chiang *et al*., 2000). Moreover, cbl-b-/- mice develop autoimmunity and T cell infiltration into multiple organs. As TCR-triggering alone in T cells from cbl-b -/- mice resulted in comparable levels of proliferation as TCR and CD28 –triggered wild type cells, it is thought that loss of Cbl-b results in lowering of the threshold for T cell activation and subsequent uncontrolled activation of Vav1 in the absence of CD28 co-stimulation (Bachmaier *et al*., 2000).

c-Cbl has also been shown to associate with Vav1 upon TCR activation in both thymocytes and peripheral T cells. This association is dependent upon tyrosine

phosphorylation of c-Cbl and the SH2 domain of Vav1, and is thought to negatively regulate the function of Vav1 (Marengere *et al*., 1997).

4.5. Downstream signalling cascades

4.5.1. Vav1 as a GEF for RhoGTPases

4.5.1.1 Rho GTPases, regulators of the cytoskeleton

The mammalian Rho superfamily consists of nine proteins which share 50-55% homology and are widely expressed. These proteins are RhoA, RhoB, RhoC, Rac1, Rac2, Cdc42, G25K, RhoG and TC10 (reviewed in Hall, 1994). Rho GTPases cycle between two conformational states, a GTP-bound state (active) and a GDP-bound state (inactive). This conformational change is regulated by GEFs, which promote the cycling from the GDP-bound inactive state to the GTP-bound active state by hydrolysing phosphate (reviewed in Hall, 1994; reviewed in Hornstein *et al*., 2004; Millan and Ridley, 2005).

Studies on the function of RhoGTPases have uncovered that they are important in the regulation of the cytoskeleton, having an integral role in adhesion, polarisation and cell migration (**Table** 5)(reviewed in Etienne-Manneville and Hall, 2002). More specifically, in a series of classic experiments, Paterson *et al*. (1990) first observed that microinjection of a constitutively active form of Rho in 3T3 fibroblasts resulted in dramatic changes in cell morphology, with a typical contracted cell body. They went on to observe that microinjection of an inactive form resulted in complete rounding up of cell, suggesting that Rho is necessary for the regulation of the actin cytoskeleton.

Similar experiments were carried out by Ridley *et al*. (1992) examining the role of Rac1. Microinjection of a mutated form of Rac1 with diminished activity resulted once again in rounding up and detachment of the injected fibroblasts, while microinjection of functional Rac1 resulted in accumulation of membrane ruffles, increase in stress fibres and formation of focal adhesions containing talin and vinculin. This once again suggested that Rac1 has a vital role in the regulation of the cytoskeleton.

Another member of the RhoGTPases family, Cdc42, was first described as being important in the budding site of dividing yeast, thus suggesting a role in the regulation of the cytoskeleton (Hall, 1994). A series of classic microinjection experiments confirmed its role in mammalian cells as well; microinjection of Cdc42 in 3T3 fibroblasts resulted in the formation of microspikes, which were found to be components of filopodia (Kozma *et al*., 1995).

Several studies have since tried to describe the individual roles that Rho, Rac1 and Cdc42 have in mammalian cells. More specifically, Nobes and Hall (1995) performed a series of microinjection experiments using all three members, and examined the effect each RhoGTPase had on the actin cytoskeleton. Their data suggested that Rho induces increased adhesion and stress fibre formation, Rac1 stimulates lamellipodia formation, while Cdc42 activation results in actin polymerisation and focal complex formation, which leads to filopodia formation. This was confirmed by another study, which observed that expression of Cdc42 in 3T3 fibroblasts resulted in filopodia formation, while constitutively active Rac1 induced lamellipodia formation (Schuebel *et al*., 1998).

The role of the three RhoGTPases in migration has also been investigated (**Table** 5). More specifically, a study using macrophages showed that Rho and Rac1 are required for cell movement, while Cdc42 is more important in polarisation and gradient perception (Allen *et al*., 1998). In addition, Rac1 has been directly visualised in migrating cells, and was found to be concentrated in the leading edge in a gradient (Kraynov *et al*., 2000).

Table 5: Summary of experimental evidence on the role of Rac1, CDC42 and Rho in the regulation of the cytoskeleton and migration

Name	Effect on cytoskeleton	Effect on migration	Reference
Rho	Adhesion and stress fibre formation	Important for cell movement	Ridley, 1992; Nobes, 1995; Allen, 1998
CDC42	Microspikes/ filopodia	Important for gradient perception	Kozma, 1995; Allen, 1998
Rac1	Lamellipodia	Important for cell movement	Patterson, 1990; Nobes, 1995; Allen, 1998

Furthermore, all three RhoGTPases have been shown to act by activating PI3kinase (Bokoch *et al*., 1996; Kumagai *et al*., 1993; Zhang *et al*., 1993), with Rac1 and Cdc42 binding to the p85 subunit of the enzyme and thus activating its kinase activity (Zheng *et al*., 1994).

In addition to their role in migrating cells, RhoGTPases have also been found to be important in the EC during the process of transendothelial migration. RhoGTPases have been found to be activated during ICAM-1 cross-linking (Adamson *et al*., 1999), suggesting that these proteins are important in the stable adhesion step of the transendothelial migration (Wojciak-Stothard *et al*., 1999). It can therefore be speculated that manipulation of RhoGTPases in the EC will interfere with lymphocyte transnendothelial migration.

Indeed, a series of experiments pharmacologically manipulating RhoGTPases in brain ECs have shown that inhibition of RhoGTPases prevents lymphocyte migration. Adamson *et al*. (1999) showed that treatment with C3 transferase, which ribosyltes RhoGTPases, rendering them inactive, resulted in inhibition of lymphocyte transendothelial migration. In addition, treatment of brain ECs with prenylation inhibitors which prevent essential post-translation prenylation of Rho proteins, resulted in inhibition of lymphocyte tranendothelial migration and prevention of T cell infiltration to the brain in a model of EAE (Walters *et al*., 2002). Finally, treatment with Lovastatin, which also interferes with the prenylation step, resulted in inhibition of lymphocyte transendothelial migration and attenuation of EAE in mice (Greenwood *et al*., 2003).

4.5.1.2 Vav1 as a GEF for Rho GTPases

The ability of Vav1 to act as a GEF for RhoGTPases was first described by Adams *et al*. (1992) who observed the homology of Vav1 with the Dbl family of Rho. This was also directly observed in a study where NIH3T3 cells were transformed with Vav1, resulting in induction of foci similar to the ones induced by other RhoGTPases (Khosravi-Far *et al*., 1994).

Vav1 was initially identified as being a GEF for Ras in activated human T cell lines and fibroblasts (Adams *et al*., 1992; Gulbins *et al*., 1994a; Gulbins *et al*., 1994b). However, evidence obtained by Bustelo *et al*. (1994) suggested otherwise. More specifically, they found that overexpression of Vav1 in rodent fibroblasts did not result in increase of GTP bound Ras. In addition, Vav1 did not display Ras GEF activity in GTP/GDP exchange assays and finally that the morphology of Vav1 transformed cells did not resemble that of Ras transformed ones (Bustelo *et al*., 1994; Khosravi-Far *et al*., 1994).

Subsequent studies showed that Vav1 could act as a GEF for RhoA, Rac1 and Cdc42 and induce mitogen activated protein (MAP) kinase signalling cascades, as microinjection of Vav1 in fibroblasts resulted in actin polymerisation similar to the one induced by the former RhoGTPases (Olson *et al*., 1996). In addition, overexpression of Vav1 was shown to increase the activity of Rac1 and Cdc42 in Jurkat T cells (Kaminuma *et al*., 2001).

Further studies have suggested that Vav1 primarily acts as a GEF for Rac1 and Cdc42 via its DH domain (Crespo *et al*., 1996; Garcia-Bernal *et al*., 2006; Garcia-Bernal *et al*., 2005; Han *et al*., 1997). This activity has been shown to require Lck or Fyn phosphorylation of tyrosine 174 which, as mentioned earlier, is thought to release the GEF activity from the autoinhibitory arm (Arudchandran *et al*., 2000; Crespo *et al*., 1997; Han *et al*., 1997). In addition, the Y174F mutation of the DH domain enhances the Vav1 GEF activity for Rac1, suggesting that tyrosine residue acts as a negative regulatory site for Vav1 (Kuhne *et al*., 2000).

Recent nucleomagnetic resonance studies have also indicated that Vav1 interacts with Rac1 via its CD domain, with residues K116 and S83 being either part of the interphase or undergoing conformational change in response to the CD binding (Heo *et al*., 2005). Vav1 has also been shown to act as a GEF through the interaction of its SH2 domain with the Mer receptor tyrosine kinase which is known to regulate immune ingestion of apoptotic cells in monocytes (Mahajan and Earp, 2003).

In addition to Vav1 being a GEF for Rac1, Vav2 has been shown to act as a GEF for RhoA and other distant members of the Rho family such as RhoG. This was observed

in a study comparing constitutively active forms of Vav1 and Vav2, which displayed phenotype similar to Rac1 and RhoG respectively (Schuebel *et al*., 1998).

The association of Vav1 with Rac1 has been shown to activate subsequent signalling pathways, such as MAPkinase and c-JUN N-terminal kinase (JNK) (Crespo *et al*., 1996; Olson *et al*., 1996). These lead to activation of transcription factors resulting in gene expression and cytoskeletal re-arrangements.

4.5.2. PI3 kinase pathway

Vav1 activation through tyrosine phosphorylation and its association with the afore mentioned adaptor molecules results in the activation of downstream signalling cascades. One of the pathways thought to be triggered is the PI3k pathway. PI3 kinases are a family of enzymes which are divided into three classes based on their structure, regulation and *in vitro* substrate (Leevers *et al*., 1999).

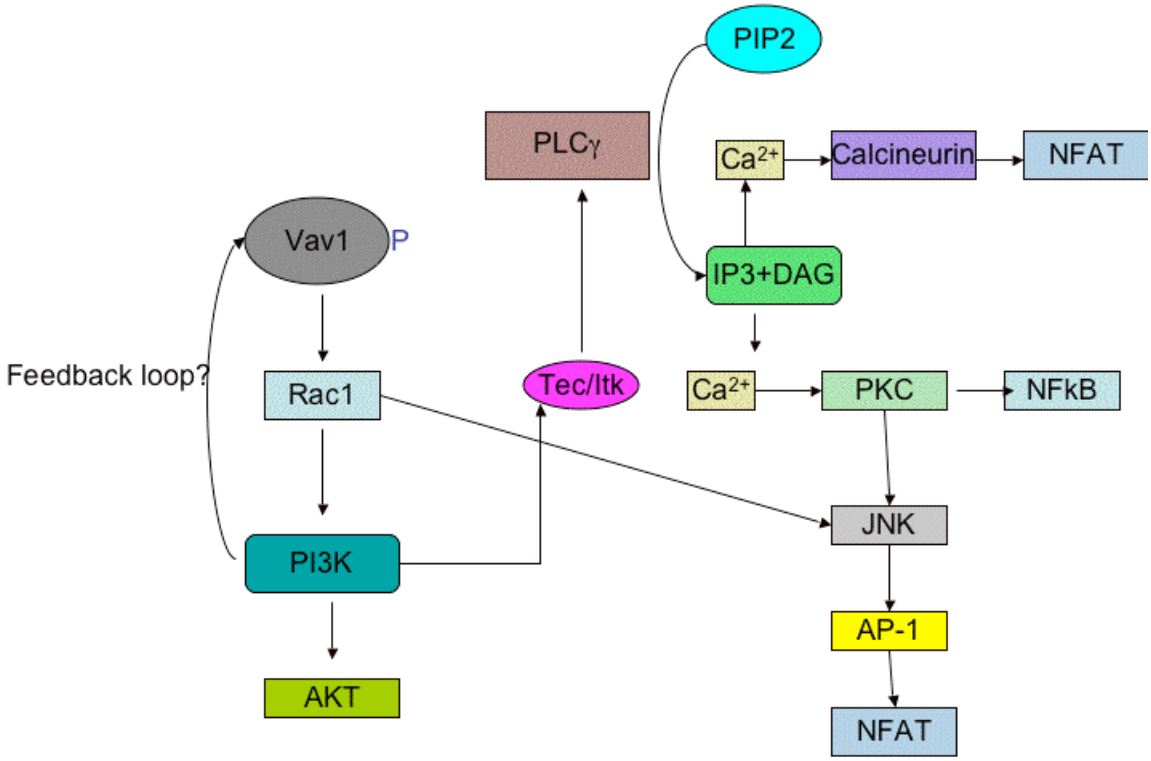
Vav1 is thought to activate the RhoGTPases Rac1 through its DH domain, which in turn is thought to activate PI3kinase, leading to AKT and Tec family kinase (Tec, ITK) phosphorylation (reviewed in Tybulewicz *et al*., 2003). Interestingly, there has been evidence that Vav1 can also act downstream of PI3kinase, as PI3 kinase inhibition can lead to reduced Vav1 phosphorylation. Thus, it has been suggested that PI3kinase and Vav1 may act in a positive feedback loop (Reynolds *et al*., 2002; reviewed in Tybulewicz *et al*., 2003).

The activation of the PI3kinase pathway leads to the activation of Tec and Itk kinases through phospholipids phosphoinositol-3-phosphotase (PIP3). Tec and Itk kinases have been shown to activate further signalling through (phospholipase C) PLCγ as well facilitate Vav1 interaction with other proteins which mediate its localisation to the plasma membrane (Dombroski *et al*., 2005). In addition, signalling through PLCγ is thought to lead to the membrane recruitment of Ras (Reynolds *et al*., 2002).

PLCγuses phosphoinositol 4.5 bisphosphate (PIP2) as a substrate for the production of inositol triphosphate (IP3) and diaglycerol (DAG). Interestingly, Vav1-/- T cells have higher levels of PIP2 than the WT, which indicates failure to activate PLCγ (Reynolds *et al*., 2002).

The two products generated from PIP2 are IP3 and DAG. They are both involved in the activation of transcription factors, the former activating NFAT and the latter NF-Kb. More specifically, IP3 leads to an increase in intracellular calcium, which mediates an increase in calcineurin, thus activating NFAT (Costello *et al*., 1999; Fujikawa *et al*., 2003). NFAT is the transcription factor for IL-2, which explains the reduction of IL-2 production in Vav1-/- mice (Costello *et al*., 1999; Fischer *et al*., 1998; Penninger *et al*., 1999). Overexpression of Vav1 has also directly been shown to lead to NFAT transcription and IL-2 expression in Jurkat T cells (Wu *et al*., 1995). Interestingly, the negative regulator of IL-2, glycogen synthase kinase (GSK)3alpha (reviewed in Cantley, 2002), is activated independently of Vav1 in a pathway regulated by PI3kinase, as Vav1-/- Jurkat and freshly isolated T cells can induce GSK3alpha phosphorylation upon CD3/CD28 stimulation (Wood *et al*., 2006). NFAT is also the transcription factor for c-Maf, which in turn is the transcription factor for IL-4, thus explaining the reduced production of the later in Vav1-/- mice (Tanaka *et al*., 2005). Note that Vav1 can activate NFAT through different signalling pathways as well (reviewed in Katzav, 2004). The downstream signalling events upon Vav1 phosphorylation as summarised in **Figure** 5.

Figure 5: Downstream signalling events



Vav1 phosphorylation leads to PI3K activation, which initiates a signalling cascade that has as an end result the activation of the transcription factors NFAT and NFkB.

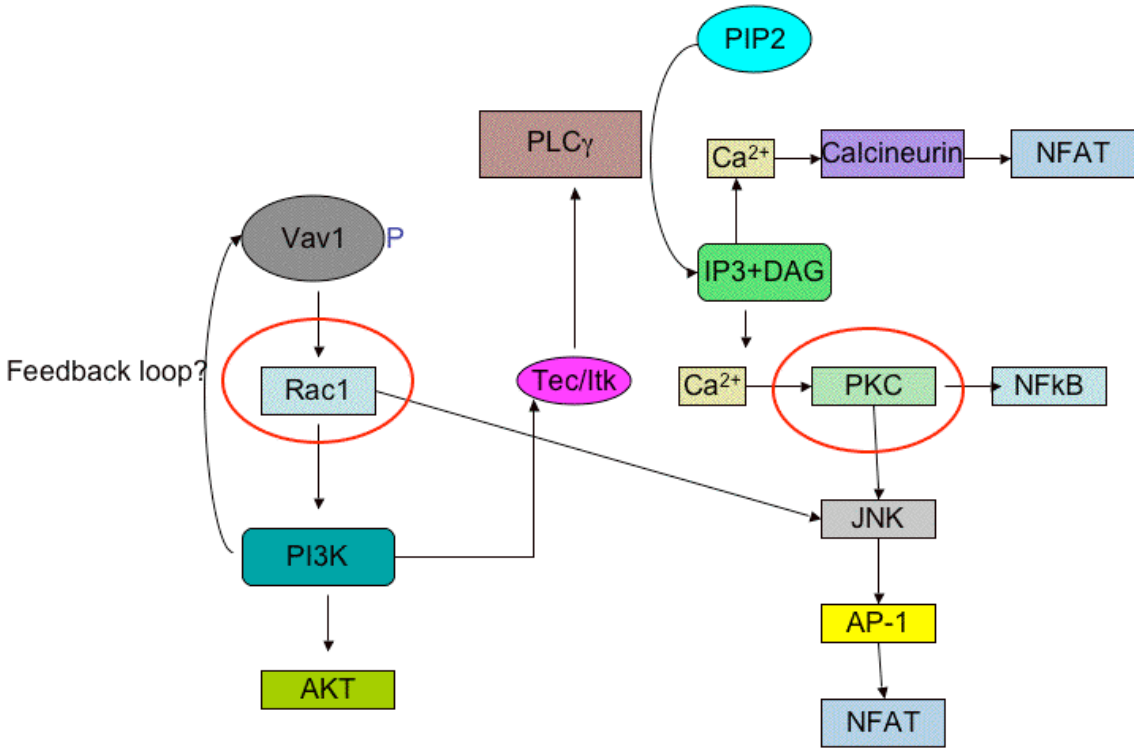
4.5.3. JNK kinase pathway

The second product generated from PIP2 is DAG, which has been shown to activate PKC, an enzyme that activates the transcription factors NFκB as well as activate JNK (reviewed in Janeway *et al*., 2001).

JNK kinase is a serine threonine kinase involved in the phosphorylation of transcription factors such as c-Jun (reviewed in Bustelo, 2000). Early studies showed that Vav1 could activate JNK to levels similar to other GEFs via its DH and CH domains (Crespo *et al*., 1996; Han *et al*., 1997). Mutation in the tyrosine regulatory sites of Vav1 have in fact been shown to induce JNK activation (Lopez-Lago *et al*., 2000).

Further studies showed that Vav1 can activate JNK by two pathways (**Figure** 6); the first is a Ca^{2+}-dependent pathway, and is controlled by Vav1 and PKCθ (Moller *et al*., 2001). The second is a Rac1-dependent pathway, where Vav1 activates JNK via its GEF activity (Kaminuma *et al*., 2001). JNK can in turn activate AP-1 by phosphorylating c-Jun (Derijard *et al*., 1994), which acts as a transcription factor activating NFAT and consequently IL-2 (Northrop *et al*., 1993). Thus, as mentioned earlier, Vav1 can activate NFAT through several pathways.

Figure 6: Activation of the JNK pathway by Vav1



Vav1 can activate JNK by two pathways. One is Ca^{2+} -dependent and is mediated by PKC and the other is Ca^{2+} - independent and is mediated by Rac1.

4.5.4. MAPKinase pathway

Vav1 has also been shown to activate the MAPK pathway. More specifically, Vav1-induced transformation is thought to involve the MAPK pathway (Khosravi-Far *et al*., 1994), while Vav1-/- T cells have been shown to have reduced extracellular signal

regulated kinase (ERK) activation (Colucci *et al*., 2001). The mechanism of activation is not really understood but it is thought that Vav1 may regulate ERK via PLCγ1, which causes release of DAG, activating the GEF for Ras, leading to a Ras-mediated activation of the MAPK pathway (reviewed in Tybulewicz, 2005). In addition, studies by Villalba *et al*. (2000) have suggested that Ras and Vav1 may act in parallel pathway with respect to the activation of ERK, where Ras plays a dominant role, overriding the effects of Vav1. The same study suggested a role for Vav1 in CD69 upregulation, the later being regulated by ERK.

4.6. Vav1 in CD28 signalling

Studies have shown that Vav1 has a pivotal role in CD28 signalling. More specifically, Vav1 is thought to be phosphorylated upon CD28 ligation, in a process dependent on residues 173-181 within the CD28 cytosplasmic tail (Klasen *et al*., 1998). Vav1 phosphorylation has been shown to require Fyn via binding of its SH2 domain and is thought to be stronger with concomitant TCR ligation (Michel *et al*., 1998; Michel *et al*., 2000). It has to be noted that a recent study by Dennehy *et al* (2007) suggests that CD28 signals are in fact dependent on TCR signals, as the latter is required for CD28-mediated phosphorylation of the adaptor Slp76 and thus recruitment of Vav1.

Interestingly, a recent study has shown that CD28 mediated methylation of Vav1 on arginine, causing it to localise to the nucleus. The localisation of Vav1 to the nucleus in this case was observed around 30 minutes after stimulation, which contrasts the seconds/minutes it takes post TCR/CD28 co-ligation and subsequent Vav1 phosphorylation. This suggests that arginine methylation, whose role is not yet fully understood, marks a different function of Vav1 (Blanchet *et al*., 2005).

Furthermore, a study by Raab *et al*. (2001) has shown that CD28-dependent activation of Vav1 also involves Slp76. Using transfected Jurkat T cells, they observed that CD28 ligation resulted in both Vav1 and Slp76 translocation to the plasma membrane, where they were found to form complexes. This CD28-dependent complex formation subsequently led to the activation of downstream effectors such as NFAT for the transcription of IL-2.

By recruiting and activating Vav1, CD28 can activate signalling pathways independently of the TCR, such as Akt, Tec and ITK kinases (reviewed in Michel and Acuto, 2002; Tuosto *et al*., 2000), which in turn can lead to gene expression. Other than the already mentioned transcription of IL-2 (Raab *et al*., 2001), CD28 ligation has been found to induce activation of NFKB in a Vav1-dependent manner (reviewed in Michel and Acuto, 2002). More specifically, NFKB is a transcription factor that belongs to the Rel family of proteins, and is important in innate immunity, as it acts to transcribe genes that encode cytokines, chemokines, enzymes and anti-microbial peptides. Upon TCR activation, the IKKβ, which is part of the I kappa B kinase (IKK) signalosome (IKKα, IKKβ, and NEMO) and functions in sequestering NFKB in the cytoplasm, becomes phosphorylated and ubiquitinated, hence degraded, thus allowing for the transcription factor to translocate to the nucleus and act on its target genes (reviewed in Ben-Neriah, 2002).

CD28-dependent activation of NFKB does not follow the afore mentioned canonic pathway. More specifically, upon CD28 stimulation, Vav1 has been found to associate with IKKα in both Jurkat and primary $CD4^+$ T cells, at the level of the CD28 clusters (Piccolella *et al*., 2003). Degradation of IκBα by the IKK signalosome leads to release of NFKB in the nucleus and consequently allows for transcription of the genes it regulates (Michel *et al*., 2000). This is thought to involve the MAPK signalling pathway (Marinari *et al*., 2002).

Vav1 activated by CD28 ligation has been found to associate with the RhoGTPase Rac1 while in a complex with Slp76, and result in remodeling of the actin cytoskeleton (Raab *et al*., 2001). In addition, CD28-activated Vav1 has been found to associate with WASp, which as mentioned earlier, is an important regulator of the actin cytoskeleton (reviewed in Sechi and Wehland, 2004).

Finally, as with TCR-dependent activated Vav1, CD28/Vav1 signalling is thought to be regulated by Cbl-b, the later acting as a ubiquitin ligase for Vav1 (Miura-Shimura *et al*., 2003).

5. Hypothesis and Aims

Vav1 acts as an essential link between TCR signalling and the re-arrangements of the cytoskeleton which are essential for T cell migration. In addition to this, Vav1 has been shown to regulate the migration of several cell types, including T cells. Based on this, we had hypothesised that Vav1 activation, occurring post-TCR triggering by the endothelium, will regulate the cytoskeletal re-arrangements resulting in recruitment and retention of T cells in the sites of inflammation. Thus, it was predicted that loss of Vav1 will result in inefficient TCR-dependent recruitment and retention of T cells in antigenic sites.

The initial aims of this study were the following:

- To establish a physiological role of Vav1 in the regulation of memory T cell trafficking *in vitro* and *in vivo*
- To establish a physiological role of Vav1 in TCR-induced T cell recruitment *in vitro* and *in vivo*.

In addition to this, Vav1 has been implicated in conjugate formation as well as CD28-mediated co-stimulation. Prompted by our findings which suggested a role for Vav1 in the regulation of the migratory stop signal in T cells, two new aims emerged:

- To establish a role for Vav1 in memory T cell conjugate formation and the tranduction of the migratory stop signal *in vitro* and *in vivo*
- To assess the contribution of Vav1 in CD28-mediated regulation of T cell trafficking *in vitro* and *in vivo*

Materials and Methods

1. Mice

129sv and C57BL/6 male and female mice aged 7 to 9 weeks were purchased from Harlan Olac (Bicester, UK) and used as tissue donors and for *in vivo* experiments. Wild type (WT) and Vav1-/- 129sv mice used for the generation of T cells were previously described (Turner *et al*., 1997). Experiments with animals were performed under the Home Office authority (PPL 70/5872).

2. Cells

2.1. Endothelial cells

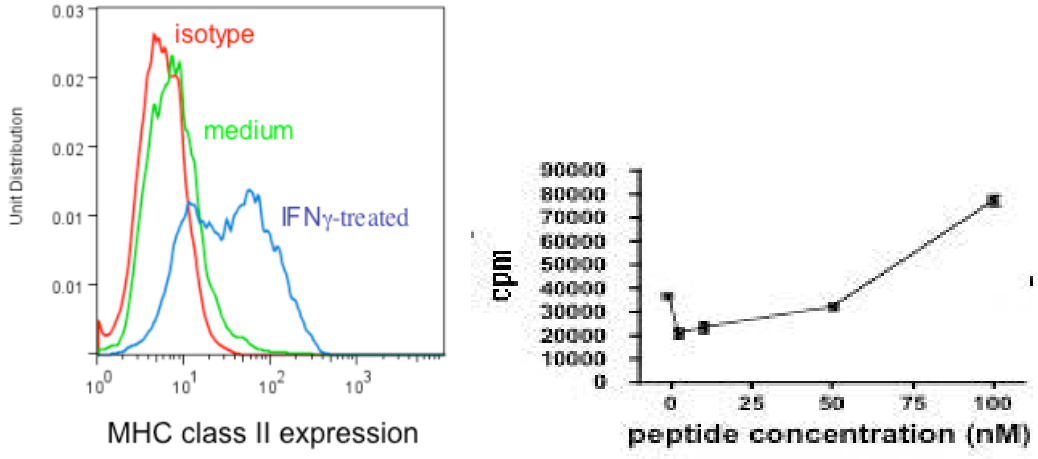
2.1.1 Generation of Endothelial cells

ECs were generated from WT 129sv male and female mouse lungs (Harlan Olac) as previously described (Marelli-Berg *et al*., 2000). Mouse lungs were isolated, minced into 2-3mm^2 square blocks and incubated with 0.5mg/ml collagenase (Sigma, Dorset, UK) for 30 minutes at 37 °C, followed by passing through a 70μm pore membrane (Marathon, London, UK) to remove undigested tissue. The digested tissue was washed in phosphate buffer saline (PBS) and further digested in trypsin/ Ethylenediaminetetraacetic acid (EDTA) (Gibco, Paisley, UK) at 37 °C for 5 minutes to create a single cell suspension. The trypsin was inactivated with foetal calf serum (FCS) (Globepharm, Guilford, UK) and the cells washed in PBS 2.5% FCS for 5 minutes. The cells were then seeded in 2% gelatinated (Sigma) 25 cm^2 culture flasks (Helena biosciences, Sunderland, UK) with EC medium as described in section 2.1.2.

2.1.2 Culture of ECs

Primary mouse ECs generated from 129sv male and female mouse lungs were cultured in 2% gelatinated 25 cm^2 flasks at 37ºC in the presence of 5% CO_2. The EC medium consisted of Dulbecco's Modified Eagle media (DMEM, Gibco) supplemented with 2 mM glutamine (Gibco), 50 IU/mL penicillin (Gibco), 50 μg/mL streptomycin (Gibco), 50 mM 2-Mercaptoethanol (2-ME) (Gibco), 1mM sodium pyruvate (Gibco), 20mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) (Gibco), 1% non-essential amino acids (Gibco), 20% FCS and 150µg/ml EC growth supplement (Sigma). Medium was replaced every 48 hours. When confluent, the cells were detached with trypsin/EDTA and passaged. For functional assays, the ECs were used between passage 2 and 6 and treated with 600 U/mL murine IFNγ (PeproTech, Peterborough, United Kingdom) for 48 to 72 hours prior to use in experiments. This led to up-regulation of MHC class I, induction of MHC class II and antigen presentation, leading to proliferation of antigen-specific T cells (**Figure** 7)

Figure 7: IFNγ treatment induced MHC class II induction and T cell proliferation



Treatment of murine ECs with 600units IFNγ for 48 to 72 hours results in MHC class II expression induction (left panel, courtesy of Eleanor Raynsford) and T cell proliferation (right panel).

2.2. T cells

2.2.1 Isolation of HY-specific T cells

Memory WT and Vav1-/- T cells specific for the male-specific minor histocompatibility antigen HY in the context of H2-D^b were obtained by two fortnightly intraperitoneal immunisations of female mice with male splenocytes in PBS. Each immunisation consisted of one whole male WT spleen in 100ml PBS suspension.

At day 28, the mice were culled and their spleens and lymph nodes extracted. The cells from the spleen and lymph nodes were detached by processing through a 70μm pore membrane and cultured *in vitro*.

2.2.2. T cell culture

HY-specific WT and Vav1-/- T cells isolated from immunised mice were further expanded *in vitro* by fortnightly re-stimulation in 24 well plates (Helena biosciences) with irradiated (60Gy) male splenocytes ($50x10^6$ splenocytes per $5x10^6$ lymphocytes) and 20U/ml rIL-2 (Roche, Hertfoshire UK) in T cell medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, 10mM HEPES and 50 mM 2-ME were cultured at 37ºC in the presence of 5% CO_2.

HY-specific WT T cells from C57BL/6 mice were already in culture in our laboratory. They were cultured as previously described and used in the skin grafting experiments.

For use in experiments, viable T cells were selected between 6 and 10 days by density gradient centrifugation with Ficoll-Paque (Amersham Biosciences, Buckinghamshire, UK).

2.3. Bone marrow-derived dendritic cells

2.3.1 Isolation of bone-marrow dendritic cells

Bone marrow (BM) -derived DCs were obtained from WT C57BL/6 mice. Femurs from 7–to-10-week-old female mice were removed and BM cells were flushed with culture medium using a 26 gauge needle (Fischer Scientific, Loughborough, UK). Red blood cells were lysed from the cell suspension with lysis buffer (Gentra systems, Minneapolis, USA). BM cells (5×10^6) were seeded per well in a 6 well plate (Helena bioscience) in DC medium as described in section 2.3.2.

2.3.2 Dendritic cell culture

Bone marrow-derived dendritic cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, 50 mM 2-ME and 8-16% murine granulocyte-macrophage colony stimulating factor (GM-CSF) obtained from the supernatant of the GMCSF hybridoma (gift from Professor Andrew George, Imperial College, London, UK). Cells were culutured at 37ºC in the presence of 5% CO_2. On days 3 and 5, fresh culture medium was added to the plates.

For functional assays, immature DCs were matured overnight with 100ng/ml lipopolysaccharide (LPS) (gift from Dr. Jian-Guo Chai, Imperial College, London, UK) and were used between 7-to-10 days post-isolation.

3. Reagents

The HY peptides Uty, Smcy and A^b were a gift from Dr. Diane Scott (Imperial College, London, UK) and used at concentrations between 0-100nM. Tritiated thymidine (Amersham) was used at 1μCi/well. Mouse IFNγ was purchased from Peprotech and used at 600units/ml or 600units/injection. rICAM-1 was purchased from R&D systems and was used between 2 and 5μg/ml diluted in Tris pH 8.5. Bovine serum albumin (BSA) was purchased from Sigma. PKH26 was purchased from Sigma. CSFE was purchased from

Molecular probes (Paisley, UK) and used between 100nM and 1μM diluted in PBS. Phalloidin conjugated with Alexa 546 (Molecular probes) and anti-tubulin conjugated with Fluorescein isothiocyanate (FITC) (Sigma) were a gift from Professor Anne Ridley (Ludwig Institute of Cancer Research, UCL, London) and were used at 5: 200 and 1:300 dilution respectively. 0.2% 100x Triton was purchased from Sigma. Vectorshield medium containing 4,6-diamidino-2-phenylindole (DAPI) was purchased from Vector laboratories (Peterborough, United Kingdom). CXCL12 was purchased from Peprotech and was used at 50ng/ml. CXCL10 was purchased from Peprotech and was used at 300ng/ml. PMA and ionomycin were purchased from Sigma and used at 5μg/ml and 2.5μg/ml respectively. Golgiplug containing Brefeldin A which prevents protein transport was purchased from BD Pharmingen (Oxford, UK), and used at 1μl per condition. Cellfix containing paraformaldehyde was purchased from BD Pharmingen and used at 1:10 dilution.

4. Antibodies

Antibody	**Clone**	**Company**
CD4-allophycocyanin (APC)	CT-CD4	Caltag, Buckingham, UK
CD8-phycoerythrin (PE)Cy5	53-6.7	BD Pharmingen
CD4-FITC	H129.19	BD Pharmingen
CD3	CT-CD3	Caltag
LFA-1-FITC	2D7	BD Pharmingen
CD25-FITC	PC61	Cambridge biosciences, Cambridge, UK
CD69-FITC	H1.2F3	Cambridge biosciences
CD62L-FITC	MEL-14	Cambridge biosciences
CXCR4-FITC	2B11	BD Pharmingen
CCR5-PE	HM-CCR5	Cambridge biosciences
CCR7	N/A	Calbiochem, Nottingham, UK
CXCR3-PE	220803	R&D systems, Abingdon, UK
CD28	37.51	BD Pharmingen
$\alpha_4\beta_7$-PE	DATK32	BD Pharmingen
PSGL-1	4RA10	BD Pharmingen
VCAM-1	N/A	From hybridoma
CD105	MJ7/18	BD Pharmingen
CD11c-PE	HL3	BD Pharmingen
A^b-FITC	25-9-17	BD Pharmingen
CD80-FITC	16-10A1	BD Pharmingen
CD86-FITC	GL1	BD Pharmingen
IFN-γ-FITC	XMG1.2	Caltag
IL-4 APC	11B11	Ebioscience (London, UK)
D^b-blocking	N/A	Gift from Maggie Millrain, Imperial College, UK

5. Proliferation assays

5.1. Cells

WT and Vav1-/- T cells were used at 10^4/well between days 6 and 10 post-stimulation. Splenocytes acting as APCs were used at $5x10^5$ per well. They were isolated from female C57BL/6 mice, passed through a 70μm membrane to create a single cell suspension and irradiated at 60Gy to prevent proliferation. ECs acting as APCs were used at 10^4 per well. 48-72 hours prior to the experiment, they were treated with 600u/ml IFNγ and subsequently irradiated at 180Gy to prevent proliferation

5.2. Proliferation assay

HY epitope specificity was assessed with thymidine incorporation assays. WT and Vav1-/- T cells were incubated with irradiated splenocytes or ECs, HY peptides UTY, SMCY and Dby (concentrations 0nM-100nM) in a 96 well plate (flat-bottom for ECs, U-bottom for splenocytes) in 200μl at 37ºC in the presence of 5% CO_2. In experiments examining MHC class I antigen presentation, 50μl D^b blocking antibody was added to the culture. The plate was pulsed 48 hours later with 1μCi/well ^3H thymidine and incubated overnight at 37ºC in the presence of 5% CO_2. The plate was harvested using the Tomtec harvester 96 and filter (Wallac/PerkinElmer, Buckinghamshire, UK), counted using the Wallac Microbeta counter for Windows. In experiments using ECs as APCs, the plate was frozen for 5 hours to detach the cells and thawed at 37ºC prior to harvesting. The results were analysed using Microsoft Excel and Prism software.

6. Flow cytometry

6.1. Cytometer and analysis software

The flow cytometer used was a two laser BD fluorescence activated cell sorter (FACS)calibur (Oxford, UK) connected to a Mac computer (OS 9) using Cellquest as acquisition software.

Acquired samples were analysed using Flowjo (Treestar, USA) by gating on live cells on the forward and side scatter plot.

6.2. Surface staining

For surface staining, cells were labelled with the appropriate volume of antibody or isotype control according to the manufacturer's instructions in 100µl PBS at 4ºC in the dark for 20 minutes. They were subsequently washed with PBS and fixed with 300µl 1xCellfix for analysis.

