CHARACTERISING LYMPHOCYTE TRAFFICKING ACROSS BLOOD VASCULAR AND LYMPHATIC ENDOTHELIAL CELLS

BY

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ABSTRACT

The recruitment of peripheral blood lymphocytes (PBL) to sites of inflammation and their subsequent traffic into the lymphatic circulation is important in host defense. However, surprisingly little is known about their recruitment from the blood vasculature into inflamed tissue, and almost nothing about their egress from inflamed tissue *via* the lymphatic circulation. We showed that both human macrovascular and microvascular endothelial cells stimulated by TNFα and IFNγ, preferentially recruited memory T-lymphocytes (CD45RO positive cells) from a mixed pool of PBL. T-cells that had migrated across vascular endothelial cells subsequently utilised a combination of β1 and β2 integrins to traverse cytokine activated lymphatic endothelium. In addition we provide evidence that PGD2 was critical for the transmigration of lymphocytes through vascular endothelium. The process of trans-lymphatic migration was also significantly retarded in the presence of a function neutralising antibody against CCR7. Most importantly, we observed that memory T-cells showed a markedly enhanced capacity to migrate across lymphatic endothelium if they had first traversed a vascular endothelial cell barrier. We have shown that addition of exogenous PGD2 to isolated lymphocytes is able to restore the enhanced migration capacity of lymphocytes that have previously migrated through a vascular monolayer. The nature of the priming signal delivered by the process of migration across blood vessel endothelium remains to be fully identified, but is likely to be important in regulating the dynamics of an inflammatory response.

List of publications arising from this work

Ahmed SR, McGettrick HM, Nash GB, Rainger GE. Migration across vascular endothelial cells primes peripheral blood lymphocytes for efficient migration across lymphatic endothelial cells. Microcirculation, 2009, 16(5): 444-486

Ahmed SR, McGettrick HM, Yates CM, Buckley CD, Ratcliffe MJ, Nash GB, Rainger GE. Prostaglandin D2 regulates CD4+ memory T cell trafficking across blood vascular endothelium and primes these cells for clearance across lymphatic endothelium. J Immunol. 2011 Aug 1;187(3):1432-9

Dedicated to my Dad

1954 ~ 2008

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List of abbreviations

OPD	1,2 Orthophenylenediamine Dihydrochloric
15d Δ12,14-PGJ2	15d Δ12,14-Prostaglandin J2
17R-HDHA	17R-hydoxy docosahexanoic acid
APES	3-aminopropyl -triethoxysilane
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
FLAP	5-lipoxygenase activating protein
ATP	Adenosine trisphosphate
ANOVA	Analysis of Variance
AA	Arachidonic acid
BSA	Bovine serum albumin
BVEC	Blood vascular endothelial cells
Ca^{2+}	Calcium
CVD	Cardiovascular disease
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CT	Cycle threshold
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenases
Cys-LT	Cysteinyl LT
CYP	Cytochrome p450 epoxygenases
DC	Dendritic cell
DAG	Diacylglycerol
DHET	Dihydroxyeicosatrienoic acids
DHA	Docosahexaenoic acid

EPA	Eicosapentaenoic acid
EC	Endothelial cell
ELAM-1	Endothelial leukocyte adhesion molecule-1
eNOS	Endothelial nitric oxide synthase
E-selectin	Endothelial selectin
ELISA	Enzyme linked immunoabsorbance assay
EGF	Epidermal growth factor
ESL	E-selectin ligand
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
FCS	Foetal calf serum
fMLP	formyl-Met-Leu-Phe
g	gram
g	Gravitational force
GI	Gastrointestinal
GlyCAM-1	Glycosylated cell adhesion molecule-1
GPI	Glycoylphosphatidylinositol
HSPG	Heparan sulphate proteoglycans
HEV	High endothelial venule
HRP	Horse radish peroxidase
h	Hour
HMVEC	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
HCL	Hydrochloric acid
HPETE	Hydroperoxyeicosatetraenoic acid

HETE	Hydroxyeicosatetraenoic acid
Ig	Immunoglobulin
IFN-γ	Interferon-gamma
IL	Interleukin
ICAM-1	Intracellular adhesion molecule-1
JAM	Junctional-adhesion molecule
LAD	Leukocyte adhesion deficiency
LMVEC	Lung microvascular endothelial cell
L-selectin	Leukocyte selectin
LTB	Leukotriene
LTB4	Leukotriene B4
LA	Linoleic acid
LPS	Lipopolysaccharide
LX	Lipoxins
LOX	Lipoxygenase
LVEC	Lymphatic vascular endothelial cell
Mem	memory
Mg^{2+}	Magnesium
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MFI	Median fluorescent intensity
mRNA	Messenger ribonucleic acid
MeOH	Methanol
µg	Microgram
µl	Microlitre

µM	Micromolar
ml	Millilitre
mm	Millimetre
mM	Millimolar
Min	Minute
MAPK	Mitogen activated protein kinase
M	Molar
MCP-1	Monocyte chemoattractant protein-1
ng	Nanogram
nM	Nanomolar
NSAID	Non-steroidal anti-inflammatory drug
NF-κB	Nuclear factor-kappaB
Pa	Pascal
PRR	Pattern recognition receptor
PBMC	Peripheral blood mononuclear cell
PBL	Peripheral blood lymphocytes
PPAR	Peroxisome proliferator-activated receptor
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline with bovine serum albumin
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipase
PAF	Platelet activating factor

PAF	Platelet activating factor
PDGF	Platelet derived growth factor
P-selectin	Platelet selectin
PECAM-1	Platelet/endothelial cell adhesion molecule
PGI2	Prostacyclin
PG	Prostaglandin
PD1	Protectin D1
PGSL1	P-selectin glycoprotein ligand 1
qPCR	Quantitative polymerase chain reaction
REU	Relative expression units
RQ	Relative quantity
Resolvin	Resolution phase interaction product
Rv-E1	Resolvin E1
RvD	Resolvins of the D series
RT	Reverse transcription
RA	Rheumatoid arthritis
SMC	Smooth muscle cells
SEM	Standard error of the mean
TXA2	Thromboxane A2
TLR	Toll like receptor
TP	T-prostanoid
TNF-α	Tumour necrosis factor-alpha
UV	Ultra violet
U	Unit
VCAM-1	Vascular cell adhesion molecule-1

VEC	Vascular endothelial cell

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CHAPTER 1
General Introduction

1 Introduction

1.1 General description of the immune system

The human body has evolved complex and elegant mechanisms for protecting itself from invading pathogens. These mechanisms utilise an array of specialised cells, leukocytes, which are the effector cells of the human immune system. There are a number of different leukocytes that have distinct functions, but all serve to protect the body from pathogens encountered throughout the host's life, and to repair damaged tissue.

Leukocytes are dispersed systemically in the blood and are recruited to a specific site of the body when they are needed [1], by a tightly controlled process called inflammation. Inflammation can recruit leukocytes very rapidly upon infection or tissue trauma, although an inflammatory response can be prolonged for days before it resolves. Indeed, in the context of chronic inflammatory disease where the inflammatory process becomes dysregulated, inflammation may persist for years, leading to inappropriate tissue remodeling and loss of physiological function [2].

The human immune system can be divided into distinct arms, the innate immune system and the adaptive immune system, each of which is served by distinct leukocyte populations. The innate immune system is the first line of defense, and the leukocytes that make up the innate immune system arrive first at the site of inflammation, recognising extracellular bacterial components and other pathogens through specialised pathogen recognition receptors[3]. The innate system is incapable of developing immune 'memory'. On the other hand, the adaptive immune system is able to recognise specific epitopes on the invading pathogens and to generate mediators (e.g. antibodies) which specifically target these for

destruction. Unlike the innate immune system, the adaptive immune system is capable of remembering past exposure to a pathogen, or its specific epitopes, so that upon re-exposure the immune response is exclusively targeted and the response is rapid. Once a pathogen has been removed by the immune system, inflammation is curtailed by the clearance of the leukocyte infiltrate, either through apoptosis and phagocytosis by tissue-resident macrophages, or by active locomotion of the leukocytes out of the inflamed tissue, this can take place *via* the lymphatic vascular system [4], although increasingly there is evidence that the clearance of leukocytes may occur by 'reverse transmigration' back into the blood vasculature [5, 6]. Efficient resolution occurs in resolving inflammation, but the underlying deficiencies in these processes that lead to chronic inflammation and the continual recruitment of immune cells is still poorly understood.

1.2 The innate immune system

Innate immunity is the first line of defense against infectious agents. It relies on immune cells recognising and responding to pathogens in a generic way. This system is reliant on both cellular and humoral components that are present from birth, and do not change their specificity during the life span of the individual [7]. They are generally non-specific in action, i.e. do not target specific pathogenic species, and following an immune response the innate system does not develop 'memory' functions that would allow expedited or exaggerated responses to subsequent re-exposure to the initiating insult.

The different components of the innate immune system have evolved to recognise common pathogen associated components (Pathogen Associated Molecular Patterns (PAMPs) [8].

These molecules are usually essential for microbial survival because they support metabolic or structural aspects of microbial physiology, and as such they cannot be easily mutated without compromising the adaptive fitness of the microbe.

The innate immune system depends on a few germ line encoded, non variant, pattern recognition receptors (PRRs) which recognise a wide variety of microbes using common PAMPs [8]. PRRs can also generally discriminate between host tissue and microbial molecules ensuring that, ordinarily, innate immune responses do not cause collateral damage to the host physiology.

The humoral aspect of the innate immune system has PRR-like functions. For example, the complement system is a cascade of plasma and tissue fluid-borne proteins, that upon activation rapidly mark foreign material for attack by phagocytic leukocytes [8]. Thus, the complement fragment C3b, binds on to any surface that lacks sialic acid thereby 'opsonising' it for recognition and expedited uptake by phagocytes [9]. The terminal pathway of the complement cascade also results in the formation of the multi-protein (C5-C9) membrane attack complex, which is capable of inserting into the plasma membrane of targeted cells and inducing death by lysis [10]. The capabilities of this powerfully destructive system are kept in check by regulatory proteins expressed on the host cells and liberated into the body fluids [11].

Early activation of the PRRs of the innate system causes the release of cytokines from cells such as tissue macrophages, which lead to the initiation of an inflammatory response. If the pathogen persists, and is able to multiply, then the strength of the cytokine signal increases due to more macrophage recruitment to the site of infection [12].

The physiological changes that then occur during the inflammatory response are crucial to the recruitment of other mediators of the innate immune response. In particular, neutrophilic granulocytes and plasma-borne factors such as complement components cross the wall of the local microvasculature in order to gain access to the site of infection/inflammation.

1.3 The adaptive immune system

The adaptive immune system has the attribute of immunological memory and it is antigen specific [13]. The first time a pathogen is encountered, the immune response takes between four to seven days to develop mature effector functions. Upon subsequent exposure to the same pathogen, the response is greater in magnitude and is more rapidly mobilised. If specific antibodies are still present in the circulation the response can be considered immediate, however, mobilisation and expansion of the cells conferring memory function requires one or two days [14]. Indeed, it is here that the importance of the combined actions of the innate and adaptive immune responses is highlighted, as the innate immune system is able to prevent the spread of the invading pathogens until the adaptive immune system is ready to mount a response. The cells of the adaptive immune system are lymphocytes (B and T cells), natural killer cells and antigen presenting cells (usually macrophages and dendritic cells (DCs)). B and T lymphocytes are derived from common haemopoietic pluripotent stem cells located in the foetal liver or bone marrow in adults[15]. B lymphocytes continue their development in the bone marrow whereas naive T lymphocytes develop from a stem cell precursor which migrates to the thymus. It is here that T

lymphocytes undergo rigorous selection so that clones recognising host antigens are deleted [16]. Due to their role in the ontogeny of lymphocytes, the bone marrow and thymus are termed the primary or central lymph organs. The secondary or peripheral lymph organs (e.g., the spleen, tonsils, adenoids, and Peyer's patches) are where the majority of the adaptive immune response is mediated. Lymph nodes are highly organised structures through which the extracellular fluid (or lymph) of the tissues is drained and returned to the blood. During transit through secondary lymph organs foreign antigens can be assimilated for presentation to lymphocytes by antigen presenting cells in the nodes. Alternatively, tissue-resident antigen presenting cells such as dendritic cells are exposed to foreign antigen and activated by pro-inflammatory cytokines so that they process and present antigen on their surface. Immature dendritic cells recruited into the sites of inflammation are extremely efficient at presenting antigenic peptides *via* the Major Histocompatibility Complex (MHC) proteins, of which there are two types. MHC class I present intracellular peptides (e.g. viral) and MHC class II present bacterial or parasite peptides [17]. These cells then rapidly emigrate from the affected tissue through the lymphatic system to the secondary lymph nodes where they can present antigen to resident and/or trafficking lymphocytes[18]. It is here that they gain access to naïve T cells and B cells.

Importantly, lymphocytes are committed to responding to a specific antigen and must encounter this in association with a number of accessory signals (including MHC molecules) delivered by antigen presenting cells to become activated and undergo population expansion to form a large pool of effector clones. The lymph nodes have a discrete architecture which aids this process. Thus, B cells are found localised within the follicles of the lymph nodes, whereas the T cells are found in the paracortical regions (or T cell zones) to

which activated dendritic cells migrate [19]. During constitutive immune surveillance naïve lymphocytes continuously circulate from the peripheral blood, through the high endothelial venules (HEV) into secondary lymph nodes and through these microenvironments. If antigen is not encountered then the lymphocytes return to the circulation *via* the thoracic duct [20].

If lymphocytes do encounter their cognate antigens they undergo differentiation into effector and memory lymphocytes. Effector T cells can leave the lymphatic system and are recruited to the inflammatory tissue where they may target specific hosts cells (e.g. virally infected cells) for destruction and/or aid in the regulation of the inflammatory response. B cells which have differentiated under the influence of antigen stimulation with T cell help, undergo clonal expansion producing a population of plasma cells that secrete antibodies into the surrounding environment [21]. A proportion of antigen experienced lymphocytes become memory cells and continue to re-circulate through lymphoid and non-lymphoid tissue patrolling for re-exposure to their specific antigen.

1.3.1 Differentiation of T cell subsets

T cells can be divided into two main subsets, the cytotoxic T cells (CD8+) or the helper T cells (Th1 and Th2 which are CD4+), however there are many different T cell populations within these subsets which play distinct roles in the immune response [22]. Activated cytotoxic CD8+ T cells recognise antigenic peptides presented by MHC class I molecules and either lyse the infected cells using perforin molecules or induce programmed cell death (apoptosis) [23]. Th1 or Th2 CD4+ T cells recognise antigens presented by the MHC class II complex [24]. Upon activation, CD4+ cells release cytokines including interleukin-2 (IL-2), which stimulate their own proliferation as well as that of nearby CD8+ T cells [25]. Th1 cells also release γ-interferon that enhances macrophage activation and phagocytosis [26]. Th2 cells activate B

cells directly *via* CD40:CD40 ligand interactions and the release of IL-4 which can induce the class switching of immunoglobin (Ig) isotypes [21]. Another major subset of T cells, the T regulatory cells (originally called T suppressor cells) dampen down the immune response by the secretion of anti-inflammatory cytokines (e.g. IL-10) [22]. Other subsets which have been identified are the Th17 and Th9 subsets which are reported to modulate local tissue inflammation, and are reviewed by Jutel et al [27].

Naive and memory cells can be distinguished phenotypically based on changes of expression and density of surface receptors in response to activation after exposure to antigen [28]. For example, the differential expression of T-cell surface adhesion molecules on naive and memory subsets have been reported. Thus memory T cells express higher levels of β1 and β2 integrins, CD2, CD44, CD54 and CD58 [29-32]. The expression of other cell surface molecules, some involved in lymphocyte migration can also be used to distinguish between subsets as they enter the primed pool of T cells [33, 34]. Thus, α_4 integrin, CD62L, CCR7 and CD45 (discussed below) and a wide range of chemokine receptors have been used as markers. For example naive cells express high levels of CD62L and CCR7 whereas memory T-cells lose expression of these surface markers [35, 36]. The functional consequences of these changes are assumed to support trafficking of antigen experienced lymphocytes into tissue during inflammation, as opposed to the re-circulation of naive cells through secondary lymphoid tissue [28].

The most commonly utilised markers used to differentiate naive and memory T cells are different isoforms of the common leukocyte antigen CD45. Naïve T cells express the high molecular weight isoform CD45RA. Conversely, memory T cells express CD45RO [37, 38]. In

addition, highly differentiated T cells within the CD45RO+ subset can be distinguished by their expression of a third isoform CD45RB [38]. In fact, there is now a number of differentiated memory lymphocyte subsets that can be separated based on their phenotypic differences. For CD8 T cell subsets, profiling has been conducted on the basis on the expression of CD27, CD28, CCR7 and CD45RA. Distinct patterns of expression have been reported to appear under the influence of different viral stimuli (bearing in mind that CD8 cytotoxic T cell responses are directed against virally infected host cells). These have recently been reviewed by Appay V et al [34]. For CD4+ T cells, CD45RO+ (Fig 1) can be further subdivided by the presence of absence of the CCR7 receptor into central memory (CCR7+; cells that are thought to patrol mainly through the lymphatics) and effector memory cell (CCR7- cells which home to tissues to carry out their immunological functions). Effector memory cells can again be further differentiated into functionally distinct subsets depending upon the profile of secretion of cytokines.

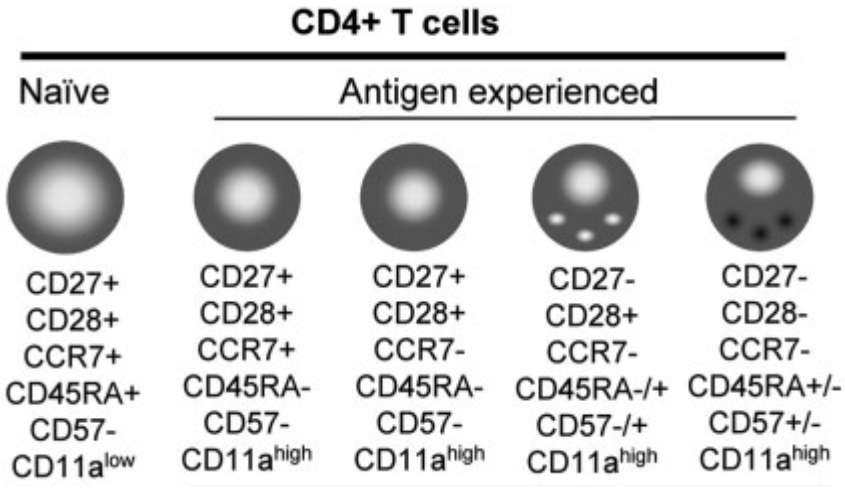


Figure 1: Common surface markers which can be used to distinguish between functionally distinct subsets[34].

Thus Th1 and Th2 cells are marked by high levels of INF-γ and IL-4 production respectively, while Th17 and Th22 cells generate IL-17 and IL-22, respectively. Such subsets are also reported to be marked by possession of distinct chemokine receptor profiles. For example Th1 cells are CCR5 and CXCR3 positive, while Th2 cells are CCR3, CCR4 and DP2 positive [39]. However, caution is required in interpreting these distinctions too literally. The receptor profiles of Th1 and Th2 cells have generally been defined in cells generated *in vitro* using polarising culture conditions and there is evidence that these distinctions are lacking, or at least blurred, in circulating peripheral blood lymphocytes [40].

In the current study we have utilised the possession of CD45RO to discriminate between memory and naïve cells, which was standard protocol within our group and a widely used means of discriminating these subsets [41, 42]. At the time these studies began, the distinction of central and effector memory cells was not common practice, although

currently this system of classification has been implemented for *in vitro* studies within our own and other groups. Classification of effector subsets (e.g. Th1, Th2 and Th17) was not conducted. However, unless studies are designed to investigate the functional characteristics of such cells, which require the use homogenous populations, such a level of discrimination is probably unnecessary.

1.4 General description of inflammation

Inflammation is a response of a tissue to injury, often caused by invading pathogens. It is characterised by increased blood flow to the tissue which leads to localised flushing if the response is in the skin, but in all other tissues leads to an increase the number of leukocytes transiting the local vascular bed [43, 44] in addition to increased temperature, swelling and pain [45]. An infiltrate of leukocytes from the blood is often a characteristic of the inflammatory response. Indeed, inflammation can be divided into different categories depending upon time (persistent or acute/ resolving); whether it is specific or non specific, type of exudates (fluid or cells), type of cellular infiltration (neutrophils/macrophages or lymphocytes); and the type of immune response that are associated with it (e.g. allergic or hypersensitivity reactions).

The number of inflammatory cells present in a tissue at any one time is dependent upon a number of factors (Fig 1.1). This balance is established by cells entering the tissue (recruitment), the number of cells leaving the tissue (emigration), the number of cells dividing (proliferation) and the number of cells that die *in situ*. Under normal homeostatic

conditions the number of leukocyte-derived cells (such as resident macrophages, DCs and lymphocytes) varies little. However, during an acute inflammatory response there is a rapid but temporary increase in inflammatory infiltrate. An important aspect of acute inflammation is the resolution phase during which leukocyte recruitment ceases and the infiltrate is removed by the modes described above [4]

In some instances, the mechanisms that ensure resolution of inflammation fail, leading to chronic inflammatory disease. Thus, leukocytes may be continuously recruited, fail to emigrate out of the tissue, replicate inappropriately or have prolonged longevity within the tissue. For example, the persistence of antigen in chronic infectious diseases such as tuberculosis [46] or host response against self antigen (autoimmune disease) could be the underlying cause of such pathology [47]. In these conditions, the inflammation can become self-sustaining.

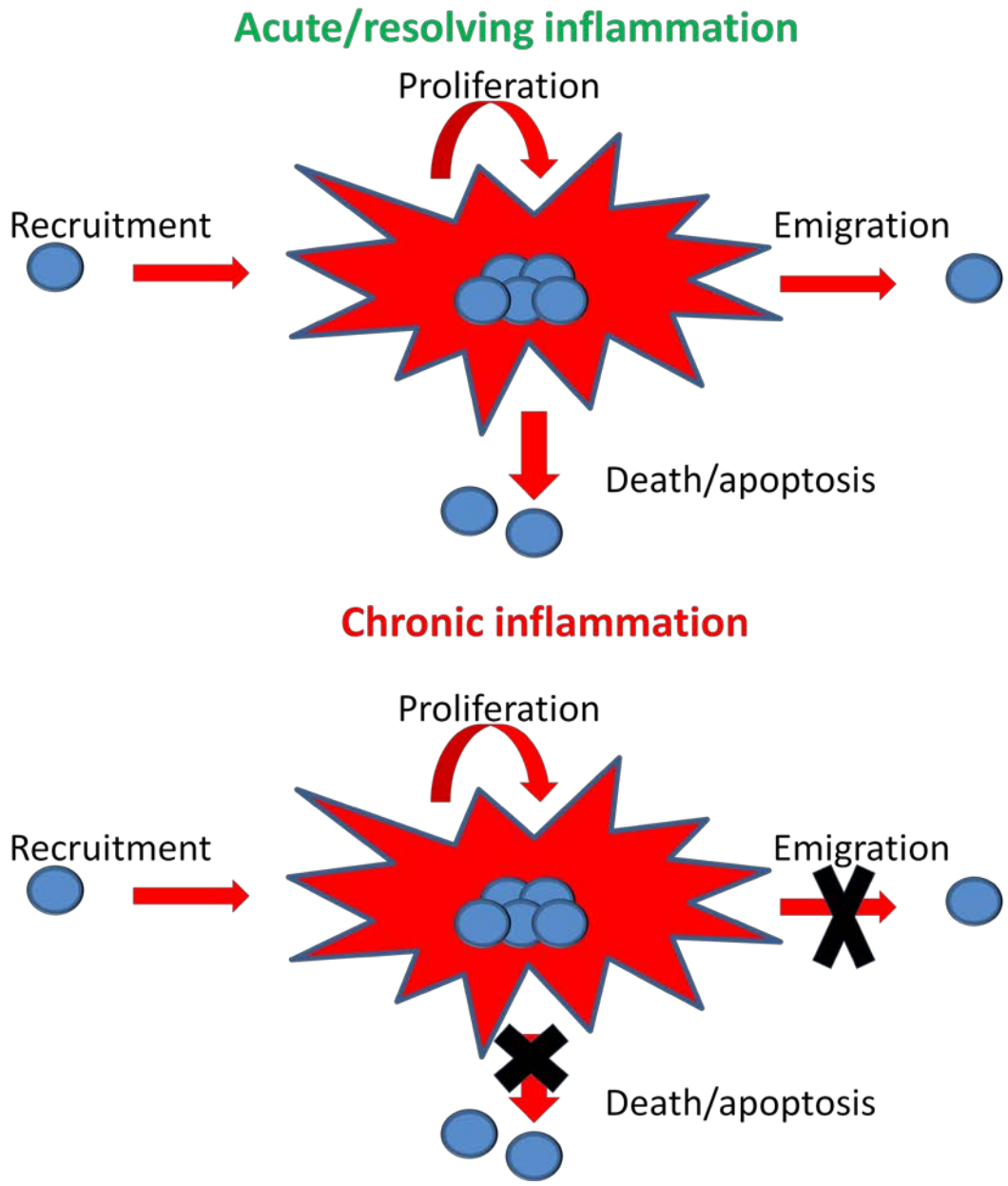


Figure 1.1: A schematic diagram of resolving inflammation and chronic inflammation. *The diagram illustrates which processes become dysregulated when resolving inflammation turns into chronic inflammation.*

1.4.1 Features of acute/resolving inflammation

Following tissue damage or infection there is an acute vascular response in localised capillary beds, leading to increased blood flow (hyperaemia) caused by the localised release of vasodilators such as bradykinins and prostanoids [48]. Vascular permeability is also altered by agents such as histamine and thrombin [49]and together these responses result in erythema (reddening) and oedema, with the resultant translocation of plasma and its soluble constituents into the site of inflammation [50]. This vascular response is associated with activation of the endothelial cells of the post capillary venules which supports a rapid infiltrate of neutrophilic granulocytes [44]. The peak response of the acute inflammatory response often occurs within a few hours of initiation, before subsiding over 24-48 hours, potentially as a result of the clearance of the infection and/or removal of the cellular debris. If the inflammatory response lasts longer, mononuclear cells such as macrophages and lymphocytes begin to infiltrate in large numbers. This stage of inflammation is described histologically as chronic [51, 52], although in reality, it will undergo appropriate resolution without leading to pathology.

1.4.2 Chronic/persistent inflammation

Chronic inflammation is a broad term that is used to describe an inflammatory response that may develop after repeated episodes of acute inflammation [53] or inappropriately prolonged *de novo* response. Chronic inflammation can be characterised pathologically by the nature of the inflammatory infiltrate, which is usually dominated by macrophages and lymphocytes, although in rheumatoid arthritis, for example, there is also a substantial neutrophil infiltrate

[54]. Chronic inflammation can last for weeks and years as opposed to hours and days, as is the case for acute inflammation. Chronic inflammation leads to tissue damage accompanied by attempts by the body to heal and repair the damage caused by the immune system [52]. For this reason, chronic inflammation has been described as a failure of wound healing [55, 56] and often results in inappropriate tissue remodeling and loss of tissue function. Although chronic inflammation is often linked with chronic infection, this is not always the case. Auto-immune diseases are often associated with chronic inflammatory responses [57], and many chronic inflammatory diseases have unknown aetiology. Indeed rheumatoid arthritis can be used as an exemplar of such a disease, as it is not associated with a known auto-antigen or aetiological agent. Other chronic inflammatory diseases are clearly associated with self antigen, e.g. Hashimotos's thyroiditis which is based on autoimmune responses to the thyroid gland [58].

1.5 The leukocyte adhesion cascade during inflammation

A multistep process dependent on specific families of adhesion receptors and chemotatic agents is widely used to describe leukocyte trafficking during acute inflammation (reviewed by Ley et al 2007). This paradigm can be divided into sequential stages that are supported by distinct cellular and molecular processes. In addition, although different leukocyte subsets may use distinct molecular machinery for recruitment during inflammation, as a generalisation these still support the distinct stages of the multistep cascade. Thus, recruitment can be broadly compartmentalised into the following scheme of sequential steps.

Stage 1: Leukocytes tether and roll on specialised EC adhesion receptors (e.g. receptors of the selectin family and/or vascular adhesion molecule-1 (VCAM-1) [20]

Stage 2: Leukocytes slow down and become activated due to interactions with peptide agonists of the chemokine family. Specifically, leukocyte receptors of the β1 and β2 integrin families are activated and mediate firm adhesion [17].

Stage 3: Leukocytes change shape due to actin polymerisation and begin to migrate along the luminal surface of the endothelial cells, predominantly using β2 integrins [59, 60].

Stage 4: Leukocytes transmigrate between intercellular junctions of the EC monolayer, although migration through the body of the endothelial cell has also been described in some vascular beds (i.e. the blood brain barrier [61]). This process may be regulated by signals distinct from those delivered by chemokines, at least in some models of inflammation. Indeed, our own laboratory has demonstrated that additional prostanoid signals are required for PMN migration across TNF-α stimulated EC [62], and in this thesis we will show a similar requirement for migrating lymphocytes.

Stage 5: Migration through the basement membrane. This process may be regulated by signals distinct from those supporting transit of the EC monolayer, and in some models has been shown to require switching of the integrin(s) supporting migration [63].

Stage 6: Movement into tissue. Little is known about the regulation of this step, but it is possible that distinct signals also regulate this aspect of leukocyte traffic.

1.5.1 Stage 1 capture and rolling

This initial stage of the leukocyte adhesion cascade requires the tethering of fast flowing leukocytes from the blood to the EC that line the luminal surface of the post capillary venules.

In inflammation, the molecules that play major roles in this process of tethering are L (leukocyte), P (platelet) and E (endothelial) selectin as well as the immunoglobulin super family (IgSF) member vascular cell adhesion molecule 1 (VCAM-1), which has integrin counter ligand in the form of α4β1 on some leukocytes. L selectin is constitutively expressed on all classes of leukocytes, whilst VCAM-1, E- and P selectin are up-regulated within a time course of hours on the luminal surface following activation of the vascular EC by pro-inflammatory mediators, such as IL-1β, TNFα, endotoxins and oxidants such as H_2O_2 released locally in the inflamed tissue [64-68]. Interestingly however, P selectin, which is stored in endothelial cell Wiebel Palade bodies, can be mobilised within minutes by agents such as histamine and thrombin [69].

The selectins and VCAM-1 are able to support rolling of the leukocytes along the lumen of the vessel due to the fast on and off rates of receptor ligand interactions. The fast rate of bond formations allows capture of fast flowing leukocytes onto the EC surface [70]. The stress of the fluid drag causes the selectin-ligand bonds to break at the rear of the cell, which allows new bonds to form at the front and so the leukocytes are able to roll along the vessel wall [71]. The structure and localisation of the selectins and their ligands may aid the capture of the leukocytes, as they are relatively long and protrude into the lumen of the vessel [20].

A further degree of subtlety in the function of selectins has also been proposed, which is the utilisation of alternative ligands. Thus, the use of different ligands may support sub-divisions of

the capture and rolling process. For example a number of E selectin ligands are expressed on leukocytes. A study using short hairpin RNA to knockdown PSGL-1 in cells genetically deficient in either ESL-1 or CD44, purports to show that the former supports tethering from flow, ESL-1 the transition to steady rolling and the latter, a process termed slow rolling. The authors suggest that close collaboration between all these ligands may be required for recruitment to vascular endothelium [72].

1.5.2 Stage 2 Activation and firm adhesion

'Firm' adhesion of leukocytes is mediated by β1 and β2 integrins. Ordinarily these receptors, which are expressed constitutively on the leukocyte surface, are in a low avidity state and do not support significant adhesion until they are activated [73]. This is generally assumed to occur upon receipt of a chemokine signal presented on the endothelial cells surface (discussed in detail in 1.8). Other agonists can also activate leukocyte integrins e.g.complement fragments C5a and C3a, eicosanoids such as leukotriene B4 (LTB_4) and other lipid mediators such as platelet activating factor (PAF) [74-77]. It is unclear, however, if all of these are active in the vasculature, or if some serve to direct migration into tissue. Indeed, most review articles consider that the first and most prominent activating stimulus presented to rolling leukocytes is a surface bound chemokine [78]. Some groups have shown that this activation step of leukocytes can be triggered by cross-linking of cell surface molecules such as L-selectin on the leukocyte membrane *in vitro* [79], the physiological significance of this is unclear, as cells such as neutrophils are able to roll indefinitely on substrates of purified or recombinant selectins,

strongly implying that ligand-receptor interactions are not directly activatory, at least for these cells [80, 81].

The kinetics of integrin activation in response to EC presented signals has been investigated [82]. In a study where EC borne IL-8 or PAF were presented to neutrophils making intermittent contacts with P-selectin at very low densities, both agonists could convert short lived rolling interactions into stable β2 integrin mediated adhesion [79]. IL-8 could activate neutrophils more quickly than PAF, the former having a median duration of rolling prior to firm adhesion of 560ms while that of PAF was 720ms [79]. These experiments indicate that activating signals can be very rapidly assimilated by selectin-bound leukocytes and that integrins can be activated within less than a second of exposure to these. As mean rolling velocities in this system were in the order of 10μm/sec, and in *intra-vital* experiments values up to 100μm/sec have been reported, this would indicate that leukocytes could be recruited well within 100μm of initial contact with the EC monolayer [79].

1.5.3 Stage 3 Shape change and migration on the EC surface

At the point of activation, which transforms rolling adhesion to firm adhesion, the leukocyte is still spherical. In order to migrate into tissue, it must undergo polarisation so that directional locomotion is possible. This process is dependent upon alterations in the leukocyte cytoskeleton, in particular dynamic formation of fibrillar (f) actin from the soluble pool of globular (g) actin localised in the cytoplasm [83, 84]. The process of spreading and shape change takes approximately 30s for both neutrophils and lymphocytes (unpublished

observations, GE Rainger). During this period the leukocyte does not undertake active migration. Indeed, in a study where the dynamics of migration of neutrophils was observed on immobilised adhesion receptors or activated platelet monolayers cells were immobile for approximately 1 minute while spreading in response to the agonists PAF and the formyl-tripetide, fMLP [79]. After this, the cells showed a marked phase of acceleration for approximately 5 minutes. Having reached maximum velocity this was maintained for the duration of the experiment (approximately 15 minutes). Interestingly, the terminal velocity of neutrophils was determined by accessory signals delivered by some adhesion molecules on the substrate. Thus, the presence of CD31 and/or P-selectin resulted in a significant increase in velocity, while ICAM-1 had no more effect than a minimal substrate of immobilised albumin [79].

The direction of migration on the endothelial cell surface is also a regulated process, at least for neutrophils [82]. Thus, on both immobilised adhesion molecules and on the EC surface, neutrophils migrate in the direction of flow. This is not a passive process of shear mediated 'pushing', as antibodies inhibitory for interactions between CD31 and αvβ3 integrin abolish directed migration even in the presence of continuous flow [85].

1.5.4 Stage 4 Migration through endothelial cells

Prior to transmigration through post capillary vessels, leukocytes have been shown to crawl inside blood vessels *via* binding of the β2 integrin CD11b/CD18 [59]. In addition, it has been demonstrated that when the process of crawling is blocked, the route of transmigration through the EC is trans-cellular and is delayed [60].

In order for transmigration across endothelial cells to occur, the binding of integrins to their IgSF ligands must be up-regulated in a manner that allow bonds to be formed at the leading edge of the cell and to be broken at the trailing edge [84, 86]. Cytoskeletal reorganisation also plays a very important role in the binding of the leukocyte integrins to their ligands, and the force behind leukocyte migration is due to the active polymerisation of globular actin to filamentous actin [84, 86, 87]. *In vitro*, the transmigration of leukocytes through the EC monolayer occurs preferentially between two EC junctions; in addition there are reports that demonstrate that a small percentage of leukocytes, mostly from lymphocyte studies, migrate through the body of the EC [88, 89]. Junctional proteins such as CD31 and VE-Cadherin may play a role in maximising the efficiency of the transmigration process [90]. Recently published work by our laboratory has revealed an additional step in the leukocyte adhesion cascade. Following the chemokine activation of neutrophils leading to the activation of their integrins, neutrophils require a prostaglandin signal, specifically Prostaglandin D_2 (PGD_2) [62]. PGD_2 is a metabolite of arachidonic acid produced by the action of the cyclooxygenase (COX) enzymes [91]. The actions of eicosanoids will be discussed below. This PGD_2 signal which signals through a receptor known as DP1 receptor following chemokine activation, allows the neutrophil to transmigrate through the HUVEC monolayer [62]- a similar requirement for PGD_2 in lymphocytes has been demonstrated in this thesis [42].

ICAM-1 and VCAM-1 also play important roles in directing leukocyte transmigration [89]. Following transmigration through the EC layer, leukocytes must migrate through a perivascular basement membrane, which appears to represent a separate transmigratory step whose regulation is not well defined [92]. *In vivo* experiments using IL-1β stimulated rat mesenteric

micro vessels have demonstrated that CD31 play a pivotal role in migration across basement membrane [93].

1.5.5 Stage 5 Migration through basement membrane

The basement membrane is made up of two protein networks composed of the vascular laminin, such as laminin-8 and laminin-10, and collagen type IV, which are connected by interactions with molecules such as nidogen-2 and the heparan sulphate proteoglycans perlecan [94].

Recent findings in the cremasteric venules have identified regions of low expression of matrix proteins particularly laminin-10 and collagen IV, and the authors found that these regions appear to be permissive to emigrating neutrophils [95] and T cells [96]. The heparan sulphate components of the basement membrane have been reported to bind chemokines and may therefore act as reservoirs for guidance molecules [97].

Trans-endothelial migration plays important roles in altering the expression of specific molecules, specifically integrins to allow their migration through the vessel wall, and the tissue [63, 98]. Ligation of CD31 is able to induce the mobilisation of the integrin $\alpha_6\beta_1$ from intracellular stores to the cell surface of transmigrating neutrophils [92]. As this integrin is the main leukocyte receptor for laminin, CD31 mediated increased expression of $\alpha_6\beta_1$ integrin on the surface of transmigrating neutrophils allows migration through the EC basement membrane [92]. Engagement of the β_2 integrin chain also induces expression of other $\beta_1$integrins that are reported to be involved in leukocyte migration in the extracellular matrix environment [99].

These responses may be facilitated by the cell surface-expressed leukocyte proteases, which expose binding sites within matrix protein constituents, with which leukocytes can interact or generate chemotactic fragments, by selective cleavage of basement-membrane constituents [100]. Transmigrating neutrophils both *in vitro* [101] and *in vivo* [102] express elastase, and there is evidence for interaction between $\alpha_6\beta_1$ and neutrophil elastase in the regulation of neutrophil transmigration *in vivo* [95, 102].

Upon transmigration into tissue, the leukocytes may then follow a chemokine gradient that is either soluble or immobilised on the ECM to the site of inflammation [103], or into lymphatic vasculature.

1.5.6 Stage 6: Migration of leukocytes into and out of tissue

Little is known about the movement of T cells in non lymphoid tissue. Collagen matrices have been employed *in vitro* to demonstrate that T cells show a high rate of motility and form short lived interactions with DCs [104]. This pattern of motility is very similar to that observed in LN with naïve T cells [105]. This suggests that T cells in tissue may also exhibit a high rate of basal motility. Also, the presence of collagen may promote migratory behavior, as exposure of lymphoid cells to collagen *in vitro* stimulates cell migration [106]. *In vitro* studies have provided conflicting results on what roles integrins play in T cell motility through collagen matrices [107, 108]. In one such study, antibody blockade of β_1, β_2, β_3, and αV Integrins had no effect on T cell movement through a collagen gel [109].

Genetic ablation of integrins on wild type mouse DCs has recently been shown to have no effect on DC migration in a 3D matrix and in the ear dermis *in vivo* [110].

The role of integrins in cell movement was further complicated following observations of DC movement [110], and the authors proposed a "flowing and squeezing model", where the protrusive flow of actin cytoskeleton drives the cell forward and a myosin II contractile module propels the trailing nucleus through confined ECM spaces. This would support the idea that integrins act to immobilise the cell and allow transmigration but do not have a roll in migration through the tissue.

The factors that control the movement of T cells out of the tissue into lymphatics are not well defined. Expression of chemokines CCL19 and CCL21 by lymphatic endothelium have been implicated in the migration of lymphocytes out of the tissue. This follows work on DCs [111, 112] and T cells [113, 114] showing the requirement of CCR7 chemokine receptor is critical for the migration out of tissue into the draining LN. Studies with integrin null DC have indicated that integrins are not required for DC trafficking out of non-lymphoid tissue [110].

Recent work has indicated a role for sphingosine-1-phosphate (S1P) receptors in the exit of lymphocytes from the lymph node [115, 116]. It was shown that S1P1 agonist FTY720 inhibited migration of T cells to the afferent lymph vessels by promoting firm adhesion of T cells to the basal (tissue) side of afferent lymphatic vessels mediated by both $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins [116]. This would suggest a possible regulatory interplay between the S1P1 system and integrins, and that changes in S1P1 levels may control T cell egress out of non-lymphoid tissue *via* effects on integrin function.

1.6 Leukocyte traffic during inflammation

The function of the innate and adaptive immune systems relies upon the traffic of leukocytes between blood and the tissues. Leukocytes are recruited into inflamed tissue by a series of

tightly regulated adhesive and signaling events which occur on the endothelial cells (EC) lining the wall of post capillary venules. This vascular bed appears to be haemodynamically permissive for the entry of leukocytes into tissue as it has a lower wall shear stress. In addition the endothelial cells of this vascular bed express specific adhesion molecules and chemoattractants in response to inflammatory signals produced by the inflamed tissue stroma [103], a situation that is not mirrored in closely associated arterioles. Importantly, during chronic inflammatory disease the phenotype of the endothelium may be altered in other vascular beds so that inappropriate leukocyte recruitment occurs [117].

The differential ability of endothelial cells of different vascular beds to recruit leukocytes is due to their phenotype which is likely to be determined by the local tissue environment, reviewed by McGettrick et al [118]. For example, EC that line the high endothelial venules (HEV) of secondary lymphoid tissue are highly specialised cells with a distinct morphology (tall and cuboidal shaped) with a thick basal lamina[97]. The adhesion receptors and chemokines that they express allow selective recruitment of lymphocytes into peripheral lymph nodes [20, 97], a process discussed in greater detail later.

Endothelial cells that line the blood brain barrier also have a distinct morphology containing highly organised tight junctions which preclude traffic of most leukocytes between the blood and brain parenchyma, which is an immune privileged tissue [119]. In contrast, the capillary like sinusoids that are found in the liver are lined with EC which have open pores [120]. These vessels lack a well developed basement membrane, but have specialisations that allow the direct movement of macromolecules from the blood to the liver parenchyma cells [120]. The

specialisations found in different EC demonstrate that the function and morphology of the EC is dictated by its position in the vascular tree and its organ of origin. Interestingly, Rainger et al (2001) showed that the stromal environment is able to dramatically alter the endothelium that lies above it [121].

1.7 Lymphatic System

The lymphatic system works in tandem with the functions of the blood vascular system by regulating tissue fluid balance, facilitating interstitial protein transport, and serving immunological functions [122]. Fluid and macromolecules that exit blood capillaries are collected from the interstitial space by lymphatic capillaries and returned back to the blood circulation through the network of larger lymphatic vessels [122]. By directing leukocytes and antigens from tissue to the lymph nodes, lymphatic vessels play an essential role in initiating the immune response. Although the blood vascular and lymphatic vascular systems depend on each other for maintenance of tissue homeostasis, they are structurally and functionally distinct entities.

Whereas the major function of the larger lymphatics is efficient transport of lymph back into the blood circulation, the lymphatic microvasculature is responsible for the uptake of components from the interstitium. Given their important role in regulating interstitial fluid pressure and cell traffic, lymphatic endothelial cells are not very well characterised, although work in the last decade is changing this (Fig 1.2).

Lymphatic capillaries are blind ended vessels, comprised of a single, non-fenestrated endothelial cell layer that is optimally adapted for the uptake of fluid, macromolecules and

cells[123]. Although LEC have many properties in common with the endothelium of blood vessels, they also have structural differences. Lymphatic capillaries generally possess a more irregular and wider lumen than blood capillaries [123]. Lymphatic vessels have an incomplete basement membrane compared to blood capillaries, and do not have pericytes [123]. Unique to lymphatic capillaries are overlapping cellular junctions. An increase in interstitial fluid pressure causes the overlapping junctions to open, so allowing free movement of fluid and particles into the lymphatic vessel [123]. As the interstitial fluid enters the lumen, the pressure differences across the vessel wall decrease and the junctions begin to close; this prevents retrograde flow back into the interstitium [122, 124].

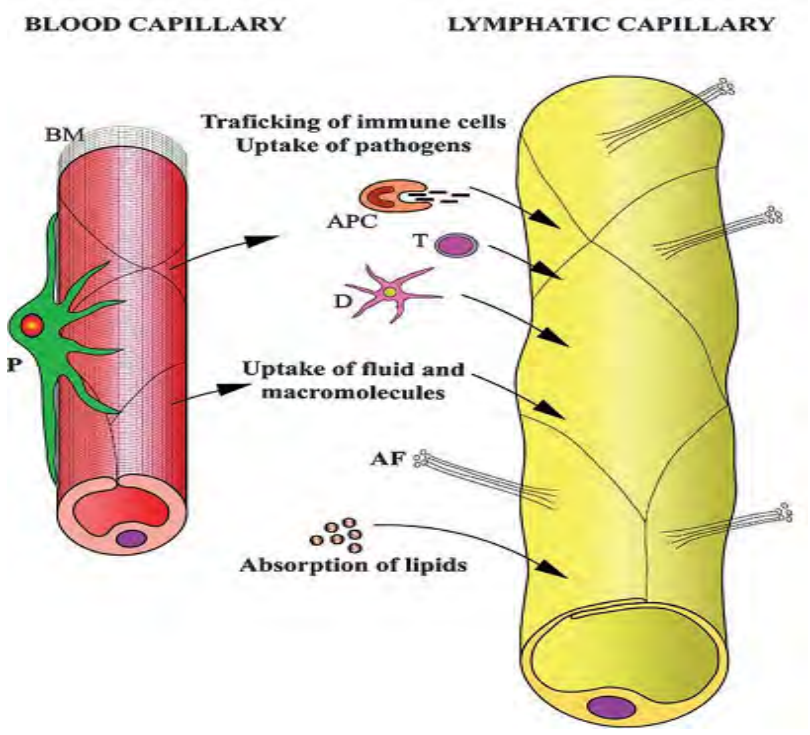


Figure 1.2: Characteristic structure and function of lymphatic microvasculature. *The lymphatic capillary is adapted for the uptake of fluid, lipids, macromolecules, and cells from the interstitium. Lymphatic capillary has a poorly developed basement membrane, and no pericytes (P). They are connected to the ECM by anchoring filaments (AF). T=T cell, APC=antigen presenting cell, D= dendritic cell. Adapted from Pepper, M.S et al [123]*

The lymphatic capillary function is dependent on its connections with the ECM. LECs are attached to the interstitial collagen by anchoring filaments, composed of elastic fibers [125], which allows the lymphatic vessel to function when interstitial pressure rises, by preventing vessel collapse.

During development and wound healing, angiogenesis generally precedes lymphangiogenesis, implying the existence of distinct yet spatially and temporally coordinated regulatory pathways [126]. Two members of the VEGF family, VEGF-C and VEGF-D have been demonstrated to play important roles in lymphangiogenesis *via* activation of VEGFR-3, which is expressed mainly by LECs in normal adult tissues [127-129]. VEGFR-3 signaling is important for development of the embryonic lymphatic system, lymphatic regeneration in the adult, and tumor lymphangiogenesis [126].VEGF-C and VEGF-D, when fully proteolytically processed, can also activate VEGFR-2 [130], but whether VEGFR-2 plays a significant role in lymphangiogenesis is not clear. Finally, Ang2 is expressed by LECs [131] and is required for the proper development of the lymphatic system [132]. Ang2 null mice displayed severe lymphoedema [132].

The homeobox transcription factor Prox-1 appears to be required for the commitment of endothelial cells to the lymphatic differentiation [133]. Prox-1 expression in embryos is localised to a subpopulation of endothelial cells in veins, which are committed to become lymphatic [133]. In adult tissues Prox-1 was exclusively shown to be expressed by LECs [133], although recent data has shown expression in the adult brain [134].

Inflammation has long been known to cause pronounced changes of the blood vasculature thereby significantly contributing to the clinical symptoms of inflammation: redness, warmth

and swelling [135]. In addition to angiogenesis, recent studies have reported, pronounced lymphangiogenesis in mouse models of chronic airway inflammation, psoriasis and rheumatoid arthritis [136-139]. In inflamed tissues, the lymphogenic factors VEGF-C and VEGF-A are secreted by immune cells, such as macrophages or tissue resident fibroblasts [140].

Lymphatic vessels play an important role in the migration of dendritic cells into draining lymph nodes where they initiate the adaptive immune response [141]. Mature inflammatory signal-activated dendritic cells up-regulate the expression of chemokine receptor CCR7 [114]. Lymphatic endothelium actively secretes its respective ligand, CCL21, causing dendritic cell chemotaxis towards lymphatic vessels [114] ; another ligand for CCR7 is the chemokine CCL19 which has been reported not to be expressed on the *in vitro* culture of LECs [142]. CCR7 may play an important role in the exit of $CD4^+$ memory T cells from tissues to lymphatic vessels [143]. Inflammatory stimuli have been reported to increase the lymph flow, which promotes dendritic cell migration to lymph nodes [144]. There is increasing evidence that inflammation-induced expression of adhesion molecules on lymphatic endothelium may facilitate the exit of dendritic cells and lymphocytes into lymphatic vessels, thus incubation of LEC with TNFα upregulates the expression on intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E-selectin, and systemic blockade of ICAM-1 and VCAM-1 has been reported to inhibit inflammation-induced lymphatic transmigration *in vivo* [145].

1.8 Chemokines

Chemokines are small secreted proteins, involved in the regulation of the motility of hematopoietic cells during their specific homing to lymphoid organs in normal haematopoiesis and during inflammation [146], through the activation of G protein coupled receptors [147].

Chemokine receptors function as allosteric molecular relays where chemokine binding to the extracellular portions modifies the tertiary structure of the receptor, allowing the intracellular part to bind and activate G-proteins. The activated G-proteins exchange guanosine diphosphate for guanosine triphosphate (GTP) and dissociate into α- and βγ-subunits. Chemokine receptors can couple to several different Gα isotypes [148, 149]. The βγ-subunits that dissociate from the pertussis toxin-sensitive $G_{i\alpha}$ mediate chemokine-induced signals [150, 151] by activating the phosphoinositide-3 kinases (PI3K), which lead to generation of phosphotidylinositol-3,4,5-triphosphate (PIP_3). The importance of this reaction is shown during the reduced migration of myeloid leukocytes to chemokines in mice lacking PI3Kγ [152]. Lymphocyte chemotaxis is not severely compromised in PI3Kγ-deficient mice, which could imply that either different signal transduction mechanisms are dominant in chemokine-induced lymphocyte migration or other PI3K isotypes, recently suggested to increase PI3Kγ-mediated reactions, could be more important in lymphocytes than myeloid cells [153, 154]

53 human chemokines and 19 receptors have been cloned and characterised to date [155] (Table 1). Chemokines are structurally very similar and have overlapping functions and often bind more than one receptor. The binding of the chemokine to its receptor leads to the tightly regulated signal transduction pathways [156]. Chemokines are divided into subfamilies by

structural and functional criteria. Structurally, chemokines are classified into four groups (C, CC, CXC and CX3C) according to the number and location of the conserved cysteine residues in the primary structure of these molecules [155]. Human CC chemokines (structurally characterised by four cysteines) include 28 members, named CCL1-28 that bind at least 10 receptors (CCR1-10) [155]. CC chemokine targets include monocytes, T cells, dendritic cells, eosinophils and basophils. The CXC group includes 21 ligands CXCL1-21 [147]. The CXC chemokines bind at least 7 receptors (CXCR1-7) and mediate neutrophil chemotaxis. Finally, the CX3C chemokines are represented by a single peptide, called CX3CL1/fractalkine, which binds the CX3CR1 receptor and regulates T cell adhesion [157]. Chemokines released upon inflammatory stimuli that induce leukocyte recruitment to damaged/infected sites are thought of as inflammatory [158] while chemokines that induce migration of leukocytes to lymphoid organs are thought to be homeostatic and constitutively secreted [159]. It is not clear whether these definitions are strictly true, as there is evidence that homeostatic chemokines can also be up-regulated in inflamed tissues [159].

Chemokine Receptor	Chemokine ligands	Leukocyte expression
CCR1	CCL3, CCL3L1, CCL5, CCL7 CCL14,CCL16, CCL23	Mo, DC, Eo, Bo, T, PMN, NK
CCR2	CCL2, CCL5, CCL7, CCL13	Mo, DC, T, Bo
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13,CCL15, CCL24 CCL26 CCL28	Eo, T, Bo, Mc
CCR4	CCL17, CCL22	DC, T, Bo, NK
CCR5	CCL3, CCL3L1, CCL4, CCL5	Mo, DC, T
CCR6	CCL20	DC, T
CCR7	CCL19, CCL21	DC, T, B, NK
CCR8	CCL1	Mo, T, NK
CCR9	CCL25	T
CCR10	CCL27, CCL28	T
CXCR1	CXCL6, CXCL8	T
CXCR2	CXCL1, CXCL2, CXCL3 CXCL5,CXCL6,CXCL7, CXCL8	No, Mo
CXCR3	CXCL9, CXCL10, CXCL11	T, B
CXCR4	CXCL12	T, B, DC, Mo
CXCR5	CXCL13	T, B
CXCR6	CXCL16	T
CXCR7	CXCL11, CXCL12	none
XCR1	XCL1	T,NK
CX3CR1	CX3CL1	T, NK, DC, Mo

Table 1: Chemokine receptors and expression by leukocytes. Adapted from Pease[160]

1.8.1 The role of GAGs in displaying chemokines

As well as chemokines being released by cells and diffusing through the tissue and blood to reach their target cells, they can also be displayed on the surface of endothelial cells by binding to glycosaminoglycans (GAGs) which include a wide range of sugars such as heparan sulphate and chondroitin sulphate [161]. The display of chemokines on the luminal surface of the endothelium establishes not only a chemokine gradient, but also a haptotactic gradient to attach rolling leukocytes, which enables T cells to bind to integrin ligands expressed on endothelial cells [103, 162].

The binding of chemokines to GAGs is not universal and there is a wide discrepancy in the ability of chemokines to bind to GAGs. This is, in part, dependent on the overall charge of the chemokine, for example CCL3 is negatively charged and is thus repelled by the highly negative heparan sulphate while most chemokines are basic [161]. In addition, the functional domains that different chemokines use for GAG binding are diverse [163]. For example, CXCL8 uses its C-terminal α helix to bind heparan sulphate [164], CCL3- three noncontiguous basic amino acids at positions 18,46 and 48 [165] and CCL5-two amino acid clusters, 44-47 and 55-59 [166]. This indicates that chemokines not only encode distinctive signaling pathways perceived through their cognate receptors, but also contain within their structure, cues as to where the chemokines will bind. The association of chemokines with GAGs also enables them to be stored in granules until they can be released. Many cells including neutrophils, eosinophils, mast cells, endothelial cells and platelets use this method to quickly release chemokines [167]. It is not clear if GAG-bound chemokines can signal as complexes through chemokine GPCRs or if

chemokines must first dissociate from GAGs to interact with their receptors. GAG-immobilised chemokines may be deposited in domains directly accessible to leukocytes, or in concealed microenvironments. Therefore, the regulation of the *in vivo* activity of chemokines may take place at the level of their GAG immobilisation and mobilisation. For example, the digestion of GAG-bearing molecules by specific enzymes may either down-regulate chemokine activity as a result of chemokine removed from the microanatomical sites that are "visible" to leukocytes [168] or expose the previously hidden activity, by releasing chemokines from sequestered stores [169].

With the exception of CX3CL1 and CXCL16, chemokines are not adhesive *per se,* but they induce leukocyte adhesion by activating integrins [170], which allows chemokines to operate at the blood-EC interface and induce leukocyte adhesion. Chemokine attachment onto GAGs on the luminal surface of ECs is a prerequisite for optimal progression of leukocyte-EC adhesion [171, 172] during both chemokine-induced inflammatory recruitment and constitutive homing of leukocytes to tissue. Irrespective of its exact function, the key aspect of GAG-mediated chemokine presentation on the EC surface is to provide a mechanism of directing the chemokine signal to leukocytes that have already initiated interactions with the EC, and away from freely circulating leukocytes. The latter, when stimulated by chemokines, lose their ability to tether and emigrate [173, 174]. Therefore, an immobilised chemokine on EC induces leukocyte adhesion, whereas a blood-borne chemokine functions as an adhesion inhibitor.

There is some evidence that specific mechanisms select tissue chemokines to appear on the luminal EC surface. For example, of the two CCR4 ligands capable of *in vivo* T lymphocyte

recruitment into skin, CCR17 and CCL22, the more potent CCL22 preferentially appears on the EC surface.[175, 176]

Endothelial cells also express the Duffy antigen receptor for chemokines (DARC) which is also expressed by red cells [177]. Although the function of DARC is still not fully understood, it is thought to provide a reservoir of chemokines, controlling the level of free chemokines within the blood and so controlling leukocyte recruitment [103]. In addition, another chemokine receptor which shows promiscuous ligand binding is D6 chemokine receptor; it was shown to be important in the resolution of cutaneous inflammation [178]. Excessive production of inflammatory chemokines may lead to uncontrolled recruitment of leukocytes which can cause collateral damage to tissues. A potential safeguard against this may be the chemokine interceptor D6, which is expressed by lymphatic ECs [179]. As D6 internalises many CC chemokines and presumably targets them for lysosomal degradation [180], it may allow only selected chemokines from the periphery to reach LNs unaltered, whereas others may arrive only after adjusting by D6. In this way D6 may control leukocyte trafficking through the lymphatics by modifying local gradients of chemokines.