6.3. Intracellular staining

For intracellular staining of IFNγ and IL-4, $1x10^6$ T cells were incubated with $5x10^6$ female splenocytes pulsed with HY peptide and Golgiplug containing BrefeldinA for 4 hours at 37ºC in the presence of 5% CO_2. . T cells were incubated with 5µg/ml PMA and 2.5µg/ml ionomycin in the presence of Golgiplug as a positive control or Golgiplug alone as a negative control. In experiments assessing cytokine secretion upon antibody stimulation, T cells were activated with plastic-bound antibody as described in section 6.4 in the presence of Golgiplug. The cells were subsequently washed and staining for intracellular antigen using the Fix&Perm kit (Caltag) according to the manufacturer's instructions. Briefly, T cells were incubated with surface antigen for 15 minutes at room temperature in the dark, followed by incubation with permeabilising reagent A for 15 minutes at room temperature in the dark. The T cells were subsequently washed and

stained for intracellular antigen in the presence of permeabiling reagent B for 20 minutes at room temperature in the dark. Cells were fixed in 300µl 1xCellfix and analysed by flow cytometry.

6.4. Activation experiments

For the activation experiments, WT and Vav1-/- T cells were activated for 24 hours with plate-bound 1µg/ml anti-CD3 (BD Pharmingen) and 5µg/ml anti-CD28 or 2µg/ml hamster IgG as a control (Sigma) and subsequently stained for analysis as described in section 6.2. Antibody coating was done in the presence of 200µl Tris pH 8.5 for 2 hours at 37^0C. The wells were washed with PBS prior to plating the T cells.

7. Adhesion assays

96 well plates were coated with rICAM-1 (5µg/l in Tris pH 8.5) for 2 hours at 37^0C. Control wells were incubated with PBS alone. The plate was subsequently blocked with 2.5% BSA in PBS at 37°C for 1 hour, and washed with 0.5% BSA in PBS. T cells were labelled with PKH26 as described in section 12.2 and were plated at 10^3 per well and incubated for 10-60 minutes. Non-adherent T cells were removed by gentle washing and the number of adherent cells was analysed with wide-field fluorescence microscopy as described in section 12.4. Control wells were not washed. The number of cells present in each well was estimated using the algorithm in the appendix. The percentage adhesion was calculated with the following formula:

$$\frac{\text{Experimental adhesion-min adhesion}}{\text{Control adhesion-min adhesion}} \times 100$$

8. Labelling the cytoskeleton

Glass coverslips (VWR, Lutterworth, United Kingdom) were coated with ICAM (5µg/ml in Tris pH 8.5) for 2 hours at 37ºC. ICAM-1-coated coverslips were blocked with 2.5% BSA in PBS for 1 hour at room temperature. Viable WT and Vav1-/- T cells,

used between days 6 and 10 post-stimulation, were plated on ICAM-1-coated coverslips at 0.5-1x10^6 cells per coverslip for 15 minutes at 37ºC in the presence of 5% CO_2. Non-adherent cells were removed by gentle washing with PBS and fixed with 1xCellfix for 10 minutes at room temperature. 1xCellfix was washed with PBS and the cells were permeabilised with 0.2% 100x Triton in PBS for 4 minutes at room temperature. The coverslips were subsequently incubated with 0.5% BSA in PBS for 30 minutes, followed by addition of Phalloidin and anti-tubulin for 1 hour at a dilution of 5:200 and 1:300 respectively. The antibodies were washed off with PBS and the coverslips were allowed to dry overnight. The coverslips were mounted with vectorshield medium containing DAPI which labels the nucleus and visualised with wide-field fluorescence microscopy as described in section 12.4.

9. DC:lymphocyte conjugate formation assays

Female DCs were matured overnight with 100ng/ml LPS at day 7 post-isolation. They were subsequently labelled with 100nM CFSE as described in section 12.2 and used at 10^5 DCs per condition. Viable WT and Vav1-/- T cells were used at day 10 post-isolation at 2.5x10^5 cells per condition and labelled with PKH26 as described in section 12.2.

For conjugate formation, female or female plus 50nM Dby peptide DCs were incubated with 2.5x10^5 WT and Vav1-/- T cells between 1 and 2 hours at 37°C in the presence of 5% CO_2. The cells were spun at 650rpm for 5 minutes to increase the possibility of cell contact. Conjugate formation was analysed with flow cytometry and Flow Jo software. Conjugates were defined as the double positive population formed from the contact between the CFSE-labelled DCs and the PKH26-labelled T cells. Single stains were used as controls for compensation.

10. *In vitro* transwell-based migration assays

10.1. Cells

ECs were treated for 48 to 72 hours with 600u/ml IFNγ. The day before the experiment, they were detached and plated on gelatinated Costar Transwell tissue-culture well inserts (diameter,12 mm), which contained polycarbonate membranes with a 3μm pore size (Sigma), at 10^4 per transwell, and allowed to adhere. On the day of the experiment, the transwells were gently washed to remove non-adherent cells.

Viable HY specific WT and Vav1-/- T cells were used between days 7 and 10 post-stimulation and were plated on the top chamber in 200μl RPMI 2% FCS medium at 3-5x10^5 cells per insert.

10.2. Induction of CD28 signalling

To induce CD28 signalling, viable 3-5x10^5 HY specific WT and Vav1-/- T cells per transwell were treated with a mixture of hamster anti-mouse CD28 (5μg/5x10^6 cells), and rabbit anti-hamster immunoglobulin (Ig) (2.5μg/5x10^6 cells) or Hamster Ig (5 μg/5x10^6 cells) and rabbit anti-hamster Ig (2.5 μg/5x10^6 cells) for 45 minutes at 37ºC in the presence of 5% CO_2. The T cells were washed in PBS prior to use in the experiments.

10.3. Induction of stop signal

To induce the stop signal, 24-well plates were coated with a mixture of 5μg/ml CD28 and 1μg/ml CD3 or hamster IgG (2μg/ml) as an isotype control in 200μl Tris pH 8.5 for 2 hours at 37ºC. The wells were subsequently washed and 3-5x10^5 HY specific WT and Vav1-/- T cells per transwell were plated on the coated wells for 45 minutes at 37ºC in the presence of 5% CO_2.The T cells were harvested and washed in PBS prior to use in the experiments.

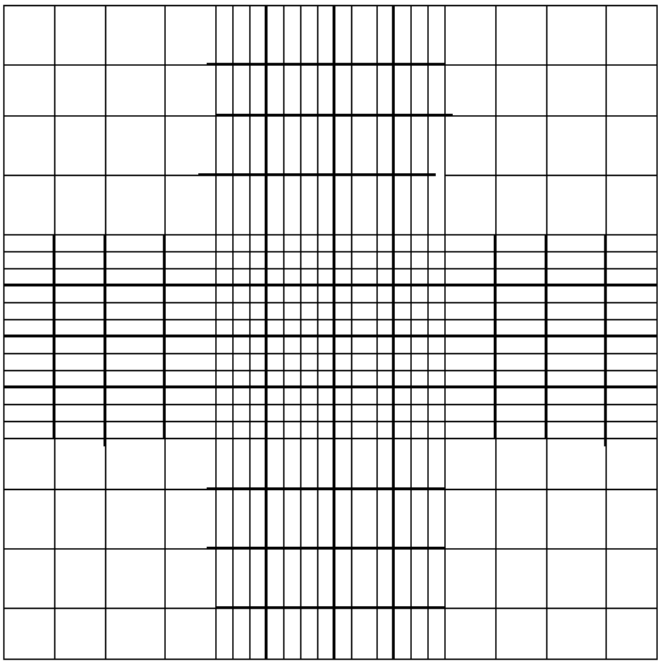
10.4. Transwell-based migration assays

Purified HY specific WT and Vav1-/- T cells (3-5x10^5/transwell) in RPMI 1640 supplemented with 2% FCS were added into each insert and left to migrate through the monolayer. In experiments examining the response to integrin, the transwells had previously been coated with ICAM-1 (2μg/ml in Tris pH 8.5 for 2 hours at 37ºC). In experiments examining the response to chemokines, CXCL12 or CXCL10 were added directly to the bottom well at a concentration of 50ng/ml and 300ng/ml respectively. In experiments examining migration in response to antigen presentation by the endothelial monolayer, the ECs were pulsed with IFNγ for 48 to 72 hours to induce MHC class II expression and antigen presentation, were seeded on 2% gelatinated transwells and allowed to form a monolayer as described in section 9.1.

The number of migrated T cells was determined by counting the lymphocytes present in the well media at different time points. Results were analysed with Microsoft Excel and Prism software and are expressed as the percentage of transmigrated cells.

10.5 Cell counting

Migrated lymphocytes were counted at 2, 4, 6 and 24 hours by sampling 10μl of well media into a haemocytometer (Sigma). Cells present in the four corners of the haemocytometer (depicted below) were counted and divided by four to take an average of migrated cells. The derived number was multiplied by 10^4 to estimate the number of cells per ml and divided by two to calculate the number of cells present in 500μl of well medium.



11. *In vitro* time-lapse migration assays

11.1. Cells

ECs were plated on 35mm dishes and were allowed to grow to confluence at 37ºC in the presence of 5% CO_2 in EC medium. 48 to 72 hours prior to the experiment they were pulsed with 600u/ml IFNγ .

Viable HY-specific T cells were selected through high density centrifugation and were serum-starved for 2 hours in RPMI 2% FCS at 37ºC in the presence of CO_2. They were subsequently plated at $1x10^6$ per 35mm dish in 1ml of RPMI 2% FCS and allowed to migrate.

11.2. Time-lapse microscopy

In time-lapse migration assays testing the response to ICAM-1, 35mm dishes were coated with 5µg/ml ICAM-1 in PBS and incubated at 4°C overnight. The plate was subsequently blocked with 2.5% BSA PBS at 37°C for 1 hour, and washed with 0.5% BSA PBS. HY-specific WT and Vav1-/- T cells were serum-starved in RPMI 2% FCS for

2 hours and subsequently seeded on ICAM-1 dishes at a concentration of $1x10^6$ in 1ml per dish. T cells were incubated for 5 minutes at 37^0 C and washed once to remove non-adherent cells.

In time-lapse migration assays testing the migration through EC monolayer, male and female IFNγ treated ECs were seeded on 2% gelatinated 35mm dishes and incubated at 37°C for 3 days. Prior to the assay, the EC monolayers were gently washed with warm medium to remove non-adherent cells. HY-specific WT and Vav1-/- T cells were serum-starved in RPMI 2% FCS for 2 hours and subsequently seeded on EC-coated dishes at a concentration of $1x10^6$ in 1ml per dish. T cell migration was visualised directly after seeding.

T cell migration was observed with time-lapse microscopy using Tempus software (Kinetic Imaging Ltd, Nottingham, UK). The movie was made with the collection of images taken by a KPM1E/K-S10 CCD camera (Hitachi Denshi, Japan) every 15-30 seconds for 25-50 minutes. The path of approximately 10 cells per movie was tracked for the whole of the time-lapse sequence using Motion Analysis (Kinetic Imaging Ltd, UK). Mathematical analysis was then carried out using Mathematica 6.0 (Wolfram Research Institute) workbooks (Wells and Ridley, 2005).

12. *In vivo* migration assays

12.1 Cells

HY-specific WT and Vav1-/- T cells were used between 5 and $10x10^6$ per iv injection or between 2 and $3x10^6$ per ip injection. For the chemokine experiment, T cells were rested for two hours in 2% FCS prior to the injection.

Female DCs were matured with LPS as previously described and were used at 2 to $3x10^6$ cells per ip injection.

12.2 PKH26 and CFSE labelling

For labelling with PKH26, T cells were washed and re-suspended at a concentration

of 10^7/mL in PBS. The PKH26 Cell Linker was added at a final concentration of 5μM, and cells were incubated at room temperature for 5 minutes. The reaction was inactivated by adding an equal volume of FCS to the cell suspension and the cells were washed in PBS 10% FCS for 10 minutes.

For labelling with CFSE, T cells were washed and re-suspended in 100nM-1μM CFSE in PBS at room temperature for 10-15 minutes. The reaction was inactivated by adding an equal volume of FCS to the cell suspension and the cells were washed in PBS 10% FCS for 10 minutes.

12.3 *In vivo* migration assays

To assess T cell trafficking, HY specific labelled WT and Vav1-/- T cells were injected intravenously (iv) at 10^7 cells/injection in male and female 129sv or C57BL/6 mice. The mice were culled 2, 6 or 24 hours post-injection.

To assess chemokine -dependent recruitment, C57BL/6 female mice were injected intraperitoneally (ip) with CXCL10 (1.2μg/ml) followed by an iv injection of 10^7 HY-specific labelled WT or Vav1-/- T cells per injection. Prior to the assay, T cells were rested for 2 hours in 2% FCS in the absence of IL-2 to promote chemokine-driven migration. The mice were culled 16 to 20 hours post-injection

To assess antigen-dependent and CD28-dependent recruitment from the bloodstream, C57BL/6 male and female mice received an injection of $6x10^5$ units IFNγ ip. On day 3, CD28-signalling was induced in labelled $10x10^6$ WT and Vav1-/- cells as described in section 5.4 and the cells were injected iv. The mice were culled 24 hours later.

To assess antigen-dependent tissue infliltration, male and female C57BL/6 mice received an injection of $6x10^5$ units IFNγ ip. On day 3, labelled HY specific $2x10^6$ WT and Vav1-/- were injected ip into male and female mice. The mice were culled 24 hours later.

To assess skin graft infiltration, female C57BL/6 were grafted on with skin from the tail of male or female C57BL/6 mice according to the method of Billingham and

Medawar (1951). On day 4, the mice received an iv injection of labelled HY-specific WT and Vav1-/- T cells. The mice were culled 24 hours later.

To assess conjugate formation *in vivo*, female C57BL/6 mice were injected with female DCs ($2x10^6$) pulsed with Dby peptide ip, followed by an iv injection of HY-specific labelled WT and Vav1-/- T cells. Mice were culled 24 hours post-injection.

12.4 Wide-field fluorescence microscopy and flow cytometry

Tissue samples were embedded in optimal cutting temperature (OCT) compound (Raymond Lamb, United Kingdom), snap-frozen, and stored until analysis. Frozen tissue samples were sectioned at 5-10μm. The number of infiltrating cells obtained in six randomly selected x10-magnified fields/sample from at least three animals were then averaged and assessed statistically.

Cryostat sections were laid onto microscope slides (VWR), left to dry overnight, and then mounted in Vectorshield mounting medium for fluorescence with DAPI which labels the cell nucleus blue.

Peritoneal membranes were laid on polysine microscope slides (VWR), left to dry overnight, and mounted in Vectorshield mounting medium for fluorescence with DAPI.

Slides were visualized with a Coolview 12-cooled CCD camera (Photonic Science, Newbury, United Kingdom) mounted over a Zeiss Axiovert S100 microscope equipped with Metamorph software (Zeiss, Welwyn Garden City, United Kingdom). 10x, 20x, 40x and 100x (oil immersion)/0.6 NA objectives and standard epi-illuminating fluorescence filter cubes were used and 12-bit image data sets were generated. Quantification of T-cell infiltrates observed by wide-field fluorescence microscopy was performed using aspecifically designed software to run in the LabView (V7.1; National Instruments, Austin, TX) environment. This automatic cell-counting algorithm is based on a combination of background subtraction, multiple thresholding, and morphologic processing approaches which allows identification of single fluorescent cells within the tissue (also see Appendix). Statistical analysis was performed using Microsoft Excel and Prism software.

Enrichment of labelled T cells in the peritoneal lavage was assessed by flow

cytometry using BD FACSDiva and analysed with Flowjo. To control for autofluorescence, the peritoneal lavage of mice injected with PBS without T cells was analyzed. Enrichment of labelled T cells in the spleen and lymph nodes was analysed by processing the tissue sample through a 70μm membrane and assessed by flow cytometry.

Results

Chapter 1: Generation and Characterisation of HY-specific WT and Vav1-/- T cells

1.1. Introduction

Histocompatibility (H) antigens are products of polymorphic genes recognised by the immune system upon exchange of tissues between genetically dissimilar individuals. In the event of MHC-mismatch, the transplanted tissue is termed an allograft and is rapidly rejected by the immune system due to allorecognition of major H antigens (Janeway *et al.,* 2001). However, T cell responses can also be observed when transplants are matched for major H antigens between donor and recipient, for example in human leukocyte antigen (HLA)-identical siblings (termed syngeneic transplant). This suggests the presence of other antigens involved in transplant rejection by immune mechanisms (reviewed in Janeway *et al.,* 2001)(reviewed in Simpson *et al.*, 2002)

Minor H antigens are derived by endogenous polymorphic peptides and are expressed on the MHC complex on the surface of the APC. Some of the minor H antigens recognised by female transplant recipients are encoded by the Y chromosome, and in mouse include the MHC class I D^b-restricted peptides Uty (WMHHNMLDI) and Smcy (KCSRNRQYL), the former thought to be immunodominant, as well as the A^b-restricted class II Dby peptide (NAGFNSNRANSSRSS) (Greenfield *et al.*, 1996; Millrain *et al.*, 2001; Scott *et al.*, 2000; reviewed in Simpson *et al.*, 2002). T cells specific for these antigens can be generated by *in vivo* immunisation of female mice with male APCs; tetramer-staining of $CD8^+$ T cells from the female recipients immunised with male splenocytes or DCs revealed that with weekly immunisations, antigen-specific populations can be observed as early as day 14 (Millrain *et al.*, 2001).

The HY system has previously been successfully used in studies examining the migration of antigen-specific T cells both *in vitro* and *in vivo* (Marelli-Berg *et al.*, 2004; Mirenda *et al.*, 2006). For this reason, HY-specific T cells were generated from WT and Vav1-/- female mice as described in the material and methods section, and were used to

investigate the role of the GEF Vav1 in T cell migration. This chapter describes the characterisation of the generated T cells in terms of specificity, surface and intracellular antigen expression as well as adhesion.

1.2. Results

1.2.1. Proliferative responses and co-receptor expression by Vav1-/- T cells

Studies on Vav1 in mice have revealed that Vav1-/- T cells have altered thymic development, defective positive selection and altered sensitivity to negative selection, which may result in expression of different variable TCR chains than the WT controls (Turner *et al*., 1997). It was therefore possible that the primed WT and Vav1-/- T cells may have had different epitope specificity.

To test epitope specificity of the WT and Vav1-/- T cells we used proliferation assays based on tritiated thymidine incorporation. WT and Vav1-/- T cells (10^4 per well) were incubated with irradiated female APCs, including either splenocytes ($5x10^5$ per well) or ECs (10^4 per well), and HY peptides, including the class I-restricted Uty and Smcy, as well as the class II Dby peptide. Different concentrations (0-100nM) of the peptides were also examined to determine the highest proliferative response.

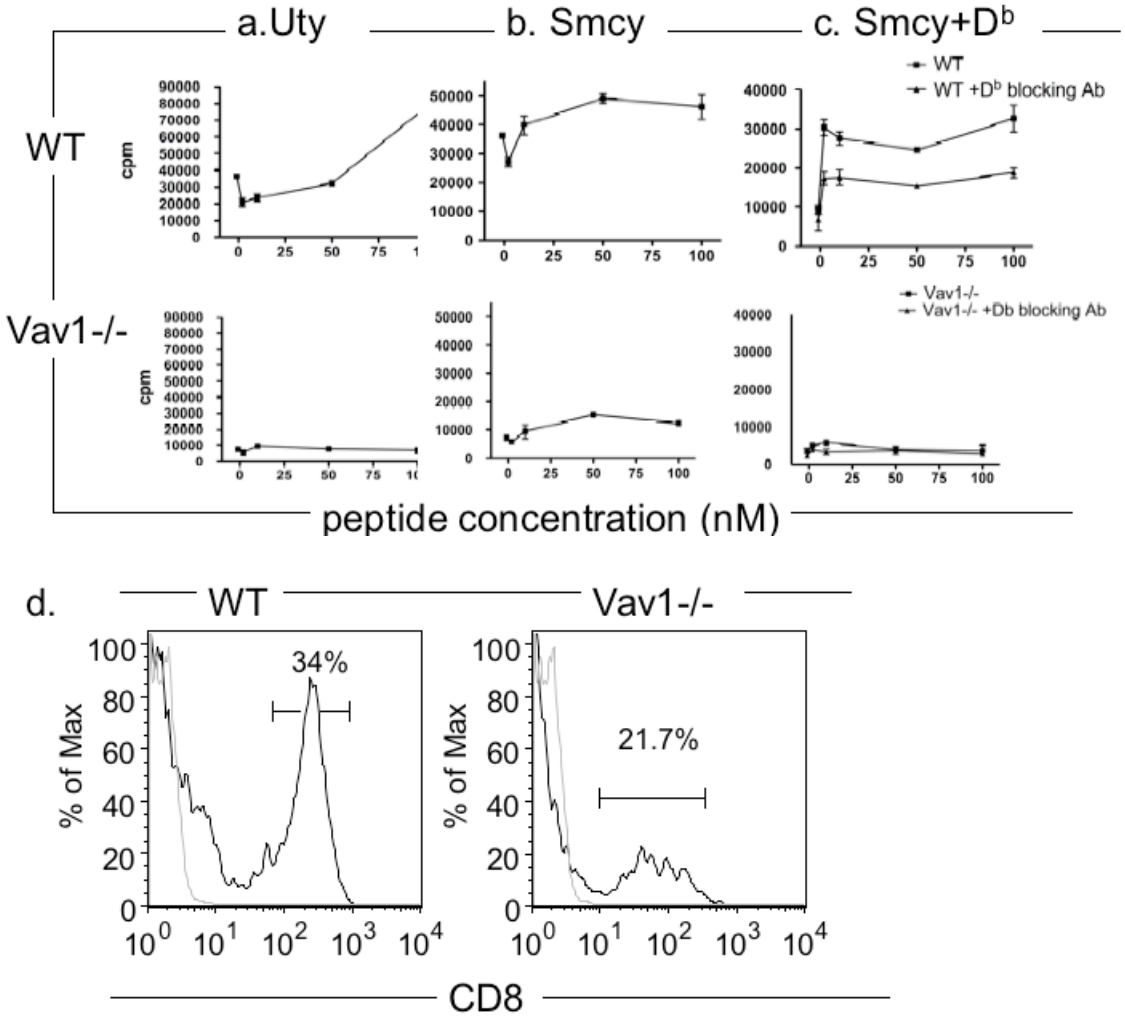
Figure 8 a,b shows the proliferation of WT and Vav1-/- T cells in response to the class I-restricted epitopes upon initial isolation. WT T cells responded with high levels of proliferation against both the immunodominant Uty as well as the Smcy epitope, while the Vav1-/- T cells showed very little proliferation against the immunodominant Uty epitope, primarily responding to Smcy. Flow cytometry analysis revealed that at this stage, both WT and Vav1-/- T cells were a mixed population of $CD4^+$ and $CD8^+$ T cells, but both expressed the CD8 co-receptor (**Figure** 8 d).

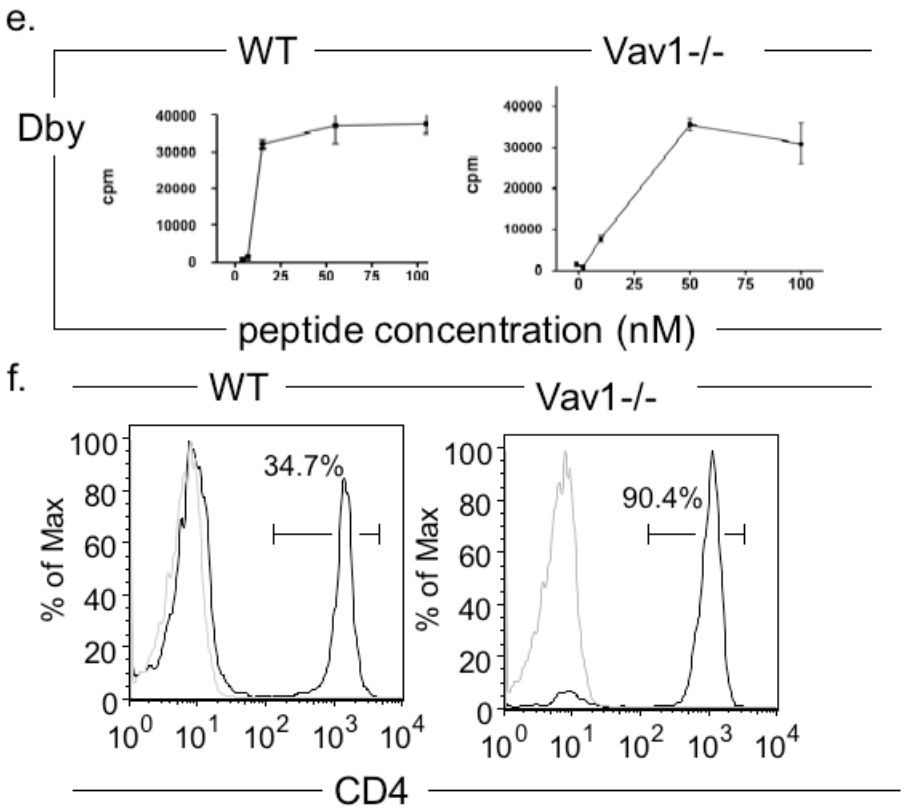
Both Uty and Smcy are MHC class I D^b epitopes (reviewed in Simpson *et al*., 2002). To examine the proliferative responses of WT and Vav1-/- T cells further, we added a D^b blocking antibody to the culture. As shown in **Figure** 8 c, proliferation of both WT and Vav1-/- T cells was significantly inhibited in the presence of the D^b blocking antibody, suggesting that the observed proliferative response was due to presentation by MHC class I.

At around passage eight, i.e. after eight rounds of re-stimulation with male splenocytes *in vitro*, $CD4^+$ T cells overgrew the $CD8^+$ ones in both WT and Vav1-/- T

cells (**Figure** 8f). For this reason, the specificity of WT and Vav1-/- T cells against the class II Dby peptide was once again examined by tritiated thymidine incorporation. As shown in **Figure** 8 e, both WT and Vav1-/- T cells were specific for the class II Dby peptide, with the highest response shown at the peptide concentration of 50nM. The specificity to this peptide was routinely examined thereafter to certify that both WT and Vav1-/- T cells remained HY-specific throughout the study. It is important to note that both WT and Vav1-/- T cells used in the *in vitro* and *in vivo* functional assays were $CD4^+$ and specific for the Dby peptide.

Figure 8: Vav1-/- T cells display reduced proliferative responses to antigenic challenge





Viable WT and Vav1-/- T cells used between days seven and ten post-stimulation were incubated with 10^4 irradiated female ECs pulsed with different concentrations of Uty (a) or Smcy (b) peptide for 48 hours, followed by pulsing with 1μCi tritiated thymidine (a)(b). WT and Vav1-/- T cells were incubated with irradiated 10^4 female ECs pulsed with peptide and with or without D^b blocking antibody for 48 hours, followed by pulsing with 1μCi tritiated thymidine (c). WT and Vav1-/- T cells were labelled with CD8 and analysed for expression by flow cytometry (d) WT and Vav1-/- T cells were incubated with irradiated $5x10^5$ female splenocytes pulsed with the A^b- Dby peptide for 48 hours, followed by pulsing with 1μCi tritiated thymidine (e). WT and Vav1-/- T cells were labelled with CD4 and analysed for expression by flow cytometry (f). The proliferative response is measured as counts per minute (cpm). Co-receptor expression in the flow cytometry plots is depicted in black while isotype control expression is depicted in grey.

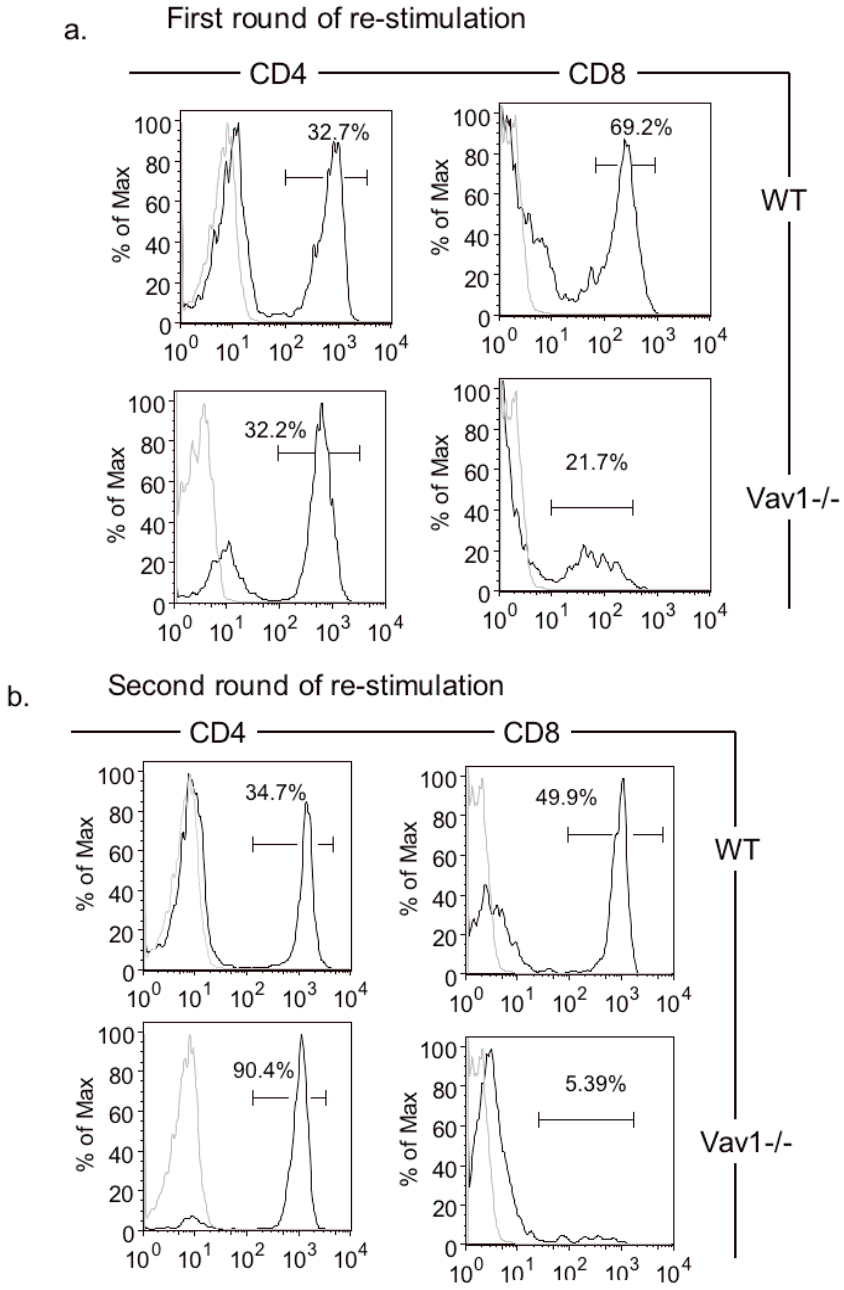
1.2.2. Expression of co-receptors and co-stimulatory molecules by Vav1-/- T cells

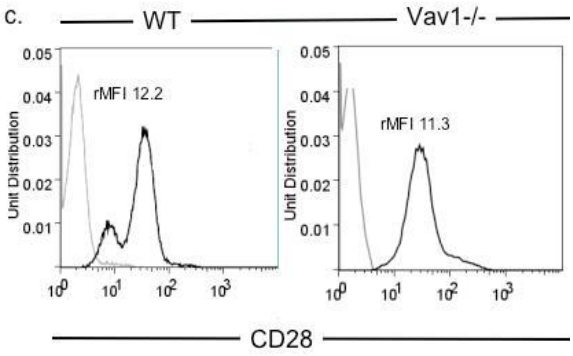
The expression of co-receptors CD8 and CD4 was routinely examined on the HY-specific WT and Vav1-/- by flow cytometry. Upon isolation, both WT and Vav1-/- T cells were a mixed population of $CD4^+$ and $CD8^+$, as shown in **Figure** 9 a. However, as mentioned earlier, around passage eight, i.e. eight rounds of re-stimulation with male splenocytes, the $CD4^+$ populations overgrew the $CD8^+$.

For this reason, we examined the kinetics of CD4 overgrowth by analysing co-receptor expression in freshly-isolated T cells as well as after the first round of stimulation with male splenocytes. As shown in **Figure** 9 a, upon isolation, HY-specific WT and Vav1-/- T cells expressed both co-receptors, but after the first round of stimulation, both populations displayed similar kinetics of $CD4^+$ overgrowth, as shown in **Figure** 9 b. This phenomenon was more readily observed in the Vav1-/- T cells. Both HY-specific WT and Vav1-/- T cells used in functional assays were $CD4^+$.

The expression of the co-stimulatory molecule CD28 was also examined because of its importance in some of the functional studies (**Figure** 9c). Both HY-specific WT and Vav1-/- T cells were found to express high levels of CD28 by flow cytometry.

Figure 9: Co-receptor and co-stimulatory molecule expression of WT and Vav1-/- T cells





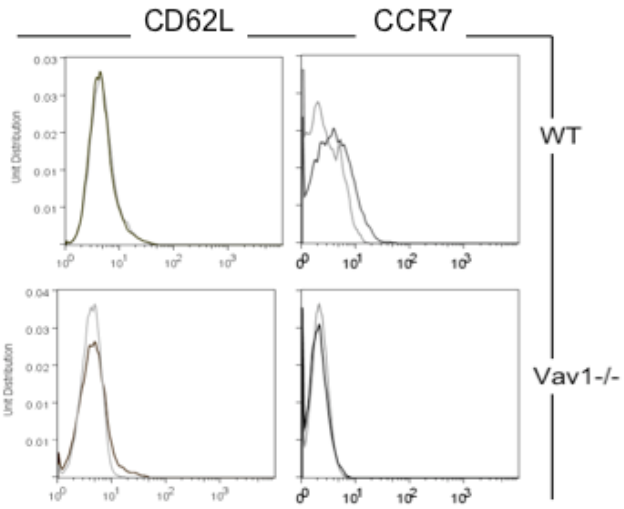
Viable WT and Vav1-/- T cells were labelled with CD4 or CD8 and analysed by flow cytometry on day zero (a) and day seven (b). WT and Vav1-/- T cells were labelled with CD28 and analysed by flow cytometry between days seven and ten post-stimulation (c). Co-receptor and co-stimulatory molecule expression is represented by the black line and isotype control by the grey line. rMFI is calculated by dividing the MFI of the labelled T cells with the MFI of the isotype control.

1.2.3. Phenotypic characterisation of Vav1-/- T cells

Memory T cells can be subdivided based on their homing potential to central memory and effector memory T cells. Central memory T cells are characterised by the expression of CCR7 and preferentially migrate to the lymph nodes, while effector memory T cells have little or no expression of CCR7 and preferentially migrate to sites of inflammation (Sallusto *et al*., 1999; Weninger *et al*., 2001).

To determine whether the HY-specific WT and Vav1-/- T cells belonged to the effector memory or central memory subset, they were labelled with CD62L (L-selectin) and CCR7 and expression was analysed by flow cytometry. **Figure 10** shows that both WT and Vav1-/- T cells expressed CCR7 in very low levels and did not express CD62L, suggesting that they belonged to the effector memory subset. The relative MFI of expression of CCR7 and CD62L is summarised in **Table 6.**

Figure 10: Vav1-/- T cells belong to the effector memory subset



Viable WT and Vav1-/- T cells were labelled between days seven and ten post-stimulation with CCR7 and CD62L and analysed by flow cytometry. Expression of CD62L and CCR7 is shown in black while the grey line represents the isotype control

Table 6: rMFI of CD62L and CCR7

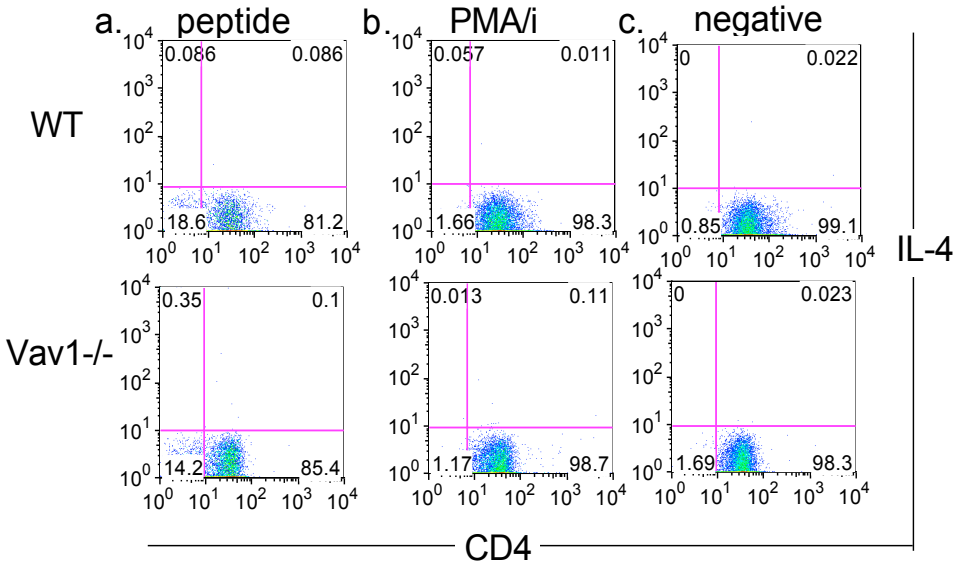
Marker	Average rMFI WT	Average rMFI Vav1-/-
CD62L	0	0
CCR7	2.3	2.2

rMFI was calculated by dividing the MFI of the sample by that of the isotype control

Memory T cells can also be categorised based on cytokine secretion. Traditionally, T cells have been classified as belonging to the Th1 or Th2 subtypes, the former characterised by secretion IFNγ and the latter IL-4 (reviewed in Janeway *et al*., 2001). Reports on cytokine secretion of Vav1-/- T cells have been confusing. One study observed that Vav1-/- T cells do not secret IFNγ or IL-4 when stimulated with their cognate peptide *in vitro* (Korn *et al*., 2003), while another report suggested that Vav1-/- T cells have a skewed Th1 profile, with impaired IL-4 production (Tanaka *et al*., 2005).