1.8.2 Turning off chemokine receptors

Chemokine receptor signaling is temporary due to its mechanism of activation; this is because the receptor has a shut off sequence in its structure. The G_{α} subunits have intrinsic GTPase activity to hydrolyse GTP and reunite with $G_{\beta\gamma}$ to return to the initial conformation or inactive heterotrimers. Specific molecules termed regulators of G-protein signaling (RGS) can modify the GTPase activity of some G_{α} subunits [181, 182]. RGS act bimodally, increasing GTPase activity of

some G_α isotypes while being inhibitory on others [183]. Other mechanisms of controlling chemokine receptor signaling include desensitisation and downregulation. Desensitisation is caused by steric hindrance of G protein activation due to receptor phosphorylation by G protein-coupled receptor kinases (GRK). Downregulation is caused by β-arrestin-or adaptin-2-mediated receptor sequestration and internalisation through clathrin-coated pits or caveolae [184-186]. Both mechanisms of receptor control are regulated separately and require the phosphorylation of different serine residues in the c-terminal tail of the chemokine receptors [187]

1.9 Integrins and their role in firm adhesion

Integrins are heterodimers made up of non-covalently associated α and β chains [188]. Each subunit consists of an extra cellular domain, a series of EGF repeats containing divalent cation binding sites, a series of transmembrane domains and a cytoplasmic tail [189]. Integrins are divided into subfamilies dependent on their common β chain and have a diverse array of ligands. Table 2 displays integrin ligands and pairs.

Table 2: Integrins and their ligands

Family	α chain		Leukocyte expression	Ligands	Ligand Expression
β_1 (CD29)	α_1(CD49a)	VLA-1	act T cells	CN, LN-1	ECM
	α_2(CD49b)	VLA-2	PMN	CN, LN-1	ECM
	α_4 (CD49d)	VLA-4	Lymphocytes	FN, VCAM-1 (CD106), MAdCAM-1	ECM, activated EC or HEV
	α_5 (CD49e)	VLA-5	PMN, mem T cells	FN, VCAM-1,	ECM or activated EC
	α_6 (CD49f)	VLA-6	PMN, lymphocytes	LN	ECM
	α_9	VLA-9	PMN	VCAM-1, osteopontin	Activated EC or ECM
	α_L (CD11a)	LFA-1	PMN, T cells	ICAM-1 (CD54), ICAM-2 (CD102) , ICAM-3 (CD50), JAM-A	Variety of cells including EC, PMN and monocytes.
β_2 (CD18)			T cells, DCs Mac		
	α_M (CD11b)	Mac-1	PMN.	ICAM-1, iC3b, fibrinogen	
β_3 (CD61)	α_V (CD51)		PMN	CD31 (PECAM-1), FN	EC, PMN
β_7	α_4 (CD49d)		Lymphocytes	MAdCAM-1	HEV

act T cells: activated T cells; CN: collagen; EC: endothelial cells; FN: fibronectin; JAM: Junctional adhesion molecule; iC3b: complement fragment; ICAM: intercellular cell adhesion molecule; LN: laminin; mem T cells: memory T cells; PECAM-1: platelet-endothelial cell adhesion molecule-1; PMN: neutrophils; VCAM: vascular cell adhesion molecule; VLA: very late antigen.

Adapted from Springer[188], Luscinskas *et al [190]* and Ostermann *et al* [191].

The main function of integrins is to support cell-cell or cell-ECM adhesion, although they also function as signal transducing receptors that can influence cell migration and proliferation [66, 192]. On neutrophils, the β2 integrins ($\alpha_m\beta_2$, $\alpha_L\beta_2$ and $\alpha_x\beta_2$) become activated following chemokine activation and allow the neutrophil to firmly adhere to the EC *via* their ligands ICAM-1 and ICAM-2, and this allows transmigration of the neutrophil [193]. β1 integrins are found on both non-haemopoietic cells and leukocytes, and have roles in adhesive receptors in cell-cell interactions as well as mediating interactions between cells and ECM proteins such as laminin and collagen [188, 193].

VCAM-1 is a member of the IgSF, which is expressed on the vascular EC as well as on cells in the tissue stroma (e.g. smooth muscle cells and synovial fibroblasts found in RA patients) [20, 194, 195]. VCAM-1 is up-regulated on the surface of cytokine stimulated EC [196]. It binds to $\alpha_4\beta_1$ (VLA-4) and is constitutively expressed on the surface of monocytes, lymphocytes and eosinophils [197]. VCAM-1 plays a dual role in the recruitment of mononuclear cells as it mediates capture and rolling onto activated vascular EC as well as firm adhesion [198]. Intermediate activation of $\alpha_4\beta_1$ mediates rolling and full activation of the $\alpha_4\beta_1$ is able to cause firm adhesion [199]. Indeed, *in-vitro* studies of cytokine-activated EC of purified VCAM-1 have shown that VCAM-1 is able to support tethering and rolling [200].

Chemokine receptor signaling activates integrins on the lymphocyte surface through G proteins and enables the lymphocytes to adhere and emigrate through VEC. The most important integrins during this process are LFA-1, α4β1, α4β7. Chemokines such as CXCL12 switch α4 integrin to a high-avidity (clustered) state resulting in the arrest of lymphocytes on

VEC within 0.1 seconds ([201]. The fact that α4 integrin is predominantly displayed on the microvilli of activated T cells supports the idea that it acts as the capture integrin on VEC, whereas LFA-1 which is expressed on the lymphocyte, takes over once the lymphocyte rolls along the EC [202, 203].

The junctional adhesion molecule (JAM)-family members are also involved in transmigration (reviewed by Muller et al [204]). JAM-A is a ligand for LFA-1 and is located apically and at tight junctions of EC [191]. Antibody blockade of JAM-A was shown to inhibit lymphocyte migration, thus indicating that LFA-1 may swap ligands from ICAMs to JAM-A as transmigration occurs.

There is increasing evidence that the function of one type of integrin can be modulated by ligation of another. For example, α4β1 can activate binding of LFA-1 to ICAM-1 [205]. Similarly, binding of ICAM-1 to LFA-1 alters the function of α4β1 and α5β1 on lymphocytes [206, 207]. One such situation where this crosstalk may occur is during TEM, when lymphocytes switch from α4-mediated rolling/adhesion to LFA-1 mediated adhesion/migration. As unstimulated VEC constitutively express ICAM-1 and ICAM-2 (at low levels) circulating lymphocytes would be constantly exposed to LFA-1 ligands and thus their ligand binding activity by α4β1 may serve this purpose.

Upon activation integrins may be found in lipid rafts. These specialised membrane microdomains are enriched in cholesterol and sphingolipids and serve as platforms for adaptor and signaling molecules [208]. Lymphocytes contain two major classes of raft defined by their glycosphingolipid content. Ganglioside GM3 containing rafts localise to the leading edge whereas ganglioside GM1 containing rafts are associated with the trailing edge

of polarised lymphocytes [209]. Human lymphocyte adhesion through LFA-1 and α4β1 [210], is abolished when lipid rafts are disrupted by extraction of cholesterol with methyl-β-cyclodextrin. This suggests that activation of integrins results in their localisation to a lipid raft and clustering within rafts is essential for integrin mediated lymphocyte adhesion.

Integrin activation as mentioned earlier, results from either clustering of integrins on the cell surface or an increase in affinity for the ligand induced by a conformational change. Ligation of the cell-surface receptors, such as the TCR or chemokine receptors, causes the generation of intracellular signals that increase LFA-1-mediated cell adhesion. This is termed inside-out activation. Addition of divalent cations Mn^{2+} or Mg^{2+} or activating antibodies which bind to the extracellular portion of LFA-1 cause a conformational change that also activates LFA-1, this is termed outside-in activation. *In vitro*, outside-in activation increases the affinity of LFA-1 for its ligands, whereas inside-out activation induces increased mobility and clustering [211, 212].

1.10 Formation and function of Eicosanoids

Eicosanoids are lipid-derived bioactive compounds most commonly synthesised from the n-6 & n-3 polyunsaturated fatty acid (PUFA), arachidonic acid (AA) [213]. Eicosanoids have been shown to play important roles in inflammation and immunity and are messengers within the central nervous system [214, 215]. AA is released from the plasma membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolaime (PE), by the enzyme phospholipase A_2 (PLA_2). Phospholipase C (PLC) is also able to release AA, specifically from phosphatidylinositol (PI), through a series of reactions involving the formation of diacylglyceral (DAG) and the enzyme DAG lipase (see figure 1.3) [216] [217]. AA is released

in response to a variety of stimuli including hormones, cytokines and acetylcholine (Ach) [91, 218]. Free AA is metabolised by three main classes of enzymes:

1) Cyclooxygenases (COX)
2) Cytochrome P-450 monooxygenases
3) Lipoxygenases (LOX)

The trans-metabolic production of eicosanoids can influence neighboring cell types; this process occurs when an intermediate of AA metabolism is generated in one cell which then passes to a second cell where it is fully processed into the final product [213].

COX-1 is constitutively expressed in most tissues, whereas COX-2 is not detectable under resting conditions but is rapidly up-regulated in cells of the immune system in response to growth factors, hormones and cytokines [219]. Hence COX-1 is regarded as the constitutive isoform responsible for synthesising prostanoids involved in homeostasis, and COX-2 as the inducible isoform, producing molecules responsible for the classical signs of inflammation [220]. Both isoforms of COX are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs).

Both COX enzymes catalyse the same process to metabolise AA. Firstly AA undergoes a cyclooxygenase reaction producing prostaglandin G2 (PGG_2), followed by a peroxidase reaction converting PGG2 to an unstable intermediate, prostaglandin H2 (PGH2) [221].

PGH2 has a half life of approx. 3 mins [222], and is rapidly converted by the prostanoid synthases to thromboxane A_2 (TXA_2), prostaglandin E2 (PGE2), Prostacyclin (PGI2), PGD2 and PGF2-α [222]. Each prostaglandin carries out a number of important functions. TXA2 for example has a role in platelet activation and is able to cause aggregation, which also acts in a

positive feedback mechanism to recruit and activate further platelets through their T prostanoids (TP) receptors [223]. PGE2 has been shown to have roles in uterine contraction prior to embryo implantation in mice [224]. PGE2 plays a major role in inflammation and the perception of pain; injection of PGE2 into the space surrounding the spinal cord potentiates carrageenan-induced inflammation in a rat hind paw model [225]. Also it has been shown that direct injection of PGE2 into the brain is able to cause hyperalgesia [214]. It has been shown that PGE2 is able to inhibit the trans-endothelial migration of T cells by raising cAMP levels within the cell [226].

PGI2 is synthesised in vascular EC and SMC and is spontaneously hydrolysed to 6-Keto-PGF2α [227]. PGI2 relaxes coronary arteries and is anti-aggregatory for platelets [215]. The balance between PGI2 and TXA2 plays an important role in the control of thrombus formation in blood vessels [228]. PGD2 is the main COX product of mast cells [229], and it has been shown to induce inflammation when injected intra-dermally into humans and rats [230]. Moreover, mice which overexpress human lipocalin-type PGD2 synthase, show increased recruitment of T-lymphocytes and eosinophils to the lung in an OVA induced murine asthma model [231]. It was shown that in a carrageenan-induced pleurisy model in rats, inhibition of PGD2 synthesis and its downstream metabolite, 15 $\Delta_{12,14}$-prostaglandin J2 (15$\Delta_{12\text{-}14}$-PGJ2), exacerbated inflammation and inhibited resolution [232]. These data suggest that PGD2 has pro and anti-inflammatory mechanisms of action. The down-stream metabolites of PGD2, Δ_{12}-PGJ2 and 15$\Delta_{12,14}$PGJ2 are thought to be responsible for some of the anti-inflammatory actions of PGD2. They are able to bind to members of the PPAR family of nuclear receptors and have been shown to suppress the ability of human monocytes to produce pro-inflammatory cytokines [233]. PGF2-α, has been shown to regulate a number of

physiological processes associated with reproduction including ovulation and maintenance of the corpus luteum during pregnancy [234]. PGF2-α has also been implicated in both acute and chronic inflammatory diseases. In chronic disease such as rheumatoid arthritis, it has been detected in the joints [235]. PGF2α has a very short half life of approximately 1 minute in the circulation and can be converted to the more stable metabolite 15-keto PGF2α and many of the actions attributed to PGF2α are due to formation of this biologically active metabolite [236].

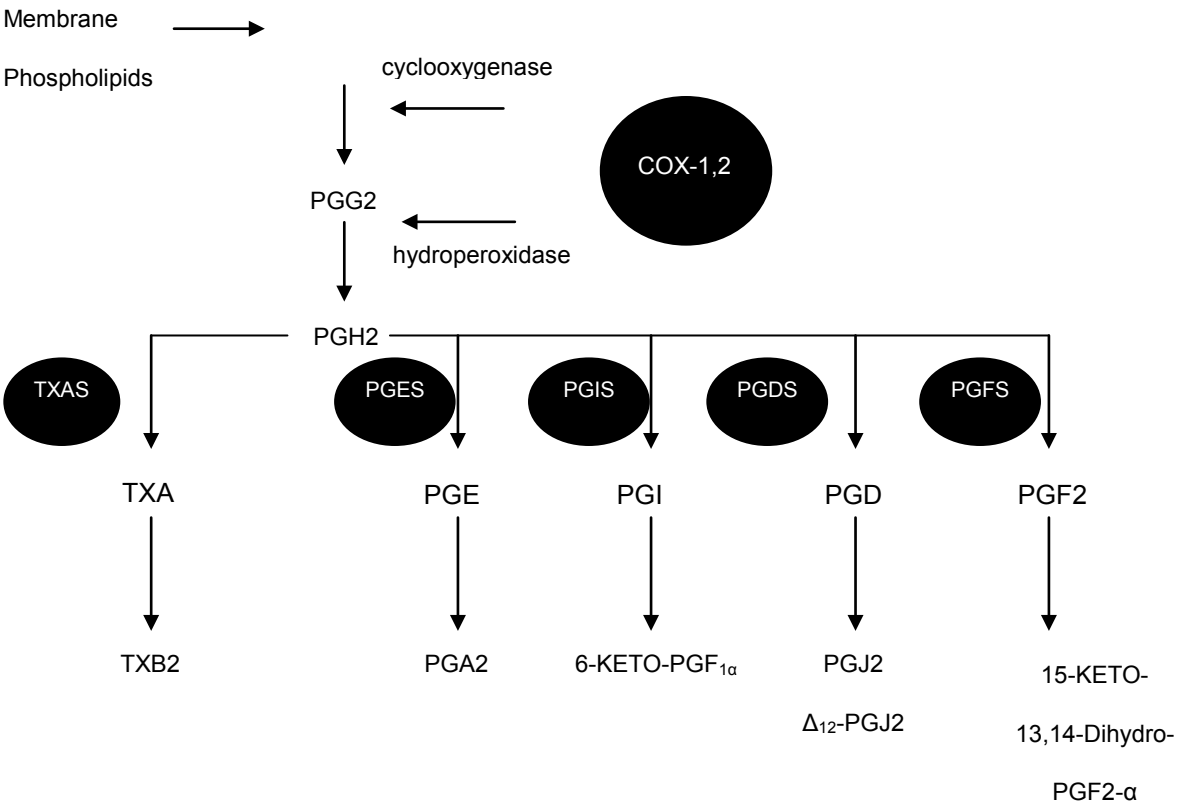


Figure 1.3 The COX pathway

COX-1, 2 metabolise AA to PGH2 by sequential cyclooxygenation and hydroperoxidation reactions. PGH2 is converted *via* specific synthases generating thromboxane A2 (TXA2) and the prostaglandins including PGE2, PGI2, PGD2 and PGF2α. These products can be non-enzymatically converted into further metabolites. TXAS, thromboxanes A2 synthase; PGES, PGE2 synthase; PGIS, PGIS synthase; PGDS, PGD synthase; PGER prostaglandin endoperoxidase synthase. (Modified from Alfranca et al[221])

1.11 Lipoxygenase enzymes and their products

In activated cells, free AA is also oxidised to the leukotriene (LT), hydroxyeicosatetraenoic acid (HETE) and lipoxins (LX) by the lipoxygenase enzymes, 5, 12 and 15-LO (see Fig 1.4A) [221]. In the presence of 5-lipoxygenase activating protein (FLAP), 5-LO converts AA into 5 hydroperoxyeicosatrienoic acid (HPETE), which is dehydrated to form LTA4 [221]. LTA4 is then hydrolysed to generate LTB4 or metabolised by LTC4 synthase to the cysteinyl LTs (Cys-LTs) [221]. LTB4 induces up-regulation of CD11b/CD18 in neutrophils, increasing adhesion to EC [237], it initiates neutrophils degranulation and acts in an autocrine manner to increase LTB4 production in neutrophils [237]. LTB4 produced by mast cells has been shown to induce chemotaxis of mouse femoral bone marrow cells, although it has no effect on mature mast cells [238]. Moreover, LTB4 produced from the mast cells during an allergic reaction, has been demonstrated to cause eosinophil degranulation which may be important in pathogenesis of allergic disease [239]. LTB4 is able to selectively recruit cytotoxic T lymphocytes to inflamed tissue [240, 241]. The Cys-LTs are synthesised in mast cells, basophils, eosinophils and macrophages. They are mediators of the allergic immune response seen in asthma, mediating bronchial smooth muscle constriction, leukocyte activation and vascular permeability [242].

12-LO generates 12(S) HETE from AA, whereas 15-LO generates 15(S) HETE. Production has been detected in vascular cells and tissues including cultured SMC, EC and monocytes [243]. Human 15-LO and the leukocytes 12-LO exhibit high homology and have been classified as 12/15LOs as they have the ability to synthesise both 12(S)-HETE and 15(S)-HETE from AA [243]. 12/15-LO has been detected in human eosinophils and airway epithelial cells [244]. It is also

detected in human monocytes activated by IL-4 or IL-13 [245]. There is evidence to support the involvement of 12(S)-HETE in hypertension as levels are increased in the urine of hypertensive human patients [246] and in rat models of hypertension [247] . Inhibition of 12-LO ameliorates hypertension in these rats [248]. Anti-inflammatory properties have been attributed to 15(S)-HETE, it inhibits superoxide production by LTB4 stimulated neutrophils [249] and inhibits LTB4 stimulated neutrophil migration across HUVEC monolayers [250]. The process by which this occurs is thought to be the rapid incorporation of 15(S)-HETE into neutrophil phospholipids which reduces the affinity of LTB4 surface receptors for their ligand [251]. This could provide a possible role for 15(S)-HETE in limiting acute inflammation.

Another family of eicosanoids generated from the AA metabolism by LOs are the lipoxins (LX), which play a major role in the resolution of inflammation [252]. The formation of lipoxins requires the action of two LOX enzymes, which can either be present in the same cell or in two different cell types, leading to transmetabolic generation of lipoxins [253]. 15-LO synthesises the formation of the intermediate 15(S)-HETE which can be reduced to yield 15(S) HETE. Both can serve as a substrate for 5-LO, generating LXA4 or LXB4. This route of synthesis has been demonstrated in the mucosa of the GI tract, where epithelial derived HETEs interact with neutrophil 5-LO [254] to generate LXA4. There is another mechanism of LX formation initiated by 5-LO, which occurs between cells of the blood and vasculature. Neutrophil 5-LO can metabolise AA to LTA4. This is released from activated neutrophils at sites of inflammation and metabolised by platelet 12-LO, generating LXA4 or LXB4 and may promote pathways required for full resolution of inflammation [255]. LXs not only moderate inflammation, but may also promote pathways required for full resolution of inflammation. It has been shown that LXA4

and LXB4 are able to inhibit the chemotaxis of neutrophils when stimulated by the bacterial wall peptide fMLP or LTB4 [256]. This could serve to reduce neutrophil recruitment to inflammatory sites during the process of resolution. LX can also promote monocyte adhesion to EC, resulting in recruitment and providing increased numbers of these cells, which are known to participate in the resolution of inflammation by removing effector inflammatory cells such as neutrophils by phagocytosis [257].

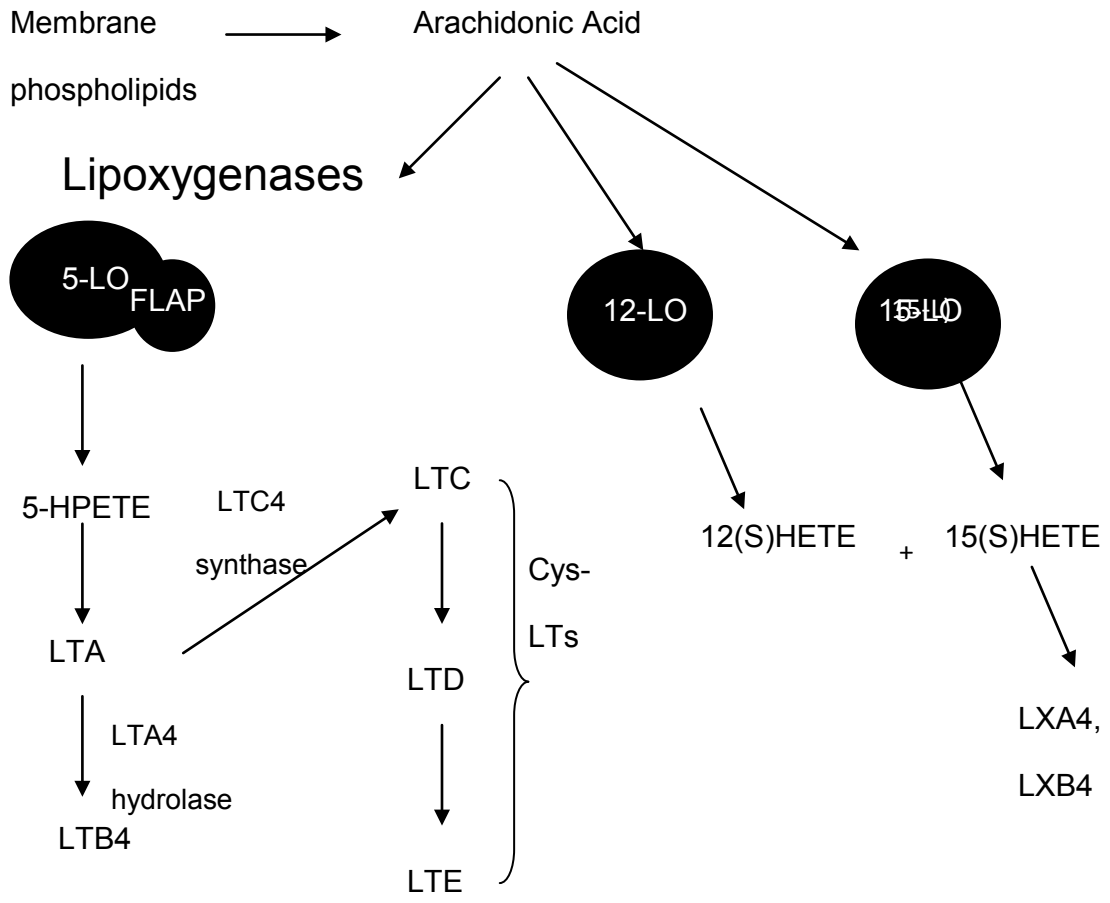


Figure 1.3A The LOX pathway: Lipoxygenases (5-, 12- and 15-LO) generate leukotrienes and lipoxins. 5-LO binds 5-lipoxygenase activating protein (FLAP) upon cell activation and converts AA to 5-HPETE, which is further metabolized to LTA4. LTA4 can also be converted into LTB4 by LTA4 synthase, or converted to the cysteinyl leukotrienes (Cys-LTs) LTC4, LTD4 or LTE4. 12-LO and 15-LO can generate 12(S)HETE and 15(S)HETE, which is the precursor for lipoxins A4 or B4(LXA4, LXB4(.Modified from Alfranca et al [221])

1.12 Project aims

The aim of this project was to investigate the ability of vascular endothelial cells to modulate the migratory behavior of lymphocytes, prior to migration through lymphatic endothelium during inflammation. Using transwell filter assays and flow based adhesion assays, we tested the hypothesis that vascular endothelium is able to modulate the behavior of lymphocytes and retard the migration of lymphocytes across lymphatic endothelial cells.

The aims of the thesis were as follows:

1) To investigate if lymphocytes migrated through TNFα and IFNγ stimulated HUVEC would allow migration through stimulated lymphatic endothelium.

2) Characterise the phenotype of lymphocytes able to migrate through HUVEC and lymphatic endothelium.

3) To investigate the ability of lung microvascular endothelial cell to recruit lymphocytes.

4) To determine the integrins involved in transmigration of lymphocytes across lymphatic endothelium.

In summary, we aim to determine the mechanisms involved in lymphocyte migration across both vascular and lymphatic endothelium.

CHAPTER 2

Migration of lymphocytes across vascular endothelium enhances migration across a lymphatic endothelium

2 Introduction

Lymphocyte recirculation and the localised recruitment of antigen specific T cells to sites of inflammation are pivotal to immune surveillance and allow T cells to execute their effector functions during inflammation. The ability of T cells to enter inflamed tissue is controlled at different levels. Firstly, pro-migratory modifications in T cell phenotype can occur during priming and differentiation in the lymph nodes. Specifically, changes in the expression of adhesion receptors and chemokine receptors [258, 259] occur following antigen stimulation. These changes may allow T cells access into non-lymphoid tissue [260]. Activated antigen specific T cells are then recruited by the endothelium to sites of inflammation. Receptor mediated interactions between T cells and the EC regulate this multistep process [258]. T cells have been reported to be captured from the blood stream by a number of receptors, e.g. selectins [66, 261], CD44 [262] and integrins such as α4β1, which binds VCAM-1 [200]. Subsequent prolonged T cell adhesion and spreading on the endothelium are regulated by chemokine dependent activation of the cells which stabilise integrin-mediated interactions, as well as promoting interactions between other counter receptors [263, 264].Following activation on EC, T cells cross the endothelial barrier and enter the tissue. Some of the chemokines that have been attributed to this are CCL3, CCL5 and CCL21 [265-267]. Some endothelial cell molecules such as CD31 may enhance the efficiency of the migratory process [268], and the enrichment of T –cells expressing surface molecules such as CD54 (ICAM-1) taken from the endothelial cells themselves has been shown [269].

Migration of the T cells into the tissues involves T cell interactions with the endothelial basement membrane and the extracellular matrix. It is probable that signals derived during passage through the EC barrier affect the subsequent interactions with basement membrane and the extracellular matrix which would lead to efficient movement into the tissue. In the first instance these could be acute responses to activation, e.g. cytoskeletal rearrangements [270] and responsiveness to the tissue derived chemotactic factors [271]. However, various groups have shown that engagement of integrin $\alpha_5\beta_1$ with the extracellular matrix protein fibronectin causes up-regulation of transcription factors such as AP-1 [272]. This could indicate that transcription dependent phenotypic changes in the T cell may be necessary to allow better interaction with either the stromal microenvironment, or to allow expedited egress into the lymphatic circulation.

In chronic inflammatory conditions and/or in patients with autoimmune disease, the lymphocytes that are recruited are almost exclusively memory T Cells [273, 274]. The phenotype of these cells is quite similar to that of lymphocytes trafficking through these sites under basal conditions. This suggests that the signals for normal lymphocyte trafficking and in inflammation may be the same. It has been shown that both antigen responsive and non-responsive lymphocytes are recruited into sites of antigenic stimulation [275]. Antigen-specific lymphocytes may accumulate in the site of chronic inflammation and be retained due to aberrant expression of local signals. Alternatively, they may not receive appropriate signals that allow them to move out of tissue into lymphatic vessels and leave the site of inflammation. This is exemplified by the work by Buckley et al [276]where it was demonstrated that SDF-1 produced by fibroblasts caused the retention of lymphocytes.

Once a lymphocyte has been recruited to the endothelium and it migrates through the EC and through the basement membrane, the signals that govern whether the lymphocyte will be able to migrate into the lymphatics or reside in the tissue are ill defined. It was thought that exit of lymphocytes from tissue was a passive process, but growing evidence has identified a specific chemokine receptor, CCR7, which appears important in regulating egress of lymphocytes from peripheral tissues. To date, there have been no experiments to investigate what effect transmigration through a vascular endothelium has on the ability of lymphocytes to migrate through a lymphatic endothelial cell monolayer. Indeed, it has long been thought that the resolution of inflammation is a passive process and simply removing the pro-inflammatory mediators would lead to the emigration of lymphocytes out of the tissue. However, it is possible that the very endothelium that recruits the lymphocyte in the first instance conditions that lymphocyte to migrate out of the tissue *via* the lymphatics in resolving inflammation. It is therefore possible that this mechanism is abrogated in chronic inflammation which leads to the continual recruitment of lymphocytes but loss of clearance mechanism.

Here we show how transmigration through a vascular endothelium delivers a signal to migrating lymphocytes which increases the efficiency of migration across lymphatic endothelium, possibly demonstrating that the endothelial cells govern the balance of recruitment and emigration of cells in and out of tissue.

2.1 Materials and Methods

2.1.1 Isolation of lymphocytes

Venous blood was obtained from healthy individuals and collected in tubes containing EDTA giving a final concentration of 1.6mg/ml (Sarstedt, Beaumount Leys, and Leicester, UK). To isolate the lymphocytes from whole blood, a two-step density gradient was employed. 15ml of Histopaque 1119 (Sigma, Poole, UK) was added to a 50ml tube. 15ml of Histopaque 1077 (Sigma, UK) was gently layered on to the top of the Histopaque 1119 so that two distinct layers were formed. 20ml of whole blood was then layered on top of the Histopaque gradients. This tube was centrifuged at 800g for 45 mins.

Following the centrifugation, three layers were formed (Fig 2). The top layer that forms at the interface of Histopaque 1077 and plasma is the mononuclear cells (lymphocytes and monocytes) the second layer that forms at the interface of Histopaque 1077 and 1119 are the neutrophils. The dark red layer contains red cells. The mononuclear cell layer was removed and placed in a 15ml tube containing 10ml of Medium 199 (M199 Gibco Invitrogen, Paisley, UK) with 0.15% bovine serum albumin (BSA; Sigma) to give a final volume of 10ml. The cells were washed twice at 500g for 5 mins in this medium and the lymphocytes were resuspended in 15ml of 199/BSA. The cell suspension was then transferred into a T75 cell culture flask to remove monocytes by adhesive panning. After incubation for 1 hour at 37°C in a humidified atmosphere, with 5% CO_2, the lymphocytes (non-adherent cells) were collected, washed in 199/BSA, and then counted using a Multisizer II Coulter Counter (Coulter UK). The cell suspension was adjusted to give a final concentration of $1x10^6$ cells/ml in medium 199 (Invitrogen) and 1% BSA (Sigma).

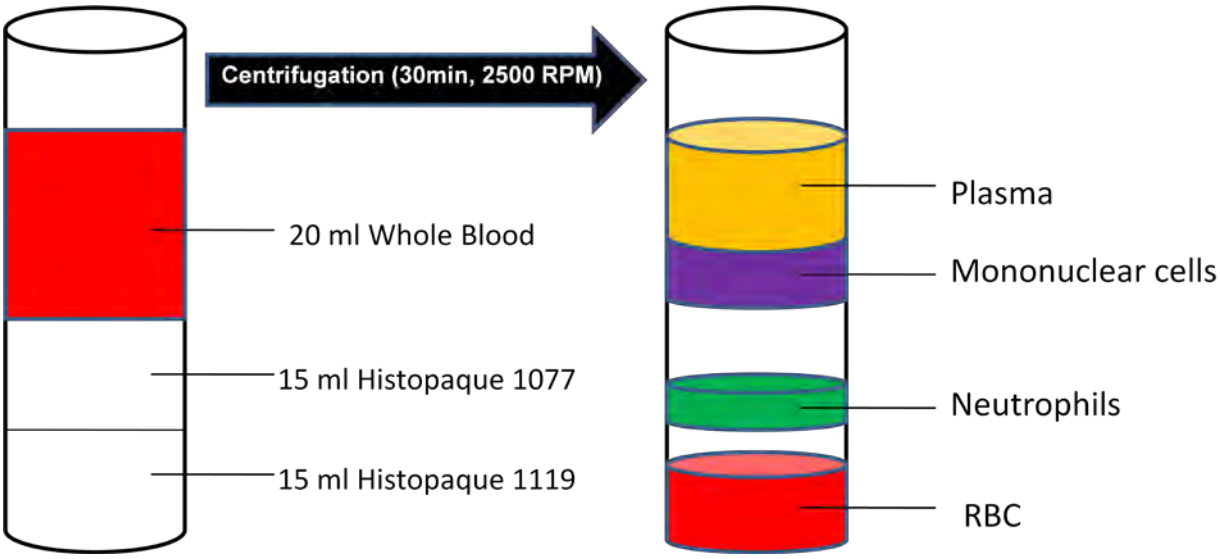


Figure 2: Diagrammatic representation of leukocyte subset location from the density interface. Whole blood was layered on top of the density gradients, Histopaque and centrifuged for 30 minutes. Following centrifugation, four layers are formed the uppermost layer containing plasma, followed by the mononuclear cells, then the neutrophil layer and final layer contains red cells.

2.1.2 Culture of HUVEC

Endothelial cells were isolated from the veins of human umbilical cords using collagenase digestion as described previously [277]. Briefly, the vein was cannulated at each end and washed with PBS to remove blood. 10ml of collagenase (type 2, Sigma, 1mg/ml) diluted in PBS was perfused through the cord until it was visible in both ends of the cannula. Cannulae were then sealed using clips and the cord incubated at 37°C for approximately 15 minutes (Figure 2.1).

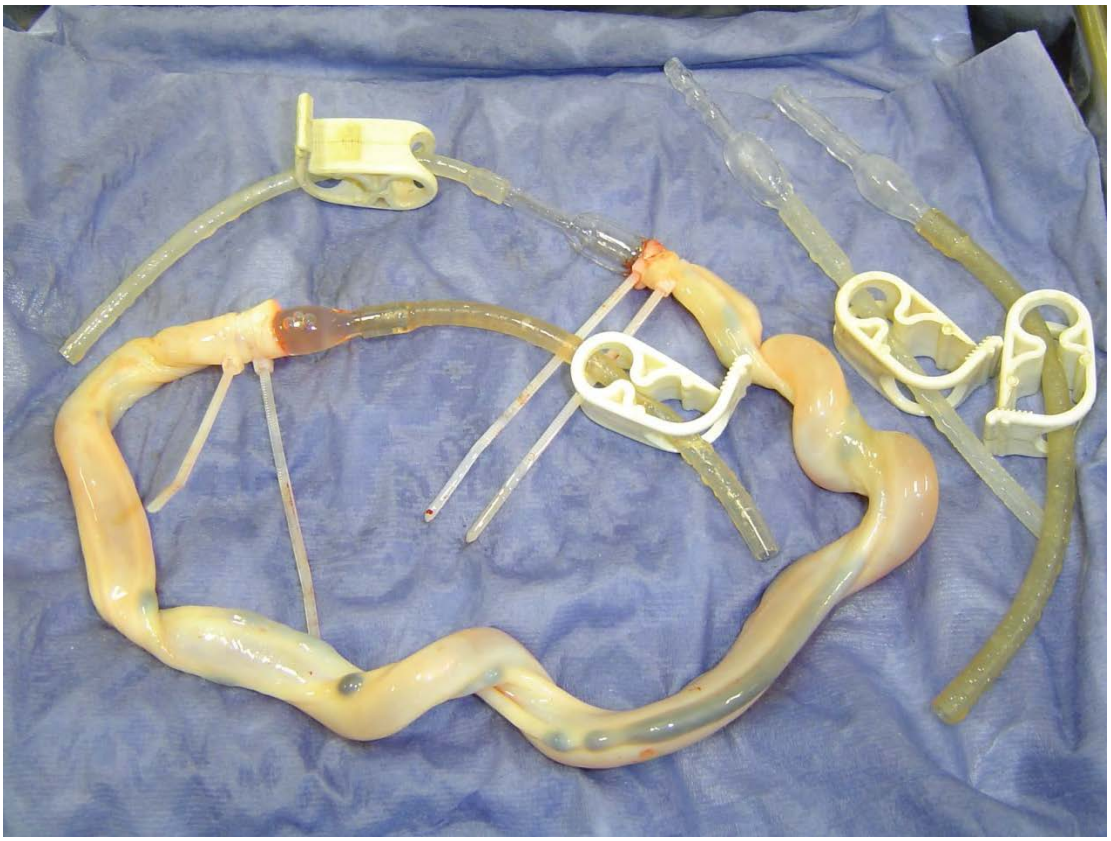
Following incubation the cells were transferred into a 50ml tube by flushing the cord through with 20ml of PBS. Following a wash at 800g for 5 mins, the cell pellet was resuspended in 5ml of complete HUVEC medium (Medium 199 (M199- Gibco Invitrogen Compounds) supplemented with 20% heat-inactivated foetal calf serum (FCS), 10ng/ml epithelial growth factor (EGF), 35µg/ml gentamycin, 1µg/ml hydrocortisone, 100U/ml penicillin and 100µg/ml streptomycin (all from Sigma) and 2.5µg/ml amphotericin B (Gibco Invitrogen Compounds)) and transferred to a T25 culture flask which had been pre-coated with 1% bovine gelatine (Sigma). The media was changed after two hours and then every two to three days, until a confluent monolayer was obtained.

2.1.3 Culture of lymphatic endothelium

Human dermal lymphatic endothelium was purchased from Promo Cell (UK). The cells were cultured in MV2 medium (Promo Cell, UK) and supplemented with 0.05% FCS, epidermal cell growth factor 5ng/ml, basic fibroblast growth factor 10ng/ml, insulin-like growth factor 2- ng/ml, vascular endothelial growth factor 165 0.5ng/ml and hydrocortisone 0.2µg/ml (all purchased from Promo Cell, UK). The lymphatic endothelium was passaged no further than P8. The cells were isolated from the dermis of human foreskin and tested for expression of CD31 and podoplanin.

2.1.4 Culture of human lung microvascular endothelial cells (LMVEC)

LMVEC cells were kindly donated by AstraZeneca, UK. The cells were cultured in MV2 medium as lymphatic endothelial cells. The LMVEC cells were isolated from a human lung and tested positive for CD31 and von Willebrand factor expression, and also tested negative for smooth muscle alpha-actin.



Courtesy of Dr E Smith

Figure 2.1. Isolation of Human Umbilical Vein Endothelial Cells (HUVEC).The HUVEC cells were isolated from the inside of the vein using enzymatic digestion of the collagen.

2.1.5 Sub-culturing endothelium

The following protocol was used for all endothelium:

Culture medium in the flask was aspirated and replaced with 5ml of PBS which did not contain magnesium or calcium ions. After a brief rinse, the PBS was aspirated and the cells were then incubated with 2.5ml EDTA 0.02% (Sigma), for approximately 1 min. The EDTA solution was aspirated and replaced with 3ml trypsin (2.5mg/ml) (Sigma) in a 2:1 ratio with 0.02% EDTA solution. Phase contrast microscopy was used to evaluate whether the cells had

detached from the flask. Once the cells had detached, 5ml of complete medium was added to the flask in order to deactivate the trypsin. The cell suspension was transferred to a 15ml tube and then centrifuged at 500g for 5 mins. The supernatant was discarded and the pellet was resuspended in complete medium and sub-cultured on appropriate culture surfaces, depending upon the experimental protocol (see below). Cells were maintained at 37°C at 5% CO_2 until confluent. Cells were sub-cultured into 12 well plates (BD, Falcon, Oxford) or onto the inner chamber of transwell filters with 3µm pores (BD, Falcon) or into ibidi chamber slides (ibidi GmbH, Am Klopferspitz 19, D-82152 Martinsried, Germany) for flow assays. All vascular EC were cultured on the apical surface of the filter to ensure correct orientation of the endothelial cells, to allow the lymphocytes to migrate through the VEC in an apical to basal direction. The concentration of the cells was chosen to give confluent monolayers within 24 hours of seeding.

2.1.6 Lymphocyte transmigration assay

In order to investigate the effect of cytokine stimulation on the ability of lymphocytes to migrate across a vascular endothelium sub-cultured on a 3µm pore transwell cell culture insert (BD, Falcon UK), an adhesion assay was developed. Briefly, 3ml of complete HUVEC medium (as detailed above) was added to 4 wells of a six-well cell culture plate (BD, Falcon UK). To each of the wells, a 3µm pore filter (BD UK) was added.

A confluent flask of HUVEC was trypsinised and resuspended to 8ml; 2ml of this cell suspension was added to the top of each of the filters. The plates were incubated for 24 hours at 37°C at 5% CO2.

The HUVEC were treated with varying concentrations of TNFα (1-500U/ml) alone, or in combination with IFNγ at a concentration of 10ng/ml for 4 or 24 hours. The monolayer was washed gently with 199/BSA to remove any residual TNFα and IFNγ, followed by the addition of 2ml PBL suspension at a concentration of $1x10^6$/ml to the top of the HUVEC monolayer and 3ml 199/BSA in the well. Following incubation, the filters were tapped on the side of the well to cause any lymphocytes that were loosely attached to the back of the insert to fall into the bottom of the well. The lymphocytes that had not migrated through the endothelium and were on the apical surface of the HUVEC were counted by removing the filter insert and placing it into an empty 6-well plate and gently resuspending the lymphocytes with 1ml of 199/BSA. The lymphocytes which had migrated through the endothelium into the lower well were also resuspended and counted. All counts were done using a coulter counter Multisizer II.

2.1.7 Identifying the locations of the PBL within the filter

Analysis of the location of the adherent lymphocytes above and below the filter was carried out. Briefly, the filters were fixed with 2% glutaraldehyde and stained with 1µg/ml bisbenzimide for 15 minutes (protected from light), and washed 3 times in order to wash out any excess fluorochrome from the pores. The filters were cut out directly onto microscope slides and mounted using Vectashield H100 (Vector Laboratories Ltd). Observations were made using UV fluorescent microscopy, by scrolling the stage up and down by approximately 10µm. This allowed us to compartmentalise the adherent lymphocytes into those apical to the filter (i.e. with the endothelium) and those basal to the filter. The total number of adherent lymphocytes were counted in ten different fields and averaged. The average was

converted to cell/mm^2 using the known field dimensions, multiplied by the area of the filter, divided by the total number of lymphocytes added and then multiplied by 100 to express lymphocyte adhesion as a percentage of those added.

2.1.8 Assaying the migration of PBL across LEC after migration across VEC

First passage HUVEC were cultured in complete HUVEC medium on six 3µm pore filters as described above. The HUVEC were cultured for 48 hours at 37˚C in 5% CO_2 to ensure confluent monolayers. The media was then aspirated from the wells and replaced with 3ml of complete HUVEC medium supplemented with 100U/ml TNFα and 10ng/ml IFNγ. The medium contained within the filter inserts was also replaced with the cytokine treated medium. The plate was cultured at 37°C and 5% CO_2 for 24 hours. Following the incubation, the cytokine treated medium was removed and the filters were washed twice using Medium 199/BSA. The medium from the apical surface of the filter was replaced with 2 ml of PBL suspension at a concentration of $1x10^6$/ml and 3ml of medium199/BSA was added to the lower well, the plate was then incubated for 24 hours. 'Control', non-migrated lymphocytes from the same donor were incubated in parallel for 24 hours on cell culture plates.

Following the incubation of the HUVEC with the lymphocytes, the filters were removed and discarded. The lymphocytes which had migrated through the endothelium and filter were pooled and centrifuged at 500g for 5 minutes. The pellet was resuspended to 1ml in Medium 199+BSA and counted using a coulter counter and resuspended to $5x10^5$ cells/ml. Lymphocytes that were cultured on plastic were collected and placed in a 50ml tube and centrifuged for 5 minutes at 500g. The supernatant was removed and the pellet was

resuspended to 1ml, this was then counted using the coulter counter. The suspension was then diluted to give a final concentration of $5x10^5$ /ml.

Lymphatic endothelial cells (LEC) were sub-cultured on the basal surface of a 12 well cell culture transwell filter; this was done so that the LEC would be in the correct orientation as would be found physiologically, i.e the lymphocytes would migrate through the LEC in a basal to apical direction. Briefly, a confluent monolayer of human primary LEC was trypsinised and resuspended as described above. Four 12-well 3µm pore filters were placed in a sterile culture box upside down and 250µl of the LEC suspension was added to the basal surface of the filter (Fig 2.2). The box was sealed and incubated at 37°C for 90 minutes to allow the cells to adhere to the surface of the filter. 1.5ml of MV2 complete endothelial basal medium (LEC medium; Promo Cell UK) was added to the well of a 12 well plate and the filter which had the LEC sub-cultured on the basal surface of the filter was placed into the well. 1ml of complete medium was added to the top of the filter. The plate was then placed in an incubator and cultured for 2 days until a confluent monolayer could be seen.

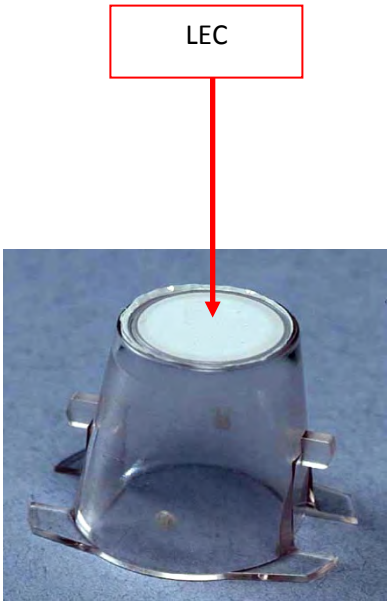


Figure 2.2: *Picture of Transwell cell culture filter. LEC cells were cultured on the basal surface of the filter as shown by the arrow*

Half of the filters bearing LEC were treated with 100U/ml TNFα and 10ng/ml IFNγ for 24 hours and half remained unstimulated. After removal of residual cytokines by washing in culture medium, 1ml of the lymphocyte suspension collected from the wells of the HUVEC plates (i.e. lymphocytes migrated through HUVEC), or the equivalent number of plastic cultured, un-migrated cells was added to LEC bearing filters. After 24 hours, lymphocytes on the apical surface of the monolayer or those that had migrated to the lower chamber were gently resuspended and placed in separate 10ml tubes which were centrifuged, at 500g for 5 minutes. The pellets were resuspended and the number of lymphocytes assessed using a coulter counter. Cells were then phenotyped by flow cytometry (see below).

2.1.9 Standard flow cytometric techniques

All flow cytometry was performed using either an EPICS XL flow cytometer or a DAKO cyan (Beckman Coulter). Levels of expression of conjugated antibody labeled cell markers were determined by FITC, PE, RPE-Cy5, APC and pacific blue excitation using an argon laser. The cytometer calibration was standardised using flow-set fluorospheres (Beckman Coulter).

Analysis of the surface molecules on fresh isolated cells was performed using standard techniques. 1-2x10^5 cells were incubated on ice for 30 minutes with conjugated antibody at pre-determined optimal concentrations in 50µl of PBS containing 2% BSA. Cells were washed twice, and further incubated on ice for 30 minutes with secondary antibody where appropriate, followed by a further two washes. The labeled cells were transferred into FACS tubes, with control tubes containing conjugated or irrelevant antibodies to establish the specificity of the staining. Antibodies were diluted in PBS containing 2% BSA, and the staining was generally performed in Eppendorf tubes. Washing steps were performed by adding 1ml of PBSA to the Eppendorf containing the labeled cells. The cells were centrifuged at 250g for 5 mins. Where 2 or 3 colour-cytometry was performed, single colour samples were also set up to act as compensation tubes for the flow cytometer.

Flow cytometry data was analysed using WinMDI software (version 2.8, Scripps Research Institute, La Jolla, CA). Results are expressed as the median fluorescence intensity (MFI) of antibody labeled cells minus the MFI of cells stained with an irrelevant isotype control.

2.1.10 DiOC6 staining of viable cells

The reduction in mitochondrial transmembrane potential was assessed as an early and irreversible step in apoptosis, measured by reduced incorporation of the lipophilic fluorochrome $DiOC_6$ [278] Lymphocytes (50µL) were incubated with 150µL of $DiOC_6$ (40nM) for 30 minutes at 37^{o}C. The reaction was stopped by the addition of one drop of ice-cold PBS. The samples were centrifuged at 800Xg for four minutes at 4^{o}C and re-suspended in 100µL of ice-cold PBS. This solution was transferred to FACS tubes containing 50µL FCS in 150µL PBS. The samples were analysed using the Coulter XL flow cytometer (Coulter, UK) and WinMDI (Windows Multiple Document Interface) version 2.8 software package (The Scripps Institute, USA) and Summit (Dako Cyan).

2.1.11 mRNA extraction from vascular and lymphatic endothelia

EC were cultured in 24-well plates until confluent. Cells were suspended using 0.02% trypsin/2.5mg/ml EDTA and RNA was isolated from the cell pellets using the Qiagen RNEasy Mini Kit 50 (Qiagen, West Sussex, UK) according to manufacturer's instructions. Briefly, EC were washed in PBS and lysed in the well using 350µl RTL lysis buffer. 350µl of 70% ethanol was added to the cell lysate, and this was vortexed and pipetted onto the supplied columns. The columns were placed in 2ml collection tubes and centrifuged at 8000g for 1 minute. The eluted buffer was discarded and 350µl of RW1 buffer was added to each column and centrifuged at 8000g for 1 minute. 500µl of RPE buffer was added to the column and centrifuged as before, this was repeated with the columns being centrifuged for 2 minutes. The columns were transferred to new 2ml collection tubes and centrifuged at 8000g for 1 minute to remove any

remaining RPE buffer. The columns were then placed in 1.5ml collection tubes and 30µl RNAse free water was added to elute the purified RNA. The purity and concentration of the RNA was determined using a Nano Drop spectrophotometer 2100 (Thermo Scientific, USA).

2.1.12 Real-Time PCR of mRNA from vascular and lymphatic EC

Real-time PCR was performed using QuantiTect™ probe RT-PCR kit according to the manufacturer's instructions (Qiagen, West Sussex, UK). In a 96 well optical reaction plate (Applied Biosystems), 24µl of the following master mix was added: 12.5µl of QuantiTect™ probe master-mix; 0.25µl of QuantiTect™ reverse transcriptase master-mix; 1µl of 20x 18s-VIC labeled (Applied Biosystems); 1µl of primer and 9.25µl of RNA water; along with 1µl of cDNA (5ng/µl). Each sample was run in triplicate. CCL21 FAM labeled primers were bought as an Assay on Demand kit from Applied Biosystems. The plate was covered with optical adhesive covers (Applied Biosystems), and pulse-centrifuged. Samples were amplified using the 7900HT Real-Time PCR machine and analysed using the software package SDS 2.2 (Applied Biosystems).

Triplicate data were averaged and expressed as relative expression units (REU) or relative quantification (RQ) calculated from the threshold cycle (C_T). The C_T value represents the cycle number at which the fluorescence crosses a threshold, normally associated with the exponential production of the PCR product. The threshold was manually adjusted for each sample to ensure that it crossed the log-linear phase of amplification and excluded outlying signals. The more cDNA in the sample, the earlier the product was detected, and the lower the C_T value. The real-time PCR reaction was run for 40 cycles, any genes that had a C_T value >35 cycles were considered to be below the limit of detection and therefore not expressed.

2.2 Results

2.2.1 The effect of cytokine stimulation on the ability of lymphocytes to migrate across HUVEC cultured on a transwell membrane

In order to investigate the ability of lymphocytes to migrate across cytokine treated endothelium cultured on transwell filters, an adhesion assay was developed and validated. The effects of changing a number of different parameters were investigated, which included the duration and pattern of cytokine stimulation and the duration of the migration phase of the assay. The manner in which these manipulations altered the location of the PBLs in the assay, in addition to their viability was assessed (Fig 2.3).

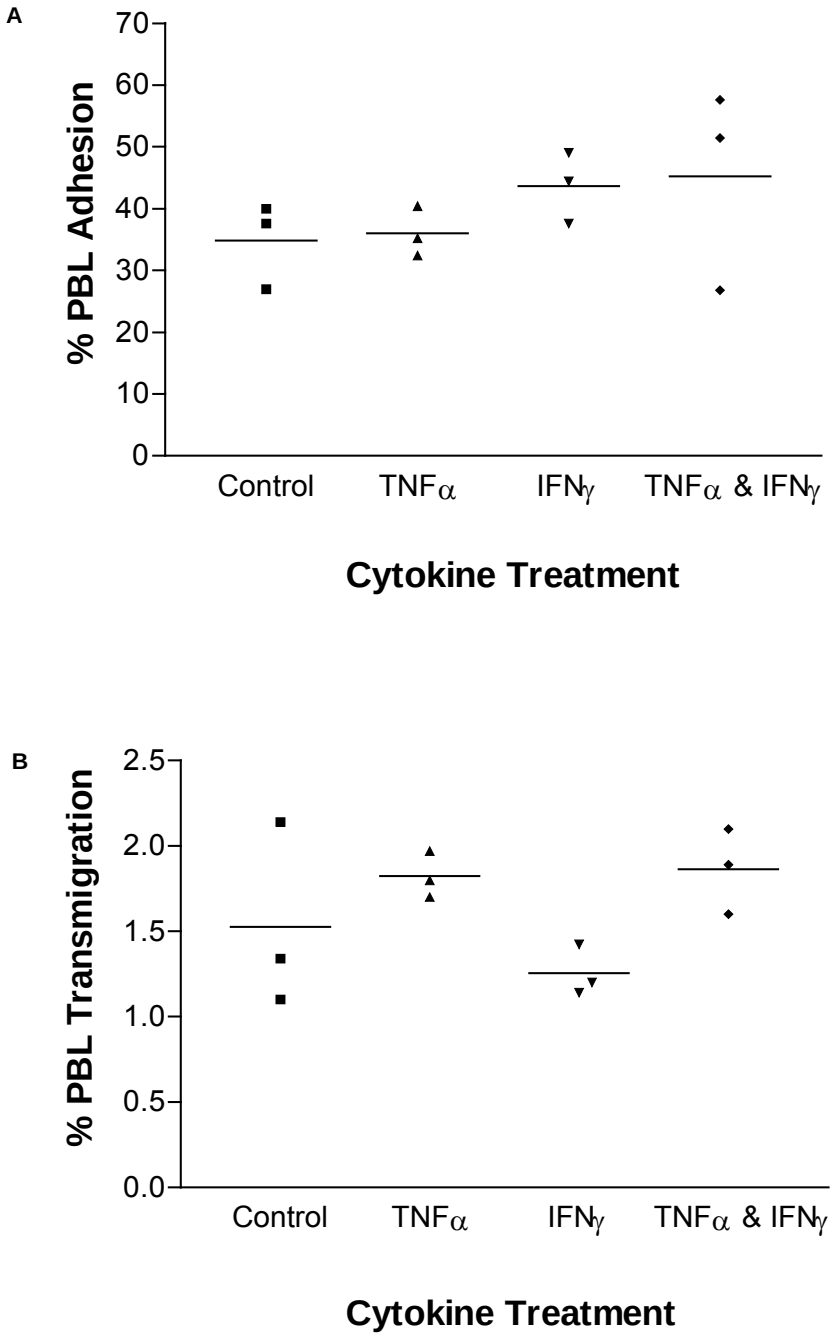


Figure 2.3: Effect of different cytokines on lymphocyte adhesion and transmigration through HUVEC. HUVEC were stimulated with 100U/ml TNFα alone or with 10ng/ml IFNγ for 24 hours. Lymphocytes were allowed to adhere and migrate for 4 hours after which lymphocyte **(A)** adhesion and **(B)** transmigration were analysed. Lymphocyte transmigration was expressed as percentage of PBL added. These data show the mean (horizontal line) from 3 independent experiments using 3 different donors.

When lymphocytes were allowed to migrate across EC for 4 hrs, we observed no significant difference in the level of lymphocyte adhesion to HUVEC that were unstimulated or to those activated for 24 hrs with TNFα or IFNγ, or a combination of these cytokines (Fig 2.3). The number of lymphocytes migrating into the lower chamber was also the same irrespective of the EC treatment (Fig 2.3). However as levels of migration were only about 1% of added PBL (Fig 2.3b), this indicated that the assay was run for an insufficient period to achieve substantial PBL migration.

When PBL were allowed to migrate for 24hr we observed some differences on EC under different regimens of activation. Thus, although we observed no significant difference in the levels of lymphocyte adhesion to EC that were unstimulated or to those activated for 24hrs with TNFα or IFNγ or a combination of these cytokines (Fig 2.4a), we noted that the efficiency of migration was increased in the presence of a combination of TNFα and IFNγ (Fig 2.4b).

As the combination of both cytokines produced the highest levels of transmigration, the regimen used for all subsequent transwell filter experiments was 100 U/ml TNFα and 10ng/ml IFNγ, and the lymphocytes were allowed to adhere and settle on the EC for 24 hours.