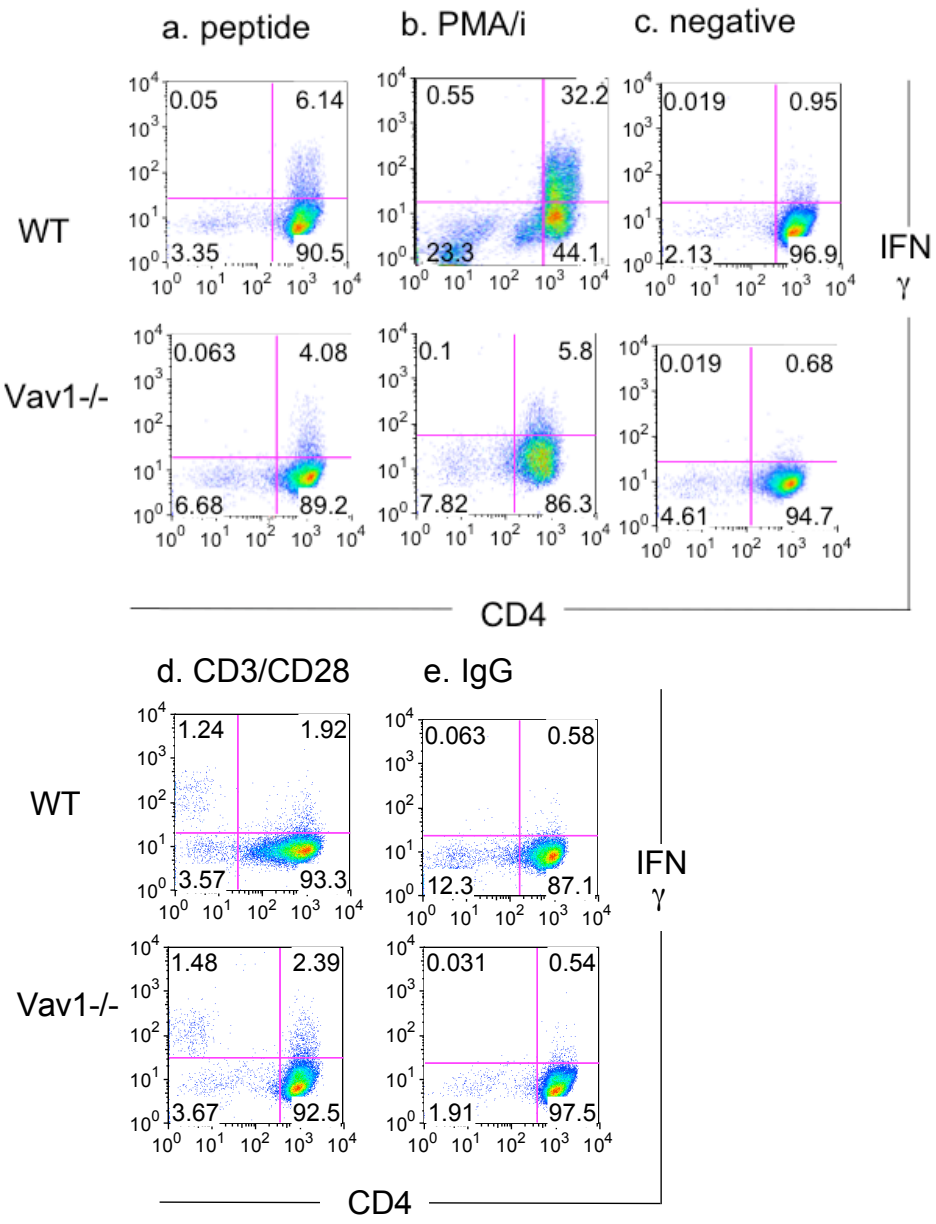
To determine their cytokine profile *ex vivo*, HY-specific A^b-restricted WT and Vav1-/- T cells (10^6 per condition) were stimulated with Dby peptide (50nM) -pulsed irradiated splenocytes ($5x10^6$ per conditions) and then analysed for intracellular IFNγ by flow cytometry. As seen in **Figure 11** and **Figure 12**, both WT and Vav1-/- T cells had a polarised Th1 phenotype, as they secreted IFNγ but not IL-4. Both WT and Vav1-/- T cells secreted IFNγ in response to peptide stimulation as well as TCR triggering through CD3 and CD28 and the non-specific mitogens PMA/ionomycin.

Figure 11: Expression of intracellular IL-4



Viable WT and Vav1-/- T cells used between days seven and ten post-stimulation were stimulated for four hours with irradiated female splenocytes and Dby peptide (a), PMA/ionomycin (b) or Golgi plug alone as a negative control (c) and labelled with CD4 and intracellular IL-4. Flow cystometry analysis was performed using FlowJo.

Figure 12: Expression of intracellular IFNγ



Viable WT and Vav1-/- T cells used between days seven and ten post-stimulation were stimulated for four hours with irradiated female splenocytes and Dby peptide (a), PMA/ionomycin (b) Golgi plug alone as a negative control (c) plastic-bound CD3 and CD28 (d) or hamster IgG (e) and labelled with CD4 and IFNγ. Flow cytometry analysis was performed using FlowJo.

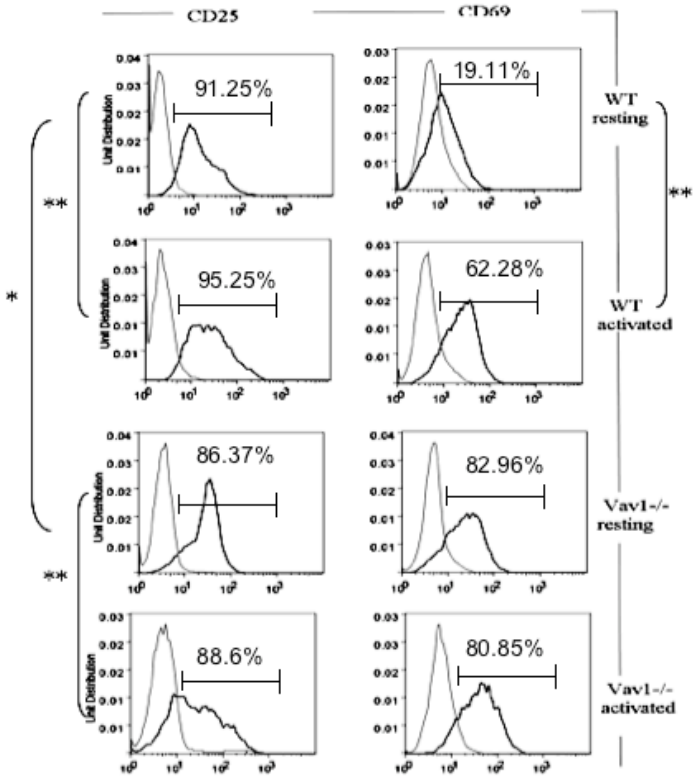
1.2.4 Upregulation of activation markers by Vav1-/- T cells

The importance of Vav1 in T cell activation has been described, with studies showing reduced TCR-induced proliferation and IL-2 production as well as reduced Ca^{2+} flux (Tarakhovsky *et al*., 1995; Turner *et al*., 1997; Fischer *et al*., 1998). In addition, Vav1 is thought to be required for CD69 upregulation upon activation (Villalba *et al*., 2000).

CD69 is thought to be the earliest glycoprotein upregulated following activation in T cells, and is involved in lymphocyte proliferation and function (Cambiaggi *et al*., 1992). Upon 24-hour activation with plastic-bound CD3 (1μg/ml) and CD28 (5μg/ml), HY-specific WT T cells significantly upregulated CD69 while CD69 upregulation was not statistically significant for Vav1-/- T cells as shown in **Figure 13**. The relative MFI of expression of CD69 in resting and activated T cells is summarised in **Table** 7.

CD25 is the receptor for IL-2 and is promptly upregulated by T cells upon activation (Leonard *et al*., 1985). In this experiment, 24-hour stimulation of HY-specific Vav1-/- T cells by plastic-bound CD3 (1μg/ml) and CD28 (5μg/ml) resulted in upregulation of CD25 to levels similar to the WT control (**Figure** 13), suggesting that Vav1 is not important for CD25 upregulation upon activation. The relative MFI of expression of CD25 in resting and activated T cells is summarised in **Table** 7. Interestingly, expression of CD25 by resting T cells was significantly higher in Vav1-/- T cells compared to WT controls.

Figure 13: Upregulation of surface markers upon activation



*Viable WT and Vav1-/- T cells were used between days seven and ten post-stimulation and were incubated with plastic-bound CD3 and CD28 antibodies or medium alone for 24 hours. The expression of the activation markers CD69 and CD25 was examined by flow cytometry. The expression of the markers on resting and activated T cells is represented in black and of isotype control in grey. Statistical analysis was performed using Student's t test comparing resting and activated rMFI (*p<0.05, **p<0.01).*

Table 7: Average relative MFI and percentage positive expression of CD25 and CD69

Marker	Average rMFI resting		Average % positive resting		Average rMFI activated		Average % positive activated	
	WT	Vav1-/-	WT	Vav1-/-	WT	Vav1-/-	WT	Vav1-/-
CD25	1.4	6.3	76.3	84.6	4.7	26	81.3	87.6
CD69	5.6	8.1	41.7	74.1	8.5	12.2	64.2	86.4

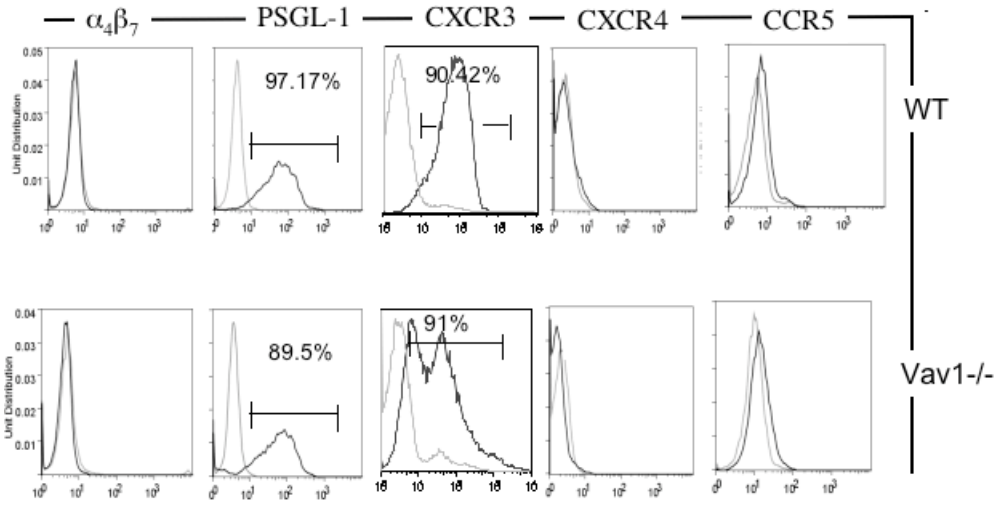
rMFI is calculated by dividing the MFI of the marker expression by the MFI isotype control

1.2.4 Expression of migration-related markers by Vav1-/- T cells

As the focus of this study is T cell migration, the expression of several migration related markers was examined by flow cytometry. The importance of integrins and chemokine receptors in T cells migration has already been described in the introduction. Briefly, CXCR4 is the chemokine receptor for CXCL12 and is postulated to have a role in migration of T cells to the lymph nodes (Okada *et al*., 2002; Scimone *et al*., 2004). CXCR3 is the chemokine receptor for CXCL10 and has an important role in migration to sites of inflammation as well as in transplantation, while CCR5 has been involved in the migration to the liver and CNS during inflammatory conditions such as cirrhosis and multiple sclerosis respectively (Grant *et al*., 2002; Guint *et al*., 2003; Hancock *et al*., 2000). Finally, $\alpha_4\beta_7$ has been shown to be the homing receptor for the gut (Schweighoffer *et al*., 1993; Campbell *et al*, 2002; Abitorabi *et al*, 1996; Hamann *et al*, 1994), while PSGL-1 directs T cells to the skin (Campbell *et al*, 2002).

As shown in **Figure 14**, HY-specific $CD4^+$ WT and Vav1-/- T cells did not express $\alpha_4\beta_7$ integrin but had high expression of PSGL-1 and high expression of the inflammatory chemokine receptor CXCR3. Both HY-specific $CD4^+$ WT and Vav1-/- had very low expression of CXCR4 and CCR5. The average relative MFI of expression of each of these markers is summarised in **Table** 8. The expression of these homing receptors directly correlates with some of the data observed in the migration studies, as will be discussed later on.

Figure 14: Expression of migration-related markers



WT and Vav1-/- T cells were used between days seven and ten post-stimulation in vitro and were labelled with antibodies against $\alpha_4\beta_7$, PSGL-1, CXCR3, CXCR4 and CCR5 and analysed by flow cytometry. Expression of the markers is represented by the black line, while the grey line represents the isotype control. rMFI is calculated by dividing the MFI of the sample with the MFI of the isotype control

Table 8: rMFI and percentage positive cells of migration-related markers

Markers	Average rMFI WT	Average % WT	Average rMFI Vav1-/-	Average % Vav1-/-
α4β7	0.07	0	0.04	0
PSGL-1	19.7	95.83	20.6	90.95
CXCR3	9.3	87.18	9	73.5
CXCR4	1.4	9	0.6	0
CCR5	4.4	37	3.3	24.3

rMFI is calculated by dividing the MFI of the sample with the MFI of the isotype control

1.2.5 Expression of adhesion and cytoskeletal molecules by Vav1-/- T cells

Vav1-/- has previously been shown to be important for the transduction of LFA-1 "inside-out" signals (Krawczyk *et al*., 2002; Ardouin *et al*., 2003). To determine whether

this would affect LFA-1 surface expression, HY-specific $CD4^+$ WT and Vav1-/- T cells were labelled with anti-LFA-1 antibody expression was examined by flow cytometry.

Figure 15 a shows the expression of LFA-1 in HY-specific $CD4^+$ WT and Vav1-/- T cells as determined by flow cytometry. Interestingly, although WT T cells had a homogeneous high expression of LFA-1, Vav1-/- T cell LFA-1 expression was heterogeneous and consistently lower than that of the WT controls (**Figure 15** b).

Integrins such as LFA-1 bind to adhesion molecules on the surface of ECs, such as ICAM-1 (de Fougerolles *et al.,* 1991). Vav1 has previously been shown to be important in the adhesion of thymocytes and peripheral T cells to different adhesion molecules and extracellular matrix proteins such as fibronectin, and collagen (Krawzcyk *et al*., 2002). As Vav1-/- T cells were shown to have lower expression of LFA-1, we examined whether this translated to reduced adhesion to ICAM-1.

HY-specific $CD4^+$ WT and Vav1-/- were examined for adhesion to recombinant ICAM-1 using wide-field fluorescence microscopy, as described in the materials and methods section. Briefly, PKH26-labelled WT and Vav1-/- T cells were plated on ICAM-1-coated 96-well plates (5μg/ml) at a concentration of 10^3 per well and allowed to adhere. Non-adherent cells were removed at different time-points by gentle washing and adhesion was examined by wide-field fluorescence microscopy.

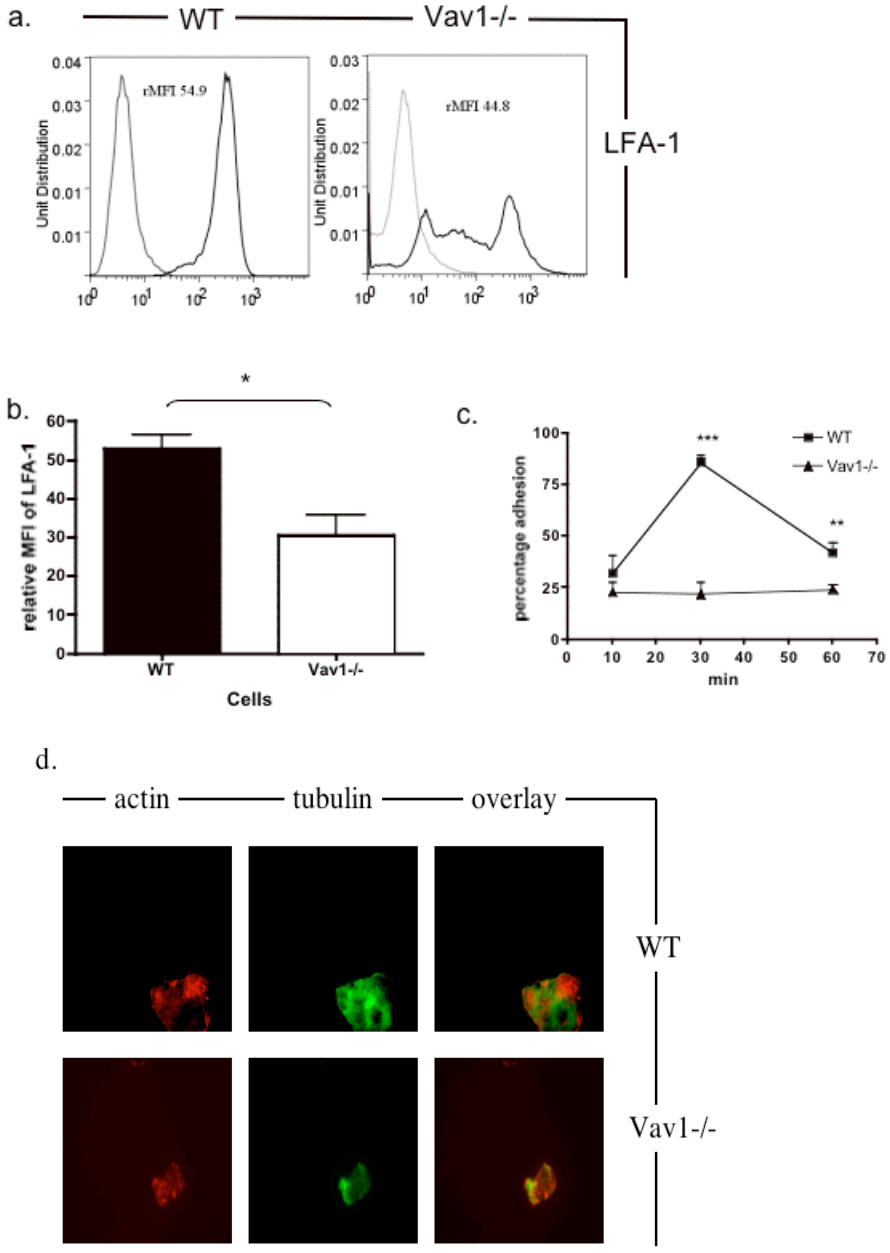
As shown in **Figure 15** c, the percentage adhesion of HY-specific $CD4^+$ Vav1-/- T cells, calculated by dividing the number of T cells adhering to ICAM-1 with the number of T cells adhering to PBS alone, was significantly lower than that observed with WT T cells at two of the three time-points tested. This suggests that LFA-1/ICAM-1-mediated adhesion of memory T cells requires Vav1.

As a GEF for Rac1, Vav1 has been extensively implicated in the regulation of the cytoskeleton. In T cells, Vav1 interacts with the actin polymerising WASp (Savoy *et al.,* 2000) while it has also been implicated in the inactivation of ERM proteins (Faure *et al.,* 2004). It was thus interesting to examine whether the HY-specific $CD4^+$ T cells had normal expression of the cytoskeletal proteins actin and tubulin.

To this effect, HY-specific $CD4^+$ WT and Vav1-/- T cells were plated on glass coverslips coated with ICAM-1 (5μg/ml) and stained for actin and tubulin for one hour as described in the materials and methods section. Both WT and Vav1-/- T cells expressed

the two proteins when examined with wide-field fluorescence microscopy with x100 magnification (**Figure 15**d). This suggests that HY-specific CD4$^+$ Vav1-/- T cells express normal levels of the cytoskeletal proteins actin and tubulin.

Figure 15: Adhesion to ICAM-1 and expression of LFA-1



Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. WT and Vav1-/- T cells were labelled with LFA-1 and analysed by flow

cytometry. Expression of LFA-1 is represented by the black line, while the grey line represents the isotype control (a). The rMFI of LFA-1 in WT and Vav1-/- T cells was calculated by dividing the MFI of the labelled sample by the MFI of the isotype control from three independent experiments (b). WT and Vav1-/- T cells were plated on ICAM-1-coated 96 well plates and non-adherent cells were removed by gentle washing after 10, 30 and 60 minutes of incubation. Percentage adhesion was measured by counting the number of cells in each well by wide-field fluorescence microscopy and dividing by the number of cells adhering to PBS alone. The graph represents the average percentage adhesion from four experiments (c) WT and Vav1-/- T were plated on ICAM-1-coated coverslips and labelled with phalloidin-Alexa 546 and anti-tubulin-fitc for one hour. Actin and tubulin expression was visualised using wide-field fluorescence microscopy (x100 oil immersion lense) (d). Error bars indicate standard error. Statistical analysis was performed using the Student's t test. ($p<0.05$, ** $p<0.01$, *** $p<0.001$)*

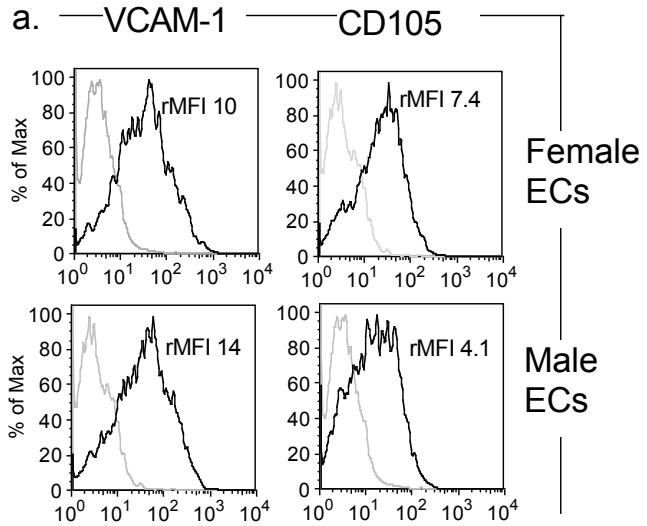
1.2.6 Characterisation of other cells used in this study

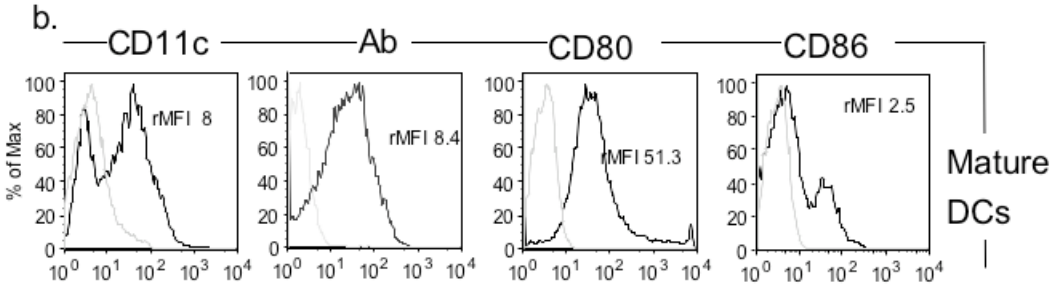
Many of the functional assays used in this study include vascular ECs generated from mouse lungs, as described in the materials and methods section. It was therefore thought important to characterise these cells by flow cytometry for markers that are typically found in ECs.

CD105, also know as endoglin, is a glycoprotein primarily expressed by ECs (Gougos and Letarte, 1988), while VCAM-1 binds to VLA-4 on T cells and is expressed on vascular ECs (Henninger *et al*., 1997). They are therefore both suitable identification markers for vascular ECs. As shown in **Figure 16** a,b, both male and female ECs expressed high levels of CD105 and VCAM-1 respectively.

Some of the functional assays have also used BM-derived DCs, as described in the materials and methods section. These cells were also characterised by flow cytometry after having been matured overnight in an optimal concentration of LPS. As shown in **Figure 16** (b), mature DCs were positive for CD11c, an adhesion molecule primarily expressed by these cells. The $CD11c^+$ cells were then found to express the MHC class II molecule A^b as well as the co-stimulatory molecules CD80 and CD86. These findings validate the use of these DCs in functional assays.

Figure 16: Characterisation of other cells





Male and female ECs (between passage two and six) were labelled with VCAM-1 and CD105 antibody and analysed by flow cytometry (a). Female DCs used seven days post-isolation were matured overnight with LPS and labelled with CD11c, A^b, CD80 and CD86 and analysed by flow cytometry (b).

1.3. Discussion

HY specific WT and Vav1-/- T cells were generated for the purpose of this study through two fortnightly intraperitoneal immunisations of female 129sv mice with male splenocytes from WT mice. We used one whole male spleen per injection of female mice, which differs from the protocol of Millrain *et al*., (2001), who used $5x10^6$ male splenocytes per female mouse. The reason for this was that Vav1-/- T cells have altered sensitivity to negative selection (Turner *et al*., 1997) and would possibly require higher dose of antigen for priming.

Korn *et al*. (2003) observed that Vav1-/- T cells were defective in priming with the MOG_{35-55} EAE peptide. However, proliferation assays based on thymidine incorporation revealed that Vav1-/- T cells showed some proliferative response to the HY peptides, albeit with lower counts than the WT controls.

Reduced proliferation of Vav1-/- T cells in response to TCR stimuli has previously been documented in several studies (Tarakhovsky *et al*., 1997; Fisher *et al*., 1998; Costello *et al*., 1999; Penninger *et al*., 1999). This could be due to loss of responsiveness to TCR stimulation in the absence of Vav1, as signals which bypass the TCR elicit higher proliferation, as well as reduced IL-2 production (Tarakhovsky *et al*., 1997; Turner *et al*., 1997; Fisher *et al*., 1998; Costello *et al*., 1999; Penninger *et al*., 1999). The latter is interesting, as proliferation of the $CD4^+$ Vav1-/- T cells to the class II Dby peptide resulted in counts comparable to the WT control, suggesting that IL-2 may compensate for reduced TCR signalling in Vav1-/- T cells.

Extensive studies by Millrain *et al*. (2001) have shown that the class I epitope Uty is immunodominant to the Smcy epitope. In this study, both WT and Vav1-/- T cells responded to the Uty peptide upon isolation, but the Smcy-specific population in both WT and especially the Vav1-/- T cells overgrew the Uty-specific one. Millrain *et al*. (2001) examined the responses of immunised HY D^b C57BL/6 mice, so it is possible that the kinetics of priming HY D^b 129sv mice are different. It would have been informative to examine HY-specificity using tetramer staining, as the method would allow us to identify the percentage of each population at different time-points, thus dissecting the "switch" from primarily Uty positive to Smcy positive. Unfortunately, these tetramers are

no longer commercially available, and pentamer staining has been ineffective for the staining of polyclonal T cells in our hands as well as others,

Both $CD4^+$ WT and Vav1-/- T cells overgrew $CD8^+$ ones in culture. This effect has been observed by others in our lab (S. Jarmin, personal communication) as well as other labs using HY-specific T cells (J. Dyson, personal communication). The importance of this is not understood, but it is possible that $CD4^+$ T cells are more likely to survive and be "naturally selected" over $CD8^+$ T cells due to higher autocrine IL-2 secretion. This may also explain why this phenomenon was observed more readily in the Vav1-/- T cells than the WT control.

Both HY-specific $CD4^+$ A^b-restricted WT and Vav1-/- T cells generated by our protocol of immunisation appear to be effector memory T cells as they consistently have little or low expression CCR7 or L-selectin (CD62L). In addition, they have a Th1 polarised phenotype, as they do not secrete IL-4 and secrete IFNγ. This is interesting, as previous reports on cytokine secretion have been confounding, with Korn *et al*. (2003) finding no IFNγ secretion upon peptide stimulation of antigen-specific cells *in vitro*, and Tanaka *et al*. (2005) observing defective IL-4 secretion in Vav1-/- T cells *in vitro*.

Previous studies have implied reduced activation of Vav1-/- T cells, with reduced proliferation, IL-2 production and Ca^{2+} flux (Tarakhovsky *et al*., 1997; Turner *et al*., 1997; Fisher *et al*., 1998; Costello *et al*., 1999; Penninger *et al*., 1999). It was interesting to observe that upregulation of CD25 in Vav1-/- T cells was comparable to the WT controls and no significant differences were seen when comparing the rMFI of both resting and activated T cells. It is possible that one of the other Vav proteins compensated for the loss of Vav1 in this case. It is worth mentioning that resting Vav1-/- T cells also had higher expression of CD25 than the WT controls. As CD25 is the receptor for IL-2, higher expression of this marker as well as comparable upregulation to the WT controls may have been a compensatory mechanism in response to reduced IL-2 secretion.

The second activation marker tested was CD69. HY-specific $CD4^+$ Vav1-/- T cells expressed CD69 when resting, which is surprising as previous studies have observed that Vav1 is necessary for CD69 expression via the MAPK signalling pathways (Villalba *et al*., 2001). However, Vav1-/- T cells did not significantly upregulate this marker when

stimulated with CD3 and CD28 antibodies. It is therefore possible that Vav1 is required for upregulation of CD69 upon activation.

Characterisation of expression of migration-related markers was an essential preliminary experiment for the future investigation of the migratory properties of the HY-specific $CD4^+$ A^b-restricted WT and Vav1-/- T cells *in vitro* and *in vivo*. Expression of $\alpha_4\beta_7$, PSGL-1, CXCR3, CXCR4 and CCR5 was comparable between both WT and Vav1-/- T cells, suggesting that Vav1 does not regulate the expression of these markers. This migratory phenotype correlates with the data obtained from the migration studies, as will be discussed in Chapter 2.

The importance of Vav1-/- in T cell adhesion had previously been highlighted by Krawczyk *et al*. (2002). Our findings confirm this observation, with HY-specific $CD4^+$ Vav1-/- T cells showing significantly lower adhesion to ICAM-1 compared to the WT control. Defects in the cytoskeleton, as implied by several studies (Savoy *et al*., 2000) (Faure *et al*., 2004), may account for this effect. The reduced expression of LFA-1, which is the integrin that binds to ICAM-1, may also partially explain this phenotype. In addition, Vav1 has been found to be required for the transduction of "inside-out" signals for LFA-1 as well as LFA-1 clustering (Krawczyk *et al*., 2002; Ardouin *et al*., 2003). Thus, diminished LFA-1 signalling and clustering may also account for reduced adhesion to its ligand, ICAM-1.

Despite having reduced adhesion to ICAM-1, HY-specific $CD4^+$ Vav1-/- T cells expressed both actin and tubulin when plated on ICAM-1 coverslips and no differences in clustering and distribution were observed when compared to the WT control. It is possible that although Vav1 is a GEF for RhoGTPases, its absence does not result in reduced expression of actin and tubulin. Quantifying the levels of actin and tubulin by Western blotting may be useful in establishing any differences in levels of expression of the two proteins. In addition, confocal microscopy may provide more detailed images where differences in organisation and clustering may be visible.

Chapter 2: Constitutive and chemokine-driven migration of WT and Vav1-/- T cells

2.1. Introduction

As part of dissecting the role of Vav1 in T cell migration, we assessed its involvement in T cell trafficking in the absence of antigen. This would allow us to uncover any underlying migratory defects which could have been incorrectly interpreted when examining the role of Vav1 in antigen-dependent T cell migration.

Migration of T cells from the bloodstream into tissues is tightly controlled by molecular checkpoints. Integrins, whose conformational changes mediated by chemokines and shear flow lead to tight adhesion, are important regulators of T cell migration (reviewed in Janeway *et al*, 2001; Garood *et al*, 2006; Alon and Dustin, 2007). They can also act as homing receptors, directing T cells to different sites of the body (reviewed in Ebert *et al*, 2005). Of the various integrin heterodimers, LFA-1 has a fundamental role in T cell migration. As discussed in Chapter 1, HY-specific $CD4^+$ Vav1-/- T cells showed reduced LFA-1 expression and reduced adhesion to ICAM-1. This part of the study will therefore aim to investigate whether these defects will translate to impaired integrin-mediated migration both *in vitro* and *in vivo*.

Chemokines also play an important role in T cell migration, mediating tight adhesion of integrins as well as acting as homing receptors. The two chemokines tested in this study are CXCL12 and CXCL10 which are known to bind to CXCR4 and CXCR3 on the surface of T cells. CXCL10 is particularly relevant in this study, as it is induced by the secretion of IFNγ (Luster and Ravetch, 1987) which will be used to induce MHC class II expression in the antigen-dependent migration studies.

This part of the study aims to investigate the requirement for Vav1 activity in constitutive migration of memory T cells. Defects in HY-specific $CD4^+$ Vav1-/- T cell migration at the cellular level are investigated using *in vitro* assays, while the *in vivo* experiments provide information on the role of Vav1 activity in a more physiological setting.

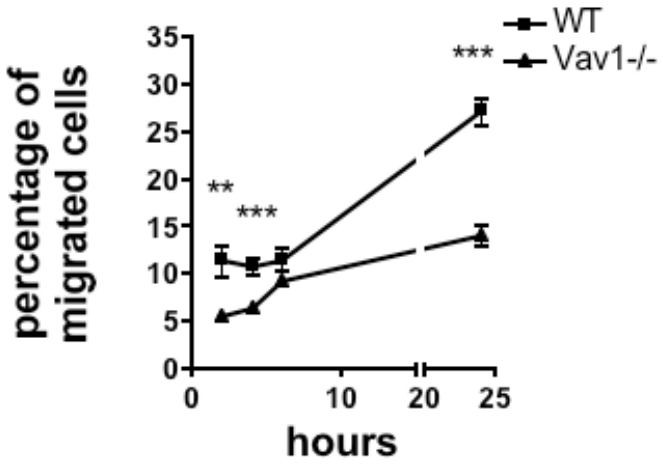
2.2. Results

2.2.1 Assessing the role of Vav1 in T cell migration in vitro

As a preliminary experiment in dissecting the role of Vav1 in T cell migration, the migration of HY-specific $CD4^+$ WT and Vav1-/- T cells was first examined through syngeneic endothelial monolayer using female-derived ECs. Briefly, female ECs were plated on the transwells at a concentration of 10^4 per transwell and allowed to form a monolayer. Viable WT and Vav1-/-T cells ($3\text{-}5x10^5$ per transwell) were used between days seven and ten post-activation to ensure that they were not cycling, as this has been found to be the optimal time for T cell migration in our laboratory. T cells were allowed to migrate for 24 hours and the number of migrated cells was counted every two hours.

As shown in **Figure 17**, HY-specific $CD4^+$ Vav1-/- T cells migrated significantly less through the syngeneic female endothelial monolayer compared to the WT control, suggesting that they have a lower baseline migration.

Figure 17: Vav1-/- T cells show reduced baseline migration compared to WT



Viable WT and Vav1-/- T cells were used between days seven and ten post-stimulation and were plated on syngeneic female endothelial monolayer. Migration was measured at

*two, four, six and twenty-four hours. Percentage of migrated cells was calculated by dividing the number of cells in the bottom chamber by the total number of plated T cells from the average of three experiments. Error bars indicate standard error. Statistical analysis was performed using Student's t test (**p<0.01, ***p<0.0001)*

2.2.2 Investigation of the role of Vav1 in integrin-mediated migration

As discussed in Chapter 1, HY-specific $CD4^+$ Vav1-/- T cells were found to have lower expression of LFA-1 in the flow cytometry assays and reduced adhesion to ICAM-1. As LFA-1 is an important regulator of T cell migration, the involvement of Vav1 activity in T cell migration was examined using transwell-based migration assays as well as time-lapse microscopy.

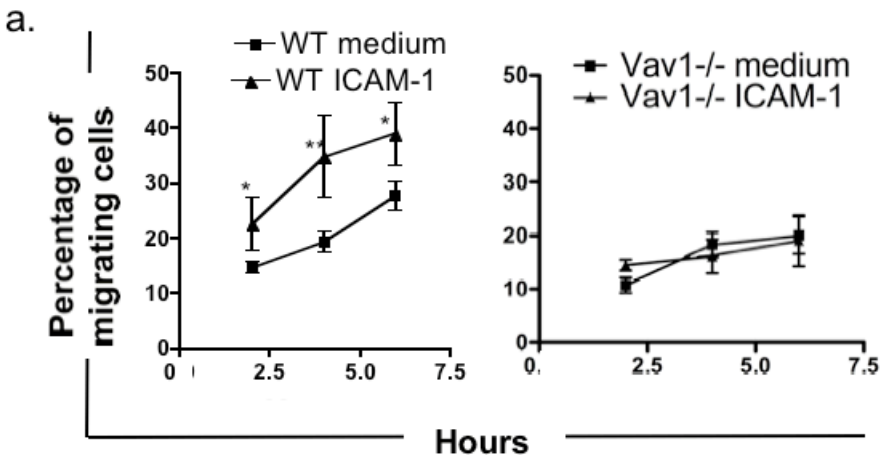
Figure 18a shows the results of the transwell-based assay. Briefly, $3\text{-}5x10^5$ T cells were plated on ICAM-1-coated transwells and allowed to migrate for six hours. WT T cells showed enhanced migration in response to ICAM-1 at all time-points tested. In contrast, Vav1-/- T cells did not respond to ICAM-1 with increased migration.

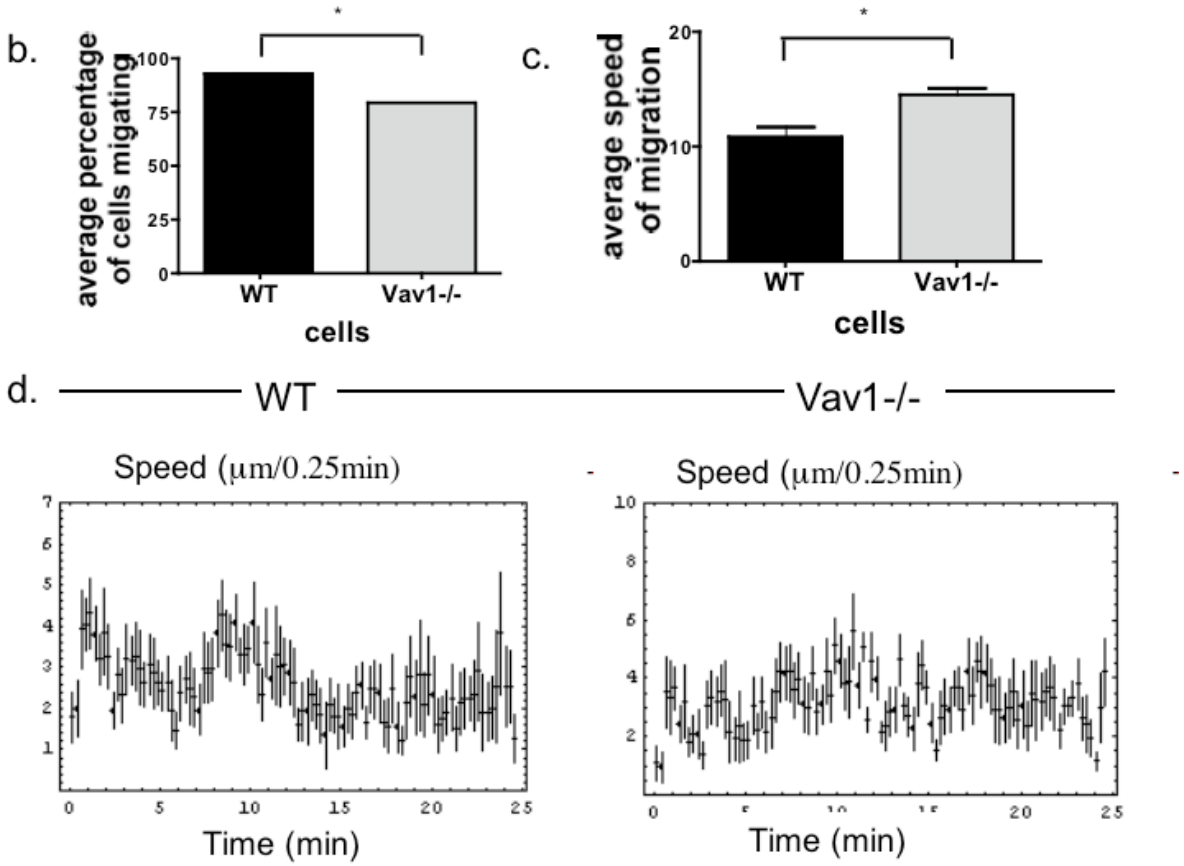
The requirement for Vav1 activity in T cell migration in response to ICAM-1 was also examined by time-lapse microscopy. 10^6 HY-specific $CD4^+$ WT and Vav1-/- T cells were serum starved in RPMI 2% FCS for one hour and were subsequently plated on ICAM-1-coated dishes. Non-adherent cells were removed by gentle washing prior to the assay. The movie was made by taking pictures every 15 seconds for 25 minutes and mathematical analysis of T cell migration was performed using Mathematica workbooks after having tracked the path of ten cells per condition.

Figure 18b shows the average number of cells migrating, calculated by counting motile HY-specific $CD4^+$ WT and Vav1-/- T cells from each video. As expected from the transwell-based assay, Vav1-/- T cells were found to migrate less than the WT control. In addition, although retraction of leading edge and uropod normally occurs in a co-ordinated fashion, Vav1-/- T cells displayed a "jumping" phenotype (Supplementary videos 1 and 2) whereby the leading edge moved forward while the uropod stayed stationary until it detached and retracted.

The speed of migration of the HY-specific $CD4^+$ WT and Vav1-/- T cells was calculated using Mathematic workbooks provided by Professor Anne Ridley (Ludwig Institute, UCL) (Wells and Ridley, 2005). As shown in **Figure 18** c, Vav1-/- T cells had a higher average migratory speed (μm/min) than the WT control. A representative example of the raw data obtained from the Mathematica workbook is shown in **Figure 18**d. Although it appears that WT and Vav1-/- T cells had similar average migratory speed in the first five minutes, the high migratory speed was maintained by the Vav1-/- T cells but not by the WT controls.

Figure 18: Vav1-/- T cells have reduced LFA-1-mediated migration but display increased migratory speed





*Viable WT and Vav1-/- T cells, used between days seven and ten post-stimulation, were plated on plastic-bound recombinant ICAM-1 coated transwells and allowed to migrate for six hours. Percentage migration was calculated from the average of four independent experiments. (a) WT and Vav1-/- T cells (1x10^6) were serum-starved in RPMI 2% FCS for one hour and were plated on ICAM-1-coated 35mm dishes. Their migration on ICAM-1 was followed by time-lapse microscopy. The number of cells migrating was quantified by counting motile T cells (b). T cells were tracked using Kinetiq tracking software and their migratory speed (μm/min) was quantified using Mathematica spreadsheets (c). Representative data from one experiment obtained from the Mathematica spreadsheet. Each line represents the average migratory speed at each time-point (μm/0.25min) (d). The average percentage of motile cells and average speed was calculated from data of three independent experiments. Statistical analysis was performed using Student's t-test (*p<0.05, **p<0.01). Error bars indicate standard error.*

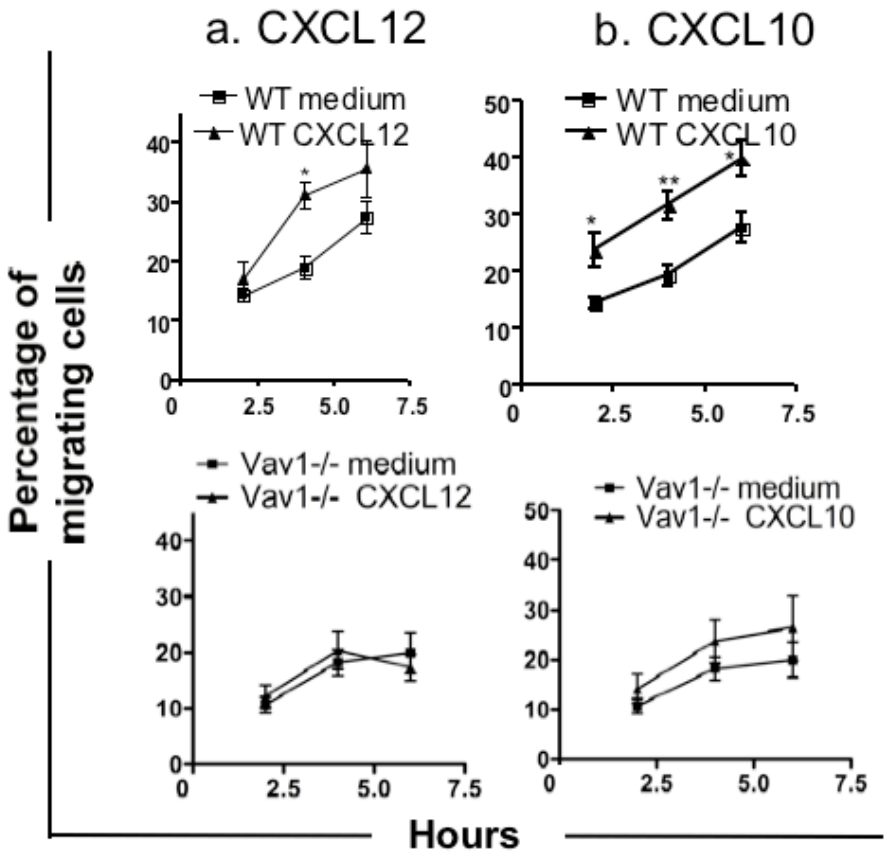
2.2.3 The role of Vav1 in chemokine-driven migration

Previous studies have implicated Vav1 in chemokine-driven migration of T cells. Vicente-Manzanares *et al*. (2005) showed that *in vitro* Vav1 is important in CXCL12-mediated T cell migration using a dominant-negative form of Vav1, while Garcia-Bernal *et al*. (2005) observed that Vav1 and Rac1 are necessary for CXCL12-mediated adhesion and transendothelial migration *in vitro*.

To examine this further, we used transwell-based migration assays as described in the materials and methods section. Briefly, 3-5x10^5 HY-specific $CD4^+$ WT and Vav1-/- T cells were plated on transwells in the presence of CXCL12 (50ng/ml) or CXCL10 (300ng/ml) in the lower chamber and allowed to migrate for six hours.

Figure 19 shows the migration of the HY-specific $CD4^+$ WT and Vav1-/- T cells in response to CXCL12 and CXCL10 at different time-points. HY-specific $CD4^+$ WT T cells showed significantly enhanced migration in response to CXCL12 (**Figure 19** a) and CXCL10 (**Figure 19** b) compared to medium alone. In contrast, HY-specific $CD4^+$ Vav1-/- T cells did not show enhanced migration in the presence of chemokines.

Figure 19: Vav1-/- T cells do not response to CXCL12 or CXCL10



*WT and Vav1-/-T cells (used between day seven and ten post-stimulation) were allowed to migrate in response to CXCL12 (a) or CXCL10 (b) for six hours. Migration was examined by counting the cells in the bottom chamber at two, four and six hours. Percentage migration was calculated by dividing the number of cells in the bottom chamber by the original number of cells plated on the transwell. Error bars indicate standard error. Statistical analysis was performed using the Student's t test (*p<0.05, ** p<0.01). The average percentage migration was calculated using data from four independent experiments.*

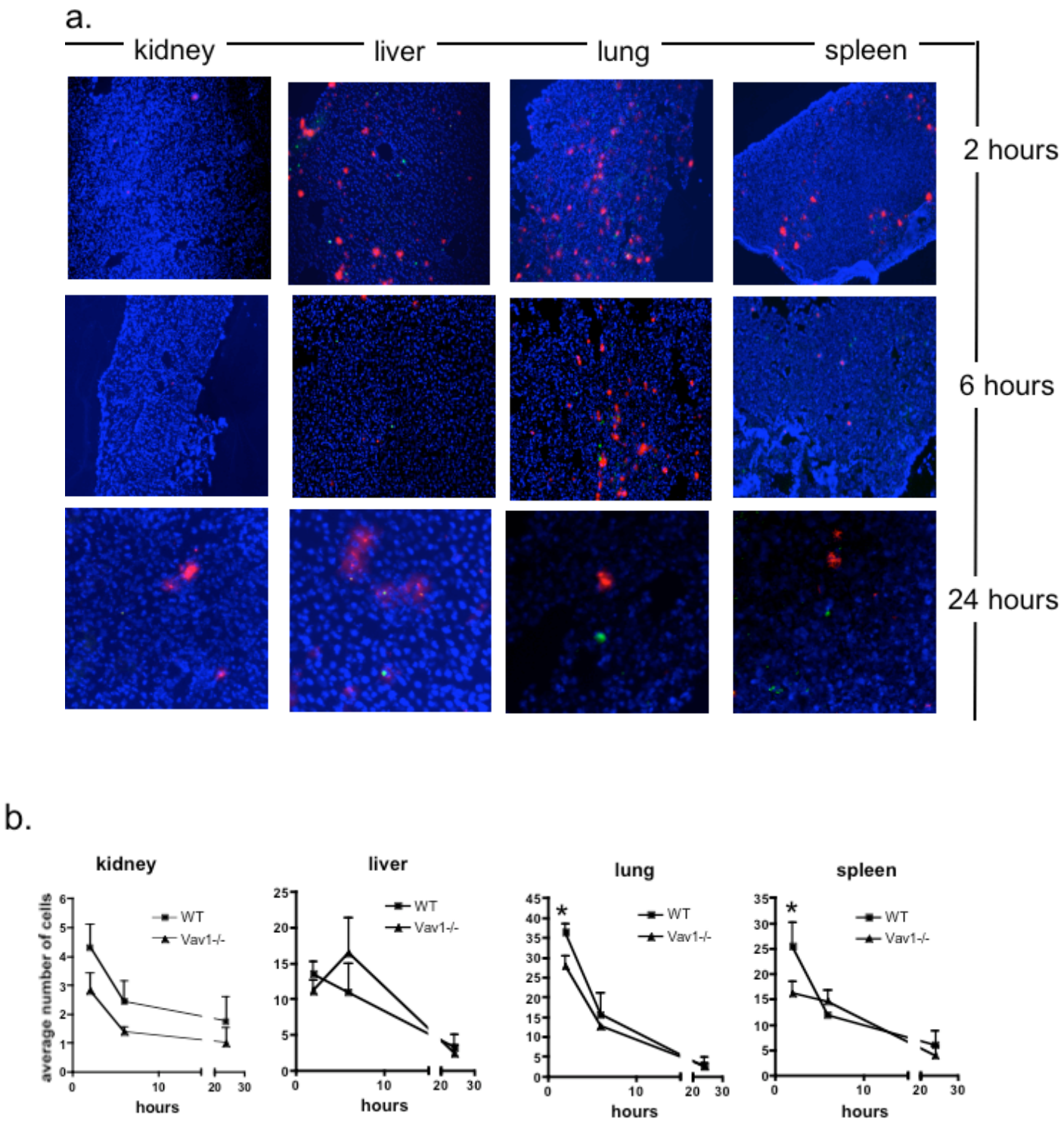
2.2.4. Constitutive trafficking of Vav1-/- T cells

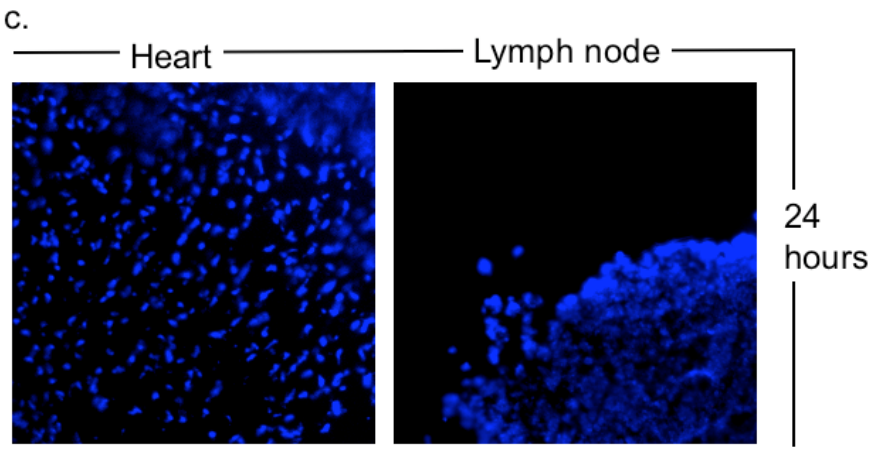
Constitutive trafficking of primed memory Vav1-/- T cells has never been examined before. We, as well as others (Vicente-Manzanares *et al,* 2005; Garcia-Bernal *et al*, 2005), have shown that Vav1-/- T cells have defects in migration *in vitro*, and the migration of Vav1-/- T cells in response to syngeneic non-antigenic ECs was shown to be significantly lower than that of the WT controls in the transwell assays. To examine whether these defects translated into altered T cell trafficking *in vivo,* PKH26-labelled HY-specific $CD4^+$ WT and CFSE-labelled HY-specific $CD4^+$ Vav1-/- T cells (5-10x10^6 T cells) were co-injected iv in female C57BL/6 mice. The mice were culled 24 hours later and T cell migration to the kidney, lung, liver and spleen was examined by wide-field fluorescence microscopy, following cryotomy.

Figure 20 a shows representative examples of sections obtained through wide-field fluorescence microscopy following cryotomy of the tissue samples at each time-point. The number of cells present in each section was quantified using the algorithm in the appendix and is represented in **Figure 20** b. Both HY-specific $CD4^+$ WT and Vav1-/- T cells migrated readily to liver, lung and spleen, with some cells present in kidney as well. No difference in number of migrated cells was observed in any of the tissues tested at six and 24 hours, however at two hours there were less HY-specific $CD4^+$ Vav1-/- T cells in the lung and spleen compared to WT control (**Figure 20** b)

Trafficking of HY-specific $CD4^+$ WT and Vav1-/- T cells to the heart and lymph nodes was also examined. Consistent with the effector memory phenotype observed in Chapter 1 (little or no expression of CCR7 and CD62L), no cells were found in either of the two tissues at any of the time points tested. **Figure 20**c shows a representative example of the sections obtained at 24 hours.

Figure 20: Constitutive trafficking of Vav1-/- T cells





Viable WT and Vav1-/- T cells (used between days seven and ten post-stimulation) were labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice. Trafficking into kidney, liver, lung and spleen was monitored at two, six and 24 hours post-injection by harvesting, snap-freezing the tissue and taking 5-10μm sections. The sections were examined by wide-field fluorescence microscopy by mounting with vectorshield medium containing DAPI which stains the nucleus blue and an average of six representative pictures was taken per sample (x10 magnification) (a). The number of cells present in each field was quantified using the algorithm in the appendix (b). Representative examples of trafficking into heart and lymph nodes at 24 hours (x10 magnification) (c). Statistical analysis was performed using the Student's t-test ($p<0.05$). Error bars indicate standard error. Each experimental group consisted of three female C57BL/6 mice.*

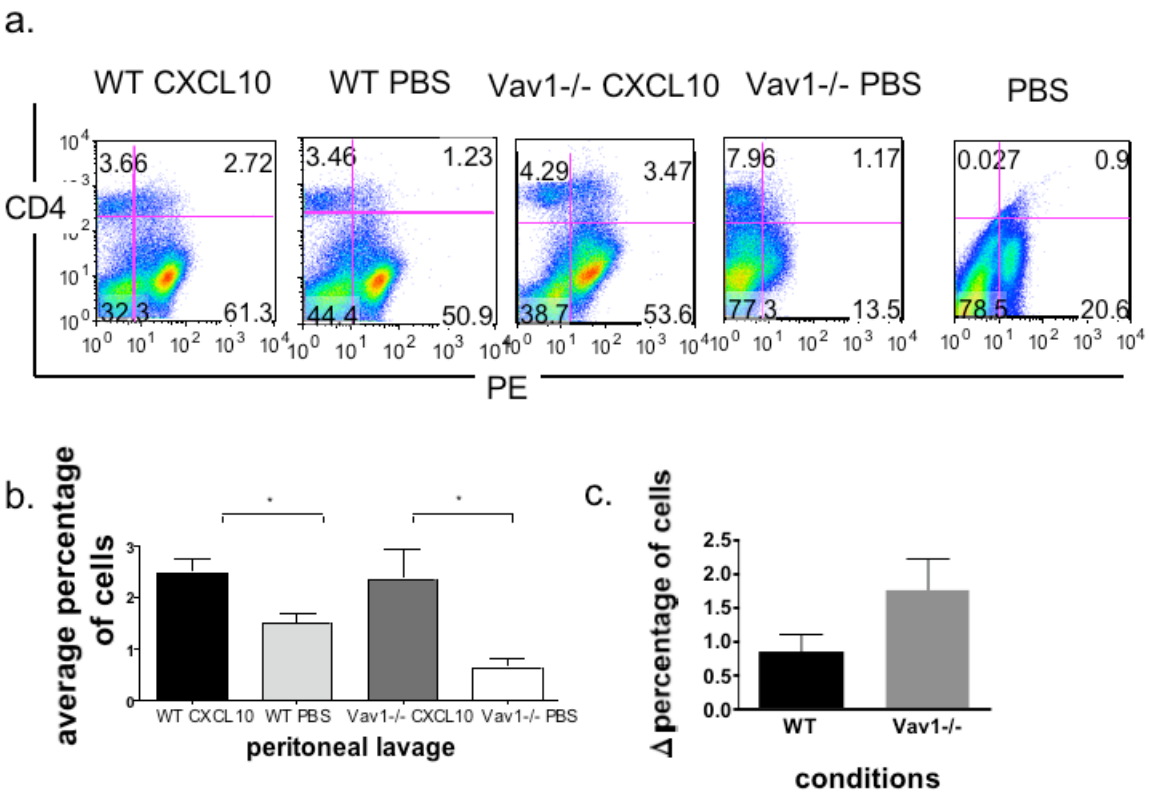
2.2.4 Chemokine-driven migration in vivo

HY-specific CD4$^+$ Vav1-/- T cells showed no defects in constitutive trafficking *in vivo* as shown in section 2.2.3. To complete this part of the study and get a more complete picture prior to commencing the antigen-dependent migration experiments, we sought to examine the recruitment of HY-specific CD4$^+$ Vav1-/- T cells in response to an inflammatory stimulus in the absence of antigen. We chose to examine migratory responses to the chemokine CXCL10 *in vivo*, as its receptor, CXCR3, was found to be expressed in very high levels by both HY-specific CD4$^+$ WT and Vav1-/- T cells in Chapter 1. In addition, CXCL10 is the chemokine induced by the secretion of IFNγ, which was also used to induce MHC class II expression in the antigen-dependent migration studies that will follow.

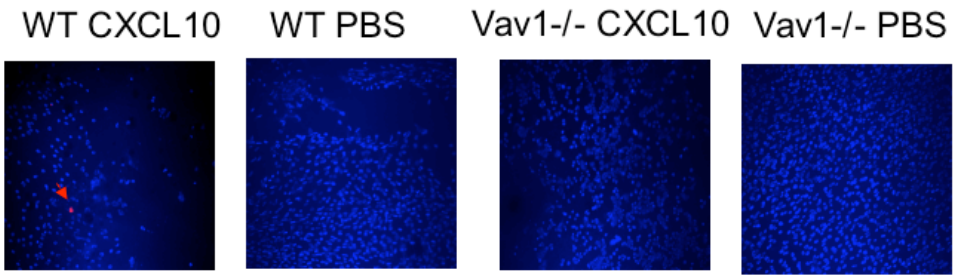
To investigate the involvement of Vav1 in chemokine-driven migration *in vivo*, a dose of CXCL10 (1.2μg/ml) that was previously found to induce chemotaxis *in vivo* (unpublished observations) was injected ip into female C57BL/6 mice, followed by an iv injection of 10x10^6 PKH26 labelled HY-specific CD4$^+$ WT or Vav1-/- T cells. Recruitment to the peritoneal cavity was analysed 20-hours post-injection.

Figure 21a,b and c shows the migration of T cells into the peritoneal lavage in mice injected with CXCL10 or PBS alone as a control. In addition, the lavage of a female mouse injected with PBS alone (i.e. no T cells) is also shown to indicate the presence of an autofluorescent population in the absence of T cells. Both HY-specific CD4$^+$ WT and Vav1-/- T cells showed significantly enhanced migration to the peritoneal cavity in the presence of the chemokine, as shown in **Figure** 21a and b. Interestingly, the Δ migration of HY-specific CD4$^+$ Vav1-/- T cells (i.e. the average percentage of T cells in the peritoneal lavage with the PBS background subtracted) was higher than that of the WT T cells (**Figure** 21c). Concomitant with this observation, more HY-specific CD4$^+$ WT T cells were found in the peritoneal membrane of mice injected with chemokine, compared to Vav1-/- T cells or the controls (**Figure** 21 d,e). The significance of this will be further examined in the discussion.

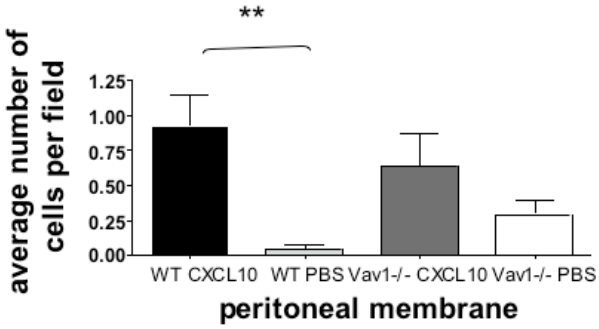
Figure 21: Chemokine-driven migration *in vivo*



d.



e.



Viable WT and Vav1-/- T cells (used between days seven and ten post-stimulation) were labelled with PKH26 (red) and injected iv in female C57BL/6 mice that had previously received an ip injection of 1.2µg CXCL10 or PBS as a control. Female mice were also injected with PBS alone (i.e. no T cells) as an autofluorescence control. The mice were sacrificed 20 hours post-injection. The peritoneal lavage was labelled with anti-CD4 antibody and analysed by flow cytometry (a). The average percentage of cells present in the peritoneal lavage (b). Δ percentage migration was calculated by subtracting the average background migration (i.e. in the presence of PBS) from the percentage of migrated cells in the presence of CXCL10. (c) The peritoneal membrane was mounted with vectorshield mounting medium containing DAPI and analysed by wide-field fluorescence microscopy (x10 magnification). Red cells are indicated by the red arrow (d). The number of cells present in the peritoneal membrane was quantified manually (e). Statistical analysis was performed using the Student's t-test ($p<0.05$). Error bars indicate standard error. Each experimental group consisted of four to five female C57BL/6 mice.*

2.3. Discussion

Phenotypic results in Chapter 1 suggested that HY-specific $CD4^+$ Vav1-/- T cells expressed LFA-1 in lower levels that the WT controls and showed reduced adhesion to ICAM-1. This, in conjunction with previous studies implicating Vav1 in adhesion to ICAM-1 and LFA-1 "inside-out" signalling (Krawczyk *et al*., 2002; Ardouin *et al*., 2003), suggested that Vav1 may also be important in LFA-1/ICAM-1-mediated migration. In line with this hypothesis, we observed that Vav1-/- T cells did not show enhanced migration in the presence of ICAM-1. It is difficult to determine whether the absence of enhanced migration in response to ICAM-1 in Vav1-/- T cells was due to reduced adhesion, as previously documented, or reduced interaction with ICAM-1 due to reduced LFA-1 expression, as indicated in Chapter 1.

To examine this further, we used time-lapse microscopy. In this case, we observed that HY-specific $CD4^+$ Vav1-/- T cells showed reduced migratory response to ICAM-1, as less Vav1-/- T cells showed a motile phenotype compared to the WT control despite the removal of non-adhering cells prior to the assay. It is possible that adhering Vav1-/- T cells may have had reduced interaction to ICAM-1 due to lower expression of LFA-1, as indicated by the findings in Chapter 1.

Interestingly, HY-specific $CD4^+$ Vav1-/- T cells were found to have enhanced speed compared to the WT controls, which has been useful in explaining some of the *in vivo* migration data. In addition, Vav1-/- T cells showed a "jumping" phenotype, i.e. a sudden and irregular retraction of the uropod while the leading edge moved forward. This phenotype suggests a defect in T cell de-adhesion from ICAM-1 and has previously been described in T cells with a mutated form of CD43 that abrogates its redistribution to the uropod (Savage *et al*., 2002). CD43 is a T cell surface glycoprotein which can bind to ICAM-1 (Rosenstein *et al*., 1991) and is thought to be involved in T cell adhesion (Manjunath *et al*., 1993) and cell polarisation (del Pozo, 1995). Interestingly, CD43 has been found to regulate T cell activation by facilitating the formation of the Vav1-Slp76 complex through Vav1 tyrosine phosphorylation (Pedraza-Alva *et al*., 1998). It is possible that in the absence of Vav1, CD43 redistribution to the uropod may be defective,

resulting in faulty de-adhesion and the "jumping" phenotype. It would therefore be interesting to examine the expression and distribution of CD43 in Vav1-/- T cells.

Previous studies have shown that Vav1 is important for T cell migration and in particular chemotaxis. Vicente-Manzanares *et al*. (2005) showed that Vav1 is important in CXCL12-mediated T cell migration using a dominant-negative form of Vav1, while Garcia-Bernal *et al*. (2005) observed that Vav1 and Rac1 are necessary for CXCL12-mediated adhesion and transendothelial migration *in vitro*. In this study, we examined T cell chemotaxis using both *in vitro* and *in vivo* models of migration. In the *in vitro* transwell-based migration assay, HY-specific $CD4^+$ Vav1-/- T cells did not show enhanced migration in the presence of CXCL12 or CXCL10 compared to medium alone, suggesting that Vav1 is involved in chemokine-mediated T cell migration *in vitro*. Interestingly, Gallego *et al*. (2006) have shown that WIP and WASp are important in CXCL12-mediated migration of T cells *in vitro*, using murine WIP-/- and WASp-/- T cells. As Vav1 is known to interact with WIP (Savoy *et al*., 2000), it is possible that the loss of responsiveness to CXCL12 in our transwell-based assays may partially be due to the reduced activation of these molecules.

Transwell-based assays are useful for identifying defects at the cellular level but their physiological relevance must be tested *in vivo*. Indeed, other factors which cannot be faithfully replicated *in vitro*, such as shear flow, have been shown to be important in T cell migration, in particular integrin activation (reviewed in Alon and Dustin, 2007). Taking this into account, we examined chemokine-mediated migration *in vivo*, by injecting CXCL10 ip in female mice prior to iv injection of T cells. Interestingly, the *in vivo* data did not replicate the *in vitro* findings, as both HY-specific $CD4^+$ WT and Vav1-/- T cells showed enhanced trafficking in the presence of the chemokine compared to the PBS control. Therefore, the defects in migration which are revealed *in vitro* must be compensated for *in vivo* by other molecules in the bloodstream as well as shear flow.

Shear flow in particular has previously been shown to have a fundamental role in T cell migration by facilitating integrin conformational changes induced by chemokines (reviewed in Alon and Dustin, 2007). To dissect this further, it would be interesting to examine chemokine-driven migration of Vav1-/- T cells in an *in vitro* flow chamber, which simulates shear flow in the bloodstream; comparable levels of chemokine-driven

migration of Vav1-/- T cells to the WT controls in these conditions would indicate that shear flow acts as an important compensatory factor for the loss of Vav1 activity *in vivo*.

Furthermore, the *in vivo* chemokine-driven migration assays indicated higher enrichment of HY-specific $CD4^+$ Vav1-/- T cells in the peritoneal lavage of female C57BL/6 mice compared to the WT controls, once the PBS background had been subtracted. This could be due to higher migratory speed of Vav1-/- T cells, as indicated by the time-lapse microscopy assays, which may have resulted in the Vav1-/- T cells migrating through the peritoneal membrane faster than the WT T cells and reaching the peritoneal cavity first. In fact, WT T cells could still be observed in the peritoneal membrane of mice injected with chemokine, suggesting that they had not yet entered the peritoneal cavity. Examining enrichment of the peritoneal lavage by HY-specific $CD4^+$ WT and Vav1-/- T cells at different time-points could provide further evidence on this, as it would be expected that at later time-points, no difference would be seen between WT and Vav1-/- T cell enrichment. However, preliminary *in vivo* data, as well as the data obtained from the *in vitro* studies, suggested that at later time-points, the chemokine loses its chemotactic effect on T cells, probably because it diffuses further into the bloodstream.

Note that the peritoneal lavage of mice injected with PBS alone also shows the presence of PE-positive non-T cells. We often observe this autofluorescent population in the peritoneal lavage cell suspension and it is therefore essential to identify recruited T cells by labelling with anti-CD4 antibody.

As mentioned earlier, *in vitro* migration assays are limited in that they do not take into account other molecules present in the bloodstream as well as shear flow, which are likely to influence T cell migration *in vivo*. The *in vitro* transwell-based assays suggested that baseline migration in response to syngeneic, non-antigenic ECs was significantly lower in HY-specific $CD4^+$ Vav1-/- T cells compared to the WT control. To examine constitutive T cell trafficking *in vivo*, HY-specific $CD4^+$ WT and Vav1-/- T cells were injected iv and their migration into different tissues was analysed at different time-points. At 24 hours, we observed no difference in tissue localisation between WT and Vav1-/- T cells, which suggested that other molecules in the bloodstream and well as shear flow may have compensated for any migratory defects of Vav1-/- T cells, as previously

discussed. In addition, the enhanced speed of Vav1-/- T cells as revealed by the time-lapse microscopy assays, may have accelerated their localisation into tissues over 24 hours.

To assess the effect of enhanced speed in Vav1-/- T cell trafficking, WT and Vav1-/- T cell trafficking was examined at different time-points. No defect was seen at six hours post-injection, however at two hours, less HY-specific $CD4^+$ Vav1-/- T cells were found in the spleen and lung of female C57BL/6 mice compared to the WT control. We have previously observed that the lung is one of the first organs infiltrated by T cells when injected iv and this has also been shown in a study by Pittet *et al*. (2007) who observed increased infiltration of the lung two hours post-injection. It is therefore possible that the enhanced speed of HY-specific $CD4^+$ Vav1-/- T cells accelerated their entry and exit from the lungs compared to the WT controls, which would explain why less Vav1-/- T cells were found there. This would also explain why no differences were observed at six and 24 hours, as the WT T cells would have had enough time to "catch up" with the faster Vav1-/- T cells.

It is possible that a similar explanation applies for the spleen as well. An intra-vital microscopy study revealed that lymphocytes in spleen vessels have higher rolling velocity than cells in the HEV of the lymph nodes, which may suggest that T cells in the bloodstream would enter the spleen faster than other tissues (Grayson *et al*., 2003).

Note that the average number of cells present in each tissue decreased at later time-points, and this is probably due to cell death, further re-distribution into the body or simply decay of the fluorescent dyes.

Chapter 3: Regulation of TCR-dependent T cell recruitment to non-lymphoid antigenic sites by Vav1

3.1. Introduction

Primed T cells recirculating in the bloodstream need to locate their cognate antigen in order to localise to antigenic sites. Recent evidence suggests that antigen presented by ECs lining the post-capillary venules of non-lymphoid antigenic tissue is recognised by circulating T cells, resulting in enhanced diapedesis and entry into the antigenic tissue (Greening *et al*., 2003) (Savinov *et al*., 2001) (Marelli-Berg *et al*., 2004)

Vav1 is activated during TCR triggering through phosphorylation by ZAP70 and acts as a GEF for Rac1, a RhoGTPase which is known to regulate actin polymerisation (Crespo *et al*., 1996; Garcia-Bernal *et al*., 2006; Garcia-Bernal *et al*., 2005; Han *et al*., 1997). Therefore, Vav1 provides an essential link between TCR signalling and the regulation of cytoskeletal re-arrangements. Based on this, it was hypothesised that loss of Vav1 activity would result in impaired T cell recruitment and retention into antigenic sites.

Previous experiments in Chapter 2 have indicated that although HY-specific $CD4^+$ Vav1-/- T cells showed impaired migration *in vitro*, they did not display gross defects in T cell trafficking *in vivo*, probably due to other compensatory factors. This would suggest that any defects potentially observed in TCR-dependent recruitment and retention to sites of antigen presentation are not due to a generic defect in migration, but specifically due to an involvement of Vav1 in antigen-dependent migration.

Due to the disparity of the *in vitro* and *in vivo* data obtained when assessing antigen-independent migration, we examined TCR-dependent migration using both *in vitro* and *in vivo* assays.

3.2. Results

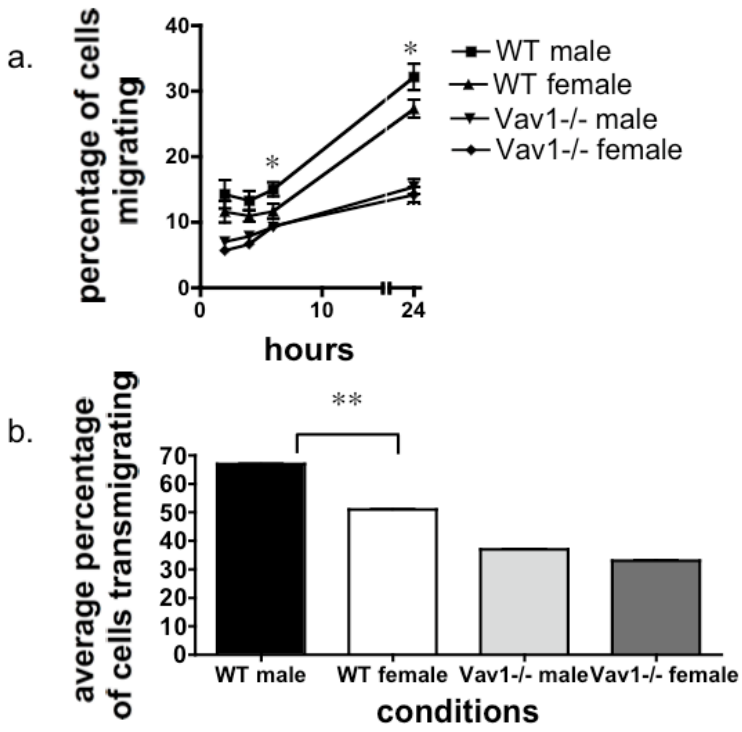
3.2.1. The role of Vav1 in TCR-dependent T cell migration in vitro

To investigate the requirement of Vav1 activity in antigen-dependent T cell migration, we first performed transwell-based and time-lapse microscopy *in vitro* assays using syngeneic antigenic (male) and non-antigenic (female) EC monolayer. For the transwell-based migration assays, 3-5x10^5 HY-specific $CD4^+$ WT and Vav1-/- T cells were plated on male or female EC monolayer-coated transwells previously pulsed with IFNγ to induce MHC class II expression, antigen presentation and proliferation, and allowed to migrate for 24 hours.