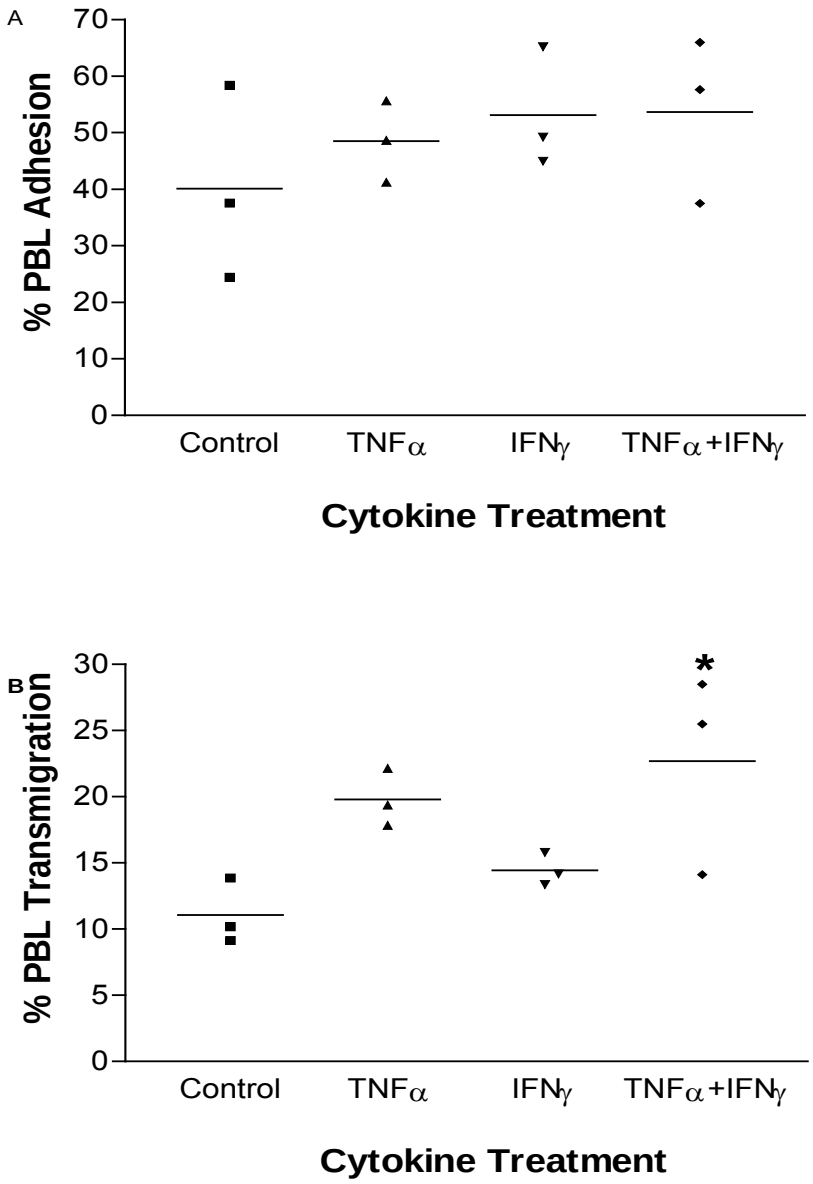


Figure 2.4: Effect of different cytokines on lymphocyte adhesion and transmigration after 24 hours. HUVEC were stimulated with 100U/ml TNFα alone or with 10ng/ml IFNγ for 24 hours. Lymphocytes were allowed to adhere and migrate for 24 hours after which lymphocyte **(A)** adhesion and **(B)** transmigration were analysed. Lymphocyte transmigration was expressed as percentage of PBL added. These data show the mean (horizontal line) from 3 independent experiments using 3 different donors for all cell types. *=P<0.05 when compared to control, Mann-Whitney test.

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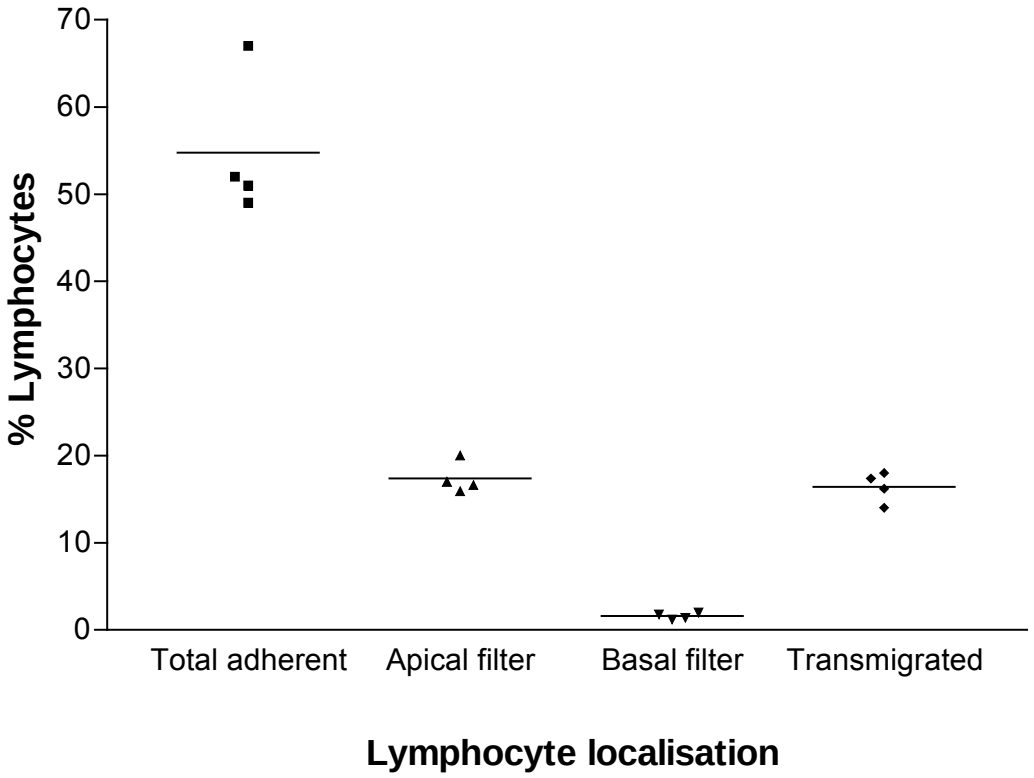


Figure 2.5: Location of lymphocytes within the filter insert. Following lymphocyte adhesion and transmigration through TNFα and IFNγ stimulated HUVEC, the filters were fixed and stained. Lymphocytes were located either apical or basal to the filter, assessed by direct microscope observation. All counts were expressed as a percentage of lymphocytes added. Transmigrated represents the percentage of lymphocytes counted in the lower chamber. 'Total adherent' denotes lymphocytes adherent to the filter or that had transmigrated into the lower chamber as assessed by subtracting the count for the non-adherent population from the total number of lymphocytes added. These data show the mean (horizontal line) from four independent experiments.

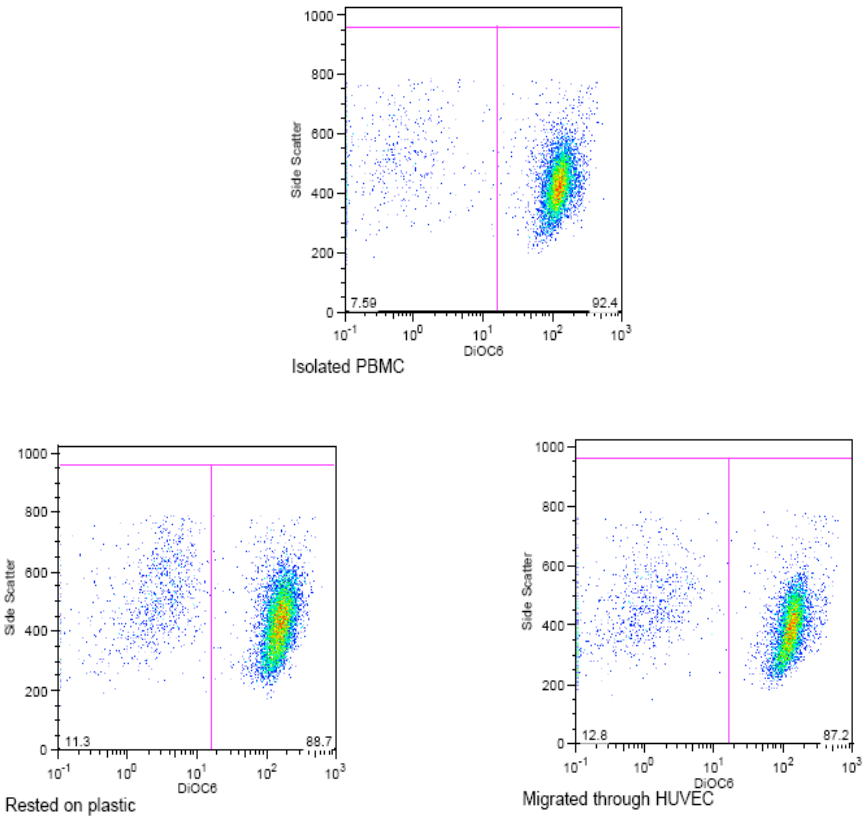


Figure 2.6: PBMC migration through HUVEC and rested on plastic for 24 hours does not affect the viability of the PBMCs. PBMCs were stained with $DiOC_6$ to ascertain the viability of the cells, immediately following isolation (isolated PBMC), following culture on plastic for 24 hours (rested on plastic) and following migration through HUVEC for 24 hours.

Direct visualisation of filters that had been fixed enabled us to determine whether the lymphocytes were located above or below the filter insert. Counting showed that significantly more lymphocytes were adherent on the apical surface of the filter (Fig 2.5). Furthermore, lymphocytes apical to the filter appeared phase dark when we flicked between the UV light and phase contrast at this magnification, supporting the suggestion that they were in a sub-endothelial location. However, of all adherent lymphocytes only approximately 30% were found retained by the filter. Approximately 30% were migrated into the lower chamber leaving a deficit of 30% of the originally adherent cells. This suggests loosely adherent cells were washed off during the staining protocol. It was also interesting to note that some endothelial cells had also migrated through the filter as we saw the presence of large lozenge-shaped nuclei basal to the filter (data not shown).

As the time length of the assay is 24 hours, it was important to verify that cells collected from the lower chamber were viable. In addition, as we wanted to use plastic cultured PBL as non-migrated control lymphocytes it was important to verify that this population was also viable. Thus, when the PBL were collected from the lower chamber following the VEC (Vascular Endothelial Cell) transmigration the PBL were stained with DioC6. We found that the percentage of lymphocytes that were Dioc6 high (i.e. cells were not apoptotic) was consistently above 90% following isolation (Fig 2.6). When cells cultured on plastic or migrated through VEC were subjected to the same protocol, we found comparable levels of Dioc6 positive staining irrespective of the culture conditions.

2.2.2 The effect of transmigration across VEC on the ability of lymphocytes to migrate across LEC

Having identified the optimal conditions for lymphocyte migration across vascular endothelial cells, we went on to determine whether passage across a VEC monolayer had any effect on the efficiency of migration across a LEC monolayer.

Firstly we wanted to confirm the phenotype of the LEC by investigating the expression of key markers which could be used to distinguish between vascular endothelial cells and lymphatic endothelial cells. Podoplanin is a key marker of LEC and is not expressed on vascular endothelial cells, and was found to be highly expressed across all donors of LEC (Fig 2.7A). The expression of podoplanin was very low on HUVEC when compared to the LEC. CD34 is expressed on vascular endothelial cells and low expression on lymphatic endothelial cells. We found that CD34 was highly expressed on HUVEC, and consistently at low levels on LEC (Fig 2.7B). The expression of both LYVE-1 and Prox-1 was barely above detectable levels both in HUVEC and LEC (Fig 2.7C and D).

In addition to the expression of cell surface proteins, we wanted to confirm that the LEC was producing CCL21 mRNA. CCL21 is a chemokine that is highly expressed by LEC and not produced by vascular endothelial cells. The levels of CCL21 mRNA in the LEC samples were very high across all donors (Table 2.1), conversely, CCL21 mRNA in HUVEC were not detectable (Table 2.2).

When PBL which had migrated across VEC, or had been cultured on plastic for an equivalent period were put onto LEC, we observed no significant differences in the numbers adhering to

the monolayer, irrespective of its activation status (i.e. with or without cytokine stimulation) (Fig 2.8A). Interesting differences in the behavior of migrated or plastic cultured PBL were however evident (Fig 2.8B). Thus, non migrated PBLs traversed LEC with an efficiency of approximately 10%, irrespective of whether the monolayer had been activated with cytokine. Interestingly, PBL that had migrated across VEC showed no increase in their ability to traverse LEC in the absence of cytokine stimulation. However, PBL that had migrated across VEC migrated in significantly greater numbers across a TNF/IFN stimulated LEC. These data demonstrate that under inflammatory conditions, lymphocyte passage across VEC primes these cells for the transmigration across a LEC monolayer, or the VEC itself is selecting a more pro-migratory lymphocyte population.

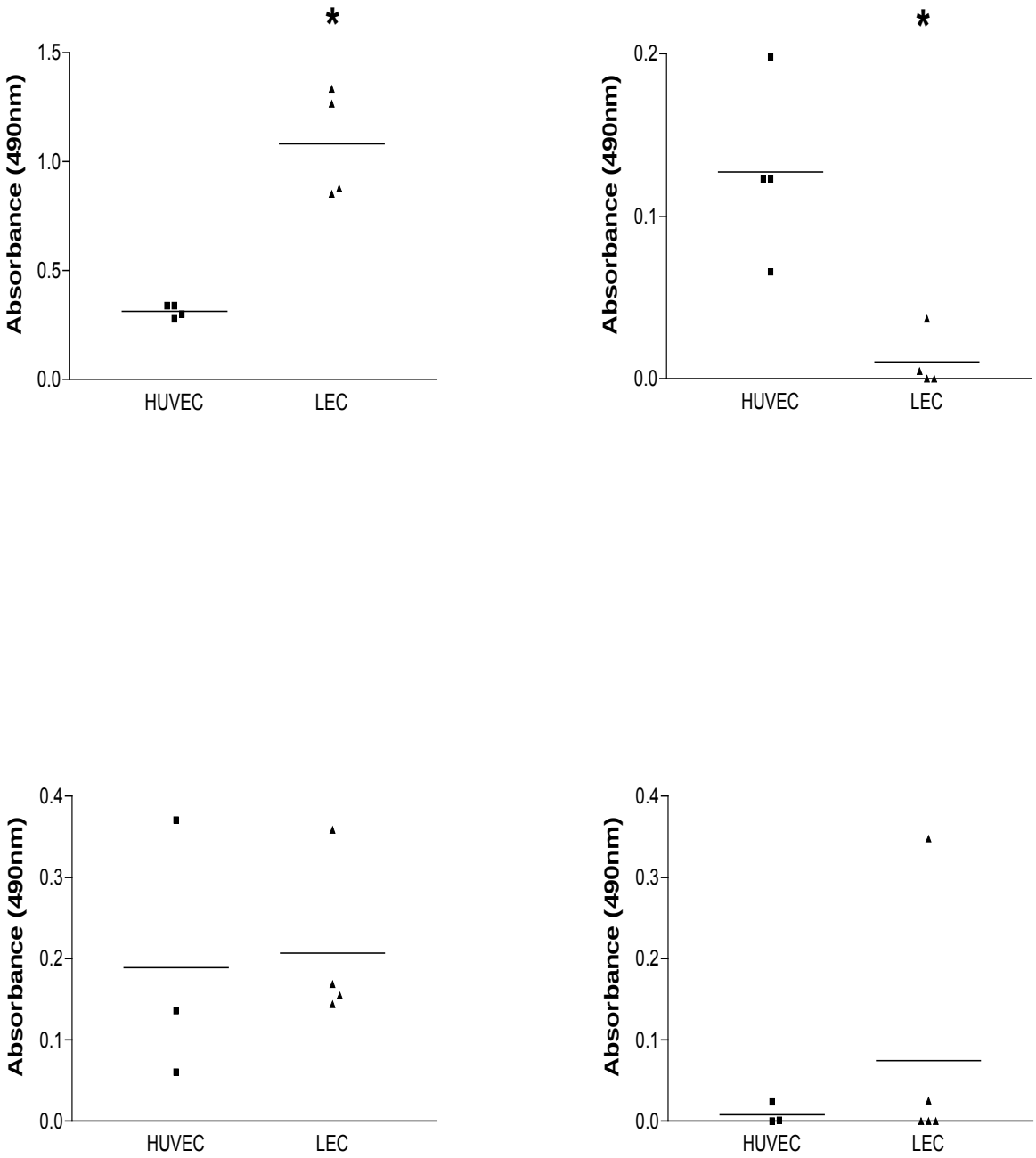


Figure 2.7: Expression of key proteins distinguishes between vascular and lymphatic endothelial cells. HUVEC and LEC were cultured in 96-well plates until confluent and fixed. The expression of podoplanin (A) a marker of LEC, CD34 (B) a marker of VEC, LYVE-1 (C), and Prox-1 (D) a marker of LEC was assessed by ELISA. The data shown is minus isotype control. *P=0.01 compared to HUVEC (A and B) Mann-Whitney test.

Table 2.1

LEC sample	18s Ct	CCL21 Ct	Delta Ct
1	16.144	26.838	10.69
2	13.075	15.341	2.266
3	13.0563	19.959	6.903

Table 2.2

HUVEC sample	18s Ct	CCL21 Ct	Delta Ct
1	16.059	None detected	N/A
2	13.134	None detected	N/A
3	15.604	36.560	20.956

Table 2.1 and 2.2: HUVEC does not express mRNA for the chemokine CCL21. LEC (Table 2.1) and HUVEC (Table 2.2) EC were cultured on plastic until confluent and mRNA was isolated. mRNA obtained from the EC were subjected to Taq-man PCR. The different LEC and HUVEC samples (numbered 1-3) are ECs from different cell donors.

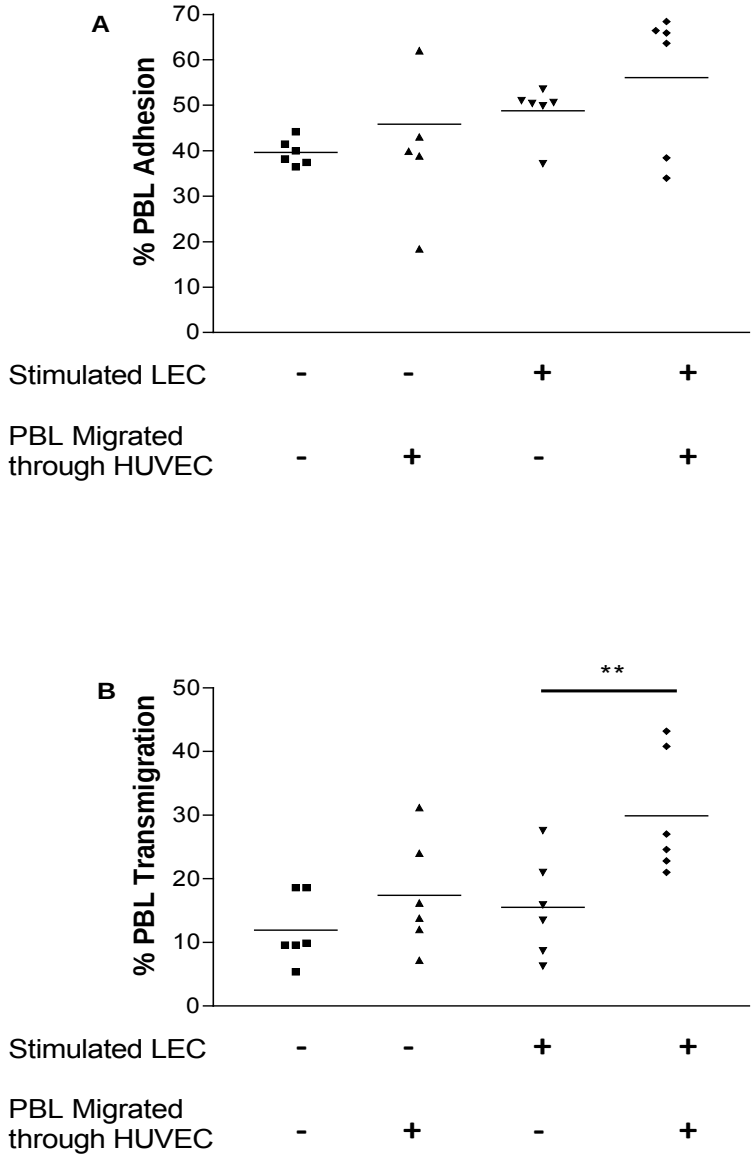


Figure 2.8: The effect of transmigration on the ability of lymphocytes to migrate through a lymphatic endothelial cell monolayer. PBL were migrated through HUVEC and a proportion cultured on plastic. Lymphocytes were added to the top of a LEC monolayer and allowed to adhere and migrate for 24 hours after which lymphocyte **(A)** adhesion and **(B)** transmigration were analysed. Lymphocyte transmigration was expressed as percentage of PBL added. These data show the mean (horizontal line) from 6 independent experiments using 6 different donors for all cell types. **=P<0.001 t-test.

2.2.3 Microvascular endothelial cells are able to condition lymphocytes to migrate across LEC.

So far we have demonstrated that macrovascular cells (HUVEC) are able to condition lymphocytes to transmigrate through a LEC monolayer with increased efficiency. We wanted to investigate if microvascular endothelial cells were also capable of conditioning lymphocytes to transmigrate across LEC with greater efficiency.

To do this we cultured primary human lung microvascular endothelial cells (LMVEC) on the apical surface of a transwell filter. Lymphocytes from healthy donors were added to either stimulated or unstimulated LMVEC to ascertain whether lymphocytes would be able to transmigrate through the LMVEC monolayer, at levels that would enable us to collect and add them on to the top of a LEC monolayer.

When lymphocytes were allowed to migrate across unstimulated LMVEC, the level of transmigration observed was approximately 7% (Fig 2.9). Following cytokine treatment of the LMVEC with TNFα and IFNγ, we observed a dramatic increase in the level of transmigration to above 20%.

Since the level of transmigration of PBL through LMVEC monolayer was high enough to allow us to collect the transmigrated lymphocytes and subject them to the same protocol as carried out in Fig 2.8, we wanted to investigate whether the microvascular cells would be able to condition the lymphocytes to allow enhanced transmigration through a LEC monolayer.

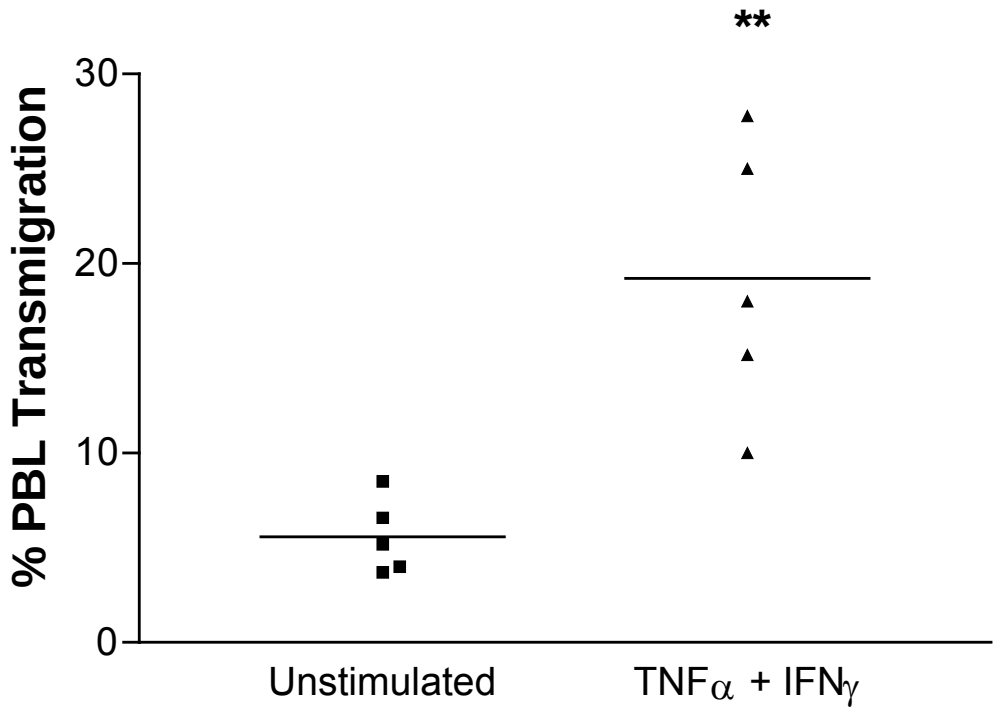


Figure 2.9 PBL Migration through LMVEC monolayer: LMVEC were cultured on the inside of a 3µm pore filter. The monolayer was then stimulated with TNFα (100U/ml) and IFNγ (10ng/ml) for 24 hours. PBL were then added to the top of the monolayer and incubated at 37°C for 24 hours. The lymphocytes were collected from the bottom of the well and counted. The data is expressed as a percentage of the total number of lymphocytes added. These data show the mean (horizontal line) from 5 independent experiments using 5 different donors for all cell types. P=0.001 t-test compared to unstimulated.

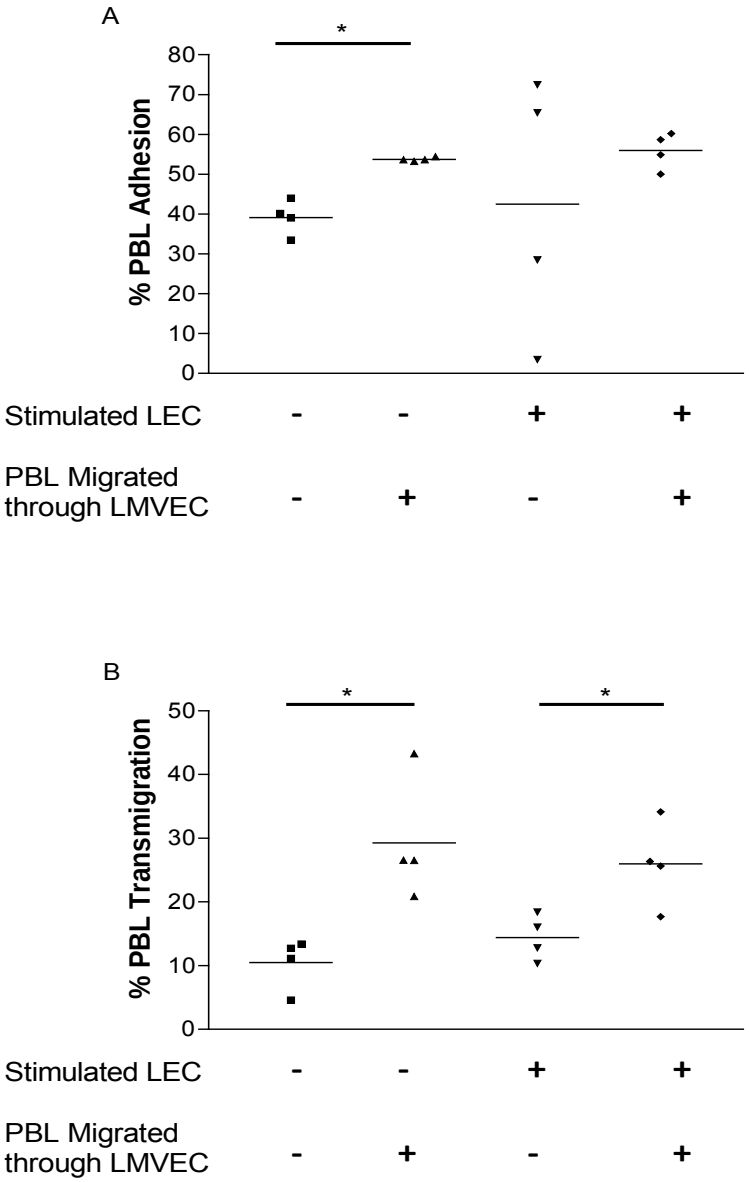


Figure 2.10: The effect of transmigration across LMVEC on the ability of lymphocytes to migrate through LEC. PBL were migrated through LMVEC and a proportion rested on plastic. The lymphocytes were added to the top of either a stimulated or unstimulated LEC monolayer and allowed to adhere and migrate for 24 hours, after which lymphocytes were collected, counted and (A) adhesion and transmigration (B) were assessed. Lymphocyte transmigration was expressed as percentage of total lymphocytes added. These data show the mean (horizontal line) from 4 independent experiments using 4 different donors.*=$p<0.05$, Mann Whitney test.

When PBL that had migrated across LMVEC or had been cultured on plastic for 24 hours were put onto LEC, we observed that a significant number of the lymphocytes had adhered to an un-stimulated LEC monolayer (Fig 2.10A). Cytokine stimulation of the LEC or migration through a LMVEC monolayer did not cause a significant increase in adhesion of the lymphocytes to the LEC. Non-migrated PBLs traversed LEC with an efficiency of approximately 10% similar to that seen with the HUVEC migrated PBL (Fig 2.10B), irrespective of whether the monolayer had been activated with cytokine stimulation. Interestingly, PBL that had migrated across LMVEC showed an enhanced ability to traverse LEC even in the absence of cytokine stimulation. This enhanced migration was not seen with the HUVEC migrated PBL.

2.2.4 Enhanced lymphocyte migration is specific to LEC.

We next wanted to investigate whether the conditioning effect imparted on to the lymphocytes following migration through VEC would enhance transmigration through a second VEC monolayer. The levels of PBL adhesion to a second monolayer of VEC were very similar to those seen adhering to LEC and did not vary significantly whether the PBL had already traversed a VEC monolayer (Fig 2.11) or whether the second monolayer had been stimulated with TNF/IFN. Although not significant, there is a trend towards more efficient migration of PBL across a second VEC monolayer stimulated with TNF/IFN. Importantly however, there is no indication that the previous passage across VEC primes the PBL for passage across a second monolayer of VEC; this indicates that the priming effect observed is exclusive to the LEC.