Figure 22 a shows the result on the transwell-based assay. As predicted, HY-specific $CD4^+$ WT T cells showed enhanced migration through the male monolayer compared to the female, non-antigenic one. In contrast, HY-specific $CD4^+$ Vav1-/- T cells did not have any significant differences in migration through male or female endothelial monolayer, suggesting that Vav1 activity is required for TCR-dependent migration *in vitro*. In addition, as mentioned in Chapter 2, HY-specific $CD4^+$ Vav1-/- T cells showed reduced baseline migration in the absence of antigen through female monolayer, compared to the WT controls.

These findings were confirmed using time-lapse microscopy (Supplementary videos 3-6). In this case, 10^6 WT and Vav1-/- T cells were serum-starved in RPMI 2% FCS for one hour and subsequently plated on male or female endothelial monolayer-coated 35mm dishes that had previously been pulsed with IFNγ to induce MHC class II expression, antigen presentation and proliferation. As shown in **Figure 22** b, HY-specific $CD4^+$ WT T cells showed enhanced transmigration when plated on male EC monolayer than when plated on female, as defined by the change of phase (i.e. turning from bright to dark when under the monolayer)(Supplementary videos 3-6). This effect was not observed in the HY-specific $CD4^+$ Vav1-/- T cells confirming that *in vitro*, Vav1 activity is required for TCR-dependent migration.

Figure 22: Vav1-/- T cells do not show enhanced migration in the presence of antigen

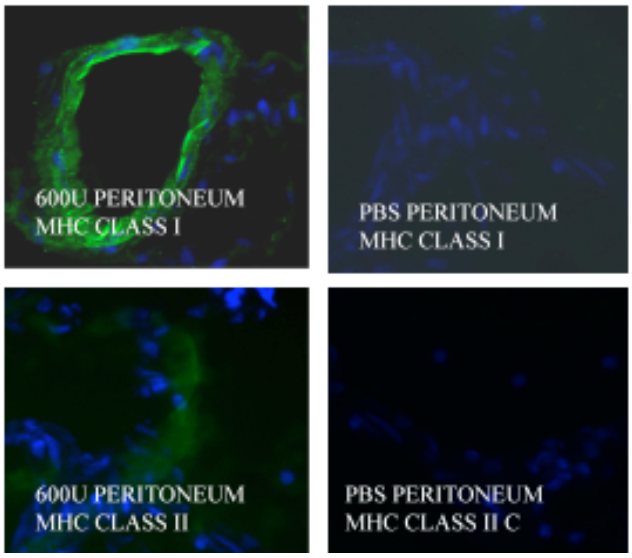


*Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation with male splenocytes and recombinant IL-2. T cells were plated on transwells coated with antigenic (male) or non-antigenic (female) 129sv ECs that had previously received an optimal dose of IFNγ to induce MHC class II expression and allowed to migrate for 24 hours. Percentage migration was calculated by dividing the number of cells in the bottom chamber by the total number of cells plated from three experiments (a). 1x10⁶ T cells were plated on 35mm dishes coated with antigenic (male) or non-antigenic (female) 129sv ECs that had previously received an optimal dose of IFNγ to induce MHC class II expression and allowed to migrate for 50 minutes. The movie was made by taking photos every 30 seconds. Transmigrating T cells were defined as changing phase i.e. turning from bright to dark once under the endothelial monolayer. Average percentage of cells transmigrating was calculated from three independent experiments from a sample of 100 cells per movie (b). Error bars indicate standard error. As the standard error was very small in this case, the error bars are not visible Statistical analysis was performed using Student's t-test (*p<0.05, **p<0.01).*

3.2.2 The role of Vav1 in TCR-dependent recruitment and retention in vivo-antigen-dependent peritoneal recruitment model

The *in vitro* migration assays indicated that HY-specific $CD4^+$ Vav1-/- T cells had impaired TCR-dependent migration, as predicted by our hypothesis. To examine whether this also applied *in vivo,* we used the peritoneal recruitment model. In this model, HY-specific T cells are injected iv in male and female mice that previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation in the peritoneal membrane (**Figure 23**). Previous studies (Marelli-Berg *et al*., 2004) have shown that iv injection of HY-specific WT T cells in mice results in recruitment to the peritoneal cavity and retention to the peritoneal membrane in male but not female mice injected with IFNγ.

Figure 23: Injection of IFNγ ip results in increased MHC class I and induction of MHC class II expression in mice



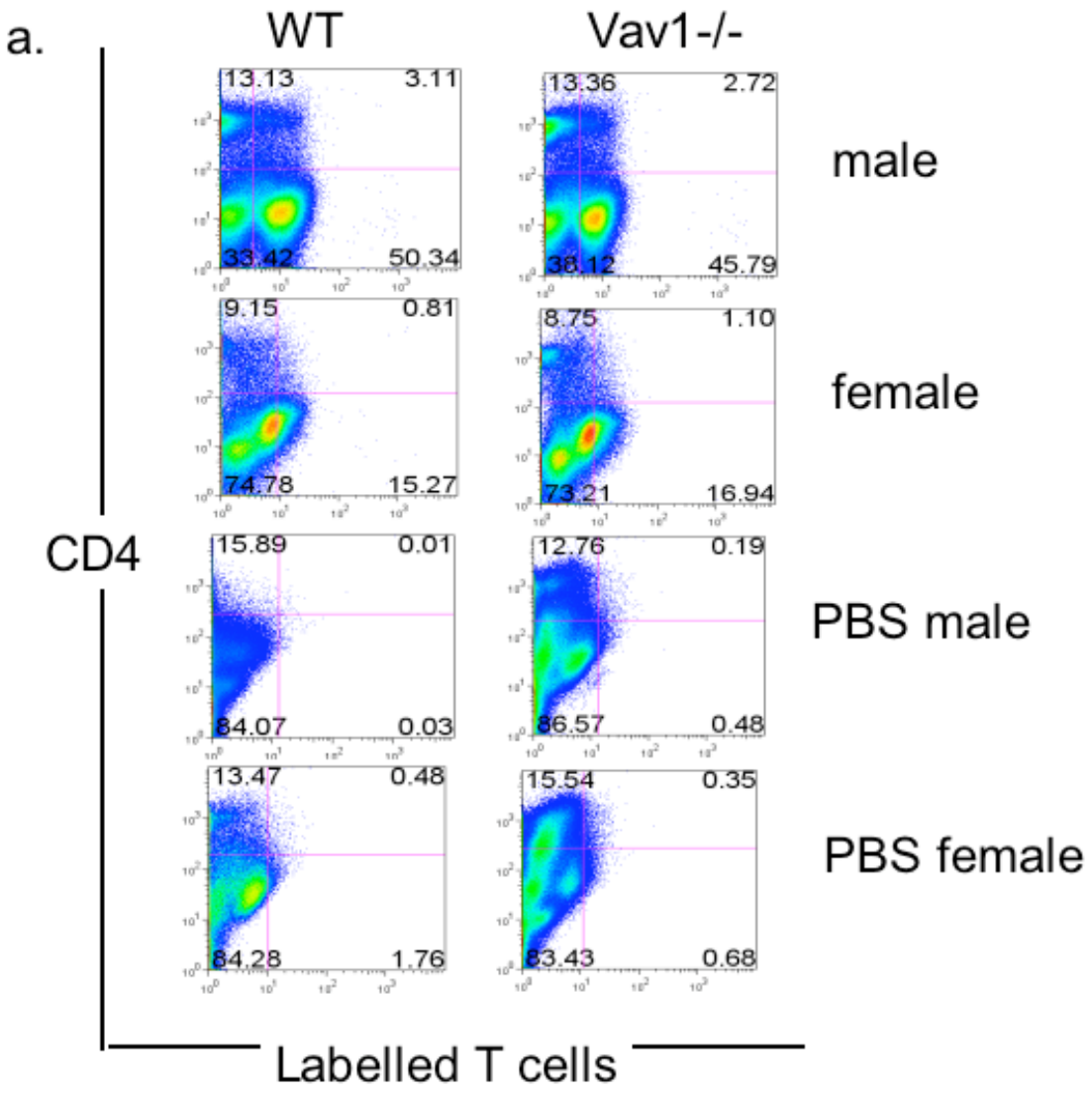
C57BL/6 mice were injected with 600 units recombinant IFNγ ip or PBS as a control. MHC class I and II expression was analysed 48 hours later by labelling with a FITC-conjugated anti-MHC class I and II antibody and mounting with DAPI which stains the nucleus blue (x40 magnification)(courtesy of Federica Marelli-Berg).

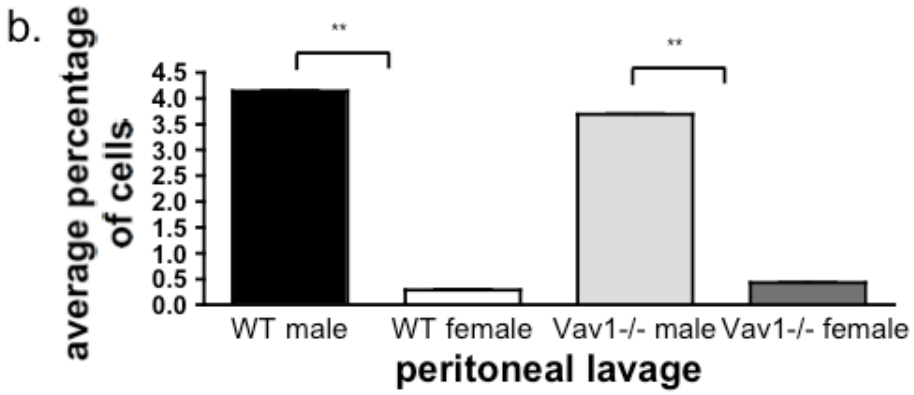
To examine antigen-dependant migration *in vivo*, we employed the afore mentioned peritoneal recruitment model. Briefly, PKH26-labelled 10^7 HY-specific $CD4^+$ WT and CFSE-labelled 10^7 HY-specific $CD4^+$ Vav1-/- T cells were co-injected iv in male and female mice that had previously received an ip injection of IFNγ to induced MHC class II expression and cause localised antigen presentation in the peritoneal cavity. Control mice were injected ip with PBS. The mice were culled 24 hours later and the enrichment of T cells to the peritoneal lavage was examined by flow cytometry, while the presence of T cells to the peritoneal membrane was examined by wide-field fluorescence microscopy.

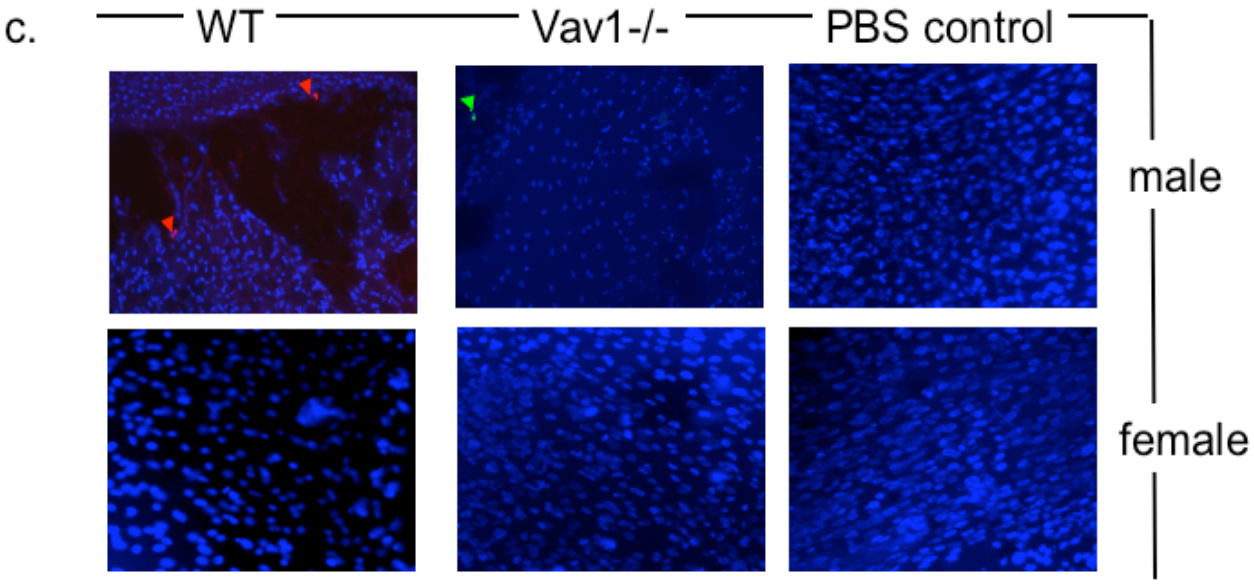
Figure 24 a shows the recruitment of T cells to the peritoneal cavity as analysed by flow cytometry. As shown in **Figure 24** b, both HY-specific $CD4^+$ WT and Vav1-/- T cells showed enhanced recruitment to the peritoneal cavity in male but not female mice, which suggests that Vav1 activity is not necessary for TCR-dependent recruitment to sites of antigen presentation. As expected, ip injection of PBS did not result in recruitment of either HY-specific $CD4^+$ WT or Vav1-/- T cells to the peritoneal cavity.

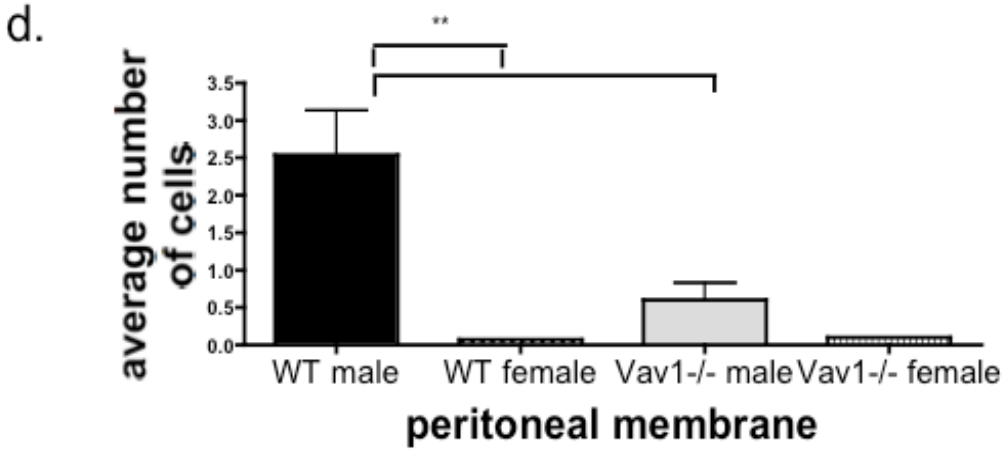
However, we observed a significantly lower number of HY-specific $CD4^+$ Vav1-/- T cells retained in the peritoneal membrane in male mice compared to the WT controls, as shown in **Figure 24** d, with representative examples of the peritoneal membrane sections shown in **Figure 24** c. This suggests that although Vav1 activity appears to be dispensable for TCR-dependent recruitment, it may be important for TCR-dependent retention to sites of antigen presentation. As expected, a negligible number of T cells was found in the peritoneal lavage and membrane of female mice injected with HY-specific $CD4^+$ WT and Vav1-/- T cells as well as the male and female mice injected with PBS instead of IFNγ.

Figure 24: Vav1-/- T cells have normal recruitment but defective retention to antigenic sites.




b.
average percentage of cells
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
WT male
WT female
Vav1-/- male
Vav1-/- female
peritoneal lavage


c.
WT
Vav1-/-
PBS control
male
female



*Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 10^7 WT and Vav1-/- T cells were labelled with PKH26 (red) and CFSE (green) respectively and co-injected iv into male or female C57BL/6 mice which had previously received an ip injection of IFNγ or PBS as a control. The mice were culled 24 hours later. Enrichment into the peritoneal lavage was analysed by flow cytometry (a). Average percentage of T cells in the peritoneal lavage (b). Retention of T cells into the peritoneal membrane was analysed by wide-field fluorescence microscopy after mounting with vectorshield medium containing DAPI which labels the nucleus blue (x10 magnification). Red arrows indicated retained WT T cells, while green arrows indicated retained Vav1-/- T cells (c). The average number of cells in the peritoneal membrane was quantified manually (d). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.05, **p<0.01). Experimental groups consisted of three to seven mice.*

3.2.3. The role of Vav1 in TCR-dependent T cell recruitment and retention- peritoneal tissue infiltration model

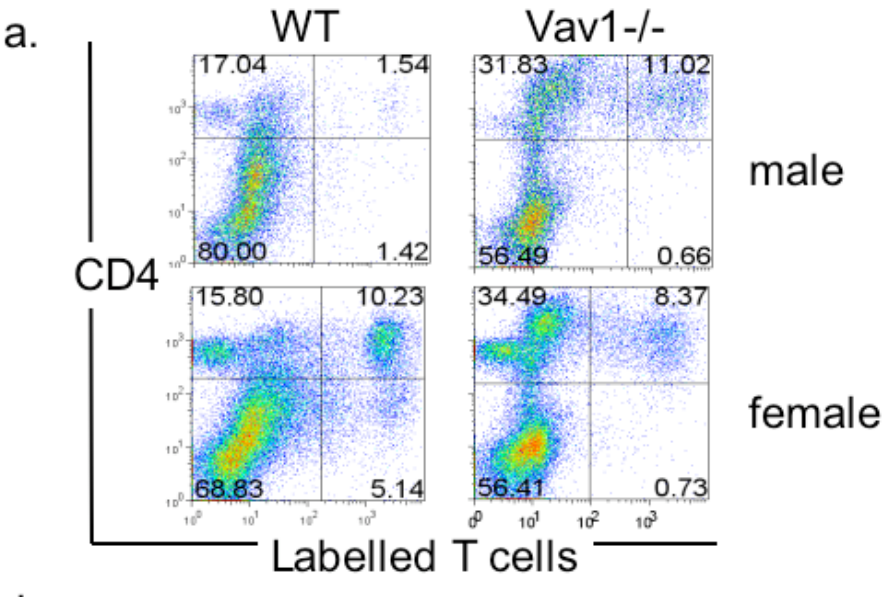
The results of the peritoneal recruitment model were surprising, as they suggested that Vav1 is dispensable for TCR-dependent T cell recruitment to sites of inflammation but may be required for retention to the antigenic tissue. As we had previously speculated that flow compensates for the loss of Vav1 activity in T cells *in vivo*, we examined T cell infiltration to antigenic tissue in static conditions. In this peritoneal tissue infiltration

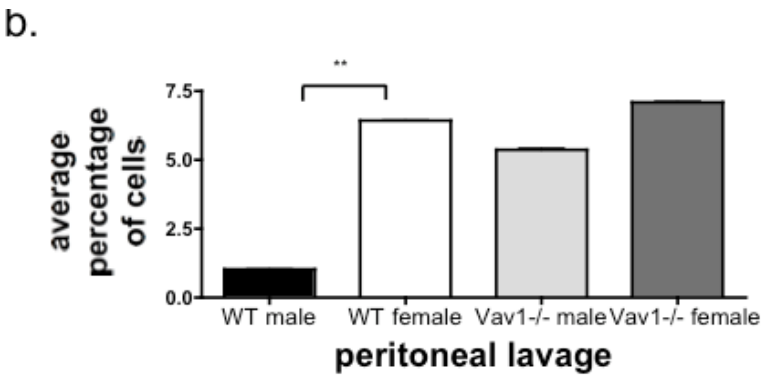
model, HY-specific T cells are injected directly into the peritoneal cavity of male and female mice that have previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation. Previous studies have shown that HY-specific WT T cells injected in male mice migrate out of the peritoneal cavity and are retained into the peritoneal membrane due to presentation by local APCs, whereas HY-specific WT T cells injected in female mice remain in the peritoneal cavity and do not infiltrate the peritoneal membrane (James *et al*., 2003).

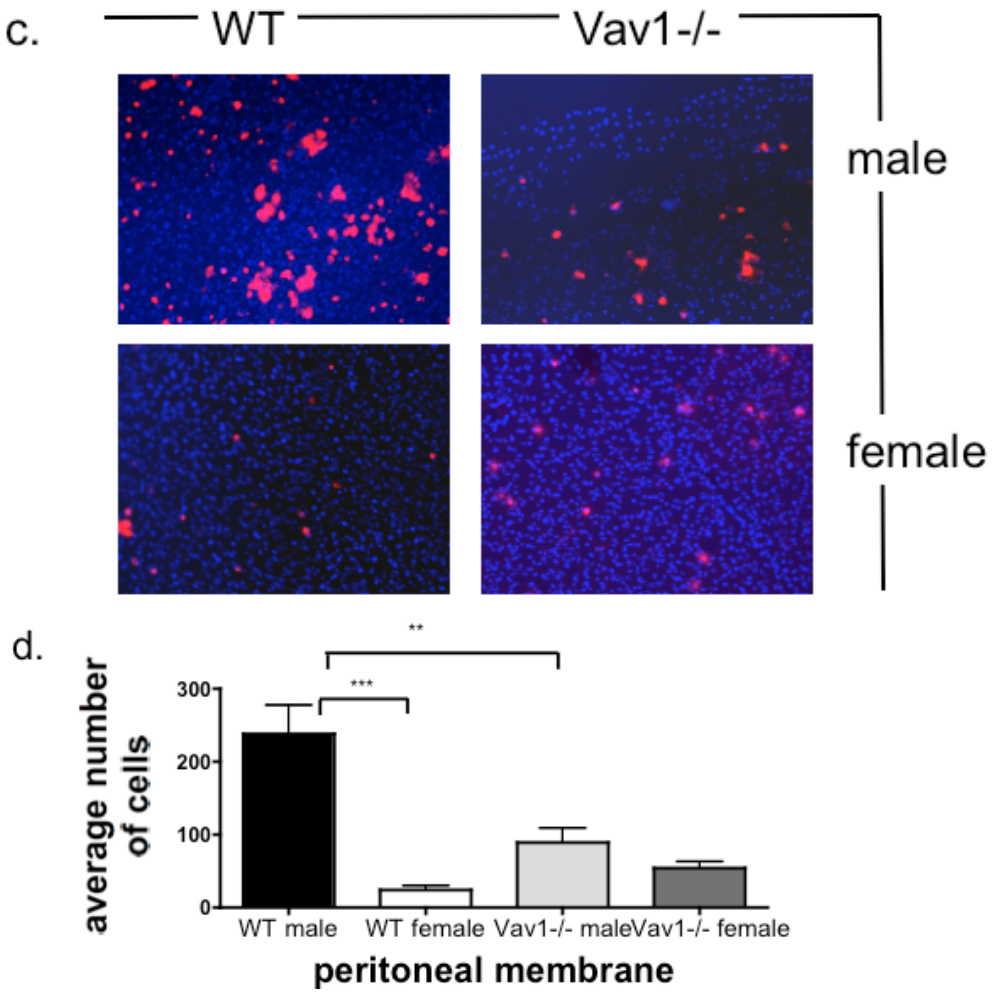
As shown in **Figure 25** a,b, significantly higher numbers of HY-specific $CD4^+$ WT T cells were found in the peritoneal cavity of female mice than that of male mice, as expected for WT HY-specific T cells. In contrast, there was no difference between the male and female peritoneal lavage when HY-specific $CD4^+$ Vav1-/- were injected ip.

To examine this further, the peritoneal membrane, which serves as the antigenic tissue in this case, was examined by wide-field fluorescence microscopy. As expected, more HY-specific $CD4^+$ WT T cells were found in peritoneal membrane of male mice compared to that of female mice (**Figure 25** c,d). However, no difference in the number of cells present in the male and female peritoneal membranes was observed when HY-specific $CD4^+$ Vav1-/- T cells were injected ip. Interestingly, significantly higher number of HY-specific $CD4^+$ WT T cells were found in the peritoneal membrane of male mice compared to HY-specific $CD4^+$ Vav1-/- T cells, suggesting that Vav1-/- T cells have a defect in infiltrating antigenic tissue in the absence of flow.

Figure 25: Vav1-/- T cells do not infiltrate the peritoneal membrane of male mice







*Viable HY-specific CD4^{+} WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 3x10^{b} T cells were injected ip in male and female mice that had previously received an ip injection of IFNγ to induce MHC class II expression. The mice were culled 24 hours later. T cells remaining in the peritoneal lavage were analysed by flow cytometry (a) Average percentage of cells in the peritoneal lavage (b). Representative examples of retained T cells in the peritoneal membrane (x10 magnification) (c) The average number of cells in the peritoneal membrane was quantified using the algorithm in the appendix (d). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.05, **p<0.01). Experimental groups consisted of four to six mice.*

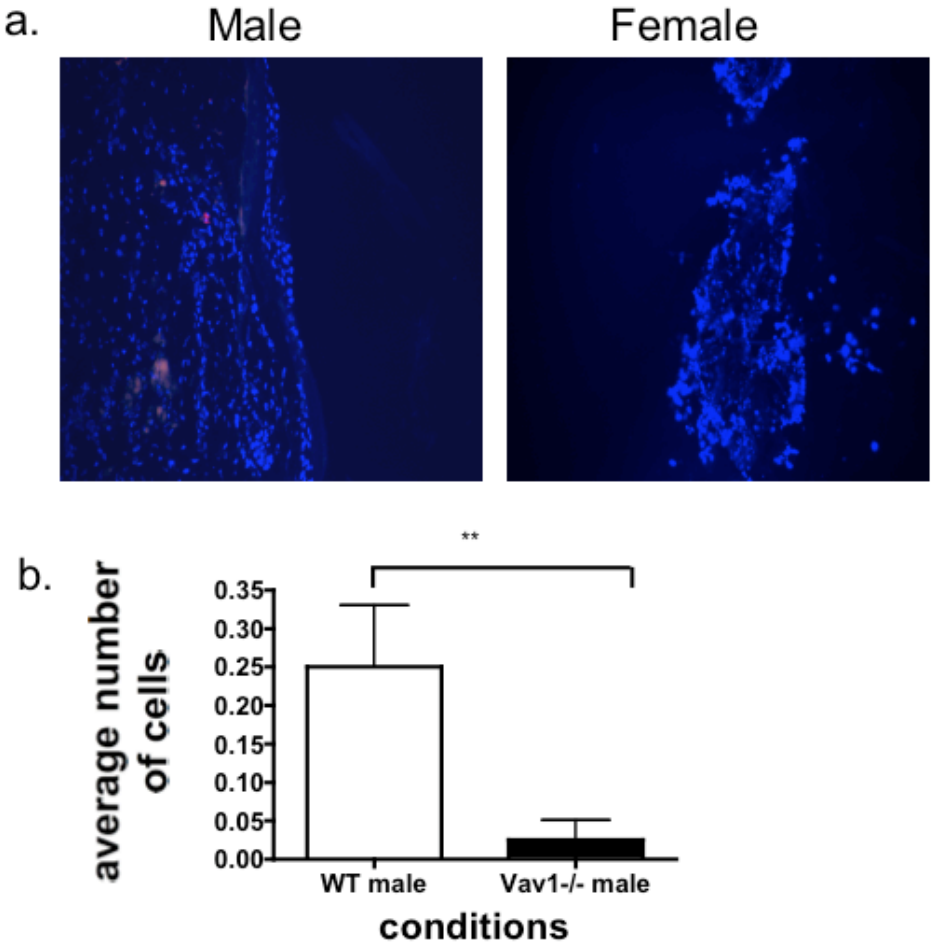
2.4. Skin graft infiltration by Vav1-/- T cells

The previous findings suggest that, although HY-specific $CD4^+$ Vav1-/- T cells have no gross defects in recruitment to the site of antigen presentation, they are not retained in the antigenic tissue *in vivo*. A recent study by Weckbecker *et al*. (2007) observed increased heart allograft survival in Vav1-/- mice compared to WT mice, with only mild cellular rejection observed upon histological examination. In addition, Wang *et al*. (2007) observed increased heart allograft survival when treating mice with 6-thio-GTP, a metabolite of azathioprine which blocks the Vav1-Rac1 interaction.

Prompted by these findings, HY-specific $CD4^+$ WT and Vav1-/- T cell graft infiltration was compared using an established skin graft model. In this model, male skin grafts, which serve as the allograft, are rapidly rejected while female grafts remain healthy (Zelenika *et al*., 1998). We have previously observed that iv injection of HY-specific WT T cells at day four post-grafting results in skin graft infiltration despite the absence of vasculature connection between donor and recipient (unpublished observations). Based on this, female C57BL/6 mice were grafted with male or female C57BL/6 tail grafts, and at day four, 10^7 HY-specific $CD4^+$ WT and Vav1-/- T cells were labelled with PKH26 or CFSE respectively and co- injected iv. Skin graft infiltration was examined 24 hours post-injection using wide-field fluorescence microscopy following sectioning by cryotomy.

Figure 26 a shows representative examples of sections obtained from the male and female skin grafts. Significantly more HY-specific $CD4^+$ WT T cells than Vav1-/- T cells were found in the male skin graft, as shown in **Figure 26** b. Neither HY-specific $CD4^+$ WT nor Vav1-/- T cells infiltrated the female skin graft.

Figure 26: Vav1-/- T cells do not infiltrate the antigenic skin graft



*Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 10^7 WT and Vav1-/- T cells were labelled with PKH26 (red) and CFSE (green) respectively and co-injected iv into female C57BL/6 mice that had previously received a tail skin graft from male or female C57BL/6 donors. The mice were culled 24 hours later and tissue infiltration was measured by wide-field fluorescence microscopy after mounting with vectorshield medium containing DAPI which stains the nucleus blue (x10 magnification). Representative examples of male and female skin grafts sections (a). The number of cells present in each section was quantified manually (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (**p<0.01). Each group consisted of three female mice.*

3.3. Discussion

Vav1 acts as an essential link between the TCR and the cytoskeleton so we had hypothesised that loss of Vav1 will result in impaired T cell recruitment and retention to sites of antigen presentation. The first part of this study aimed to test this hypothesis using *in vitro* migration assays. Indeed we observed that the HY-specific $CD4^+$ Vav1-/- T cells did not show enhanced migration in the presence of antigen in the transwell-based migration assays, as no significant differences in transendothelial migration through antigenic male and non-antigenic female EC monolayer were detected. This observation was further supported by the time-lapse microscopy assays. As expected, the HY-specific $CD4^+$ WT T cells showed enhanced transmigration through male endothelial monolayer in both assays.

Our previous findings in Chapter 2 showed disparity between the *in vitro* and the *in vivo* findings, whereby any defects in migration observed *in vitro* appeared to be compensated for *in vivo* by other factors, possibly shear flow. It was therefore essential to assess whether *in vitro* findings on the role of Vav1 in TCR-dependent migration were reflected *in vivo*.

In the first part of the *in vivo* study we used an antigen-driven peritoneal recruitment model, previously established in our laboratory (Marelli-Berg *et al*., 2004); ip injection of IFNγ causes localised antigen presentation in the peritoneal membrane, which results in HY-specific WT T cells being recruited to the peritoneal cavity and membrane of male but not female mice when injected iv. Based on our preliminary data as well as the *in vitro* assays, we hypothesised that HY-specific $CD4^+$ Vav1-/- T cells would show inefficient recruitment to the peritoneal cavity in male mice. Surprisingly, HY-specific $CD4^+$ Vav1-/- T displayed normal recruitment suggesting that Vav1 activity is not required for TCR-dependent T cell recruitment to antigenic sites *in vivo*.

We have previously speculated that shear flow may compensate for the loss of Vav1 activity in T cell migration *in vivo*. To confirm this hypothesis, T cell migration was examined in the absence of flow using an *in vivo* peritoneal tissue infiltration model. In this case, T cells are injected directly into the peritoneal cavity and infiltration of the antigenic tissue, the peritoneal membrane, is only observed in male mice (James *et al*.,

2003). Indeed the HY-specific $CD4^+$ WT T cells infiltrated the peritoneal membrane in male mice, while in female mice, they remained in the peritoneal lavage. However HY-specific $CD4^+$ Vav1-/- T cells showed significantly lower infiltration of the peritoneal membrane of male mice compared to the WT controls, confirming that flow may compensate for the loss of Vav1 activity in TCR-dependent T cell migration *in vivo*.

In the peritoneal recruitment model, more HY-specific T cells are retained in the peritoneal membrane of male mice compared to female controls. It is thought that retention to the peritoneal membrane is mediated by the presentation of antigen by resident APCs, which has previously been shown to result in temporary loss of migratory ability, allowing the T cell to polarise and proliferate; this is known as the stop signal (Dustin *et al*., 1997).

An interesting possibility explaining the reduced number of HY-specific Vav1-/- T cells in the peritoneal membrane is reduced susceptibility to the migratory stop signal. In this case, engagement of the TCR through presentation of antigen by resident APCs in the peritoneal membrane does not result is loss of migratory ability, allowing the Vav1-/- T cells to continue migrating to the peritoneal cavity. This possibility will be explored further in Chapter 4.

One of the first studies to demonstrate a role for Vav1 in T cell migration to sites of inflammation *in vivo* was by Korn *et al*. (2003) who observed reduced brain infiltration of MOG peptide-primed Vav1-/- T cells in a model of EAE. This could be explained as reduced recruitment of T cells from these Vav1-/- mice to sites of inflammation, but our studies suggest that Vav1-/- T cells show normal recruitment. It is therefore possible that reduced brain infiltration observed in this study may have been due to reduced retention to the antigenic brain tissue.

Previous studies of transplant tolerance have observed that Vav1-/- mice show increased heart allograft tolerance with significantly reduced histological damage compared to WT mice, suggesting a role for Vav1 in T cell-mediated transplant rejection (Weckbecker *et al*., 2007). This was further supported by a study by Wang *et al*. (2007), who used 6-thio-GTP, an inhibitor of the Vav1/Rac1 interaction to prolong heart allograft tolerance in mice. Based on these findings, we hypothesised that Vav1-/- T cells may

migrate to the transplant but are not retained following antigen presentation by graft APCs, leading to reduced transplant rejection.

To test this hypothesis, we used tail skin grafting of syngeneic male and female grafts on female C57BL/6 mice. We have previously observed that although the vasculature of donor and recipient are not connected, HY-specific WT T cells are able to infiltrate the graft when injected at day four post-grafting. Other studies have also demonstrated that $CD8^+$ T cells infiltrate the skin graft between days three and seven post-transplantation (Jones *et al.*, 2001) suggesting that vascularisation of the skin graft, which is thought to start at day three and progress until day 21 post-transplantation, is not necessary for the entry of T cells to the graft. Therefore, retention may play a dominant role in mediating T cell localisation to the skin in this model.

Using this tail skin grafting model, we observed reduced infiltration of HY-specific $CD4^+$ Vav1-/- T cells to the male skin graft compared to the WT control. This suggests that in the absence of Vav1 activity, T cells either did not migrate to the male skin or were not retained due to reduced responsiveness to the migratory stop signal, mediated by resident APCs. As we have previously shown that HY-specific $CD4^+$ Vav1-/- T cells are able to traffic to different organs in the body, are efficiently recruited to sites of antigen presentation and express high levels of the skin homing marker, PSGL-1, we can hypothesise that HY-specific $CD4^+$ Vav1-/- T cells migrated to the male skin graft but were not retained. Nevertheless, as the skin graft was not vascularised, it is possible that in the absence of flow, HY-specific $CD4^+$ Vav1-/- T cells showed impaired infiltration to the site of antigen presentation, as previously described in the peritoneal infiltration model.

Chapter 4: Regulation of the migratory stop signal by Vav1

4.1. Introduction

The data presented in Chapter 3 suggest that HY specific $CD4^+$ Vav1-/- T cells were recruited but not retained into the antigenic tissue. Retention to antigenic tissue is likely to be sustained by two factors; enhanced adhesion due to upregulation of integrins such as LFA-1 by memory T cells as well as migratory stop signals induced by presentation of antigen by resident APCs. These two factors are not actually mutually exclusive; Dustin *et al*. (1997) showed that engagement of the TCR results in increased adhesion to ICAM-1. This suggests that presentation of antigen by resident APCs governs retention to antigenic tissue by engagement of the TCR leading to increased adhesion and loss of migratory ability.

The functional consequences of antigen presentation differ significantly depending on the context they occur. Co-engagement of the TCR and co-stimulatory molecules, mediated by conventional APCs which may be found in the peritoneal membrane, has been shown to result in loss of migratory ability, allowing the T cell to polarise, interact with the APC and proliferate. This phenomenon is known as the stop signal (Dustin *et al*., 1997). In contrast, antigen presentation by ECs does not result in proliferation in the absence of cytokines (Lodge and Haisch, 1993; Pardi and Bender, 1991) and in fact has been shown to increase migration (Marelli-Berg *et al*., 2000).

Vav1 has previously been implicated in T cell: APC conjugate formation. Vav1-/- thymocytes were defective in transducing LFA-1 signals for the formation of conjugates (Ardouin *et al*., 2003) while T cells from TCR transgenic Vav1-/- mice showed reduced ability to form peptide-specific conjugates (Krawczyk *et al*., 2002). Given the reduced retention of HY-specific $CD4^+$ Vav1-/- T cells to the antigenic tissue, we investigated the involvement of Vav1 in the regulation of the stop signal.

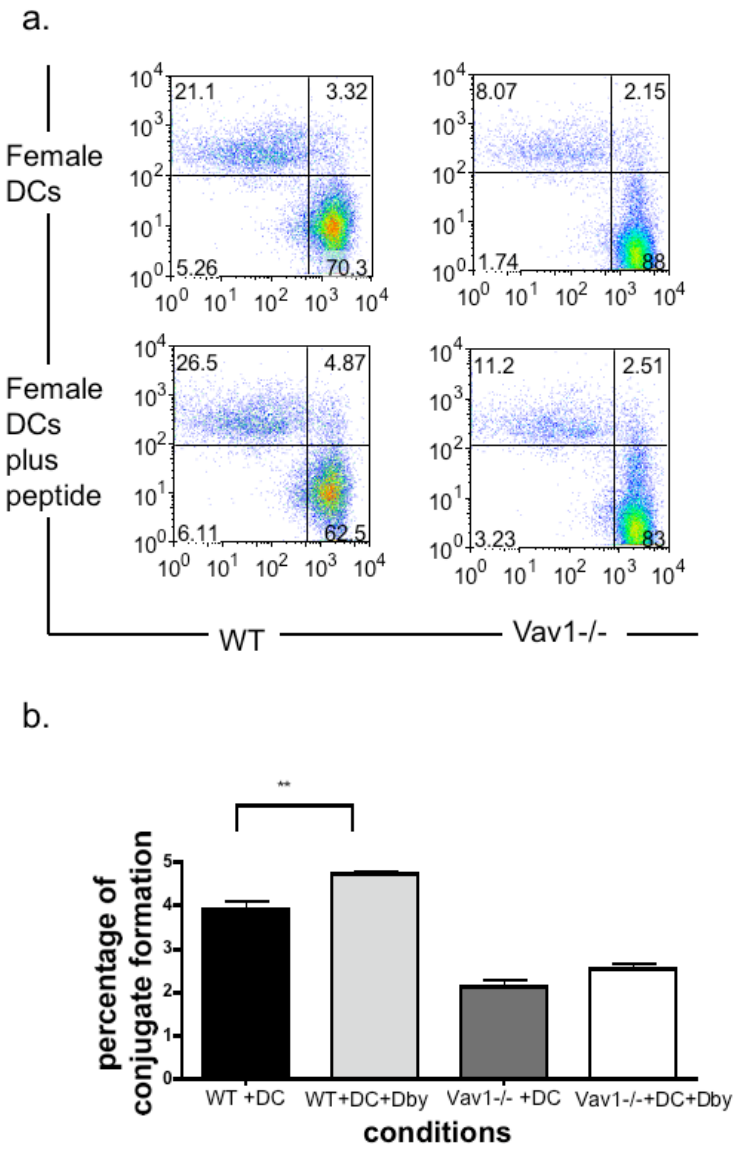
4.2. Results

4.2.1 Regulation of antigen-induced conjugate formation between DCs and T cells by Vav1

Previous studies have implicated Vav1 in conjugate formation; Ardouin *et al*. (2003) observed reduced transduction of LFA-1 signals required for conjugate formation in Vav1-/- thymocytes, while Krawczyk *et al*. (2002) observed reduced peptide-specific conjugate formation in naïve T cells from TCR-transgenic Vav1-/- mice. To extend these findings to memory T cells, we examined conjugate formation between antigen-specific memory T cells and peptide-pulsed DCs using HY-specific $CD4^+$ Vav1-/- T cells. More specifically, $2.5x10^5$ HY-specific $CD4^+$ WT and Vav1-/- T cells were incubated for two hours with 10^5 female DCs pulsed with an optimal dose of Dby peptide (50nM), previously established in the proliferation assays, or female DCs alone as a control and conjugate formation was analysed by flow cytometry. Single stains were used as controls and conjugates were defined as the double-positive population formed between the CFSE-labelled DCs and the PKH26-labelled T cells.

As shown in **Figure 27** a,b, HY-specific A^b-restricted $CD4^+$ WT T cells showed enhanced conjugate formation in the presence of the class II Dby peptide. In contrast, HY-specific A^b-restricted $CD4^+$ Vav1-/- T cells displayed no significant differences in conjugate formation in the presence or absence of peptide. This indicates that Vav1 is required for antigen-induced conjugate formation between memory T cells and DCs *in vitro*.

Figure 27: Vav1-/- have impaired conjugate formation *in vitro*



*Viable HY-specific A^b-restricted $CD4^+$ WT and Vav1-/- T cells were used at day ten post-stimulation. DCs were isolated from the bone marrow of female C57BL/6 mice and cultured for seven days, followed by overnight LPS maturation on the day before the experiment. T cells were labelled with PKH26 and incubated with DCs labelled with CFSE and pulsed with 50nM Dby peptide for two hours. DCs alone were used as a control. Conjugate formation was analysed by flow cytometry (a). Average percentage of conjugate formation from four experiments (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (**p<0.01, ***p<0.001).*

4.2.2 Regulation of the migratory stop signal by Vav1 in vitro

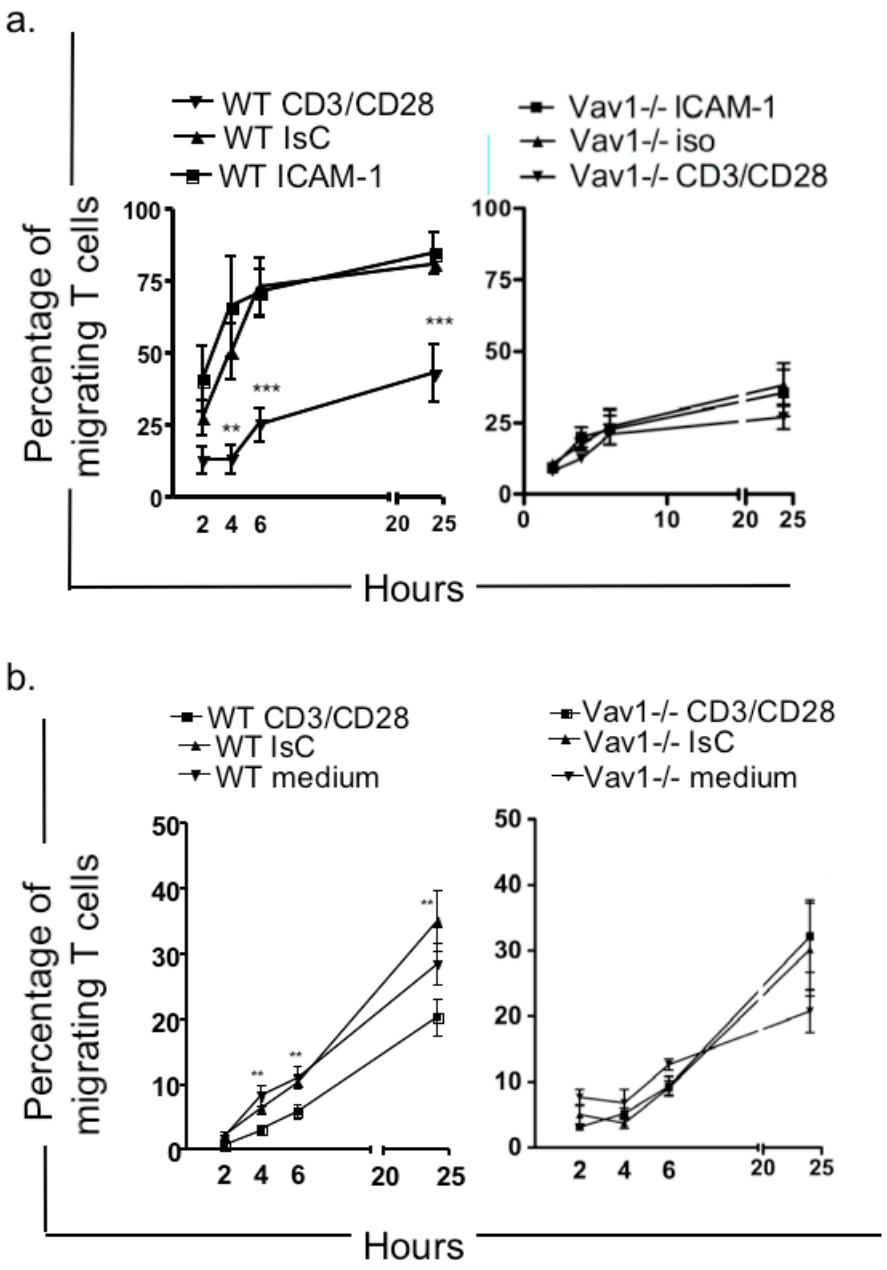
Findings in Chapter 3 implied that HY-specific $CD4^+$ Vav1-/- T cells may have lost susceptibility to the migratory stop signal induced by TCR engagement, resulting in reduced retention to the antigenic tissue. To examine this further, we used *in vitro* transwell-based migration assays. Briefly, 24-well plates were coated with 5μg/ml CD28 and 1μg/ml CD3 or hamster IgG (2μg/ml) as an isotype control in 200μl Tris pH 8.5 for two hours at 37ºC. The wells were subsequently washed and HY-specific $CD4^+$ WT and Vav1-/- T cells ($5x10^5$ per transwell) were plated for activation for 45 minutes at at 37ºC. Following activation, T cells were plated on ICAM-1-coated tranwells and migration was measured at different time-points over 24 hours.

Figure 28 a shows the migration of HY-specific $CD4^+$ WT and Vav1-/- T cells through ICAM-1-coated transwells. When stimulated with plastic-bound CD3 and CD28, WT T cells showed a significant reduction of migration in response to ICAM-1 compared to medium alone or the isotype control. In contrast, ligation of the TCR and CD28 did not result in significantly reduced migration of Vav1-/- T cells migration on ICAM-1 compared to the medium and isotype controls.

We then proceeded to examine the migration of activated HY-specific $CD4^+$ WT and Vav1-/- T cells through syngeneic ECs using transwell-based migration assays. HY-specific $CD4^+$ WT and Vav1-/- T cells were activated with plastic-bound CD3 and CD28 antibody or an isotype control, then plated on syngeneic EC-coated transwells and allowed to migrate for 24 hours.

As shown in **Figure 28** b, HY-specific $CD4^+$ WT T cells showed a significant loss of migratory ability following ligation of the TCR and CD28 compared to the isotype control and medium alone. Once again, Vav1-/- T cells did not respond to CD3/CD28 stimulation, as their migration through the syngeneic endothelial monolayer was comparable to the isotype and medium alone controls. This suggests that *in vitro*, Vav1 is required for the transduction of the migratory stop signal.

Figure 28: Vav1-/- T cells are not susceptible to the stop signal



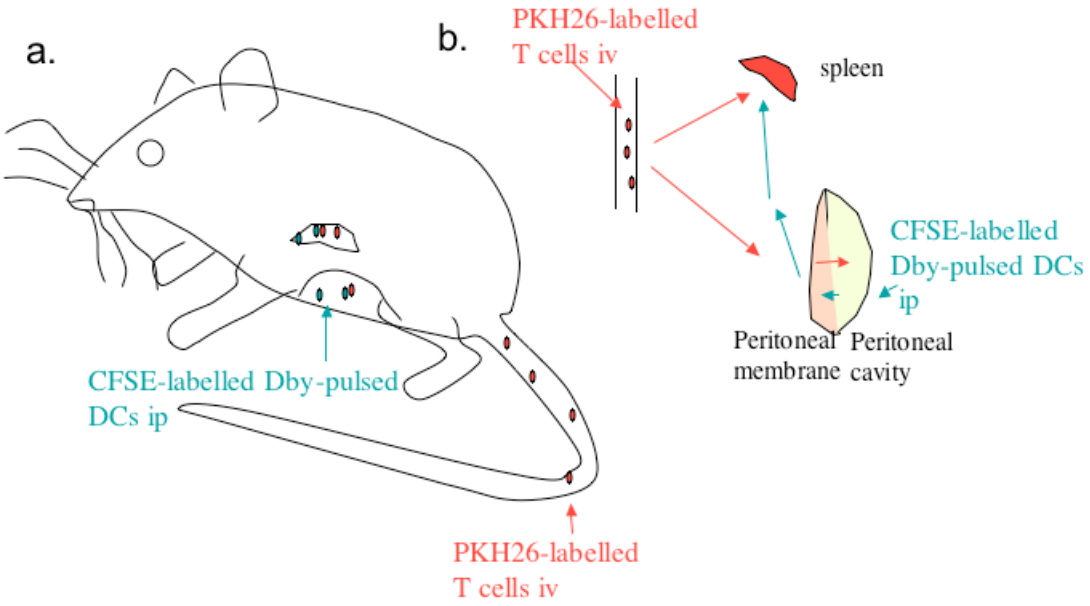
Viable HY-specific A^b-restricted WT and Vav1-/- T cells were used between days seven and ten post-stimulation. T cells were activated with plastic bound anti-CD3 and anti-CD28, hamster IgG isotype control or medium alone and plated on ICAM-1-coated transwells (a) or syngeneic endothelial monolayer- coated transwells (b). Migration was measured at two, four, six and 24 hours. Percentage of migrated cells was calculated by dividing the number of cell in the lower chamber with the original number of cells plated

*on the traswells. Error bars indicate standard error. Statistical analysis was performed from the average of at least three independent experiments using Student's t-test comparing CD3/CD28 with isotype control and medium (** p<0.01, *** p<0.001).*

4.2.3 Regulation of conjugate formation and the transduction of the migratory stop signal by Vav1 in vivo

Our findings in the first two sections of this chapter suggested that *in vitro*, loss of Vav1 activity impaired both conjugate formation as well as transduction of the migratory stop signal in T cells. To examine the physiological relevance of these observations, we developed an *in vivo* model to assess the interaction of antigen-specific T cells and peptide-pulsed APCs in both lymphoid and non-lymphoid antigenic tissue (depicted in **Figure** 29). C57BL/6 female mice were injected with 10^7 PKH26-labelled HY-specific A^b-restricted $CD4^+$ WT or Vav1-/- T cells iv and $2x10^6$ CFSE labelled LPS-matured DCs pulsed with an optimal dose (50nM) of Dby peptide ip. The mice were sacrificed 24 hours later and the presence of conjugates in lymphoid and non-lymphoid tissue was examined by wide-field fluorescence microscopy. We defined conjugates as the presence of yellow fluorescence formed by the interaction between the membrane of the red PKH26-labelled T cells and the green CFSE-labelled DCs.

Figure 29: Conjugate formation in *vivo* model

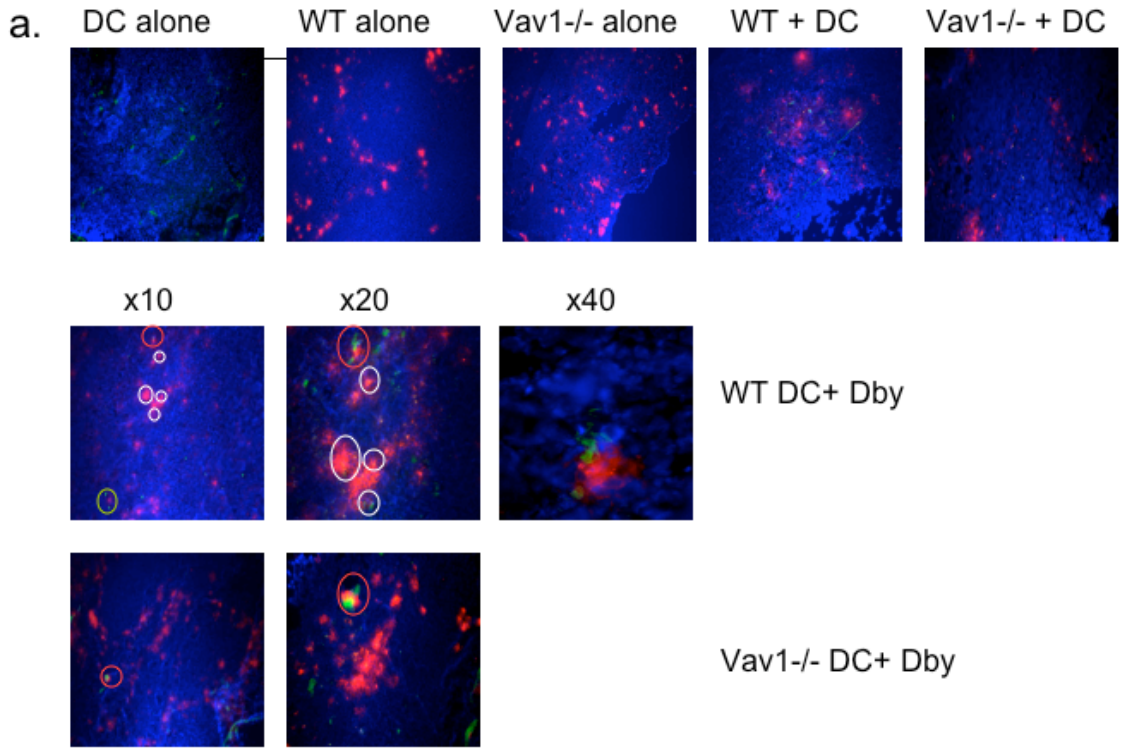


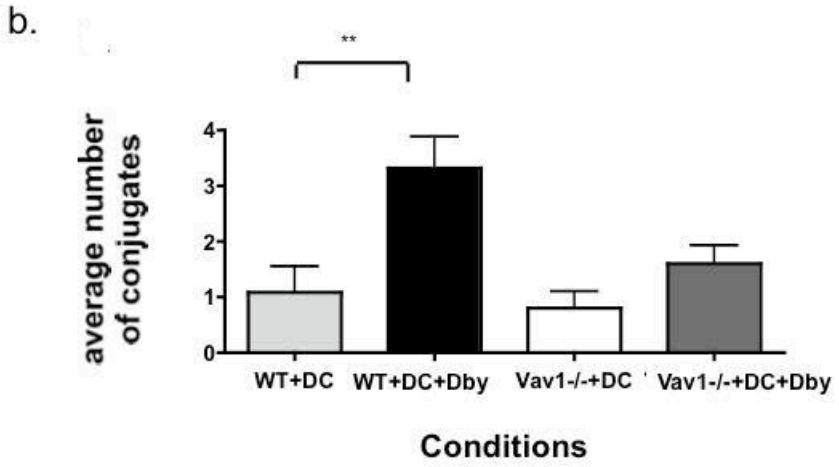
10^7 PKH26-labelled T cells were injected iv in female C57BL/6 mice that simultaneously received an ip injection of Dby peptide pulsed DCs labelled with CFSE, or DCs alone as a control (a). T cells travel into the bloodstream and reach the peritoneal membrane as well as the spleen, while DCs travel out of the peritoneal cavity, through the peritoneal membrane, enter the bloodstream and reach the spleen. T cells and peptide-pulsed DCs may form conjugates in both lymphoid (i.e. spleen) or non-lymphoid (i.e. peritoneal membrane) tissue, resulting in the transduction of the migratory stop signal and tissue retention.

Being of an effector memory T cell phenotype, HY-specific $CD4^+$ WT and Vav1-/- T cells readily migrate to the spleen but not the lymph nodes, as described in Chapters 1 and 2. As a secondary lymphoid organ, the spleen is a premier site of antigen presentation (reviewed in Janeway *et al*, 2001). In addition, DCs injected ip have previously been shown to promptly circulate in the bloodstream and reach secondary lymphoid tissues (Mirenda *et al.*, 2005). Based on these observations, we initially examined conjugate formation between HY-specific $CD4^+$ WT and Vav1-/- T cells and peptide-pulsed DCs in the spleen.

As shown in **Figure** 30 a,b, WT T cells showed interaction with the peptide-pulsed DCs, appearing as yellow fluorescence, compared to DCs alone. In contrast, the presence of peptide did not result in enhanced conjugate formation between HY-specific A^b-restricted $CD4^+$ Vav1-/- T cells and DCs in the spleen. This confirms that Vav1 activity is required for conjugate formation between memory T cells and APCs in lymphoid tissue *in vivo*. As expected, no conjugates were observed in the spleens of female mice injected with T cells alone or DCs alone.

Figure 30: Vav1-/- T cells form less conjugates in the spleen





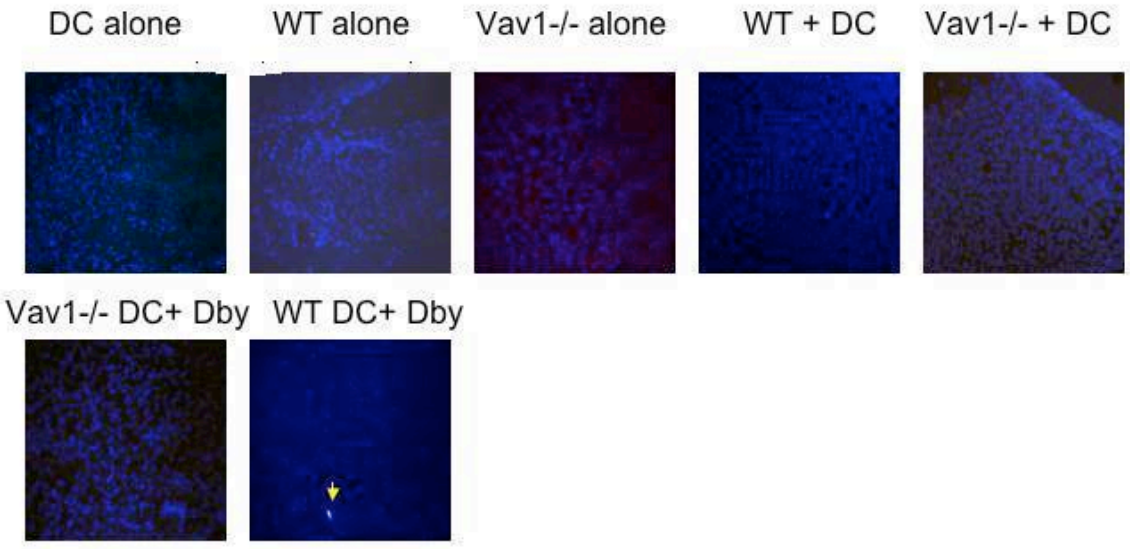
*Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. Female DCs were isolated from the bone marrow of female C57BL/6 mice and were matured overnight with LPS after having been placed in culture for seven days. HY-specific CD4⁺ WT and Vav1-/- T cells were labelled with PKH26 (red) and injected iv in female C57BL/6 mice that simultaneously received an ip injection of CFSE (green)-labelled Dby peptide pulsed DCs or DCs alone as a control. HY-specific CD4⁺ WT and Vav1-/- T cells and DCs were injected alone as a control. The mice were culled 24 hours later. The spleen was snap-frozen in OCT medium, cut in 5-10μm sections and analysed by wide-field fluorescence microscopy following mounting with vectorshield medium containing DAPI which stains the nucleus blue. Representative examples of sections from each condition. Conjugates are indicated by a yellow circle, white if magnified to x20 and red if magnified to x40 (a) Average number of conjugates in the spleen (b) Error bars indicate standard error. Statistical analysis was performed using the Student's t-test (**p<0.01). Experimental groups consisted of six female mice.*

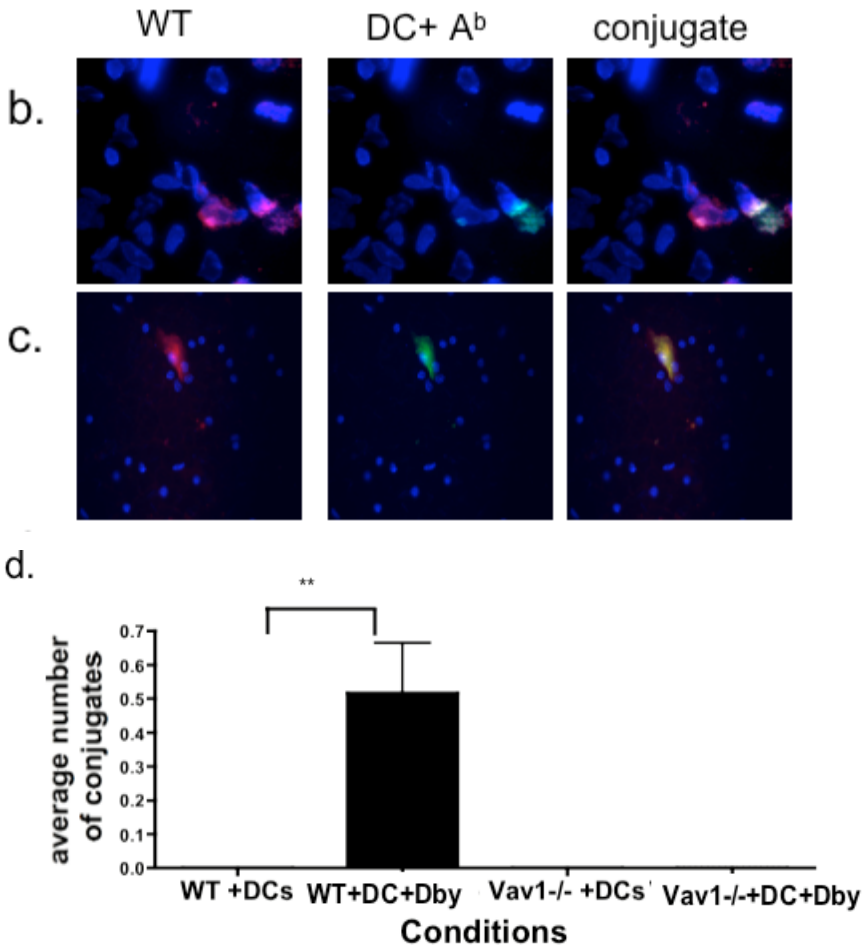
We then proceeded to examine conjugate formation in peritoneal membrane (i.e. non-lymphoid tissue containing APCs). **Figure 31** a shows the peritoneal membrane of female mice in all the conditions tested. As shown in **Figure 31** b,c,d, the peritoneal membrane of female mice injected with HY-specific CD4⁺ WT T cells and Dby peptide pulsed DCs showed the presence of yellow fluorescence which, as mentioned earlier, we defined as conjugates. This effect was not observed in any of the other conditions, including the peritoneal membrane of female mice injected with HY-specific CD4⁺ WT T

cells and DCs alone, as well as female mice injected with HY-specific $CD4^+$ WT T cells alone or DCs alone (**Figure 31**a,d). In addition, yellow fluorescence was not observed in the peritoneal membrane of female mice injected with HY-specific $CD4^+$ Vav1-/- T cells, including those injected with Dby peptide pulsed DCs, DCs alone and Vav1-/- T cells alone (**Figure 31**a,d). This suggests the HY-specific A^b-restricted $CD4^+$ WT T cells were interacting with the Dby-peptide pulsed DCs, which appeared as yellow fluorescence in the non-lymphoid antigenic tissue.

Figure 31: Vav1-/- T cells do not efficiently interact with peptide-pulsed APCs in non-lymphoid tissue

a.





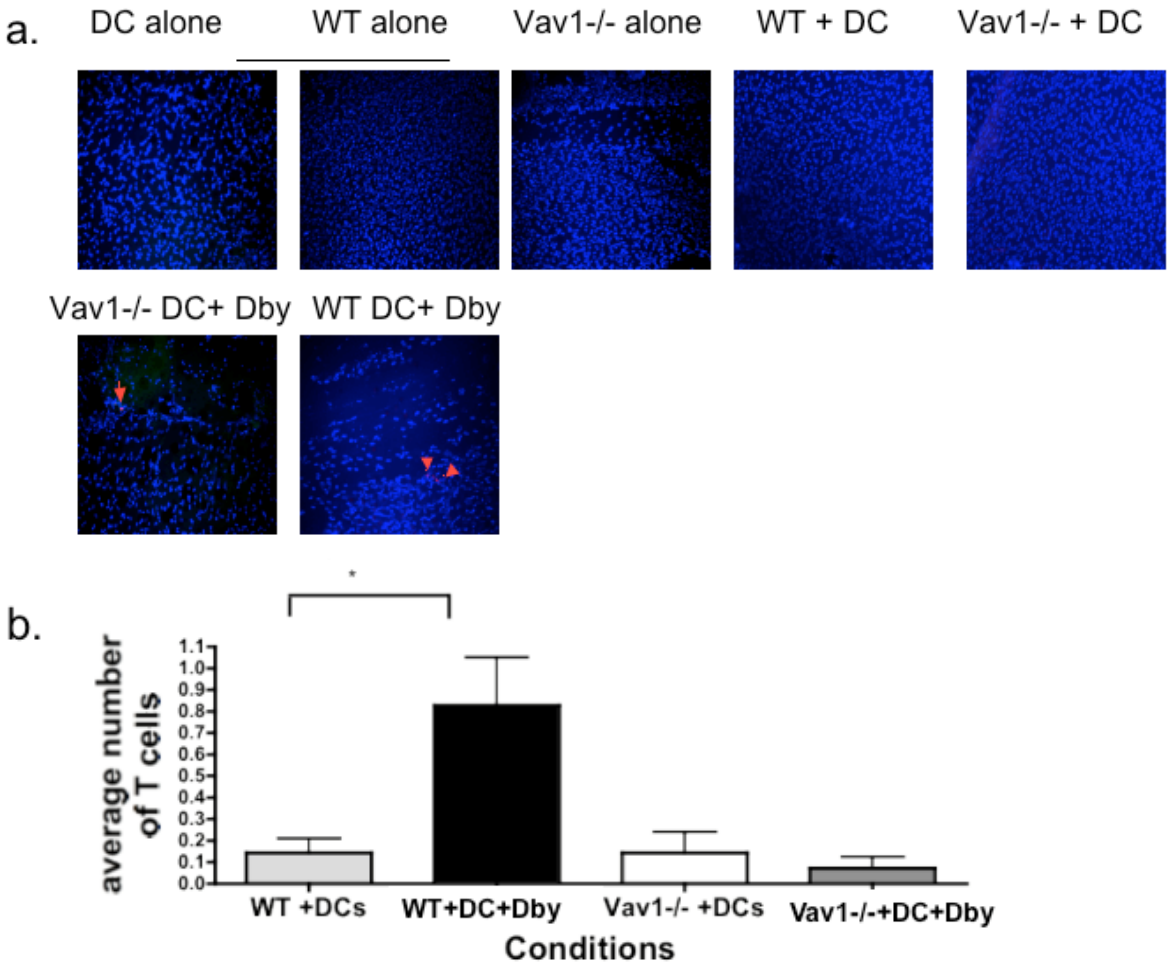
Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. Female DCs were isolated from the bone marrow of female C57BL/6 mice and were matured overnight with LPS after having been placed in culture for seven days. HY-specific CD4⁺ WT and Vav1-/- T cells were labelled with PKH26 (red) and injected iv in female C57BL/6 mice that simultaneously received an ip injection of Dby-peptide pulsed DCs or DCs alone as a control labelled with CFSE (green). HY-specific CD4⁺ WT and Vav1-/- T cells and DCs were injected alone as a control. The mice were culled 24 hours later. The peritoneal membrane was analysed by wide-field fluorescence microscopy following mounting with vectorshield medium containing DAPI which stains the nucleus blue. Representative examples of each condition (x10 magnification). The yellow arrow indicates the presence of a conjugate (a) Representative examples of conjugates between HY-specific CD4⁺ WT T cells and Dby-peptide pulsed DCs at x40

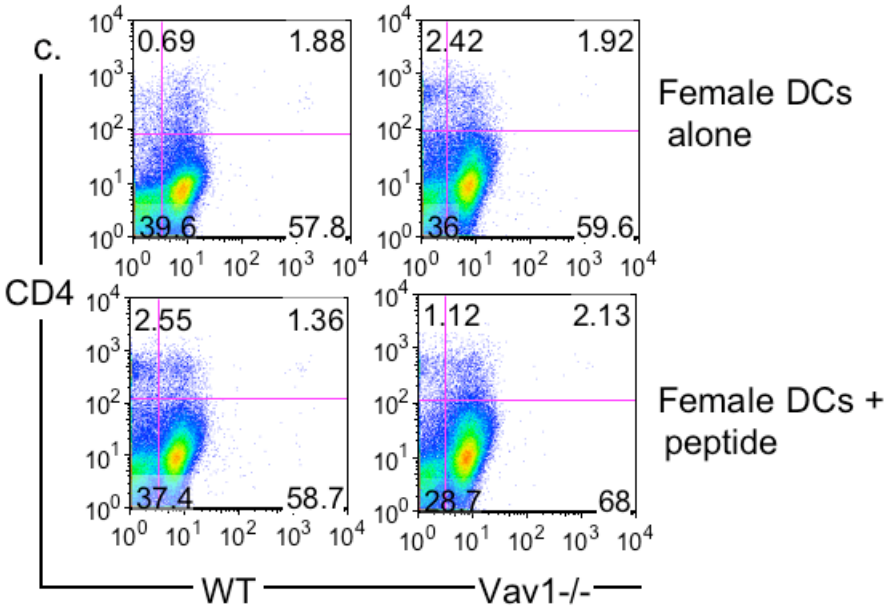
*magnification (b,c). The HY-specific CD4$^+$ WT T cell (red) and the Dby-peptide pulsed DC (green) have a tight interphase, which appears yellow (b) The HY-specific CD4$^+$ WT T cell (red) is on top of the Dby-peptide pulsed DC (green) resulting in yellow fluorescence (c) Average number of conjugates in the peritoneal membrane (d). Error bars indicate standard error. Statistical analysis was performed using the Student's t-test (**p<0.01, ***p<0.001). Experimental groups consisted of six female mice*

We have thus far observed that in the presence of antigen, HY-specific CD4$^+$ WT T cells form conjugates with peptide-pulsed DCs in both lymphoid and non-lymphoid tissue. In addition to this, we observed increased retention of HY-specific CD4$^+$ WT T cells to the peritoneal membrane of female mice injected with peptide-pulsed DCs, as shown in **Figure 32** a,b. This effect was not observed in any of the other conditions tested, suggesting that these cells may have previously formed conjugates with peptide-pulsed DCs and would have thus received the migratory stop signal.

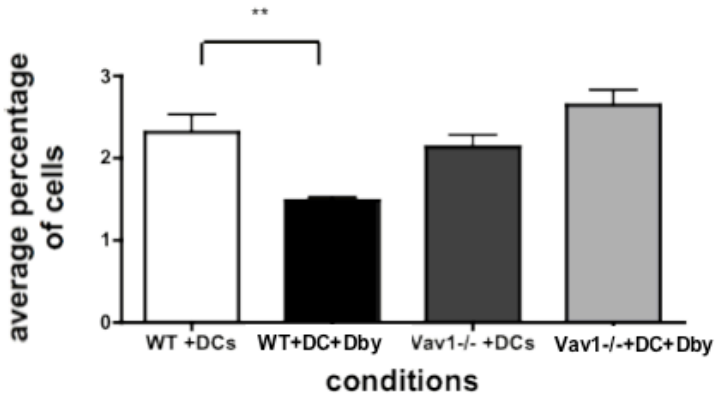
To examine whether this correlated with reduced migration to the peritoneal cavity, we analysed T cell enrichment of the peritoneal lavage by flow cytometry and FlowJo software. As shown in **Figure 32** c,d HY-specific CD4$^+$ WT T cells showed significantly reduced infiltration of the peritoneal cavity of female mice injected with Dby-peptide pulsed female DCs compared to DCs alone. In contrast, no significant difference was observed in the recruitment of HY-specific CD4$^+$ Vav1-/- T cells to the peritoneal cavity on female mice injected with DCs in the presence or absence of peptide.

Figure 32: Vav1-/- T cells do not respond to the stop signal *in vivo*





d.



Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. Female DCs were isolated from female C57BL/6 mice and were matured overnight with LPS after having been placed in culture for seven days. HY-specific CD4⁺ WT and Vav1-/- T cells were labelled with PKH26 (red) and injected iv in female C57BL/6 mice that simultaneously received an ip injection of CFSE-labelled (green) Dby-peptide pulsed DCs or DCs alone as a control. HY-specific CD4⁺ WT and Vav1-/- T cells and DCs were injected alone as a control. The mice were culled 24 hours later. The peritoneal membrane was analysed by wide-field fluorescence microscopy following mounting with vectorshield medium containing DAPI which stains the nucleus

*blue (x10 magnification). Red cells are indicated by red arrows. (a). Average number of cells retained in the peritoneal membrane (b). The peritoneal lavage was analysed by flow cytometry (c) Average percentage of cells present in the peritoneal lavage (c). Error bars indicate standard error. Statistical analysis was performed using the Student's t-test (*p<0.05, **p<0.01, ***p<0.001). Experimental groups consisted of at least three female mice.*

4.3. Discussion

In Chapter 3 we observed reduced retention of HY-specific $CD4^+$ Vav1-/- T cells to antigenic tissue, the peritoneal membrane, despite accelerated recruitment. In this chapter we have examined the possibility that reduced retention of Vav1-/- T cells to antigenic tissue may be due to lack of responsiveness to the migratory stop signal delivered by local conventional APCs. Loss of migratory ability occurs upon strong engagement of the TCR by professional APCs, such as DCs, so we commenced this investigation by examining the role of Vav1 in memory T cell conjugate formation.

Indeed HY-specific $CD4^+$ Vav1-/- T cells showed impaired conjugate formation *in vitro*, with no significant difference in percentage of conjugates in the presence or absence of antigen. This is not surprising as Vav1 has previously been shown to be involved in conjugate formation between TCR-transgenic T cells and APCs (Krawczyk *et al*., 2002). In addition, Vav1 has been found to be necessary for the formation of the immunological synapse; Vav1 activity has been found to be required for the activation of LFA-1 upon TCR triggering (Krawczyk *et al*. 2002, Ardouin *et al*., 2003) and for the translocation of lipid rafts to the immunological synapse (Villalba *et al*., 2001). Finally, Vav1-/- T cells have previously been shown to have defective accumulation of TCR/MHC II in the immunological synapse, impaired actin cap formation and reduced interaction of actin with CD3ζ (Fischer *et al*., 1998; reviewed in Hornstein *et al*., 2004). Our findings therefore confirm that Vav1 activity is required for stable conjugate formation during the interaction of the TCR with MHC-bound peptide.