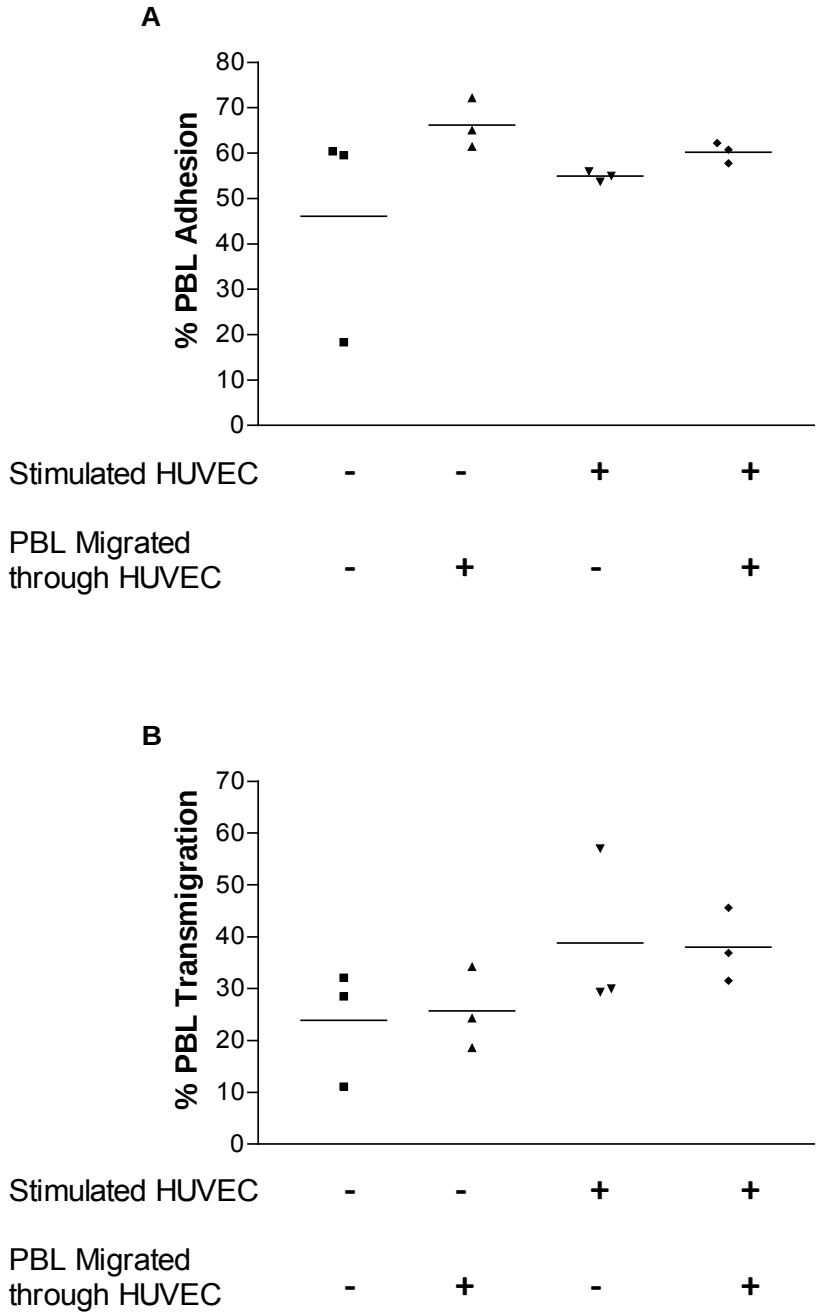


Figure 2.11: The effect of transmigration on the ability of lymphocytes to migrate through two HUVEC monolayers. PBL were migrated through HUVEC and a proportion rested on plastic for 24 hours. The lymphocytes were then added to the top of a second HUVEC monolayer and allowed to adhere and migrate for 24 hours after which lymphocytes were counted and **(A)** adhesion and **(B)** transmigration were analysed. Lymphocyte transmigration was expressed as percentage of total PBL added. These data show the mean (horizontal line) from 3 independent experiments using 3 different donors for all cell types.

2.3 Discussion

Using an *in vitro* transwell filter assay, we have demonstrated that the migration of PBLs across HUVEC and LMVEC conditions the PBLs such that when placed on lymphatic endothelial cells, they are able to migrate through the LEC better than those PBLs that have been cultured on plastic. This effect was seen after migration across both macro-, and microvascular endothelial cells. The conditioning effect was most apparent when cells had migrated through micro-vascular endothelium, as migrated cells could cross LEC more efficiently whether or not the LEC had been stimulated with inflammatory cytokines. We confirmed that this conditioning effect did not increase the migration of PBLs across a second HUVEC monolayer, thus showing that the process improved the efficiency of migration across LEC exclusively.

To confirm the phentotype of the lymphatic endothelium the expression of podoplanin and CCL21 was investigated and found to be highly expressed on LEC and not HUVEC (Table 2.2). In addition, it most be noted that in one of the HUVEC samples tested, we did see expression of CCL21, but due to the high cycle number this is most likely due to genomic DNA contamination. The expression of LYVE-1 on LEC was not detectable. This observation is unusual as LEC have been reported to express LYVE-1 [279]. The lack of LYVE-1 expression could potentially be explained by inadvertently activating the LEC when culturing the EC prior to the ELISA, as it was shown that activation of lymphatic endothelium can cause the uptake and degradation of LYVE-1 on lymphatic vessels [279]. In addition, the lack of Prox-1 expression is also unusual, as the continual expression Prox-1 is required for LEC differentiation and maintenance [280].

However, as the LEC used in all these studies have been passaged, this could have caused a change in the LEC resulting in the reduced expression of Prox-1.

When HUVEC were cultured on transwell inserts, we observed similar levels of PBL adhesion on cytokine stimulated HUVEC compared to that seen on unstimulated EC. This result has been reported previously in such transwell assays. For PBLs to transmigrate they must first become firmly adhered to the EC; this has been shown to occur through the action of chemokines displayed on the EC surface which in turn activate integrins. In fact, it was previously shown that treatment of the EC with IFNγ caused increased adhesion of lymphocytes to HUVEC in assays lasting more than 60 mins [281]. In such assays it has also been shown that treatment of endothelial cells with IFNγ alone caused more adhesion of lymphocytes than that seen with TNFα alone [282]. The variation in adhesion seen when the EC are stimulated with TNFα compared to IFNγ could be attributed to the different chemokine profiles that the two cytokines would cause the EC to produce. However, differential effects of the two cytokines on the production of adhesion molecules on the surface of the EC could also play a role. It has been shown that IFNγ stimulation of HUVEC causes the up-regulation of CXCL10 (IP-10), CXCL9 (MIG) and CXCL11 (I-TAC) [283, 284] which attract T-lymphocytes specifically. The receptor for these IFNγ-inducible chemokines is CXCR3. CXCR3 has been shown in our laboratory to play important roles in the adhesion of lymphocytes to endothelium under flow conditions [42], and it has been shown to play a role in lymphocyte transmigration on liver sinusoidal EC, again under conditions of flow [285]. In agreement with our own observations on HUVEC, others have shown that the efficiency of transmigration was dependent upon the cytokine stimulus [286] and that a potential formation of a gradient of CXCL11 played an important role in T-

lymphocyte recruitment [286]. Indeed, chemokines present at the luminal (apical) surface of the EC appeared to change the levels of leukocyte adhesion but not migration, while chemokines on the abluminal (basal) surface of EC induced transmigration [287]. This would suggest that there is a requirement for a chemokine gradient induced by IFNγ to allow T-lymphocytes to migrate through the EC in our system.

We observed that migration of PBLs through an EC monolayer enhances their ability to migrate through a LEC monolayer, and this conditioning effect seems to be exclusive to passage of a LEC monolayer as migrated PBL added to the top of a second HUVEC monolayer did not demonstrate enhanced transmigration. This observation is contrary to what was observed by Berg et al [288] who demonstrated that recently transmigrated T-lymphocytes migrated through a second HUVEC monolayer faster than non-transmigrated lymphocytes, although the authors used purified memory T cells and not unfractionated PBLs. Purifying the T cell population may result in the selection of a subpopulation of memory T cells that is more motile than a normal mixed population of PBLs.

Although the effect of transmigration across VEC on the phenotype and function of lymphocytes is unclear, it is possible that depending on the type of the endothelium through which the lymphocyte migrates (e.g. macrovascular (HUVEC), microvascular (LMVEC) or HEV), different phenotypic changes may ensue, which aid interaction with the tissue stroma and lymphatic vasculature. In contrast to our lack of understanding of such changes during leukocyte migration during immune surveillance, i.e. in the absence of inflammation, there is a lot of data demonstrating that phenotypic changes occur in lymphocytes under inflammatory

conditions. In fact, it has been shown that lymphocyte migration through cytokine treated HUVEC caused expression of CD69, which leads to the production of IFNγ [289]. Furthermore, leukocytes obtained from synovial fluid from RA patients and bronchoalveolar lavage fluid from asthmatics show altered expression of cell surface markers, such as adhesion molecules e.g. CD11b and ICAM-1. In a further example, Sedgwick et al [290] demonstrated that eosinophils from bronchoalveolar lavage fluid had increased expression of CD11b/CD18 compared to cells isolated from blood. Chemokine receptors have also been shown to be altered following recruitment in to inflamed joints in RA. For example, Brul et al [291] showed that T cells isolated from the synovial fluid showed increased expression of CCR5 and CCR2 compared with their peripheral blood counterparts. In addition, it has been shown that monocytes isolated from the joint display increased expression of CCR5 [290-293]. Similar findings have been reported in animal models of inflammatory renal disease, where increased expression of CCR5 and CCR2 were evident on infiltrating T lymphocytes [294].

ECs are a source of vasoactive and leukocyte stimulatory and inhibitory molecules that can both promote and suppress leukocyte transmigration [283, 295]. With respect to lymphocytes, numerous chemokines can influence the phenotype, by altering integrin expression and prolonged changes in integrin expression or function could well favor enhanced migration into tissue or across lymphatic endothelium as observed here. For example CCL5 has been shown to cause β1 dependent adhesion to extracellular matrix [296]. Interestingly, it has been shown that activated T lymphocytes acquire endothelium derived molecules, including CD31, ICAM-1, α_4 and $\beta_3$integrins during transmigration [297]. This transfer of molecules between EC and

lymphocytes could potentially contribute to adhesive interactions of lymphocytes with the stromal environment, including the LEC.

The conditioning effect observed in our experiments might suggest involvement of molecules expressed at the junctions between adjacent endothelial cells in triggering signaling events leading to transcriptional changes within the migrating lymphocyte, a process reviewed by Rossetti [298, 299]. Hence, the interaction of the EC junctional molecules, such as ICAM-1, ICAM-2, CD31, CD99 and JAM family members, with their leukocyte counter ligands (by homophilic interplay or *via* integrins), may stimulate the induction or regulation of leukocyte adhesion and/or co-stimulatory molecules and proteases. These changes may promote migration of leukocytes through the vessel wall, as well as aid their cellular functions in the extracellular tissue. With respect to lymphocytes, there is much known about integrin-mediated control of expression of genes, which leads to phenotypic and functional changes following engagement of EC. For instance, this process has been shown to regulate the function of the transcription factor AP-1 [288, 300]. In addition transcriptionally-induced expression of the costimulatory molecules CD80 and CD86 has been described in human CD4 T lymphocytes following contact with EC [301]. This would suggest that the EC itself is important in priming the T lymphocyte for co-stimulation by APCs within the lymph node. Importantly, CD31 is a key regulator of leukocyte function, both during and after transmigration through the EC [302]. In addition to its adhesive role, CD31 is an important signaling molecule, that when engaged in homophilic interactions on the surface of migratory leukocytes, can lead to activation of β_1 [303], β_2 [304] and β_3 [305]integrins. Also, CD31 is capable of inducing mobilisation of integrins to the membrane, as exemplified by its effects on integrin $\alpha_6\beta_1$ in neutrophils [92]. CD31

engagement on leukocytes also leads to other changes that could explain the enhanced migration observed in our studies, such as induced expression of matrix metalloproteinase-9 (MMP-9) [306], and enhanced leukocyte directionality of migration and motility [307], both of which could allow the lymphocytes to migrate through the LEC with greater efficiency. Taken together, migration of the PBL through HUVEC or LMVEC appears to cause alterations in leukocyte phenotype. These changes may be mediated by complex signaling and/or gene expression programs, which collectively lead to fundamental changes in the repertoire and distribution of the cell surface and intracellular molecules that change the capacity of the cells to interact with their stromal environment, including the lymphatic vasculature.

Further, it is possible that migration through a VEC monolayer causes an upregulation of a lymphocyte chemokine receptor which causes the enhanced migration through the LEC. It has been shown that LEC constitutively secretes CCL21 [114], and an upregulation of the chemokine receptor CCR7 would account for the enhanced transmigration. This mechanism would also explain our finding that the enhanced migration across LEC is not recapitulated, when migrated PBLs are added onto a second HUVEC monolayer, as these cells do not synthesise the CCR7 ligands CCL19 or CCL21 therefore not permitting lymphocyte migration through the LEC. We test this hypothesis in greater detail in chapter 4.

Another factor that could account for the enhanced migration across LEC is the preferential recruitment of a sub-set with an inherent enhanced capacity for efficient migration across LEC. In other words, there is in fact no priming during the passage across VEC just selective recruitment of a highly migratory fraction of the added lymphocytes. Indeed similar results

have been previously reported by Mackay (1992), who showed that during inflammation the endothelium can specifically recruit a subset of memory T lymphocytes. Again, we will deal with this hypothesis in chapter 3.

CHAPTER 3

Vascular endothelium selectively recruits lymphocyte subsets

3 Introduction

Lymphocyte trafficking through the body plays a major role in the physiology of the immune system. There is a general consensus that the extent to which lymphocytes participate in the process of recirculation is not random [308, 309]. It has been shown that naïve T lymphocytes selectively migrate to secondary lymphoid organs and memory T lymphocytes that have encountered their specific antigen, preferentially shuttle through non-lymphoid tissue [308]. This observation could account for the enhanced migration through LEC, as memory T cells may be responsible for the increased cell numbers we have observed.

Lymphocytes circulating in the blood enter secondary lymphoid tissue *via* specialised post – capillary venules with morphologically distinct high cuboidal endothelial cells, the high endothelial venules (HEV) [310]. Lymphocytes then return to the blood *via* the efferent lymphatics and thoracic duct. Lymphocyte extravasations across this and other vascular beds is a multi-step process which is, by and large, mediated by specific interactions of endothelial cell adhesion molecules (CAM) with their leukocyte ligands [311], followed by leukocyte activation by chemokines. HEV have been shown to express specific adhesion molecules that have been designated as vascular addressins and these addressins selectively bind subsets of circulating lymphocytes *via* their "homing receptors", thereby mediating the first step in lymphocyte migration across the HEV wall [312]. The peripheral lymph node (PLN) addressins PNAd [313] specifically target the homing of lymphocytes into peripheral lymphoid tissue by binding the peripheral homing receptor L-selectin, expressed on circulating lymphocytes [314]. HEV in mucosa-associated lymph nodes specifically express the mucosal addressin, MADCAM-1 which

targets the homing of another lymphocyte subset, which expresses the mucosal homing receptor α4 β7-integrin, into mucosal lymphoid tissue [315, 316]. Although these addressins confer some specificity on the identity of the recruited cells, downstream activation by chemokines is also important. Thus, for entry into secondary lymphoid tissue CCL19/21 and CXCL12 appear to confer an additional filter of specificity for entry into lymph nodes *via* the HEV.

During inflammation, certain endothelial adhesion molecules may also serve as specific addressins on post capillary venules in non-lymphoid tissue and could cause the recruitment of distinct lymphocyte subsets into inflamed tissue [317, 318]. However it has been shown that preferential expression of specific adhesion molecules does not automatically lead to tissue-specific migration [318]. This would indicate that additional intercellular interactions between endothelial cells and circulating lymphocytes must therefore be involved in lymphocyte recruitment into peripheral tissue or into the lymphatic circulation. Again, these could be based on expiration of endothelial cell chemokines and the profile of counter receptors borne by lymphocyte subsets. There is a large body of data that shows that lymphocytes present at sites of chronic inflammation are predominantly of the memory phenotype as characterised by their expression of CD45R isoforms and their expression of CD44, LFA-1 and ICAM-1 [308]. This is taken as evidence that memory T cells acquire an intrinsic migratory capacity to migrate into non-lymphoid organs [319, 320]. Therefore, memory T cells have been considered to be the major lymphocyte population which routinely migrates through un-inflamed non-lymphoid tissue in order to perform immune surveillance functions.

In order to investigate whether the cytokine-treated EC would specifically recruit memory T-cells and whether their reported inherent migratory capacity allow them to migrate across a LEC monolayer with greater efficiency than non-migrated T cells, we wanted to phenotype the cells that were migrating through the VEC monolayer, and finally through the LEC monolayer.

3.1 Materials and Methods

3.1.1 Isolation of lymphocytes

Lymphocytes were isolated from whole blood as detailed in section 2.1.1

3.1.2 Culture of HUVEC and LMVEC

Endothelial cells were isolated as detailed in section 2.1.5, 2.1.4. LMVEC were purchased from Promo Cell (UK) and used at passage 5-8.

3.1.3 Sub-culturing endothelium

EC was sub-cultured onto trans-well filters as detailed in 2.1.5

3.1.4 Assaying the migration of lymphocytes across LEC after migration across VEC

The assay was performed as described in section 2.1.8

3.1.5 The isolation of untouched CD4 memory T lymphocytes

CD4+ memory T lymphocytes were isolated using a negative selection kit (Miltenyi Biotech) and the manufacturer's protocol was followed; briefly, PBMC was isolated as detailed in 4.2.1. The PBMCs were counted and resuspended to 10^7 PBMC in 10 ml of isolation buffer. The PBMCs were centrifuged at 300Xg for 10 minutes. The supernatant was aspirated and the pellet was resuspended in 40µl PBS with CD4 memory T cell antibody cocktail (Miltenyi Biotech, Surrey), and incubated in the fridge for 10 minutes. Following the incubation, 30µl of isolation buffer and 20µl of Anti-Biotin Microbeads were added and gently mixed by pipette. The cell suspension was incubated in the fridge for an additional 15 minutes. The cells were then

washed by the addition of 3ml of buffer and centrifuged at 300g for 10 minutes. The supernatant was completely aspirated and 500µl of isolation buffer was added.

A LS MACs column was placed into a MACS separator and was primed by adding 3ml of isolation buffer to the top of the column, and the buffer was allowed to flow through the column, which was collected and discarded. The cell suspension was then added to the top of the column and was collected in a 10ml tube. A further 3ml of the isolation buffer was added to the top of the column; this was repeated twice. The purified memory T lymphocytes were then centrifuged at 400g for 5 minutes and counted. Cell purities of >95% only, were used in experiments.

3.2 Results

3.2.1 Investigating the phenotype of the lymphocytes which have transmigrated through the HUVEC monolayer

To investigate the phenotype of the lymphocytes that were migrating through a cytokine treated HUVEC monolayer on a transwell filter, an adhesion assay was performed. The lymphocytes that had migrated into the bottom of the well were stained for expression of CD4, CD8 and CD45RA (a marker of naïve lymphocytes).

When lymphocytes were isolated from whole blood and stained for phenotypic cell surface markers, it was observed that the proportion of CD8 naïve cells does not significantly reduce following migration across a HUVEC monolayer, although this decrease was not significant, a trend towards a decrease was observed (Fig 3.1A). Conversely there was a significant increase in the percentage of CD8 memory cells (Fig 3.1B), which transmigrated across the HUVEC. We also observed a significant decrease in the percentage of naïve CD4 lymphocytes which had transmigrated through the HUVEC and into the bottom of the well (Fig 3.1C), with a concomitant increase in the proportion of CD4 memory lymphocytes (Fig 3.1D). Indeed, CD4 memory cells made up more than 80% of all CD4+ lymphocytes that migrated across BVEC.

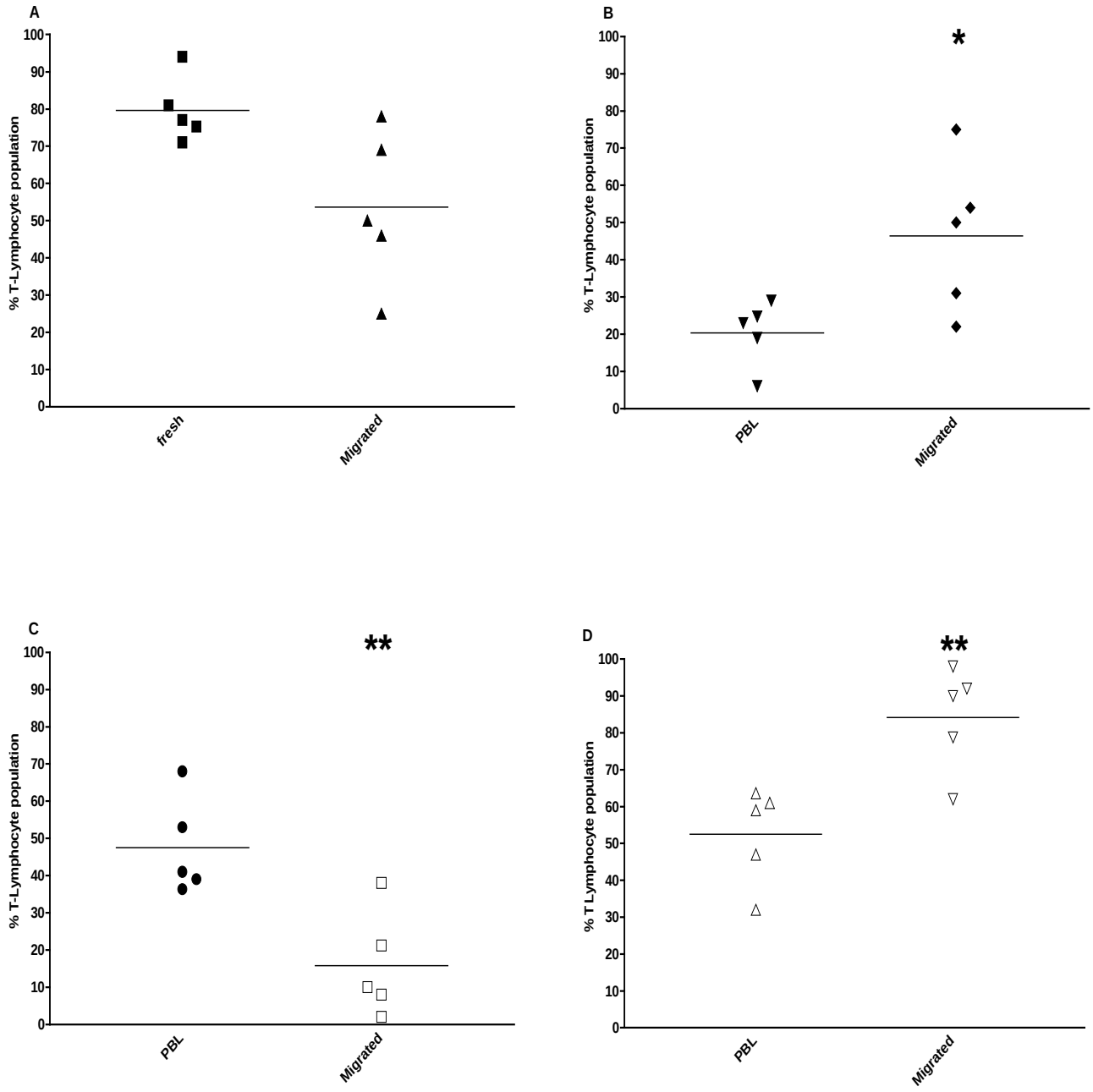


Figure 3.1 Lymphocyte migration through a HUVEC monolayer enhances memory lymphocyte populations. Lymphocytes were isolated from whole blood (PBL) then added to the top of a stimulated HUVEC monolayer activated with 100U/ml TNFα and 10ng/ml IFNγ (Migrated). The lymphocytes, which had migrated were stained for CD8 naive (A), CD8 memory (B), CD4 Naive (C) and CD4 Memory (D). These data show the mean (horizontal line) from 5 independent experiments. t test *P<=0.05 or **P<0.001 Migrated compared to PBL.

3.2.2 Investigating the phenotype of the lymphocytes which have transmigrated through the LMVEC monolayer

Having shown that migration through a cytokine treated HUVEC monolayer recruits memory cells and causes a reduction in the naïve cell population, we wanted to investigate if micro-vascular EC (LMVEC) would also show similar patterns of recruitment. These experiments essentially showed a similar pattern to those conducted on HUVEC with only minor changes in the proportions of CD8, CD4 memory and naïve cells (Fig 3.2).

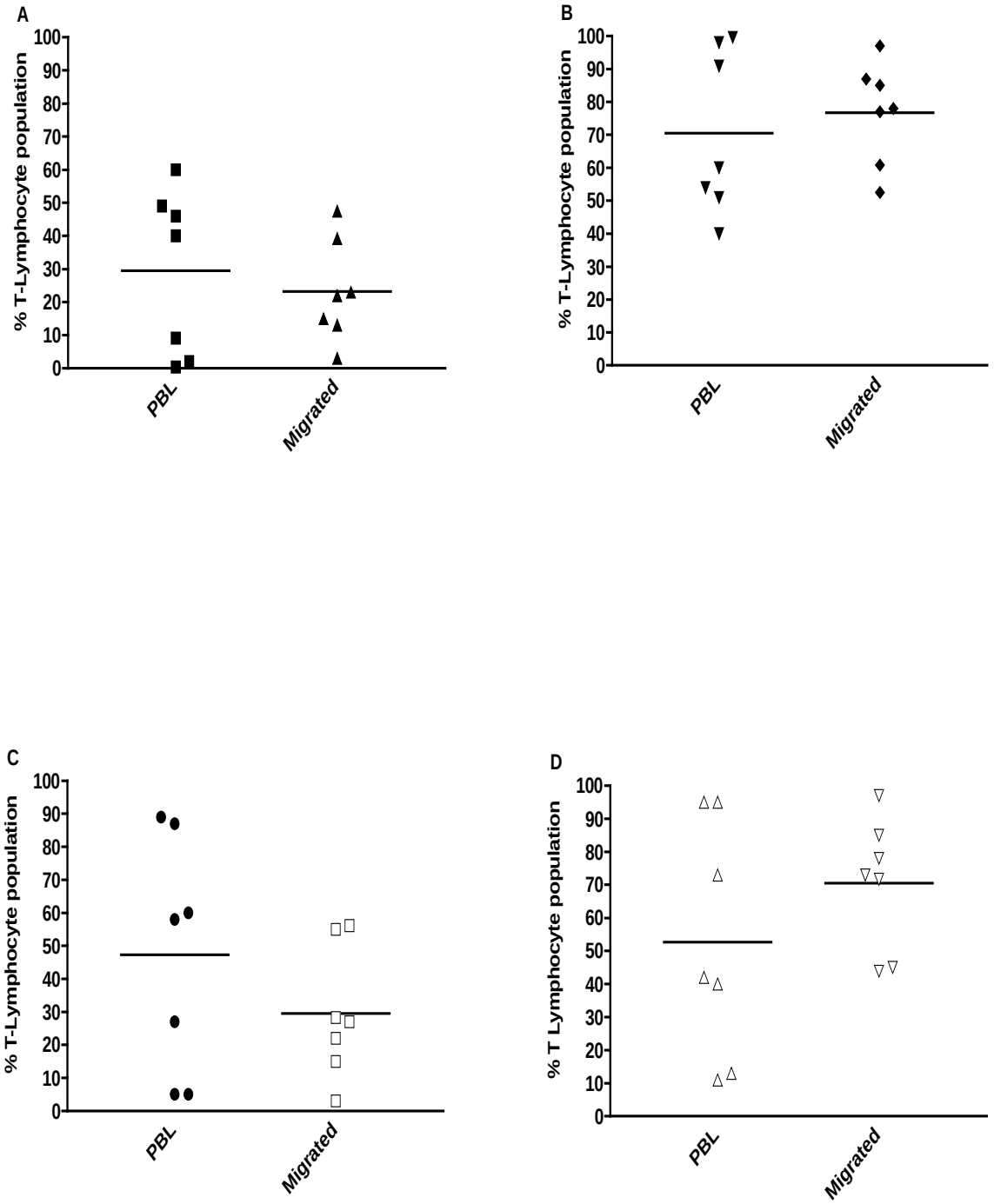


Figure 3.2 Lymphocyte migration through a LMVEC monolayer enhances both CD8 and CD4 memory lymphocyte populations. Lymphocytes were isolated from whole blood (PBL), and then added to the top of a stimulated LMVEC monolayer activated with 100U/ml TNFα and 10ng/ml IFNγ (Migrated). The lymphocytes which had migrated were stained for CD8 naive (A), CD8 memory (B), CD4 Naive (C) and CD4 Memory (D). These data show the mean (horizontal line) from 7 independent experiments.

3.2.3 Investigating the phenotype of the lymphocytes which have transmigrated through the HUVEC monolayer and then through A LEC monolayer.