Conjugate formation in non-lymphoid tissue has not been extensively examined *in vivo* and certainly never for Vav1-/- T cells. Given the disparity of *in vitro* and *in vivo* findings due to physiological factors affecting T cells in the body, we tried to examined the requirement for Vav1 activity *in vivo* using two approaches; conjugate formation in the spleen, which is known to occur as the spleen is a secondary lymphoid organ where memory T cells migrate, and conjugate formation in non-lymphoid antigenic tissue, which has not been extensively described. Due to the abundance of T cells and DCs in the spleen, examination of conjugate formation was relatively easy. We defined conjugate

formation as the presence of yellow fluorescence between a red T cell and a green DC. As expected, HY-specific A^b-restricted $CD4^+$ WT T cells showed enhanced conjugate formation in the spleen in the presence of peptide-pulsed DCs compared to DCs alone. This was not observed with HY-specific A^b-restricted $CD4^+$ Vav1-/- T cells, which confirms our *in vitro* findings on the requirement for Vav1 activity for T cell:APC conjugate formation.

Conjugate formation in the peritoneal membrane, which serves as the antigenic tissue in this model, was rather more difficult to assess due to the scarcity of both DCs and T cells in the tissue; DCs have in fact previously been shown to promptly leave the peritoneal cavity when injected ip (Mirenda *et al.*, 2005). In spite of the low numbers of cells, areas of yellow fluorescence in the peritoneal membrane of female mice injected with HY-specific WT T cells and Dby-peptide pulsed DCs were observed, validating our model for the evaluation of conjugate formation in non-lymphoid tissue. These findings indicate that Vav1 activity is required for conjugate formation in non-lymphoid antigenic tissues *in vivo*. Confocal microscopy may provide more information on the interaction as it has depth resolution, which allows distinguishing the overlap of two cells (Y. Gu, personal communication). This would allow for better visualisation of the interaction between the T cell and the peptide-bound DC. However, as we primarily wanted to quantify the number of conjugates present in the tissues, wide-field fluorescence microscopy was a more appropriate method of analysis.

A previous study on conjugate formation *in vivo* was able to visualise the interaction of lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells with LCMV targets in the brain using tetramer staining of brain sections from mice (McGavern *et al.*, 2002). This study also defined conjugates as the yellow interface between the red $CD8^+$ T cells and the green tetramer staining, validating our definition. It would have been interesting to use a similar approach, however there are no MHC class II tetramers. The authors of this paper have also justly noted that their observation of conjugate formation is just a "snap-shot" in time, which also applies is our case. Real-time *in vivo* imaging would provide more information of the dynamics of this interaction.

We have observed a requirement for Vav1 in conjugate formation both *in vitro* and *in vivo*. Conjugate formation is an integral part of the loss of migratory ability in T cells,

as only strong engagement of the TCR results in the transduction of the stop signal (Dustin *et al*., 1997). As HY-specific $CD4^+$ Vav1-/- T cells showed reduced conjugate formation, we hypothesised that they would show impaired responsiveness to the migratory stop signal.

Previous findings by Berg *et al*. (2002) have indicated that antibody engagement of the TCR and CD28 can lead to loss of migratory ability *in vitro*. Based on this observation, we examined loss of migratory ability *in vitro* using a transwell-based migration assay. We detected that engagement of the TCR and CD28 by plastic-bound antibody resulted in loss of migratory ability in HY-specific $CD4^+$ WT T cells migrating through ICAM-1-coated transwells. In contrast, HY-specific $CD4^+$ Vav1-/- T cell migration was unaffected by ligation of the TCR and CD28. We further confirmed our findings using syngeneic endothelial monolayer-coated transwells, and once again observed loss of migratory ability in HY-specific $CD4^+$ WT T cells treated with CD3 and CD28 antibody, but not in HY-specific $CD4^+$ Vav1-/- T cells. This suggests that *in vitro*, Vav1 activity is required for the transduction of the migratory stop signal due to TCR and CD28 engagement.

To examine the physiological relevance of our findings, we used the afore mentioned *in vivo* model, whereby HY-specific $CD4^+$ T cells were injected iv in female mice that had simultaneously received an ip injection of Dby-peptide pulsed DCs. In addition to increased conjugate formation, we observed increased retention of HY-specific $CD4^+$ WT T cells, compared to Vav1-/- T cells, which may have been the result of loss of migratory ability due to prior interaction with peptide-pulsed DCs. HY-specific $CD4^+$ Vav1-/- T cells also showed enhanced recruitment to the peritoneal cavity compared to HY-specific $CD4^+$ WT T cells, suggesting that they had impaired interaction with the Dby peptide-pulsed DCs in the peritoneal membrane, which did not result in strong engagement of the TCR, and thus did not result in the transduction of the migratory stop signal. These findings suggest that Vav1 activity is required for the loss of migratory ability due to engagement of the TCR both *in vitro* and *in vivo*.

Chapter 5: Regulation of T cell migration by CD28 and Vav1

5.1. Introduction

It is clear from Chapter 3 that the role of Vav1 in the regulation of T cell trafficking is more complex than we originally hypothesised. HY-specific $CD4^+$ Vav1-/- T cells showed normal recruitment to antigenic tissues under flow, but defective antigenic tissue retention and defective antigenic tissue infiltration under static conditions. These results were similar to findings by Korn *et al*. (2003) who observed reduced brain infiltration in a model of EAE in Vav1-/- mice. Administration of an anti-CTLA-4 antibody which increases CD28 signalling partially increased disease incidence, suggesting that dominant CD28 signalling may moderately compensate for the impaired tissue localisation of Vav1-/- T cells.

Entry into peripheral tissues is dependant on diapedesis through ECs on the surface of post-capillary venules, which have little or no expression of B7 molecules (CD80/CD86) in humans and mice respectively. On the other hand, retention to antigenic tissues is dependent on antigen presentation by resident APCs, which are B7 positive, and are thus capable of inducing co-stimulatory signalling (reviewed in Marelli-Berg and Jarmin, 2004). This indicates that CD28 signalling contributes to the delivery of stop signals required for the retention of T cells into antigenic tissues.

Induction of Vav1 activity by CD28 signalling has previously been described. CD28 ligation has been shown to activate Vav1 by both phosphorylation and arginine methylation (reviewed in Michel and Acuto, 2002; Blanchet *et al*., 2005). This allows CD28 to activate signalling pathways independently of TCR signals, including the non-canonical NfkB activation pathway (Picollela *et al*, 2003). In addition, Vav1 activated by CD28 ligation has been found to associate with Rac1 as well as WASp, thus mediating cytoskeletal re-modelling (Raab *et al*., 2001; reviewed in Secchi and Wehland, 2004).

We have recently shown that CD28 signalling can act in conjunction with the TCR to enhance T cell recruitment to antigenic sites. In addition, CD28 was also found to act independently of TCR signalling for the regulation of T cell migration (Mirenda *et al*., 2006).

Prompted by the apparent importance of Vav1 in CD28 signalling and our observations of a requirement for Vav1 activity in T cell tissue retention and the transduction of the migratory stop signal, we examined the relative importance of CD28 co-stimulatory signals in HY-specific $CD4^+$ Vav1-/- T cell migration.

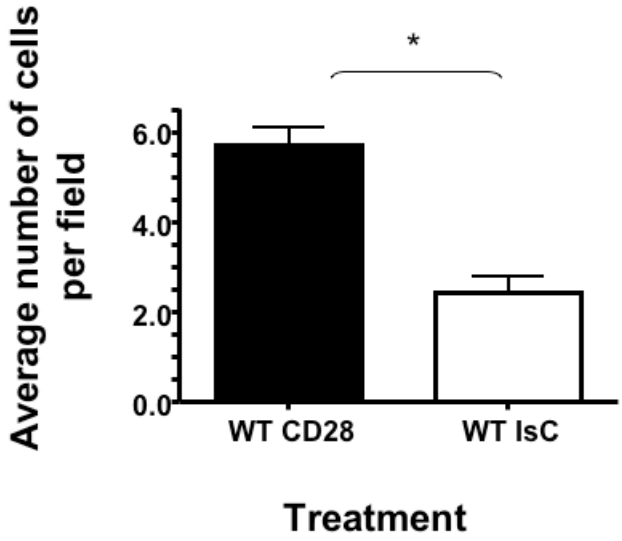
5.2. Results

5.2.1 The role of CD28 in skin graft infiltration

Recent evidence from our laboratory has indicated a role for CD28 in T cell migration; CD28 co-stimulatory signals were required for T cell localisation to antigenic sites following priming, while CD28 over-stimulation resulted in aberrant tissue infiltration (Mirenda *et al*. 2006). As part of those studies, we examined the role of CD28 in T cell skin graft infiltration. Briefly, female C57BL/6 mice were grafted with syngeneic antigenic male tail skin grafts and were iv injected four days post-grafting with 10^7 HY-specific WT T cells which had either been treated with a mixture of hamster anti-mouse CD28 (5μg/5x10^6 cells), and rabbit anti-hamster Ig (2.5μg/5x10^6 cells) to induce CD28 signalling and labelled with PKH26 or an isotype control of Hamster Ig (5 μg/5x10^6 cells) and rabbit anti-hamster Ig (2.5 μg/5x10^6 cells) and labelled with CFSE. Infiltration of the skin grafts was examined 24 hours later by wide-field fluorescence microscopy following sectioning by cryotomy. As discussed in Chapter 3, T cells injected on day 4 post-grafting are able to infiltrate the skin graft in the absence of vasculature connection between donor and recipient.

Figure 33 shows the average number of HY-specific WT T cells infiltrating the male tail-derived skin grafts. Induction of CD28 signalling resulted in T cell infiltration of the antigenic skin graft compared to the isotype control. This suggests that CD28 signalling in conjunction with TCR triggering can enhance T cell localisation to antigenic sites.

Figure 33: CD28 cross-linking enhances WT T cell localisation into antigenic tissue



*Viable HY specific WT T cells from C57BL/6 mice were used between days seven and ten post-stimulation. Female C57BL/6 mice were grafted with tail skin grafts from male C57BL/6 mice. On day 4, HY-specific WT T cells were cross-linked with CD28 and labelled with PKH26 or an isotype control and labelled with CFSE, and injected iv into the grafted female mice. Skin graft T cell infiltration was examined on day five. Error bars indicate standard error. Statistical analysis was performed using the Student's t-test (*p<0.05). Experimental groups consisted of two female mice.*

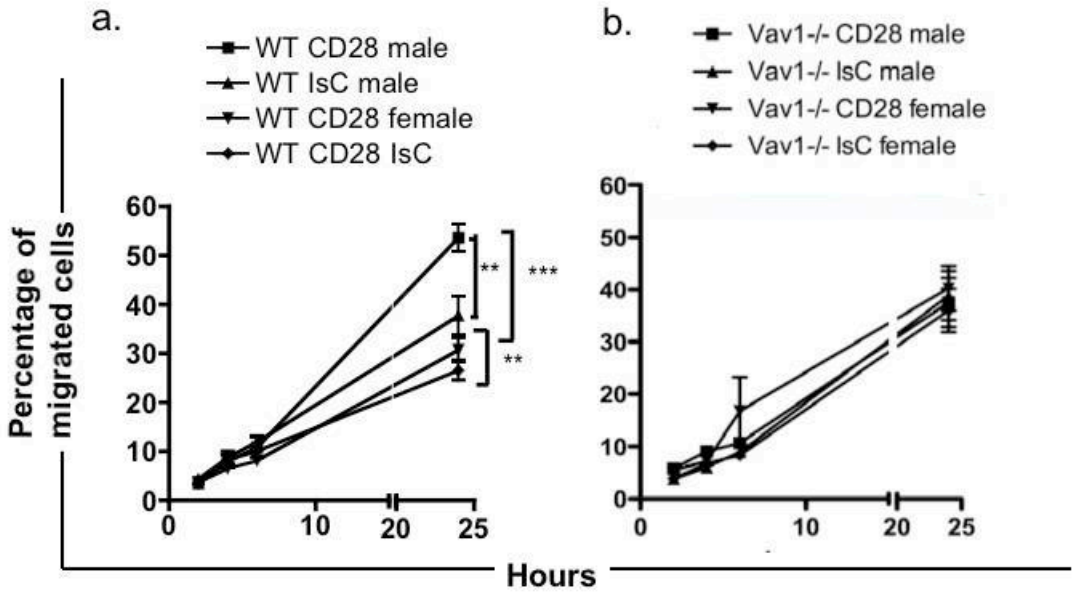
5.2.2 The role of CD28 signalling in Vav1-/- T cell migration in vitro

To investigate the requirement for Vav1 in CD28 regulation of migration in the presence or absence of TCR triggering, we examined HY-specific $CD4^+$ Vav1-/- T cell migration using *in vitro* transwell-based migration assays. 3-5x10^5 HY-specific $CD4^+$ WT and Vav1-/- T cells were cross-linked with a mixture of hamster anti-mouse CD28 (5μg/5x10^6 cells), and rabbit anti-hamster Ig (2.5μg/5x10^6 cells) or Hamster Ig (5 μg/5x10^6 cells) and rabbit anti-hamster Ig (2.5 μg/5x10^6 cells) as an isotype control for 45 minutes to induce CD28 signalling and were subsequently washed and allowed to migrate on male and female endothelial monolayer treated with IFNγ to induce MHC class II expression.

Figure 34 shows the results of the *in vitro* transwell-based migration assay. As expected, HY-specific $CD4^+$ WT T cells migrated more through the male monolayer than the female in both isotype control and CD28 antibody treatments. In addition, HY-specific $CD4^+$ WT T cells showed enhanced migration upon induction of CD28 signalling through the male but not the female monolayer, compared to the isotype control (**Figure 34** a). Note that enhanced migration due to CD28 triggering occurs at 24 hours, which might be due to enhanced expression of chemokine receptors (Walker et al., 1999).

In contrast, as shown in **Figure 34** b, CD28 triggering of HY-specific $CD4^+$ Vav1-/- T cells did not result in enhanced migration through either male or female monolayer. Concomitant with results in Chapter 3, HY-specific $CD4^+$ Vav1-/- T cells did not show enhanced migration through the male monolayer compared to the female control, and CD28 cross-linking did not compensate for this defect. These findings indicated a requirement for Vav1 activity in CD28-mediated enhancement of TCR-dependent T cell migration *in vitro*.

Figure 34: CD28 cross-linking does not enhance Vav1-/- T cell migration *in vitro*



*Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. WT (a) and Vav1-/- T cells (b) were cross-linked with CD28 or an isotype control for 45 minutes to induce CD28 signalling and were subsequently plated on male and female endothelial monolayer treated with IFNγ to induce MHC class II expression. T cells were allowed to migrate for 24 hours. Percentage migration was calculated by dividing by the number of cells plated on the transwell. Error bars indicate standard error. Statistical analysis was performed using Student's t-test (**p<0.01, ***p< 0.001) from the data from at least three experiments.*

5.2.3 The role of Vav1 in CD28-mediated T cell trafficking in vivo

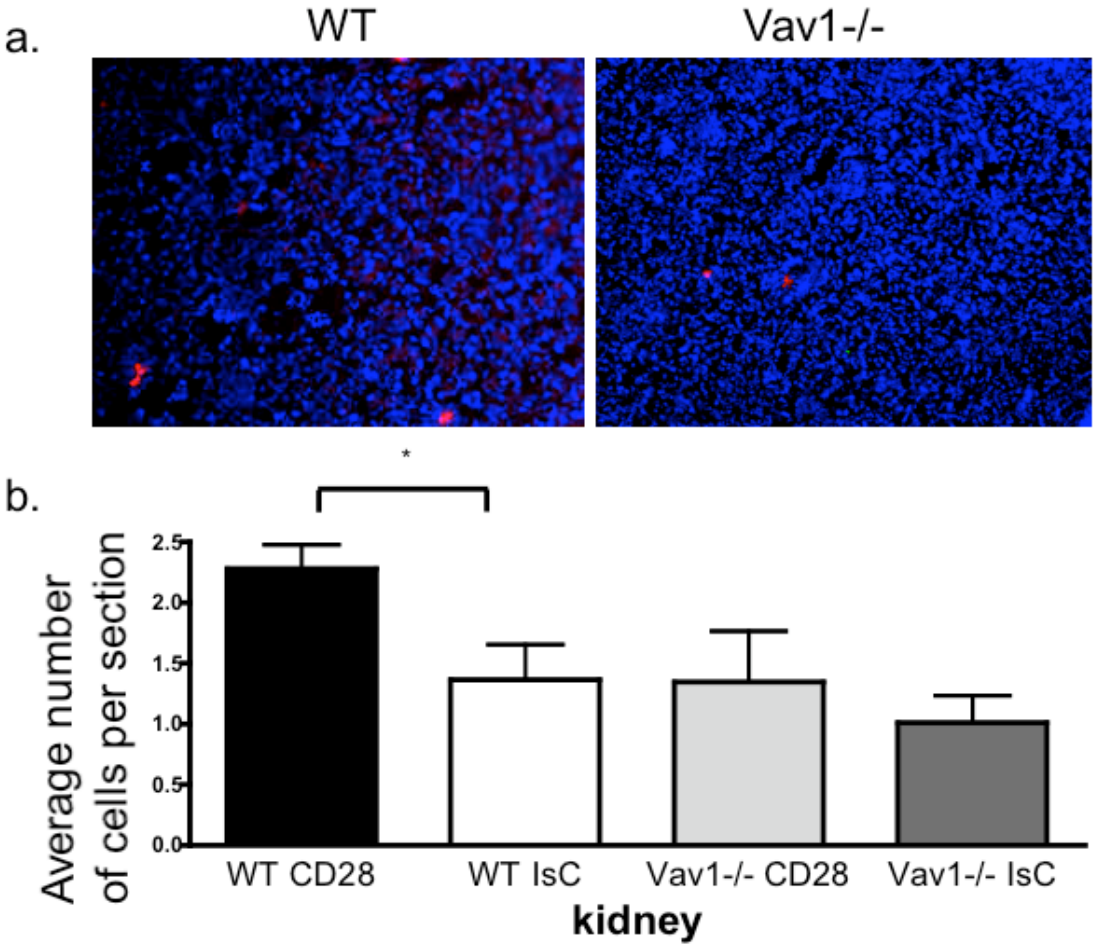
Previous studies have shown that CD28 triggering results in enhanced T cell infiltration to non-lymphoid tissue in the absence of inflammation (Mirenda *et al*., 2006). We have already shown that HY-specific CD4⁺ Vav1-/- T cells do not show any gross impairments in tissue trafficking *in vivo*. As CD28 triggering did not affect HY-specific

$CD4^+$ Vav1-/- T cell migration *in vitro*, we examined whether this translated to a defect *in vivo*.

HY-specific $CD4^+$ WT and Vav1-/- T cells were cross-linked with a mixture of hamster anti-mouse CD28 (5μg/$5x10^6$ cells), and rabbit anti-hamster Ig (2.5μg/$5x10^6$ cells) to induce CD28 signalling and subsequently labelled with PKH26, or Hamster Ig (5 μg/$5x10^6$ cells) and rabbit anti-hamster Ig (2.5 μg/$5x10^6$ cells) as an isotype control and labelled with CFSE. CD28 and isotype control-treated T cell were co-injected iv in female mice (10^7 T cells per mouse) and allowed to traffic for 24 hours. Infiltration into different organs was examined by wide-field fluorescence microscopy.

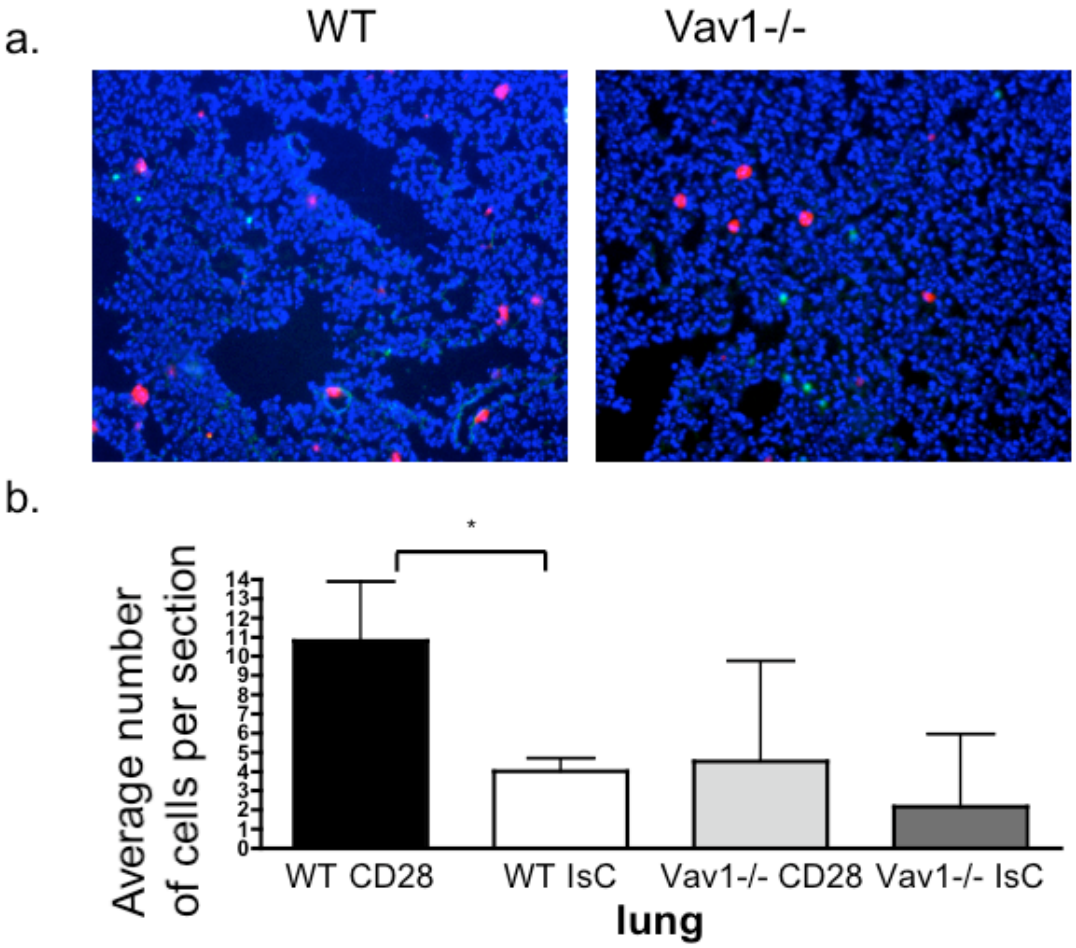
Induction of CD28 signalling by antibody cross-linking resulted in enhanced trafficking of HY-specific $CD4^+$ WT T cells to kidney (**Figure 35**), lung (**Figure 36**), spleen (**Figure 37**), peritoneal membrane (**Figure 38)** and liver (**Figure 39**), as expected from previous studies (Mirenda *et al.,* 2006). In contrast, CD28 cross-linking did not enhance the trafficking of HY-specific $CD4^+$ Vav1-/- T cells in kidney (**Figure 35**), lung (**Figure 36**), spleen (**Figure 37**) and peritoneal membrane (**Figure 38**. Interestingly, induction of CD28 antibody triggering induced increased localisation of HY-specific $CD4^+$ Vav1-/- T cells to the liver compared to the isotype control, as shown in **Figure 39**, possibly due to enhanced phagocytosis mediated by Fc receptors. These findings suggest that Vav1 is importat in transducing CD28-mediated signals regulating memory T cell migration.

Figure 35: CD28 cross-linking does not enhance Vav1-/- T cell migration to the kidney



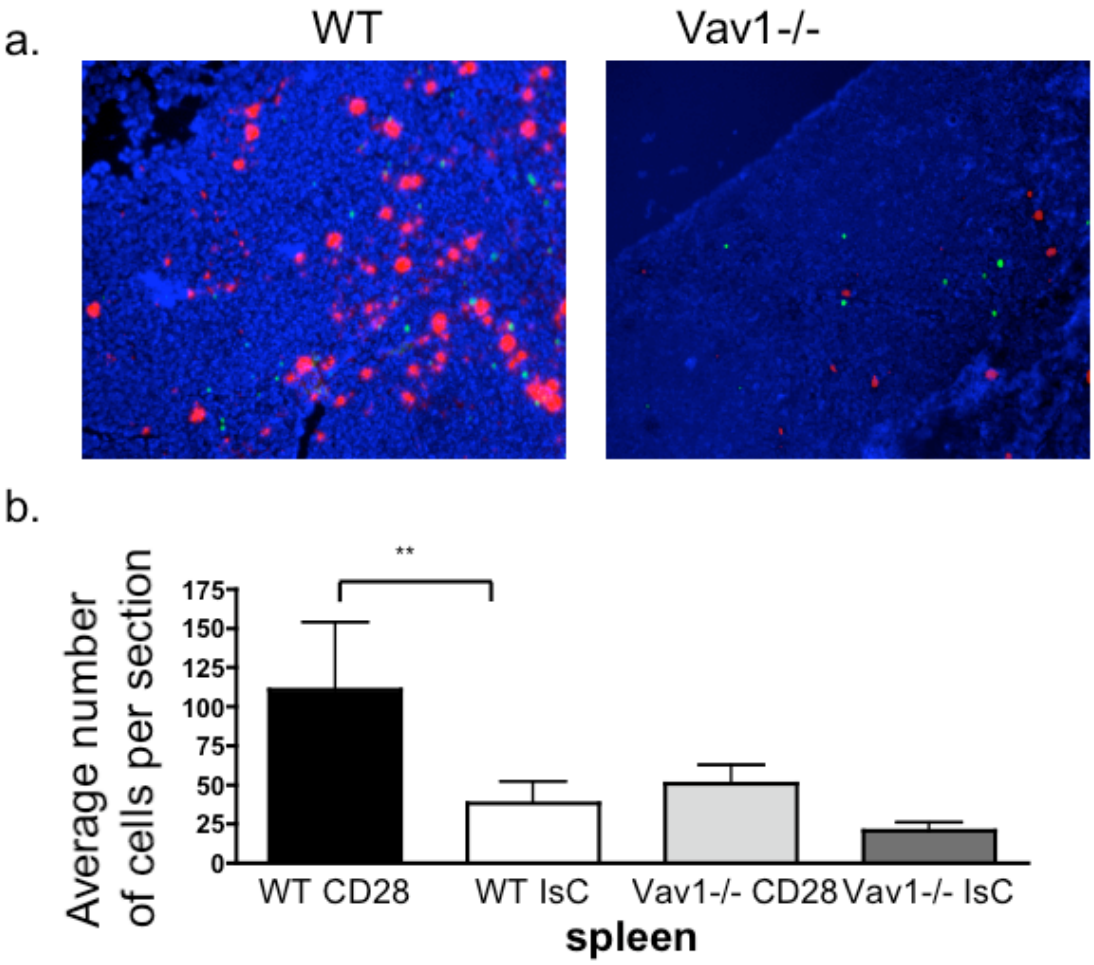
Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 10^7 WT and Vav1-/- T cells were cross-linked with CD28 or an isotyoe control (IsC), labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice. Tissue infiltration was examined 24 hours later (x10 magnification) (a). The number of cells in each section was quantified using the algorithm in the appendix (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test ($p<0.05$). Experimental groups consisted of five female mice.*

Figure 36: CD28 cross-linking does not enhance Vav1-/- T cell migration to the lung



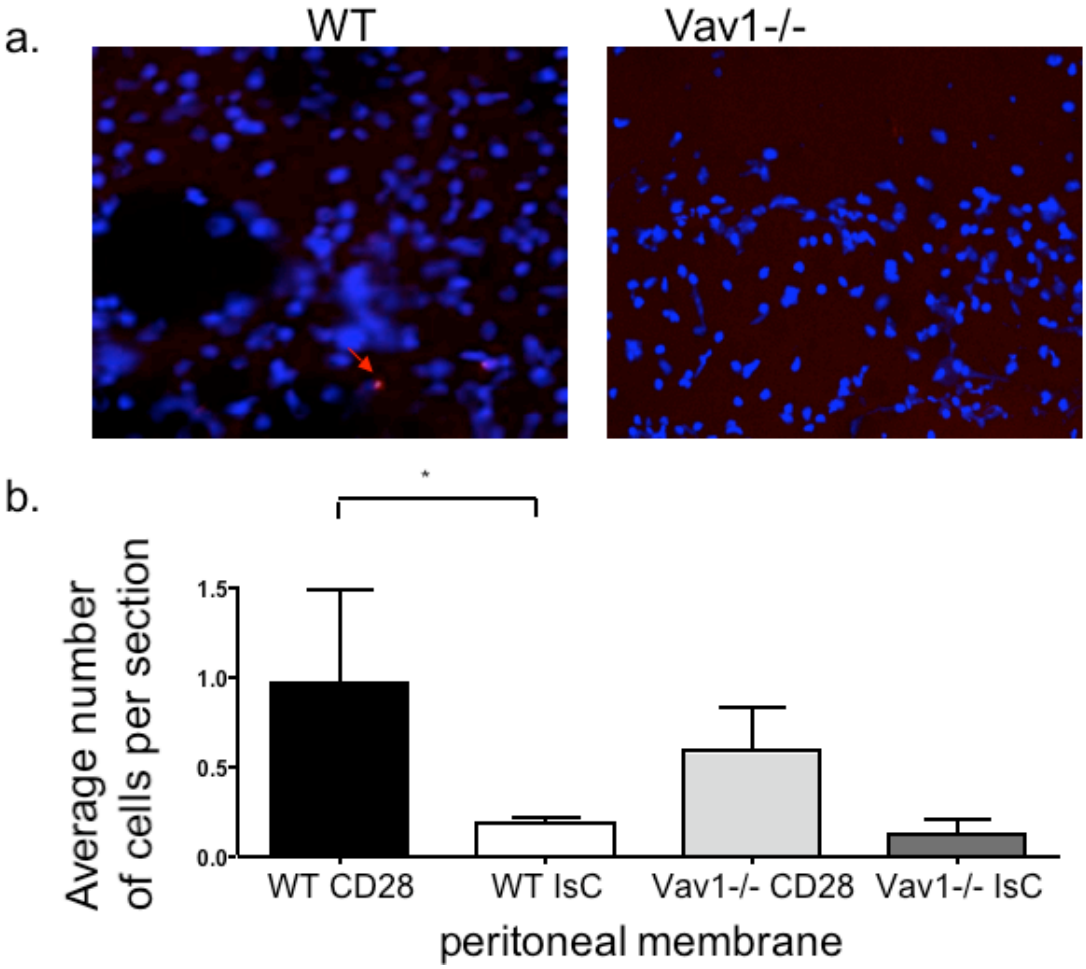
*Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 10^7 WT and Vav1-/- T cells were cross-linked with CD28 or an isotype control (IsC) labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice. Tissue infiltration was examined 24 hours later (x10 magnification) (a). The number of cells in each section was quantified using the algorithm in the appendix (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.05). Experimental groups consisted of five female C57BL/6 mice.*

Figure 37: CD28 cross-linking does not enhance Vav1-/- T cell migration to the spleen



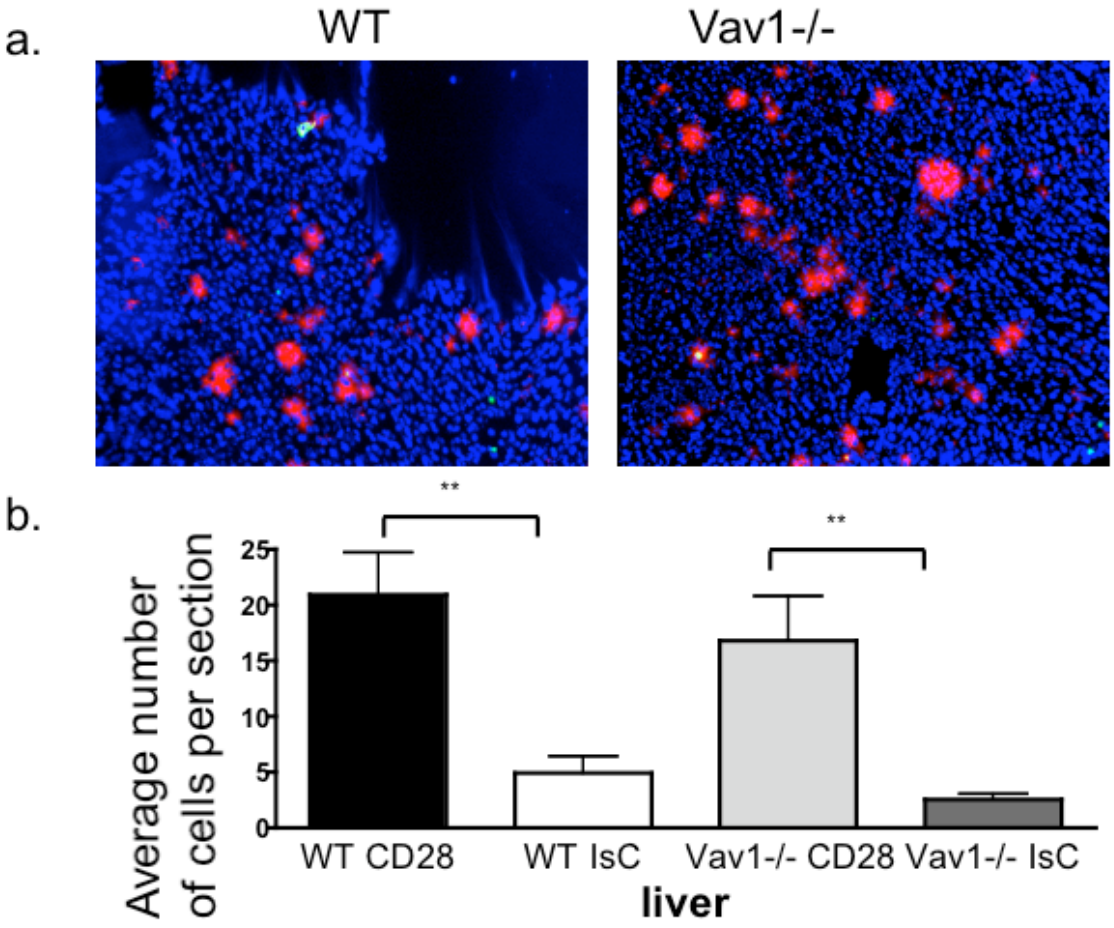
*Viable HY-specific CD4$^+$ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. 10^7 WT and Vav1-/- T cells were cross-linkedwith CD28 or an isotype control (IsC), labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice. Tissue infiltration was examined 24 hours later (x10 magnification) (a). The number of cells in each section was quantified using the algorithm in the appendix (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.05). Experimental groups consisted of five female mice.*

Figure 38: CD28 cross-linking does not enhance trafficking to the peritoneal membrane of female mice



*Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. WT and Vav1-/- T cells were cross-linked with CD28 or an isotype control, labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice that had previously received an ip injection of IFN. Tissue infiltration of the female peritoneal membrane was examined 24 hours later (x10 magnification). Red arrows indicate CD28-cross-linked T cells while green arrows indicate the isotype control (a) The number of cells in the female peritoneal membrane was quantified manually (b) Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.05). Experimental groups consisted of three to four mice.*

Figure 39: CD28 cross-linking enhances Vav1-/- T cell migration to the liver



*Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post-stimulation. WT and Vav1-/- T cells were cross-linked with CD28 or an isotype control (IsC), labelled with PKH26 (red) or CFSE (green) respectively and injected iv in female C57BL/6 mice. Tissue infiltration was examined 24 hours later (x10 magnification) (a). The number of cells in each section was quantified using the algorithm in the appendix (b). Error bars indicate standard error. Statistical analysis was performed using Student's t-test (*p<0.01). Experimental groups consisted of five female C57BL/6 mice.*

4.2.4 Role of CD28 signalling in Vav1-/- T cell migration to sites of inflammation

Previous studies have suggested that CD28 co-stimulatory signals can act in conjunction with TCR-dependent T cell migration to antigenic sites. More specifically, in the peritoneal recruitment model, cross-linking of HY-specific WT T cells with CD28 antibody resulted in induction of recruitment and retention to the peritoneal membrane of male mice that had previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation (Mirenda *et al*., 2006).

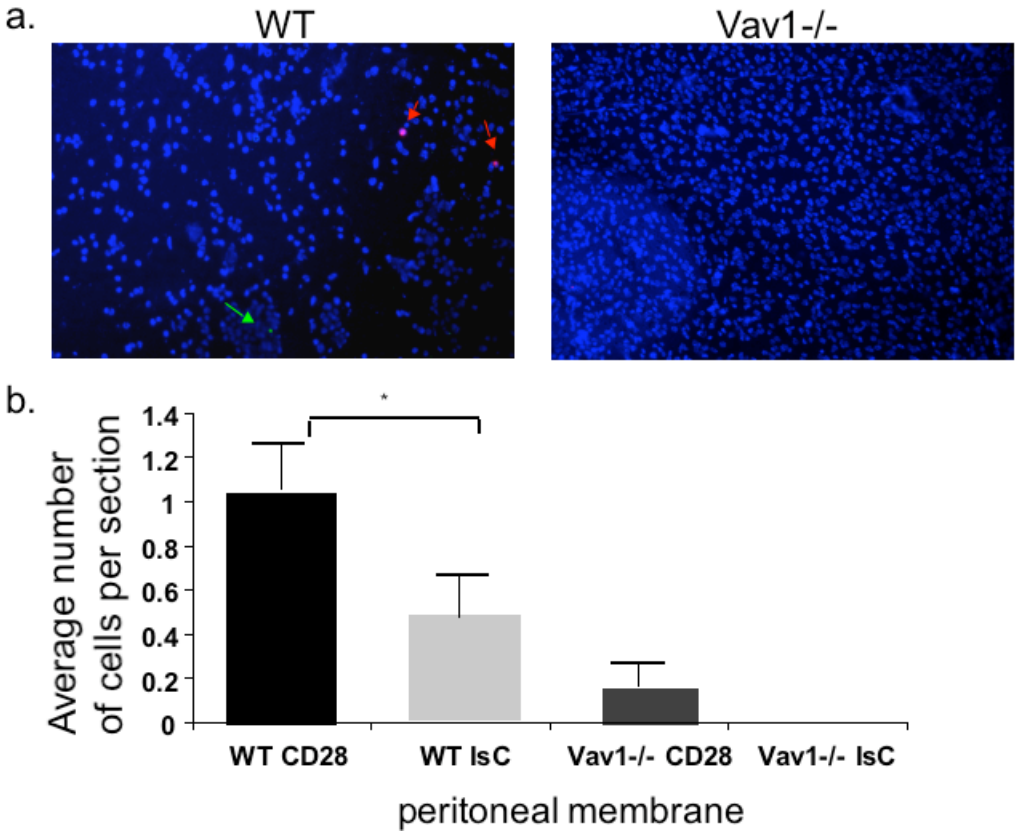
To examine whether Vav1 activity is required for the induction of TCR-dependent, CD28-mediated T cell migration, 10^7 HY-specific $CD4^+$ WT and Vav1-/- T cells were treated with a mixture of hamster anti-mouse CD28 (5μg/5x10^6 cells), and rabbit anti-hamster Ig (2.5μg/5x10^6 cells) to induce CD28 signalling and labelled with PKH26, or hamster Ig (5 μg/5x10^6 cells) and rabbit anti-hamster Ig (2.5 μg/5x10^6 cells) as an isotype control and labeled with CFSE. T cells were subsequently co-injected iv in male C57BL/6 mice that had previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation. Infiltration and retention to the peritoneal membrane was analysed by wide-field fluorescence microscopy, while recruitment to the peritoneal cavity was analysed by flow cytometry and FlowJo software.

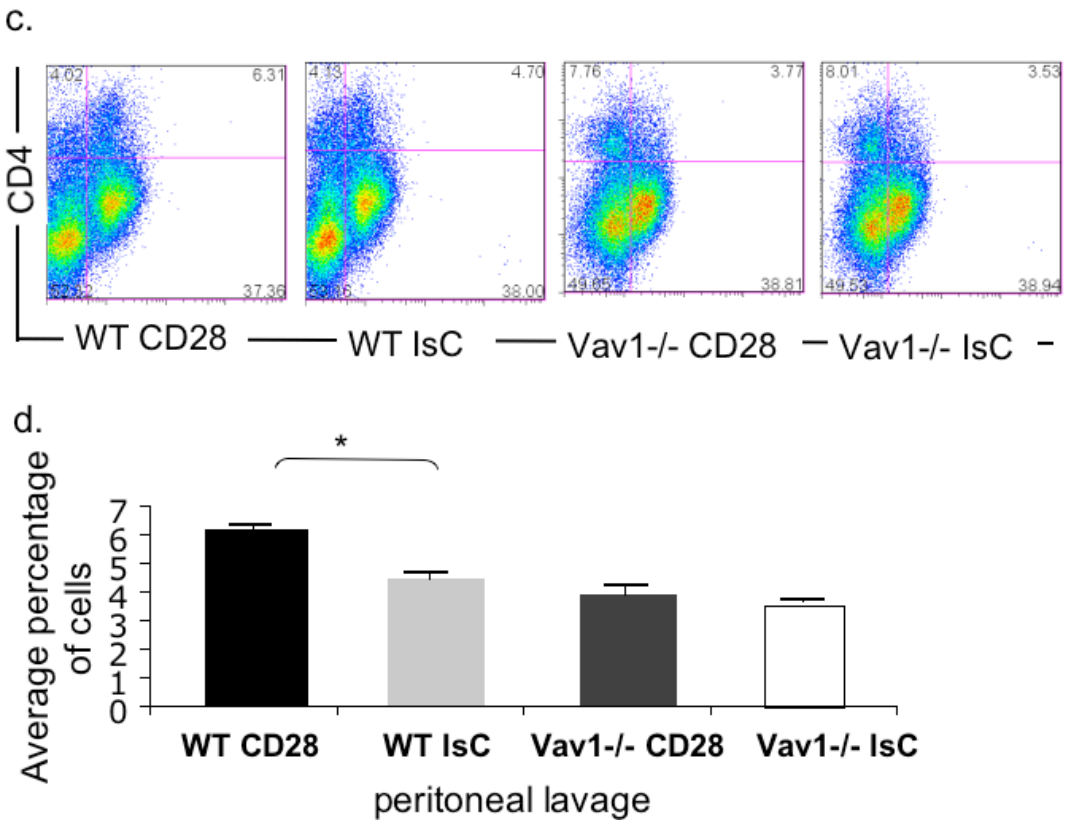
Figure 40 a,b shows the infiltration of the male peritoneal membrane by HY-specific $CD4^+$ WT and Vav1-/- T cells. As expected, induction of CD28 signalling by antibody cross-linking resulted in enhanced migration and retention of HY-specific $CD4^+$ WT T cells to the peritoneal membrane, suggesting a co-operative role for CD28 and TCR signalling in the regulation of migration. In contrast, CD28 cross-linking of HY-specific $CD4^+$ Vav1-/- T cells did not induce migration to the male peritoneal membrane, indicating that Vav1 activity is required for TCR-dependent, CD28-mediated regulation of T cell migration.

To examine this further, enrichment of T cells in the peritoneal lavage was also analysed. As shown in **Figure 40** c,d, CD28 cross-linking of HY-specific $CD4^+$ WT T

cells resulted in increased recruitment to the peritoneal cavity, compared to the isotype control. However, induction of CD28 signalling did not enhance the recruitment of HY-specific $CD4^+$ Vav1-/- T cells to the peritoneal cavity, once again confirming that Vav1 activity is not only necessary for the transduction of migratory stop signal but also for CD28-mediated recruitment to antigenic sites.

Figure 40: CD28 cross-linking does not enhance non-lymphoid antigenic tissue infiltration by Vav1-/- T cells





Viable HY-specific CD4⁺ WT and Vav1-/- T cells were used between days seven and ten post stimulation. WT and Vav1-/- T cells were cross-linked with CD28 or an isotype control, labelled with PKH26 (red) or CFSE (green) respectively and injected iv in C57BL/6 mice that had previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation. Retention to the peritoneal membrane was examined 24 hours later (x10 magnification). Red arrows indicate CD28-cross-linked T cells while green arrows indicate the isotype control (a) The number of cells in the peritoneal membrane was quantified manually (b). Recruitment of T cells to the peritoneal cavity was examined by flow cytometry using FlowJo software (c). Average percentage of CD4⁺ labelled T cells in the peritoneal lavage (d). Error bars indicate standard error. Statistical analysis was performed using Student's t-test ($p<0.05$). Experimental groups consisted of at least three mice.*

5.3. Discussion

Previous studies had demonstrated a role for CD28 in T cell migration, with enhanced CD28 signalling or blockade of negative co-stimulatory CTLA-4 signals resulting in enhanced and aberrant tissue infiltration (Chang *et al*., 1999); Walker *et al*., 1999; Girvin *et al*., 2000; Mirenda *et al*, 2006; reviewed in David and Marelli-Berg, 2007). Vav1 can be activated directly by CD28 in the absence of TCR triggering, which makes it an interesting candidate for the transduction of the CD28 migratory signals (reviewed in Michel and Acuto, 2002).

To assess the potential role for Vav1 in CD28-mediated regulation of T cell migration, CD28 signalling was induced in the HY-specific $CD4^+$ Vav1-/- T cells by antibody cross-linking as previously described in Mirenda *et al*. (2006). In the *in vitro* transwell-based migration assays, we observed that CD28 cross-linking did not enhance HY-specific $CD4^+$ Vav1-/- T cell migration through male or female endothelial monolayer suggesting that Vav1 is probably utilised by CD28 to enhance TCR signals driving T cell migration.

In the *in vitro* assays, induction of CD28 signalling did not enhance HY-specific $CD4^+$ WT T cell migration through the non-antigenic female monolayer which suggests that TCR triggering is also required in this context. This is not in agreement with findings by Mirenda *et al*. (2006), who observed enhanced migration upon CD28 signalling in the absence of TCR triggering *in vitro*. It is possible that this is due to different experimental systems, as this study used human blasts cross-linked with a human CD28 antibody which may have induced a stronger signal than the mouse one used in this study.

However, when confirming the physiological relevance of our findings *in vivo*, we observed that CD28 cross-linking enhances WT T cell trafficking in female mice. As expected, and in agreement with previous findings by Mirenda *et al*. (2006), HY-specific $CD4^+$ WT T cells showed enhanced infiltration of all the tissues examined, including kidney, heart, liver, lung and the peritoneal membrane of female mice. This effect was not observed with the HY-specific $CD4^+$ Vav1-/- T cells, where no significant difference in infiltration of CD28 and isotype cross-linked T cells was observed. Interestingly, we did observe enhanced infiltration of the liver when CD28 signalling was induced in HY-

specific $CD4^+$ Vav1-/- T cells. The liver is enriched with macrophages and Kupffer cells bearing Fc receptors which are used for the phagocytosis of immune system complexes, resulting in their clearance (reviewed in Janeway *et al*., 2001). It is therefore possible that CD28 cross-linked HY-specific $CD4^+$ Vav1-/- T cells were endocyted by these cells to levels higher than the HY-specific $CD4^+$ Vav1-/- T cells cross-linked with isotype control.

The involvement of CD28 signalling in TCR-dependent T cell recruitment and retention to antigenic sites was further investigated *in vivo*. Mirenda *et al*. (2006) had previously shown that CD28 cross-linking resulted in recruitment and retention to the peritoneal membrane of male mice that had previously received an ip injection of IFNγ to induce MHC class II expression and localised antigen presentation. This suggested that CD28 signalling can act co-operatively with TCR triggering to enhance T cell migration. Indeed we observed enhanced recruitment and retention of HY-specific $CD4^+$ WT T cells to the peritoneal membrane and cavity of male mice, which confirms previous findings. However, induction of CD28 signalling by antibody triggering in Vav1-/- T cells did not result in enhanced retention to the peritoneal membrane or increased recruitment to the peritoneal cavity. This confirms that Vav1 is utilised by both TCR and CD28 for the transduction of the migratory stop signal which is required for T cell retention to antigenic sites.

Our findings suggest that Vav1 plays a pivotal role in transducing CD28 migratory signals, even in the absence of TCR triggering. Recruitment to sites of antigen presentation is dependent on antigen presentation by ECs which in mice express little or no levels of B7 molecules (CD80/CD86), the ligands to CD28. However, retention is dependent on antigen presentation by resident APCs which are B7 positive, suggesting that CD28 signalling may be crucial for the retention of antigen-specific T cells. As HY-specific $CD4^+$ Vav1-/- T cells showed defective retention to the peritoneal membrane, an effect not compensated for by the induction of CD28 signalling, as well as reduced responsiveness to the migratory stop signal, we can conclude that Vav1 is utilized by both the TCR and CD28 for the transduction of migratory signals.

Discussion

1. Summary of findings

The GEF Vav1 has previously been shown to be an important link between TCR signalling and the regulation of cytoskeletal re-arrangements. Vav1 is activated upon TCR trigerring through tyrosine phosphorylation, and acts as a GEF for the RhoGTPase Rac1, the latter being an important regulator of actin polymerisation (reviewed in Tybulewicz, 2005). In addition, Vav1 interacts with WASp to mediate cytoskeletal re-arrangements (reviewed in Secchi and Wehland, 2004), is required for the formation of the immunological synapse (Fischer *et al*., 1998; Wulfing *et al*.,2000; Villaba *et al* 2001; Krawczyk *et al*., 2002; Ardouin *et al*., 2003) and has been shown to influence motility in various cell types including T cells (Ticchioni *et al*., 2002) (Kim *et al*., 2003; Korn *et al*., 2003) (Gakidis *et al*., 2004; Garcia-Bernal *et al*., 2005) (Vicente-Manzanares *et al*., 2005) (Vedham *et al*., 2005) (Wells *et al*., 2005) (Bartolome *et al*., 2006) (Wilsbacher *et al*., 2006). These findings, in conjunction with preliminary data showing loss of responsiveness to TCR-mediated regulation of motility in Vav1-/- splenocytes, led us to hypothesise that loss of Vav1 will result in impaired recruitment and retention to sites of antigen presentation.

Our first aim was to establish a physiological role for Vav1 in memory T cell trafficking *in vitro* and *in vivo*. Using HY-specific $CD4^+$ effector memory T cells, we observed that loss of Vav1 activity impairs baseline T cell migration *in vitro*, as well as ICAM-1 and chemokine-driven migration *in vitro*. This was in agreement with previous studies that have observed reduced CXCL12-mediated chemotaxis in Vav1-/- T cells *in vitro (*Ticchioni *et al*., 2002; Vicente-Manzanares *et al*., 2005 ; Garcia-Bernal *et al*., 2005), suggesting that *in vitro*, Vav1 activity is required for T cell motility.

Interestingly, the *in vitro* findings did not translate to a defect *in vivo*. HY-specific WT and Vav1-/- T cells showed comparable levels of constitutive trafficking at two of the three time-points tested and comparable recruitment to sites of non-specific inflammation. As we observed increased speed by Vav1-/- T cells *in vitro*, we

hypothesised that this may have partially compensated for the loss of Vav1 activity. Examination of Vav1-/- T cell speed *in vivo* through real-time imaging would provide an answer to this question. Another possibility is that shear flow may have compensated for the loss of Vav1 activity *in vivo*, which could be further examined by the use of *in vitro* flow chambers.

Based on these findings, we can conclude that although Vav1 activity is required for T cell migration *in vitro*, loss of Vav1 is compensated for *in vivo* by other factors, possibly Vav1-/- T cell enhanced speed or the presence of shear flow.

We then proceeded to examine the requirement for Vav1 activity in TCR-dependent T cell migration. We observed that in the absence of Vav1 activity, T cells lost susceptibility to antigen-dependent migration, as HY-specific $CD4^+$ Vav1-/- T cells did not show enhanced migration through the antigenic male monolayer compared to the female one in *in vitro* transwell-based and time-lapse migration assays.

Nevertheless, our *in vitro* findings once again were not reflected *in vivo*. T cells showed normal recruitment to antigenic sites in the absence of Vav1, suggesting that Vav1 is dispensable for this process. We hypothesised that this may have been due to a compensatory effect by shear flow, and investigated this further using the peritoneal tissue infiltration model which examines T cell migration under static conditions. In the absence of flow, HY-specific $CD4^+$ Vav1-/- T cells showed reduced infiltration of the antigenic tissue, the peritoneal membrane, suggesting that flow is essential for TCR-dependent migration of T cells in the absence of Vav1 activity.

Thus far, we have observed a requirement for Vav1 activity for T cell retention to antigenic tissue. The latter is regulated by antigen presentation by resident APCs, which results in temporary loss of migratory ability, allowing the T cell to polarise and proliferate (Dustin *et al.,* 1997). In this part of the study, we decided to examine whether Vav1 is required for the transduction of the migratory stop signal.

As loss of migratory ability is regulated by engagement of the TCR by resident APCs, we commenced this investigation by examining conjugate formation in the absence of Vav1. Furthering previous findings by Ardouin *et al*. (2003) and Krawczyk *et al*. (2002), we observed reduced conjugate formation in memory Vav1-/- T cells

compared to WT controls. This was further examined *in vivo* in the spleen and the antigenic tissue, the peritoneal membrane, and confirmed the *in vitro* findings.

We hypothesised that reduced ability to form conjugates may result in reduced susceptibility to the migratory stop signal. We observed that *in vitro*, antibody triggering of the TCR and CD28 did not result to loss of migratory ability in the absence of Vav1 in T cells. We further extended this finding *in vivo,* whereby the presence of peptide-loaded DCs did not result in enhanced retention to the antigenic tissue, the peritoneal membrane. We can therefore conclude that Vav1 activity is necessary for conjugate formation and the transduction of migratory stop signals both *in vitro* and *in vivo*.

Although HY-specific $CD4^+$ Vav1-/- T cells showed normal recruitment to sites of antigen presentation, they displayed reduced retention to the antigenic tissue. Recruitment to antigenic sites is thought to be regulated by antigen presentation by ECs lining post-capillary venules, which are B7 (CD80/CD86) negative and thus do not activate the co-stimulatory molecule CD28. In contrast, retention to antigenic sites is mediated by antigen presentation by resident APCs which may activate CD28 signalling pathways as they are B7 positive (reviewed in Marelli-Berg and Jarmin, 2004). As Vav1 is thought be to be utilised by CD28 for the transduction of signals independently of the TCR (reviewed in Michel and Acuto, 2002) we examined the possibility that reduced retention was due to the absence of co-stimulatory signal transduction.

To investigate the requirement of CD28 signalling via Vav1 in T cell migration we first employed *in vitro* transwell-based migration assays. In the absence of Vav1 activity, CD28 triggering did not enhance T cell migration through non-antigenic female endothelial monolayer, suggesting that Vav1 is required for the transduction of TCR-independent CD28 migratory signals *in vitro*. In addition, Vav1 activity was found to be necessary for both TCR-dependent and independent CD28 migratory signals in T cells *in vivo*, as in the absence of Vav1, T cells did not show enhanced trafficking in female mice or enhanced recruitment and retention to the peritoneal lavage and membrane in male mice. These findings suggest that Vav1 is utilised by both the TCR and CD28 to transduce migratory signals.

2. Regulation of the migratory stop signal by CD28 and CTLA-4

The co-stimulatory molecules CD28 and CTLA-4 have both been shown to affect T cell motility, as discussed in the previous chapters. We have previously speculated that CD28 and CTLA-4 do not simply act as positive and negative regulators of migration, as they do with activation. More specifically, CD28 engagement has been shown to be required for entry of T cells to non-lymphoid antigenic tissue following priming. CTLA-4 on the other hand seems to prevent slowing down of activated T cells when they come in contact with antigen, thereby preventing recruitment and entry to antigenic sites (reviewed in David and Marelli-Berg, 2007).

The mechanism by which they can differentially affect T cell motility is at present unclear. Although both have been shown to increase adhesion, CD28 and CTLA-4 may differentially regulate cell orientation and de-adhesion, which may explain their differential effects on T cell motility. CD28 is known to directly affect the cytoskeleton by activating Vav1 in the absence of a TCR signal (Michel *et al*, 2000), thus activating the Rho GTPase Cdc42 and WASp. As CTLA-4 is thought to prevent the transduction of the migratory stop signal (Schneider *et al*., 2006), it may regulate motility by mediating a de-adhesion process, possibly by activating one of the three Rho proteins which have been shown to promote detachment by acting at the rear of the cell (reviewed in Wheeler and Ridley, 2004).

Recent findings by Wei *et al*. (2007) have proposed a role for Vav1 in CTLA-4-mediated regulation of T cell motility. In this study, transduction of CTLA-4-expressing hybridomas with Vav1 mutants resulted in the conclusion that CTLA-4 utilises Vav1 to induce T cell polarization.

Despite the rather artificial nature of this study, it is interesting to suggest that perhaps both CTLA-4 and CD28 mediate migratory signals via Vav1. CD28 activates Vav1 upon TCR triggering as well as in the absence of a TCR signal (Michel *et al*., 2000), mediating T cell retention to antigenic sites, T cell infiltration to peripheral tissues and the migratory stop signal, while CTLA-4 may utilize Vav1 in the absence of a strong enough TCR stimulus mediating de-adhesion and preventing unnecessary encounters with APCs.

3. Differential regulation of T cell motility by Vav1 and PI3kinase

Vav1 activation through tyrosine phosphorylation and its association with adaptor molecules such as Slp76 and Grb-2 results in the activation of downstream signalling cascades. One of the pathways thought to be triggered by this interaction is the PI3k pathway (reviewed in Tybulewicz, 2005).

PI3kinases are a family of heterodimeric lipid kinases divided into three classes (IA, II, III) that are involved in signalling in a variety of cell types and are thought to regulate various cell functions, including motility (Leevers *et al*., 1999). Class IA PI3kinases have been extensively implicated in immune cell signalling, and of the different isoforms, p110δ is thought to be the most important in regulating immune cell function (Okkenhaug *et al*., 2006).

The interaction between Vav1 and PI3kinase p110δ is at present unclear. Vav1 is thought to activate the RhoGTPases Rac1 which in turn is thought to activate PI3kinase, (reviewed in Tybulewicz *et al*., 2003). Interestingly, there has been evidence that Vav1 can also act downstream of PI3kinase, as PI3 kinase inhibition can lead to reduced Vav1 phosphorylation. Thus, it has been suggested that PI3kinase and Vav1 may act in a positive feedback loop (Reynolds *et al*., 2002; reviewed in Tybulewicz *et al*., 2003).

A recent study in our laboratory has investigated the role of the PI3kinase p110δ subunit in TCR-dependent T cell migration (accepted for publication in the Journal of Clinical Investigation). We observed that loss of p110δ through mutation or pharmacological inhibition resulted in impaired recruitment and retention to sites of antigen presentation. In addition, HY-specific p110δ-/- T cells did not infiltrate the peritoneal membrane of male mice in the absence of flow and showed reduced infiltration of the male skin graft compared to the WT control, suggesting an essential role for PI3kinase p110δ in TCR-dependent T cell migration.

The requirement for PI3kinase in CD28 –mediated migration was also examined through the use of a T cell clone with a mutation on the cytoplasmic tail of CD28 which abrogates the interaction with PI3kinase (Y170F). Using the antigen-dependent peritoneal recruitment model, we observed that mutation of the cytoplasmic tail of CD28 resulted in

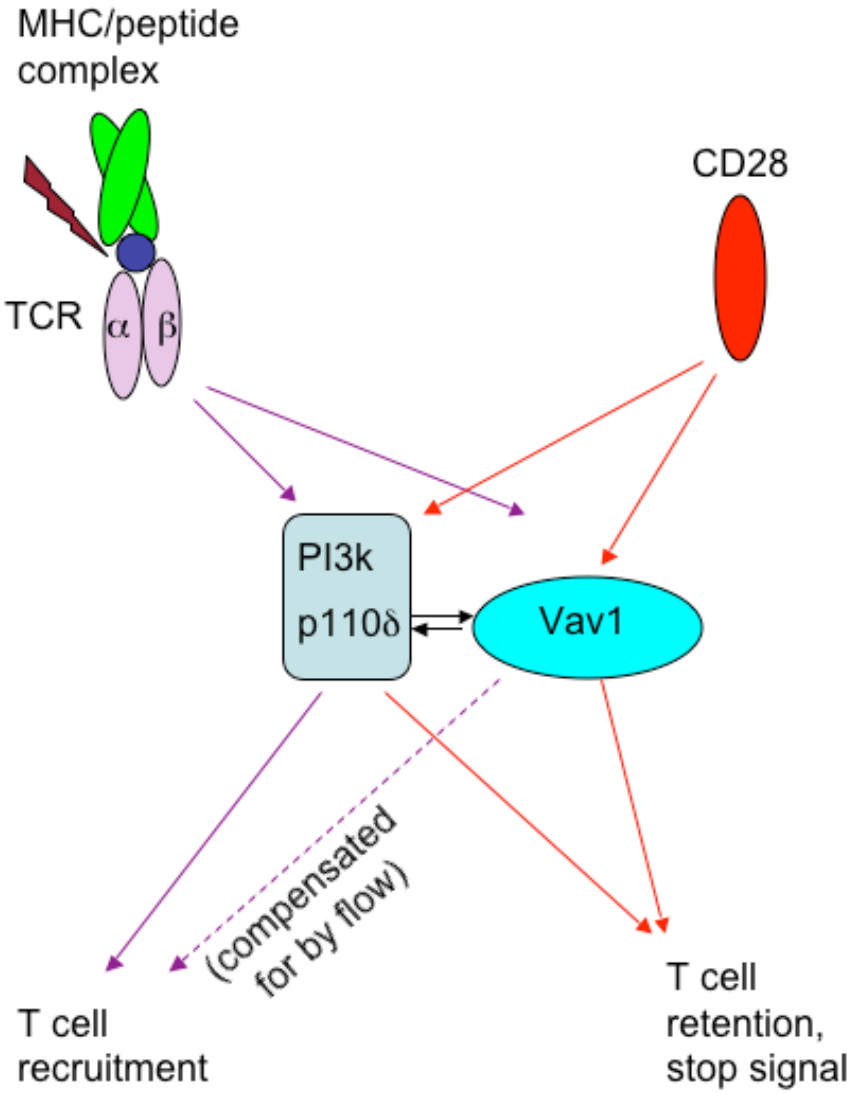
moderate impairment of T cell recruitment to the peritoneal cavity, whereas p110δ PI3kinase deficient T cells were virtually undetectable in the peritoneal lavage or membrane.

Contrary to these results, the present study has observed that loss of Vav1 activity only affects retention to non-lymphoid antigenic sites. This suggests that Vav1 and PI3kinase have differential effects in TCR-dependent T cell motility. However, evidence from these studies suggests that both Vav1 and PI3kinase can be utilised by CD28 to mediate retention of primed T cells to non-lymphoid antigenic sites.

Although the interaction between PI3kinase and Vav1 is at present unclear, from our findings, we can hypothesise that p110δ PI3kinase and Vav1 act in parallel pathways in the regulation of T cell migration; p110δ PI3kinase primarily transduces TCR-mediated signals for the regulation of T cell migration while Vav1 is utilised by CD28, mediating T cell retention to antigenic sites and the migratory stop signal.

PI3kinase can in fact regulate T cell migration independently of Vav1; Rac1 has been shown to be activated by the PI3kinase signalling product PIP3 which has a PH domain that can bind to other Dbl-homology GEFs (reviewed in Barber and Welch, 2007). It is therefore possible that the TCR-dependent signals regulating T cell recruitment utilise this pathway of activation of Rac1.

Figure 41: Proposed model of the regulation of T cell migration



TCR signalling (indicated by purple arrows) utilises PI3kinase to mediate T cell recruitment to sites of antigen presentation, while CD28 (indicated by red arrows) mediates T cell retention to antigenic tissue via Vav1. Vav1 also transduces the migratory stop signal mediated by triggering of the TCR and CD28.

To examine this further, we would have to inhibit p110δ PI3kinase in Vav1-/- T cells using a pharmacological inhibitor, which would provide information on whether these pathways act independently of each other in the context of T cell migration. One

would hypothesise that pharmacological inhibition of p110δ PI3kinase in Vav1-/- T cells would result in a more severe phenotype than loss of either molecule separately.

4. Clinical implications of this study

We have proposed a model whereby Vav1 is required for the retention of T cells to antigenic sites as well as for the transduction of the migratory stop signal upon strong activation of the TCR. It therefore is an interesting candidate for pharmacological inhibition for the prevention of T cell retention into antigenic sites.

Indeed, we observed that loss of Vav1 resulted in reduced antigenic graft infiltration in female mice grafted with male tail-derived skin. In addition, Weckbecker *et al*. (2007) observed enhanced heart allograft survival in Vav1-/- mice, suggesting that loss of Vav1 activity may be promising way of inducing transplant tolerance.

A potential inhibitor of Vav1 activity is the azathioprine metabolite 6-thio-GTP. More specifically, a study by Tiede *et al*. (2003) reported that administration of 6-thio-GTP in human $CD4^+$ T cells blocked the binding of GTP to Rac1, resulting in increased levels of Vav1. The same group subsequently described that this effect was Rac1 specific, as it did not affect other RhoGTPases, and resulted in blockade of ERM protein inactivation. This effect was due to specific inhibition of the Vav1/Rac1 interaction upon CD28 signalling, which resulted in reduced lamellipodia formation and inhibition of the T cell:APC conjugate formation (Poppe *et al*., 2006).

More recently, 6-thio-GTP was used to induce heart allograft tolerance in mice. Daily administration of 6-thio-GTP in mice transplanted with Balb/c hearts resulted in significant increase in allograft survival compared to the PBS control. In addition, they observed reduced leukocyte infiltration in the heart allograft and reduced *ex vivo* proliferative responses to alloantigen in splenocytes from mice treated with 6-thio-GTP. Therefore, inhibition of the Vav1/Rac1 interaction may have beneficial effects in prolonging transplant survival (Wang *et al*., 2007).

Other than graft survival, inhibition of Vav1 activity may also be advantageous in the treatment of autoimmune and inflammatory diseases such as rheumatoid arthritis. We have observed that Vav1-/- T cells show reduced migration compared to the WT controls

in vitro. In addition, although Vav1-/- T cells showed normal recruitment to antigenic sites, they were not retained, and were not susceptible to the migratory stop signal mediated by strong TCR engagement, suggesting that inhibition of this molecule would potentially prevent damage caused by the accumulation of T cells.

In addition to the potential uses of 6-thio-GTP as a Vav1 inhibitor, a patent has been filed for the development of Vav1 inhibitors for graft survival. This patent details potential inhibitors of Vav1 which may interfere with Vav1 phosphorylation by binding to the SH2 domain or by mimicking Vav1 phosphorylation through binding of Tyr174, or may interfere with Vav1 binding of Grb2 by binding its SH3 domain (United States Patent 20070042948). This patent also details the potential uses of Vav1 inhibitors, which include prolonging graft survival alone or in conjunction with other treatments such as cyclosporine, as well as treatment of autoimmune diseases and lymphoproliferative disorders, such as lupus and leukaemia.

5. Conclusion

This study aimed to establish a physiological role for the GEF Vav1 in T cell trafficking, T cell recruitment and retention to antigenic sites as well T cell susceptibility to the migratory stop signal. Based on our findings, we have concluded that although Vav1 is necessary for T cell migration and antigen-dependent T cell recruitment *in vitro*, other factors have compensated for the loss of Vav1 *in vivo*, possibly shear flow. In addition, Vav1 is required for the retention of T cells to antigenic tissue *in vivo*, potentially suggesting its importance in CD28-mediated control of migration. Finally, Vav1 is a mediator of the migratory stop signal in T cells, which occurs upon engagement of the TCR and co-stimulatory molecules by antigenic tissue resident APCs. We therefore propose a model, whereby Vav1 may primarily be utilised by CD28 for the transduction of migratory signals in T cells. These findings provide useful information for the development of pharmacological inhibitors for Vav1, which could potentially be used for prolonging transplant survival as well as treatment of autoimmune and inflammatory disorders.

Appendix . Automatic cell counting algorithm

The automatic cell counting algorithm is an intensity profile based approach for the calculation of single and double fluorescence labelled cell population. As illustrated in fig 1, cells in an image can be represented as groups of spatially clustered glowing pixels with certain intensity distribution. Assuming each cell has a single-peak fluorescence profile, the brightest pixel in each cell may be used as a countable feature of cell population.

The approach consists of three major steps, including image pre-processing, region masking and cell counting. Image pre-processing uses auto-level technique to eliminate uniform background and normalize the intensity scale of the image. Region masking is used to define the counting area. In a dual-colour (fluorophore) application, one colour is usually used for non-specifically cell labelling; while the other colour labels the positive areas with its fluorescence profile defining cell clusters. We use the positive indicator to mask the counting area in the non-specifically labelled channel, and count cells within the unmasked area by their maximum pixels. To find the maximum pixel in a cell, the pixel values of a counting area are segmented into a number of fluorescence levels (fig.1a), through which locations of the brightest spots are searched reiteratively. In details, all intensity pixels above a certain threshold are classified into groups according to their spatial distribution, from which the maximum pixel of each group is located. Repeating the search level by level, all cell profiles will be examined, and all maximums will be included. The number and location of the maximum pixels should be exactly the same between two adjacent loops, unless lowering threshold includes new cells (fig 1b, and 1d). Finally, cell counts from each level are combined, and a filter is applied to remove all repeatedly counted maximum pixels. The number of maximums left represents the number of cells.

The algorithm is robust to uneven cell expression level, and poor contrast tissue images with non-uniform background. It is not sensitive to the variation of cell size and shape, neither critical to extend of cell overlapping. The algorithm can identify and ignore holes in a cell, and eliminate small artefacts and noise influences, making the counting more accurate. However, it is less capable to identify a cell with multi-

peak profile, although adjusting the number of thresholding levels will reduce the rate of miscount.

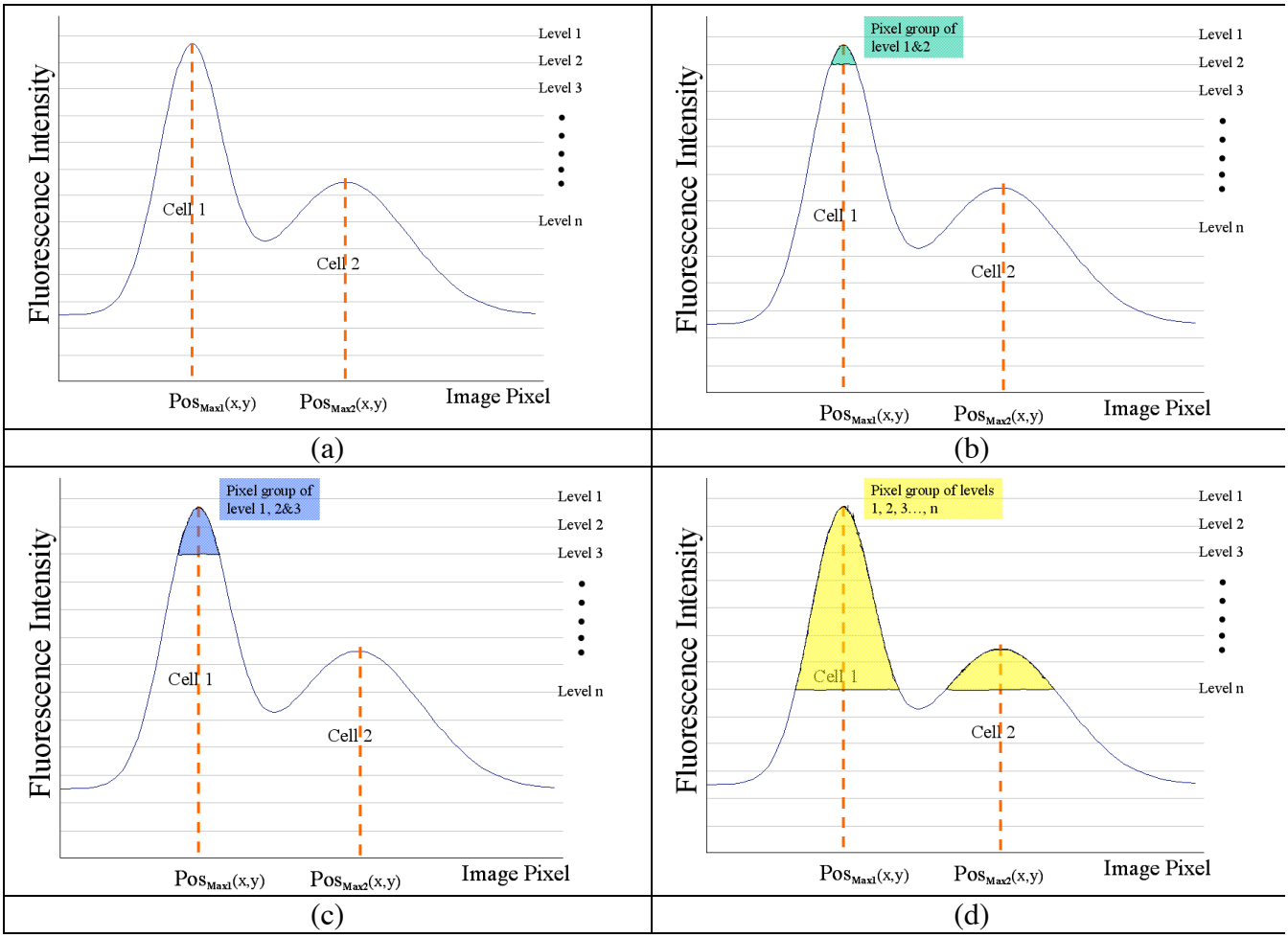
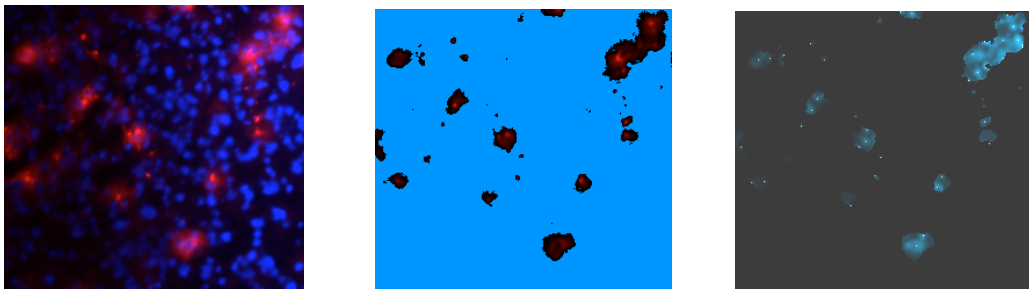


Fig 1. Schematic of cell counting algorithm, (a) no cell is above Level 1, (b) Cell 1 is above Level 2 from which its peak at $Pos_{max1}(x,y)$ can be detected, (c) Cell 1 is above Level 3 with the calculated peak at the same position as (b), and (d) Cell 1 and Cell 2 are above Level n, and a 2nd peak at $Pos_{max2}(x,y)$ is detected.

An example of image pre-processing, region masking and cell counting is illustrated in panels e-g.

e) f) g)



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