Having demonstrated that an activated vascular endothelium recruits memory T lymphocytes in preference to naïve lymphocytes, we wanted to investigate the possibility that the LEC also shows preference to a specific subset of lymphocytes. To this end, the adhesion assay described in 2.2.3 was carried out, and the lymphocytes which migrate through LEC collected and phenotyped.

Firstly we observed the migration of lymphocytes that had been cultured on plastic for 24h as a control for endothelial cell-migrated lymphocytes. It is important to note that the proportional distribution of subsets in this preparation reflect those found in the peripheral blood. When lymphocytes which had been cultured on plastic were put on to LEC, there was an increase in memory CD8 lymphocyte populations able to traverse the LEC. This occurred on LEC which were not stimulated with cytokines (Fig 3.3A), and a very similar pattern was evident when EC had been stimulated for 24h with cytokines (Fig 3.3C), although consistent between donors these observations were not statistically significant. This has not been investigated previously, but it appears that LEC also have a small capability to select for leukocyte subpopulations which are permitted to transit the monolayer. It is important to bear in mind of course, that the BVEC operate as a pre-filter for these populations and we investigated this in the next experiment. Thus, when we put HUVEC experienced lymphocytes on LEC, i.e. enriched for CD8 and CD4 memory cells (fig 3.3B and 3.3D), we found that the LEC could further enrich for CD8 memory, such that CD8 naïve cells were almost completely excluded. Interestingly, for CD4 lymphocytes

no such further enrichment was evident, and LEC permitted migration of cells so that the transmigrated population of CD4 cells reflected the population added to the surface of the LEC(fig 3.3A-D). These patterns of lymphocyte migration were similar whether or not the LEC were stimulated with cytokines. It is however important to note that the observations were not statistically significant.

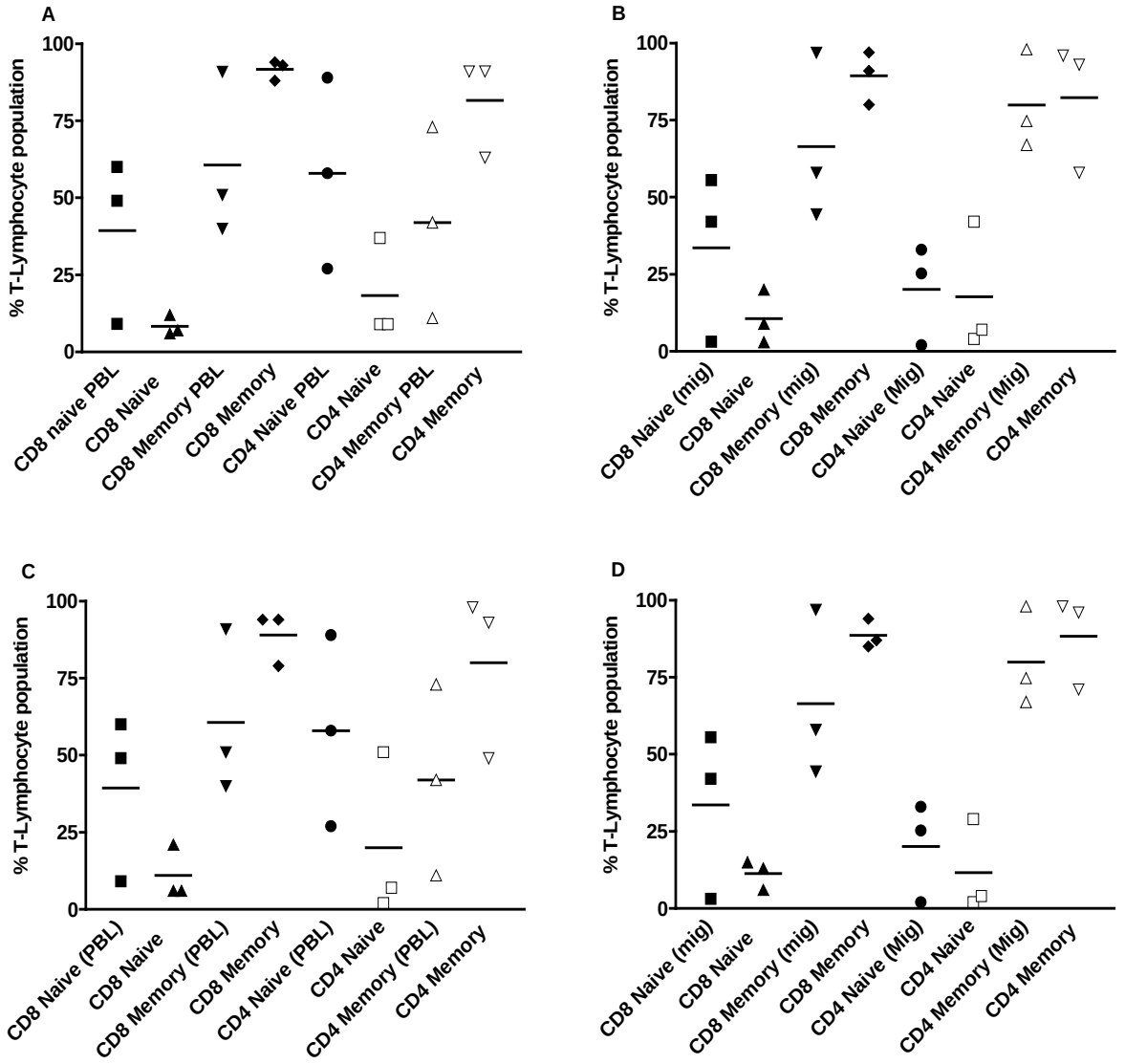


Figure 3.3 The effect of migration across HUVEC and across LEC: (A) Lymphocytes that had been cultured on plastic (PBL) and added to an un-stimulated LEC monolayer were collected and phenotyped following migration and are compared to subset frequency in peripheral blood (PBL); (B) Lymphocytes that had migrated through a HUVEC monolayer (mig) were added to the top of an un-stimulated LEC monolayer were collected and phenotyped after migration and are compared to subset frequency in peripheral blood (PBL).; (C) Lymphocytes which had been cultured on plastic (PBL) were added to the surface of cytokine stimulated LEC and phenotyped after migration and are compared to subset frequency in peripheral blood (PBL); (D) lymphocytes which had migrated through a HUVEC monolayer (mig) were added to the surface of cytokine stimulated LEC and phenotyped after migration. These data show the mean (horizontal line) from 3 independent experiments.

3.2.4 Investigating the phenotype of the lymphocytes which have transmigrated through LMVEC monolayer and then through a LEC monolayer

Since we have demonstrated that lymphocytes that have migrated across a vascular endothelium recruit specifically memory lymphocytes, and placing these cells on to LEC further selects for memory CD8 lymphocytes to a small degree, we wanted to investigate if migration of lymphocytes across a micro-vascular endothelium would cause a different pattern of migration across a LEC monolayer. An adhesion assay was performed as described in 2.2.3.

Firstly, we observed the migration of lymphocytes that had been cultured on plastic for 24 hrs as a control for endothelial cell-migrated lymphocytes. It is important to note that the proportional distribution of subsets in this preparation reflect those in peripheral blood. When lymphocytes which had been cultured on plastic, were put on to LEC, we did observe an increase in CD8 memory lymphocytes able to traverse the LEC, although this change was not significant. This occurred on LEC which were not stimulated with cytokines (Fig 3.4A), and a similar pattern was evident when EC had been stimulated for 24 hours (Fig 3.4C). As seen with lymphocyte migration through HUVEC we found there was consistently lower level of naïve lymphocyte able to migrate through the LEC (Fig 3.4A-D), seen in all culture conditions investigated. In addition, as seen with HUVEC experienced lymphocytes we found that the LEC was recruiting both CD8 and CD4 memory lymphocytes; again, this was true regardless of the activation state of the LEC (Fig 3.4A-D). It is however important to mention that these observations although consistent between donors were not statistically significant.

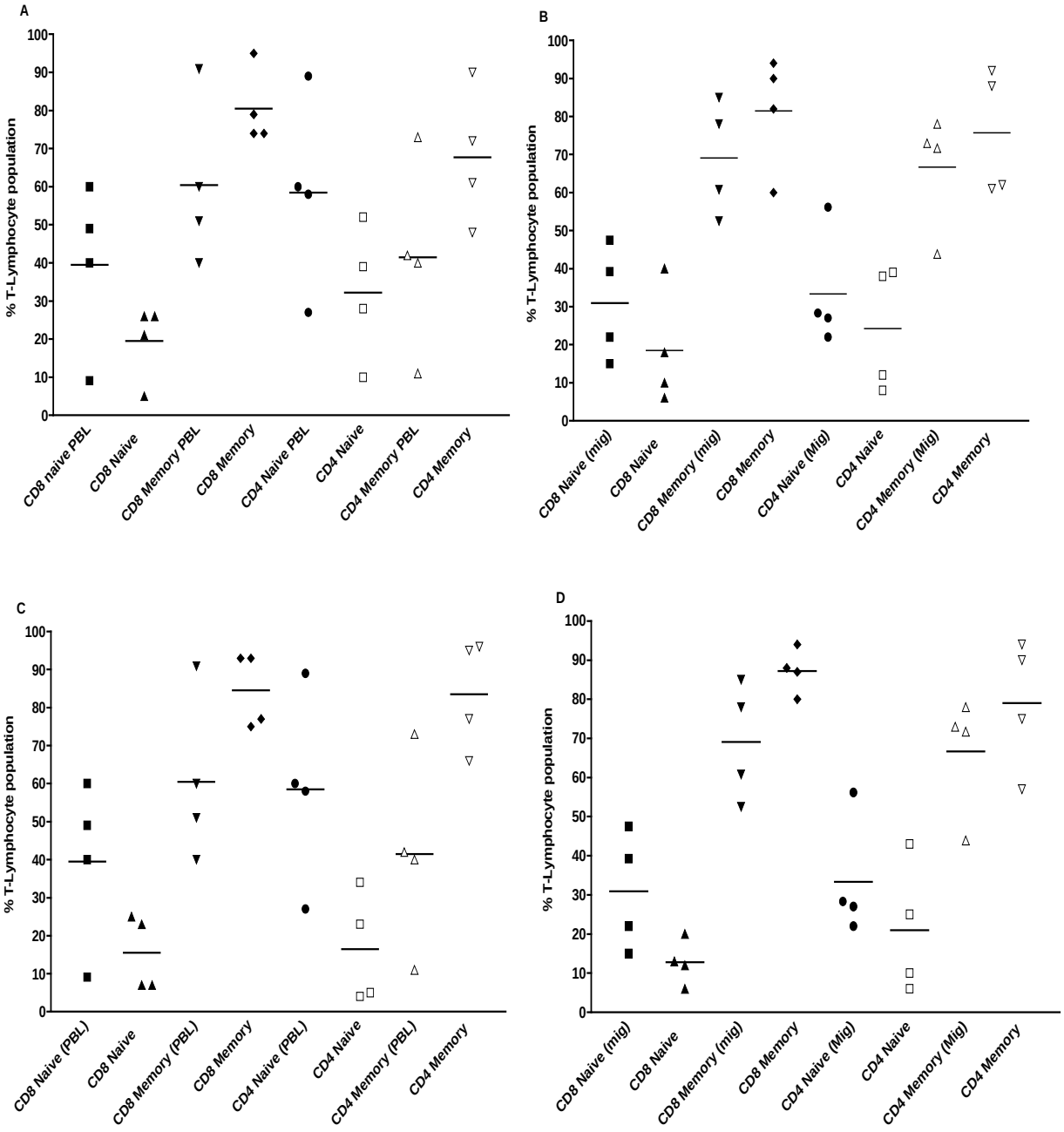


Figure 3.4 The effect of migration across LMVEC and across LEC: (A)Lymphocytes which had been cultured on plastic (PBL) and added to an un-stimulated LEC monolayer were collected and phenotyped following a 24 hour incubation.; (B) Lymphocytes that had migrated through a LMVEC monolayer (Mig) were added to the top of an un-stimulated LEC monolayer were collected phenotyped following a 24 hour incubation; (C) Lymphocytes which had been cultured on plastic (PBL) were added to the surface of a stimulated LEC monolayer were collected and phenotyped following a 24 hour incubation;(D) Lymphocytes which had migrated through a LMVEC monolayer (Mig) and added to the surface of a stimulated LEC monolayer were collected and phenotyped following a 24 hour incubation. These data show the mean (horizontal line) from 4 independent experiments.

3.2.5 Does increasing the duration of the transmigration assay change the lymphocyte subsets recruited across HUVEC?

Having demonstrated that a stimulated HUVEC monolayer preferentially recruits memory lymphocytes after 24h incubation, we wanted to investigate if lymphocytes added to a stimulated HUVEC monolayer and allowed to migrate for 24, 48 and 72 hours would allow other subsets to migrate across the HUVEC monolayer. This would also allow us to exclude the possibility that memory lymphocytes are inherently more migratory and thus explaining the enrichment of this population through the endothelium.

When the lymphocytes were allowed to migrate across a stimulated HUVEC monolayer for 24 hours, we found that the biggest population of lymphocytes was the CD8 (35%) and CD4 memory (46%) (Fig3.5B and 3.5D). When the time allowed for lymphocytes migration across HUVEC was increased to 48 hours, there was a significant decrease in the CD8 naïve lymphocyte population from 16% to 5% (Fig3.5A) and a significant increase in the CD8 memory population (Fig3.5B). In addition, a significant decrease in the CD4 naïve lymphocyte population was seen (Fig3.5C). The CD4 memory lymphocyte population did not significantly change, irrespective of the duration lymphocytes were allowed to migrate across the HUVEC. When the time length of the assay was increased to 72 hours, we observed similar changes at 48 hour time points, any changes in the sub populations recruited, occurred after 48 hours and did not alter when the length of the assay was increased (Fig3.5A-D). In essence, there was a greater enrichment of memory cells as more of these were recruited with even greater efficiency over a 48h duration, while naïve cells still failed to recruit after these extended periods.

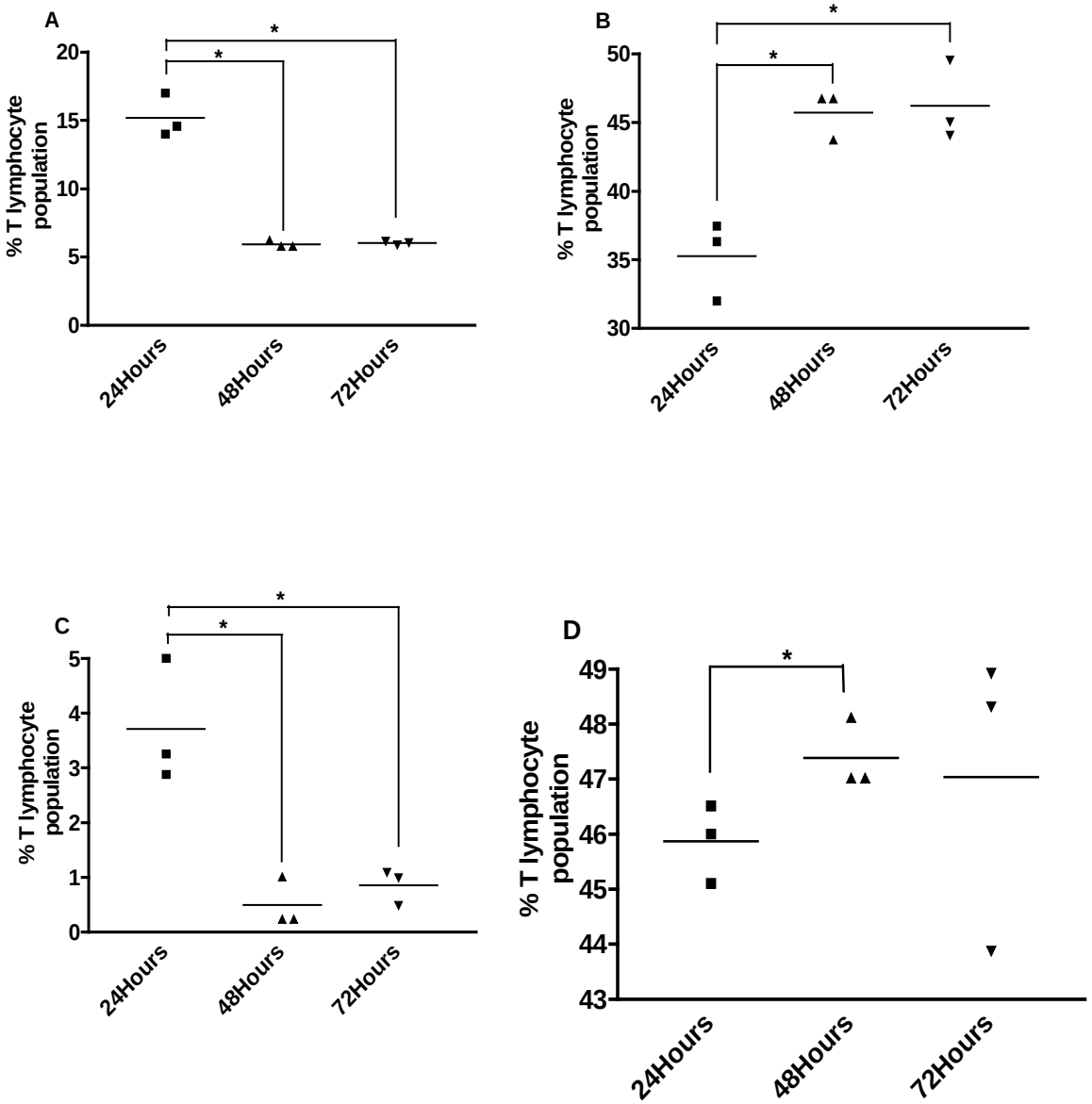


Figure 3.5 Extending lymphocyte migration across HUVEC recruits more CD8 memory T lymphocytes. Lymphocytes were isolated and added to the top of a stimulated HUVEC monolayer for time period of 24, 48 and 72 hours, after which the lymphocytes were assessed for their phenotype CD8 naïve (A), CD8 memory (B), CD4 Naïve (C) and CD4 memory (D). These data show the mean (horizontal line) from 3 independent experiments. *=P<0.05 Mann-Whitney test.

3.2.6 Purified CD4 memory T lymphocytes show enhanced transmigration across LEC following migration through HUVEC

We demonstrated that when lymphocytes migrate across a cytokine stimulated HUVEC or LMVEC monolayer, they acquired the ability to transmigrate across a LEC monolayer with greater efficiency than lymphocytes that were cultured on plastic. We found that the majority of the lymphocytes migrating across the activated vascular endothelium were of the memory phenotype. Thus, we then wanted to investigate if the enhanced migration we had seen across LEC was simply due to the memory lymphocytes having an inherently superior capacity to migrate across LEC compared to naïve lymphocytes. To this end, we negatively isolated CD4 memory T cells and subjected them to the same adhesion protocol as described in 2.1.8.

When CD4 memory T lymphocytes that had been cultured on plastic were added to an un-stimulated LEC monolayer (Fig 3.6) the level of transmigration was very similar to what was observed previously with PBLs. Memory lymphocytes which had previously migrated through a VEC did show some enhanced transmigration though an unstimulated LEC, but this was not significant. When the LEC was stimulated and memory lymphocytes which had migrated through a VEC monolayer were added to the LEC, we found that the level of transmigration was higher than any other condition, although not statistically significant. This would suggest that the VEC are influencing the lymphocytes in some way to allow faster migration through the LEC and the greater efficiency of migration of this population is not an intrinsic property of CD4 memory lymphocytes.

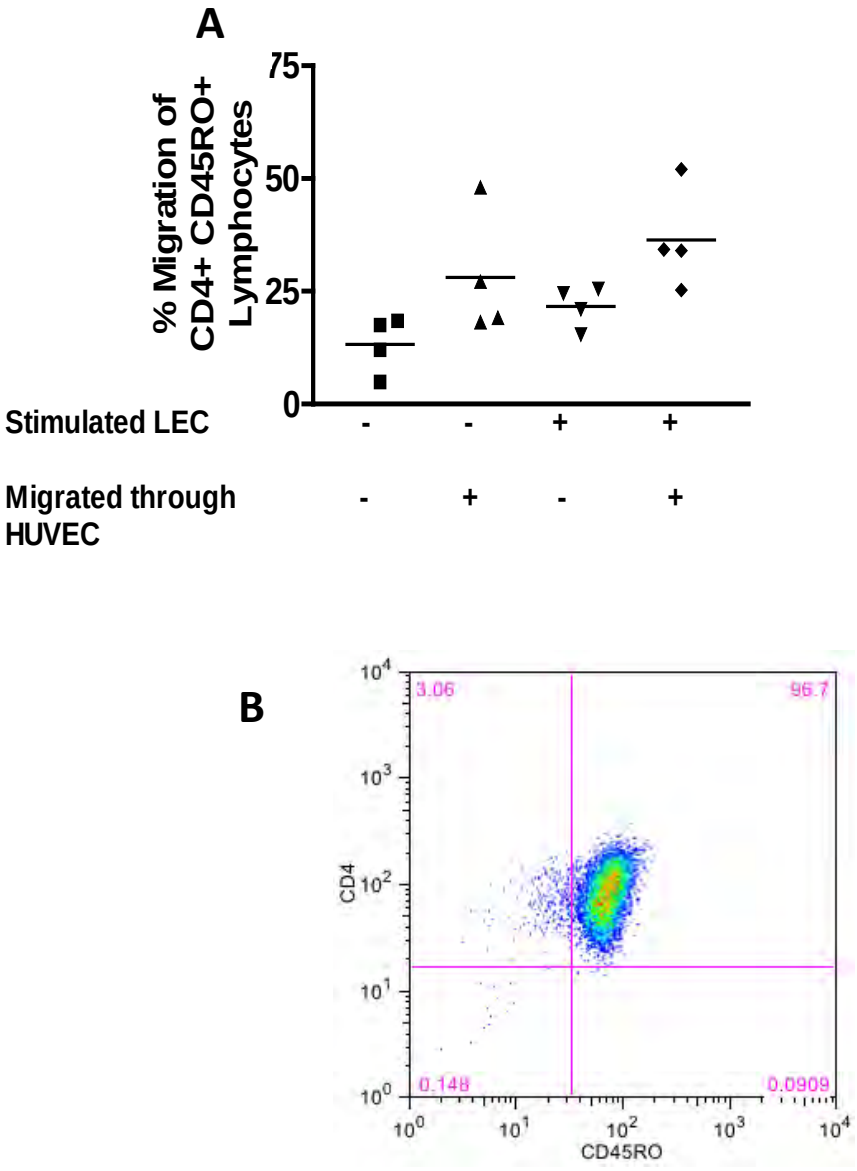


Figure 3.6: CD4 memory T cells show enhanced recruitment to LEC following migration through HUVEC. Negatively isolated CD4 memory T cells were either migrated through HUVEC or rested on plastic (A). Lymphocytes were added to the top of a LEC monolayer and allowed to adhere and migrate for 24 hours after which lymphocyte transmigration was analysed and expressed as percentage added. These data show the mean (horizontal line) from 4 independent experiments. (B) Representative dot plot of isolated CD45RO lymphocytes.

3.3 Discussion

Using a transwell filter assay we have demonstrated that HUVEC or LMVEC selectively recruit memory lymphocytes so these are greatly enriched compared to their proportional frequency in peripheral blood. In addition, we demonstrated that there was also some selectivity at the level of migration across LEC. Lymphatic endothelium enriched for the recruitment of CD8 and CD4 memory lymphocytes. When purified CD45RO+ (memory) T lymphocytes were isolated and allowed to migrate across a vascular endothelium, they could migrate across a LEC monolayer more efficiently than plastic cultured CD45RO+ T lymphocytes, potentially demonstrating the increased efficiency in LEC transmigration was imparted as a function of migration across a vascular EC and was not an intrinsic property of memory lymphocytes.

When lymphocytes were added to the apical surface of a HUVEC monolayer which was stimulated with both TNFα and IFNγ for 24 hours, and subsequently collected and stained for their phenotype, we found the lymphocytes which had migrated through the HUVEC were largely CD45RA- (memory). This recruitment of memory lymphocytes was also shown by other groups in a series of papers, where they determined the phenotype of cultured T cells following transmigration through unstimulated endothelium *in vitro* [259, 269]. This selectivity remained following cytokine stimulation of the endothelial monolayer [259, 269]. In addition, CD4+ CD45RO+ lymphocytes were captured more efficiently from flow by TNFα stimulated HUVEC than CD4+ CD45RA+ lymphocytes [321]. This observation may also account for the reduction in naïve CD4+ lymphocytes recruited to the HUVEC as it is possible that naïve lymphocytes do not express the correct adhesion molecules to be able to adhere and transmigrate through the

HUVEC. In addition, the reduction in the CD4+ naïve lymphocytes may be explained by the naïve lymphocytes not having the correct chemokine receptor or integrins profile needed to traverse the vascular endothelium. In fact, it is well documented that naïve lymphocytes change their chemokine receptor expression and up-regulate specific integrins following transmigration, antigenic stimulation and their differentiation into memory T cells which probably accounts for the differential efficiency of recruitment during inflammation [317].

The recruitment patterns seen with HUVEC were very similar to the patterns observed when the lymphocytes transmigrated through a LMVEC monolayer. Interestingly the number of naïve CD4 lymphocytes was higher, following migration through the LMVEC monolayer compared to HUVEC. This difference in recruitment may be explained by structural and functional differences between endothelial cells, as it is possible that the microvascular endothelial cells express the correct junctional adhesion molecules that would be more permissive for lymphocyte migration. Furthermore, differences in cytokine regulation of adhesion molecules and integrins have also been observed in different endothelia. In fact Swerlick et al (1992) reported that cytokine stimulation of micro-vascular cells caused an up-regulation of VCAM-1 but this was transient and peaked after 16 hours, whereas in HUVEC, this response persisted for significantly longer periods [314, 322, 323]. This observation was confirmed by another group who additionally found that TNFα stimulation of microvascular endothelial cells caused not only an elevated expression of VCAM-1, but of E-selectin, which reached a maximum after six hours and remained persistently high, in contrast, HUVEC cells which expressed E-selectin peaked in expression at six hours but then declined.

When the lymphocytes were allowed to migrate for 48 and 72 hours, we observed that the frequency of CD8+ memory cells increased at 48 hours and were not altered further at 72 hours, indicating that some of the CD8+ CD45RO+ lymphocytes exhibit the ability to transmigrate only after a prolonged incubation with the EC. In contrast, memory CD4+ CD45RO+ lymphocytes did not change in proportion at the longer time-points, an observation that is consistent with earlier reports, where lymphocytes were incubated with EC for up to 36 hours. Here, the authors found that the number of cells increased but this did not change the predominance of the major subpopulation that migrated initially [259].

When lymphocytes were added to the LEC following migration through a HUVEC or LMVEC monolayer, or cultured on plastic for an equivalent period of time, we observed that the CD8+ CD45RO+ lymphocytes (memory) were selectively recruited irrespective of whether the lymphocytes had previously migrated through a VEC. This recruitment may be attributed to CD8+ lymphocytes having an inherent capacity to migrate faster than CD4+ T lymphocytes [324]. In addition, we observed that when plastic cultured lymphocytes were added to a stimulated LEC there was a significant decrease in the proportion of CD4+ CD45RO+ lymphocytes following migration through LEC. This observation could be attributed to the sub population that exists in memory T cells [34, 325], where memory T cells are divided up into effector and central memory; effector memory T cells have been shown to express low levels of CCR7 and L-selectin both of which are required for entry into lymphatic endothelium whereas central memory T cells have been shown to express high levels of CCR7 [326, 327]. This suggests that the majority of the recruited memory CD4+ lymphocytes are of the effector

memory phenotype, although further characterisation would be required to confirm that this is the case in our assay.

We have shown that VEC selectively recruits memory lymphocytes, and this raised the possibility that the VEC does not condition but simply enriches for lymphocyte subsets that have an inherent capacity to migrate through LEC. We have demonstrated that this may not be the case, as negatively isolated CD4+ lymphocytes show some enhancement of transmigration through a stimulated LEC monolayer after migration across VEC, but not after culture on plastic for an equivalent duration. Berg *et al* (2002) found that isolated CD4 memory lymphocytes showed enhanced transmigration through a second VEC monolayer which is contrary to what we observed, although Berg used purified memory lymphocytes and not PBL, which could explain the difference. Berg *et al* attributed this enhanced transmigration to increased expression of α_L β_2 interaction with ICAM-1.

CHAPTER 4

CCR7 and β1 and β2 integrins play roles in the migration of lymphocytes across LEC

4 Introduction

The interplay between leukocytes in the circulation and EC in the vessels is an important control point in the trafficking of specific subsets of lymphocytes. This interaction is mediated by a multi-step process that, in many instances, involves lymphocyte rolling, rapid activation of leukocyte integrins, and adhesion to endothelial ligands *via* the activated integrins. The display of specific activation signals by the endothelium, maybe the most active component in controlling the specificity of lymphocyte traffic. The vascular endothelium is diversified at a number of levels, from the different chemokines they produce, to the adhesion molecules displayed on the surface. All of these could potentially contribute to the volume and specific subsets of lymphocytes recruited by the endothelium.

As previously discussed, the enhanced migration of lymphocytes across LEC could be attributed to the different phenotype of EC that the lymphocytes have to traverse, as the display of specific inflammatory ligands on the venous side would cause the capture of memory lymphocytes and the actual process of transmigration across the vascular endothelium could cause the expression of specific adhesion receptors and/or chemokine receptors that allow the lymphocytes to interact with the stromal microenvironment or migrate through the LEC after recruitment. The consequences of chemokines binding their receptors have been studied extensively both *in vivo* and *in vitro* [328]. One of the major roles of chemokines is to support the chemotaxis of the cells expressing the specific counter receptor along concentration gradients of the chemokine. An exception to this paradigm might be fractalkine, which has been

demonstrated to support adhesion and induce migration in a manner similar to adhesion molecules [329]. However chemokines maybe secreted, and to activate leukocytes must be immobilised on cell surfaces or extra-cellular matrix by binding to negatively charged glycosaminoglycans. Interestingly, certain chemokines bind different types of glycosaminoglycans with differing affinities [161]. The type of glycosaminoglycans can vary with different cell types, location and inflammatory status, therefore, specific lymphocyte subsets may be recruited to specific endothelium or different subsets could be recruited by the same endothelium depending upon its activation status.

Chemokines and their receptors vary in terms of their selectivity. Certain chemokines will only bind one receptor and vice versa, such as the exclusive interactions of CXCR4 with CXCL12 [330], CXCR5 with CXCL13(BCA-1) [331], CCR6 with CCL20[332] and CXCR6 with CXCL16 [333]. Another pattern of pairing involves chemokines receptors that bind two or three chemokines, as demonstrated by CCR7 binding both CCL19 and CCL21 [334, 335], CXCR3 binding CXCL9-11 [284, 336]. Many other chemokines and receptors are far more promiscuous, for instance CCR3 has been reported to bind at least 9 chemokines [271]. In general, chemokines and their receptors that are involved in inflammatory trafficking and activation of cells tend to be involved in overlapping and redundant pairing but those involved in homeostatic processes tend to display more exclusive interactions. It is generally accepted that only activated or memory lymphocytes respond to inflammatory chemokines, because naïve cells typically do not express receptors for the inflammatory chemokines [337].

Chemokine receptors fall into two groups of expression, those expressed exclusively on a small number of leukocyte types and those that are broadly expressed. For example, CXCR4, is present on T-cells, B cells, monocytes, neutrophils, DCs and others, and is probably the most widely expressed leukocyte borne chemokine receptor. CCR1 is expressed on cells of the lymphoid lineage, which include T lymphocytes B lymphocytes and plasma cells [338]. CCR1, CCR2 and CCR4-CCR10 are expressed on lymphocytes, monocytes and monocyte derived DCs [339]. CCR3 has an expression pattern which is unique, as it is found on eosinophils, mast cells, basophils, Th2 lymphocytes and certain DC populations [340, 341].

Chemokine receptor expression has been reported to be regulated by a number of inflammatory stimuli [337]. T-cell expression of CCR1, CCR2 and CXCR3 is induced and maintained by IL-2, but it is inhibited by activation through the CD3 complex [342, 343], whereas CCR3 expression requires both IL-2 and IL4 [344]. CCR5 has been shown to be up-regulated by Th1 cytokines, but can be suppressed by IL-10 [345]. Transforming growth factor β decreases CCR1,CCR2,CCR3 and CCR5, but up-regulates CCR7 on T lymphocytes [346]. Taken together, these observations appear to demonstrate that chemokine receptor profile expressed by lymphocytes can be modified by the environment the lymphocyte finds itself in. Thus, we believe that interaction with endothelial cells during recruitment could alter the chemokine receptor expression of infiltrating lymphocytes so that they interact with the stromal microenvironment or move into the lymphatic system efficiently.

4.1 Materials and Methods

4.1.1 Isolation of lymphocytes

Lymphocytes were isolated from whole blood as detailed in section 2.1.1

4.1.2 Isolation of HUVEC

Endothelial cells were isolated as detailed in section 2.1.2

4.1.3 Sub-culturing endothelium

EC was sub-cultured on to transwell filters as detailed in 2.1.5

4.1.4 Assaying the migration of lymphocytes across LEC after migration across VEC

1) The assay was performed as described in section 2.1.8, with the following modifications to investigate the effects of lymphocyte migration across HUVEC and LEC on; 1) chemokine receptor expression by flow cytometry 2) integrin expression by flow cytometry 3) chemokine receptor blockade on lymphocyte recruitment 4)integrin receptor blockade on lymphocyte recruitment. To investigate the chemokine receptor expression of lymphocytes isolated directly from peripheral blood, following migration through HUVEC and/ or after migration across LEC, lymphocytes were removed from the relevant stage of the experiment, transferred to a 96 well plate and incubated with 50µl of the primary antibody at a final concentration of 1µg/ml made up in 2% BSA, (details of the antibodies see appendix 1) at 4°C for 30 mins. The plate was then centrifuged at 300g for 5 mins, supernatant shaken off and washed with 100µl of 2% BSA. 50µl of the

relevant secondary antibody was added to each well and incubated at 4°C for 30 minutes in the dark.

2) To investigate the expression of integrins on lymphocytes which have migrated through a HUVEC monolayer and those that have been rested on plastic, freshly isolated lymphocytes were incubated with 50µl of a β1 integrin, β2 and β3 antibody, in conjunction with CD4, CD8 and CD45RA (see appendix for details of the antibodies) for 30 mins on ice and analysed for their expression. Lymphocytes from the same donor were allowed to migrate through a stimulated HUVEC monolayer and some of the lymphocytes were simply rested on plastic for an equivalent period of time. The lymphocytes were collected and subjected to the same protocol as before.

3) To investigate chemokine receptor blockade on lymphocyte recruitment, lymphocytes were isolated and allowed to migrate through a HUVEC monolayer for 24 hours. These lymphocytes were then collected and resuspended to $500x10^3$/ml of M199+BSA. Neutralising CCR7 and CCR8 (R and D systems, see appendix for antibody details) antibody was added to the collected lymphocytes at a final concentration of 5µg/ml or CXCR3 blocking antibody at a final concentration of 5µg/ml [41, 42] or AMD3100 (R and D Systems) a CXCR4 Selective antagonist at a final concentration of 1mg/ml [347]. 1ml of the cell suspension was then added to the top of a transwell insert which had cultured LEC on the basal surface. The LEC had been cytokine stimulated previously with TNFα and IFNγ for 24 hours. The plate was then incubated for 24 hours at 37°C. The lymphocytes were then collected from the well and counted.

4) To investigate the effects of integrin blockade on lymphocyte migration through the LEC, lymphocytes were isolated and allowed to migrate through a HUVEC monolayer for 24 hours. These lymphocytes were then collected and resuspended to $500x10^3$/ml of M199+BSA. The relevant neutralising antibodies were then added to the cell suspension and added to the apical surface of a transwell insert which had LEC sub-cultured on the basal surface. Following a 24-hour incubation at 37°C, the lymphocytes were collected and counted. The concentrations of the β1 integrin was 10μg/ml [348]; β2 integrin was 10μg/ml [347, 348]; β3 integrin 10μg/ml [349].

4.1.5 ELISA to assay for protein expression of adhesion molecules on the endothelium

Confluent HUVEC and LEC were trypsinised, suspended in medium and plated into gelatin coated 96 well plates, and grown until confluent. Both endothelial cells were then treated with 100U/ml TNFα and 10ng/ml IFNγ or treated with vehicle for 24 hours. The cells were then washed with PBS and mixed with 4% formaldehyde and the ELISA for ICAM-1, VCAM-1, CD31 and E-Selectin were carried out (see appendix for antibodies used). Primary antibodies were diluted in 0.15% PBSA /2% goat serum to prevent non-specific binding. 50μl of the antibodies were added to duplicate wells of fixed cells and incubated for 1 hr. The negative control used for each experiment was mouse IgG (1 in 2000; DAKO). Cells were then washed 5 times with PBS and 50μl of HRP-conjugated, goat-anti-mouse antibody (1 in 2000; DAKO) added to each well and incubated for 1 hour, cells were again washed 5 times with PBS. The enzyme substrate 1,2 Orthophenylenediamine Dihydrochloric acid (OPD, in tablet form) (DAKO) was made to working concentrations in distilled water containing 0.02% hydrogen peroxide (Sigma, UK). 50μl

of OPD substrate was added to each well until a colour change was observed (approx. 15-30mins). The reaction was stopped with 50µl of 1M sulphuric acid. The absorbance of each plate was read on an automated plate reader (Synergy 2 Microplate Reader, BioTek UK) at a wavelength of 490nm.

4.2 Results

4.2.1 Chemokine receptor screen of lymphocytes

We wanted to investigate whether the enhanced transmigration of lymphocytes through a LEC monolayer after traversing a monolayer of vascular endothelial cells, could be attributed to an up-regulation of specific chemokine receptors. We hypothesised that the act of transmigration though the VEC would cause up-regulation of chemokine receptors which supported expedited migration across LEC.

When mixed peripheral blood lymphocytes were isolated and stained for chemokine receptors, we found that there was a basal level of expression of the entire panel of chemokine receptors tested (Fig 4.1). Some receptors showed modest levels of modulation after migration across HUVEC. For example, there was a small reduction of CCR3 expression following migration through the HUVEC monolayer and this increased again following migration through the LEC, although these changes were not statistically significant. There was a marked increase in CCR10 after each stage of migration in our assay, but again this was not statistically significant (Fig 4.1C). Another receptor that changed dramatically was the fractalkine receptor, which decreased following migration through the HUVEC and remained at the lower level following migration through the LEC. In addition, we observed a dramatic increase in the expression of

the CCR8 following migration through the LEC from two donors (see Fig 4.1A for dot plot). This, however, was probably due to non-specific binding of the antibody (personal communication from R and D systems). However, due to large inter-donor variation in expression levels, none of these changes were significant. The only receptor that showed significant changes, despite inherent variability in the assay was CXCR4 (see Fig 4.1B for dot plot). This receptor showed a 3-4 fold increase in expression following migration through HUVEC and remained persistently high following migration through the LEC. Interestingly CCR7, which is reported to support lymphocyte migration across LEC, was not up-regulated by passage across VEC, implying that the mechanism by which the efficiency of migration is achieved is not the increased expression of this receptor. In reality, a more exacting analysis based on chemokine receptor expression on a sub-set specific basis would have been more informative. However, such experiments were not conducted and are good targets for additional work.

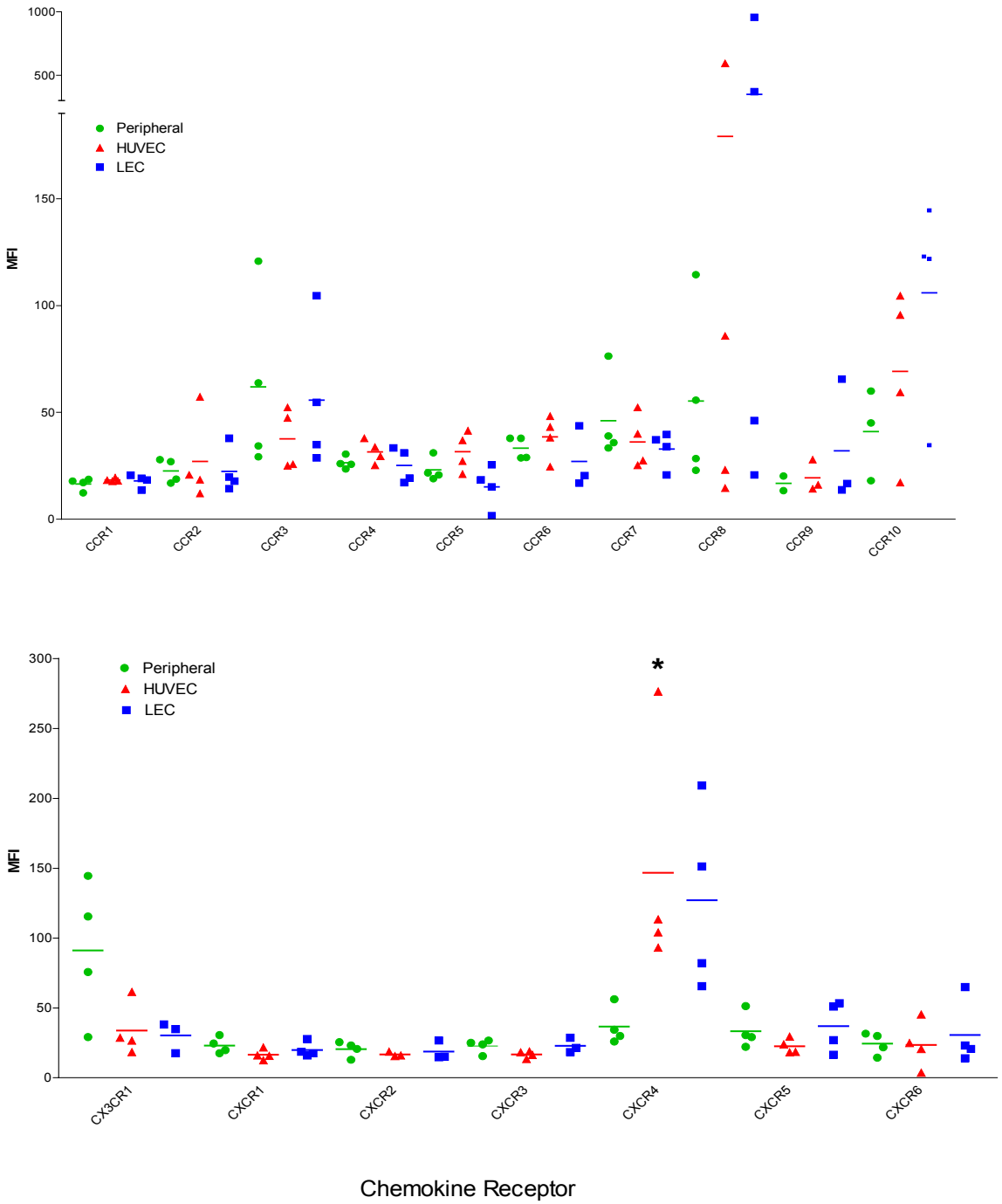


Figure 4.1: Chemokine receptor expression of lymphocytes following migration through HUVEC and LEC. Lymphocytes were isolated and immediately stained for chemokine receptors (Peripheral), a proportion of the lymphocytes were then added to the top of a stimulated HUVEC monolayer allowed to transmigrate for 24 hours, cells were collected and stained for chemokine receptors (HUVEC), and again following migration through LEC (LEC). These data show the mean (horizontal line) from 4 independent experiments.*p=<0.05 Mann-Whitney test, compared to peripheral blood lymphocytes.

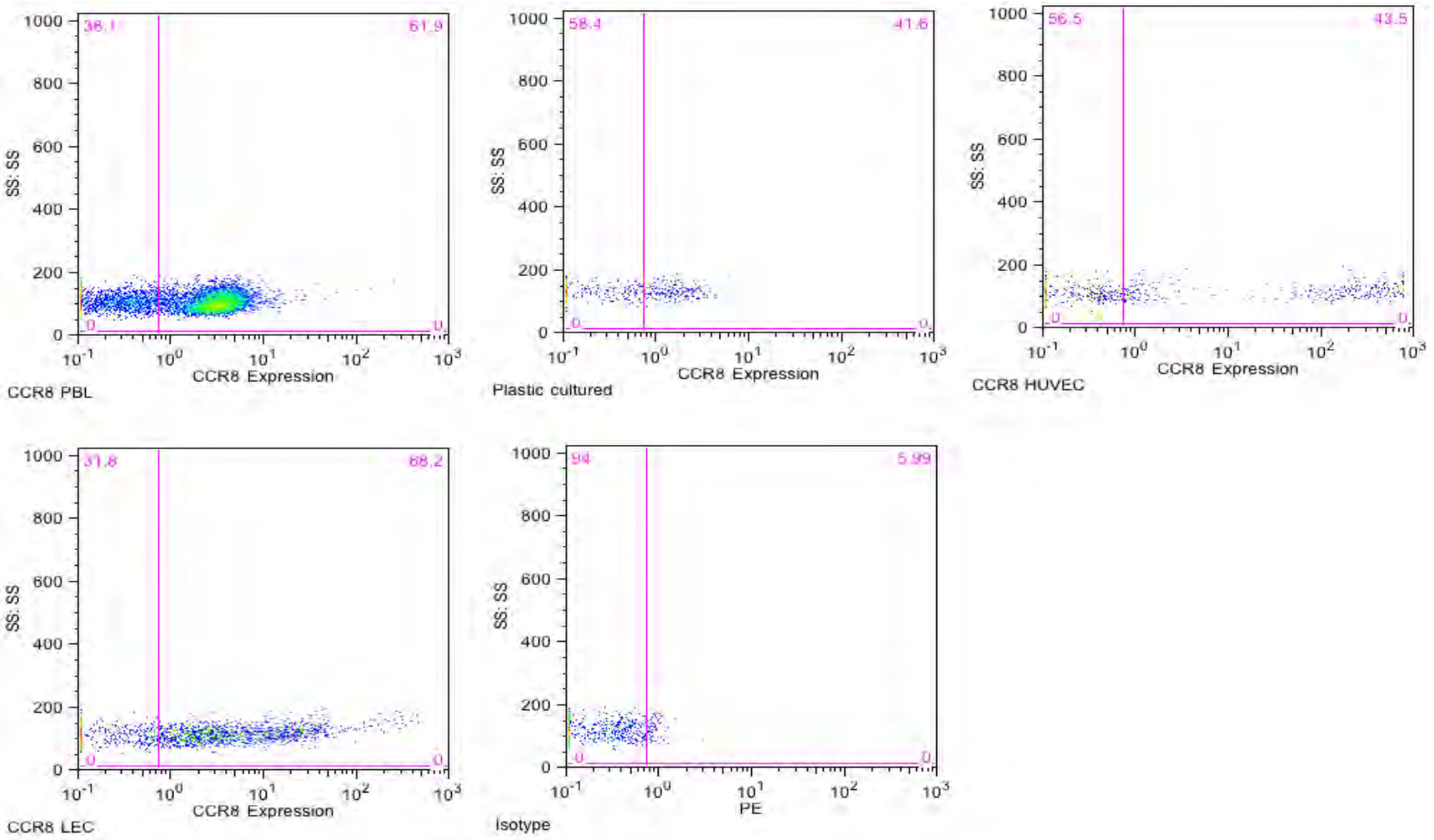


Figure 4.1A: Representative dot plot for CCR8 chemokine receptor expression. Lymphocytes were assessed for CCR8 expression following isolation from whole blood (CCR8 PBL), after culture on plastic for 24 hours (Plastic cultured), following migration through TNF and IFN stimulated HUVEC (CCR8 HUVEC) and following migration through TNF and IFN stimulated LEC (CCR8 LEC).

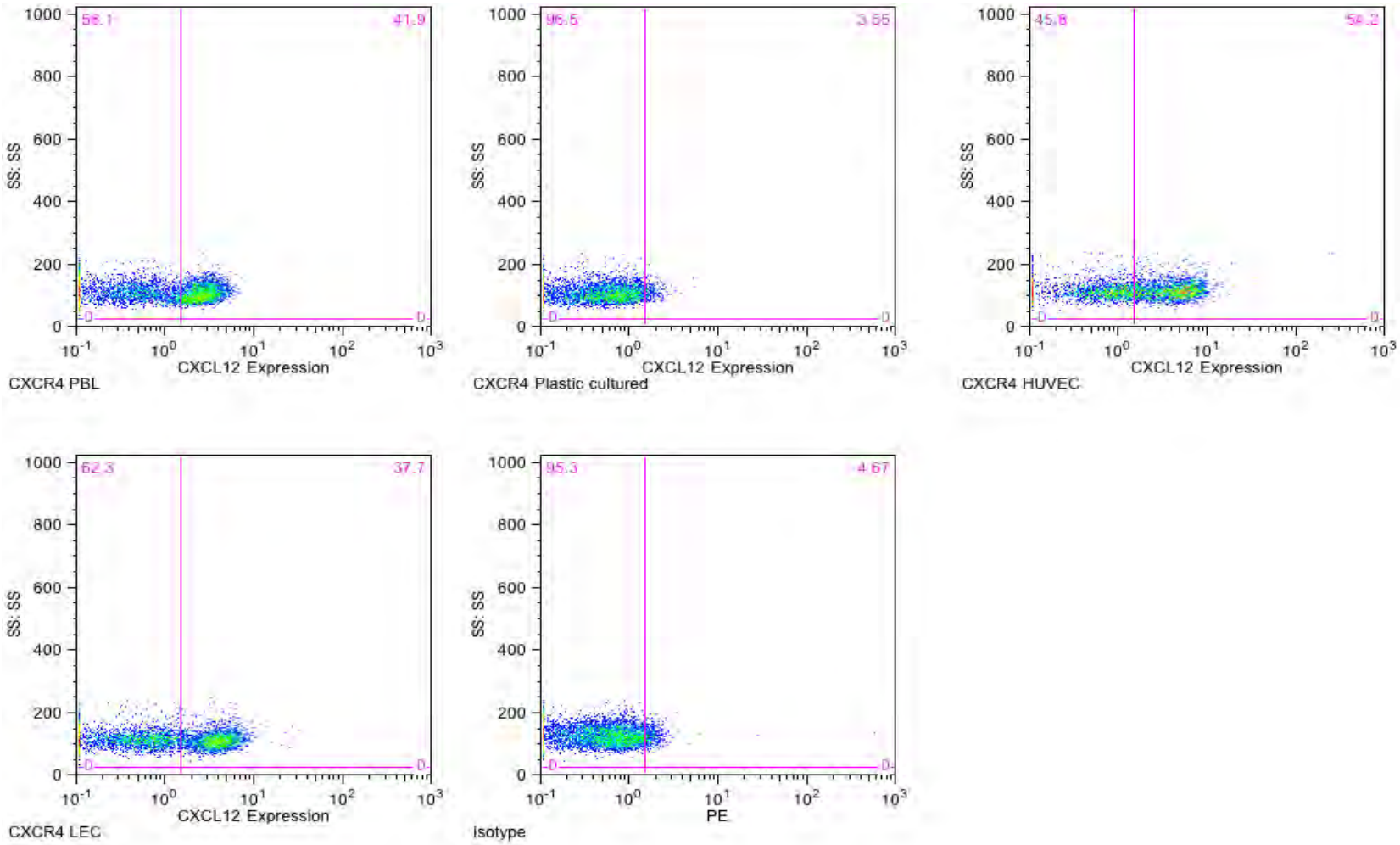


Figure 4.1B: Representative dot plot for CXCR4 chemokine receptor expression. Lymphocytes were assessed for CXCR4 expression following isolation from whole blood (CXCR4 PBL), after culture on plastic for 24 hours (Plastic cultured), following migration through TNF and IFN stimulated HUVEC (CXCR4 HUVEC) and following migration through TNF and IFN stimulated LEC (CXCR4 LEC).

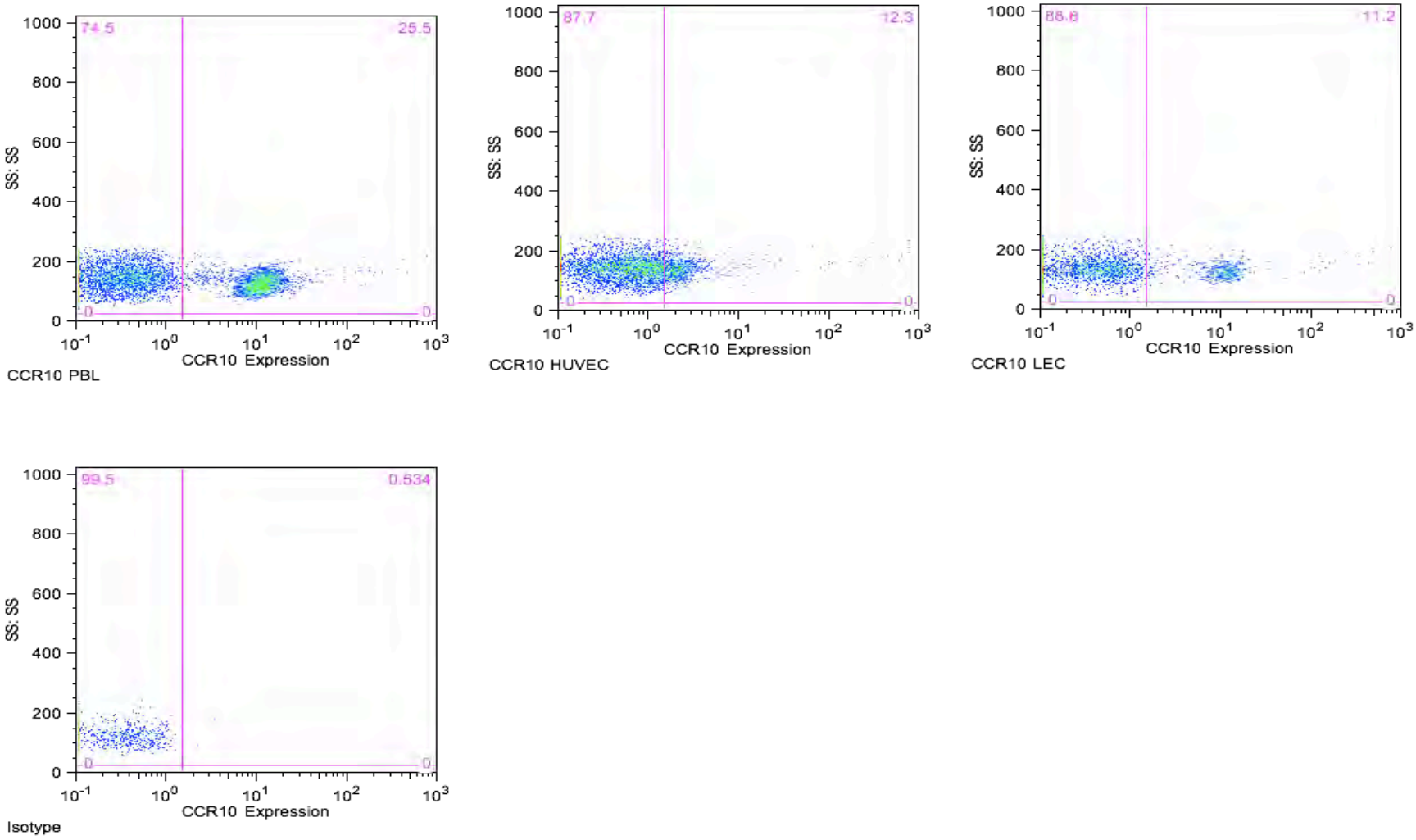


Figure 4.1C: Representative dot plot for CCR10 chemokine receptor expression. Lymphocytes were assessed for CCR10 expression following isolation from whole blood (CCR10 PBL), migration through TNF and IFN stimulated HUVEC (CCR10 HUVEC) and migration through TNF and IFN stimulated LEC (CCR10 LEC).

4.2.2 Integrin expression of lymphocytes following migration through HUVEC and LEC

We next wanted to investigate if the enhanced transmigration could be explained by the increased expression of integrins. To this end, lymphocytes were isolated and stained for β1 and β2 integrins from freshly isolated lymphocyte preparations, following HUVEC migration and those rested on plastic for 24 hours.

We observed no effect of migration on integrin expression following either migration through HUVEC or when the lymphocytes were rested on plastic for an equivalent period of time for any of the subsets (Fig 4.2). However, when the β1 expression is compared to other subsets following isolation from whole blood, we found that CD4 naïve lymphocytes express β1 at significantly higher levels than on CD4 memory cells (Fig 4.3a). CD8 memory cells showed similar expression pattern of β1 integrins. Following migration through the HUVEC we found that the levels of β1 did not change (Fig 4.2b), although we did observe a small increase of β1 integrin expression on the CD4 memory and the CD8 naïve populations, but this was not significant. When the lymphocytes were rested on plastic we found that there was a small reduction of β1 integrins across all subsets (Fig 4.3c), this decrease in expression was most apparent on the CD4 naïve populations, but again was not significant. We also observed that following isolation from whole blood, the expression of β2 integrins on CD4 naïve population was almost double the expression found on the CD4 memory population (Fig 4.4a). The level of expression on the CD8 memory population was significantly higher than that found on the CD4 memory population and also tended to be higher on the CD8 naïve population compared to the CD4 memory population (Fig 4.4a). Following migration through the HUVEC (Fig 4.4b) we found

the level of expression was, again, significantly higher on the CD8 memory population than on the CD4 memory population even following migration through the HUVEC. We also observed that the level of expression on CD8 naïve subset was higher than that on the CD4 memory cell population. When the lymphocytes were rested on plastic, we found similar levels of expression as we saw when the lymphocytes had migrated through the HUVEC, again, the CD8 memory population was found to have the highest level of expression and this was consistent, irrespective of the treatment to the lymphocytes. To summarise, although we found differences in integrin expression between subsets, the process of culture on plastic for 24 hours or of migration across HUVEC had no significant effect on integrin expression for cells within individual subsets.

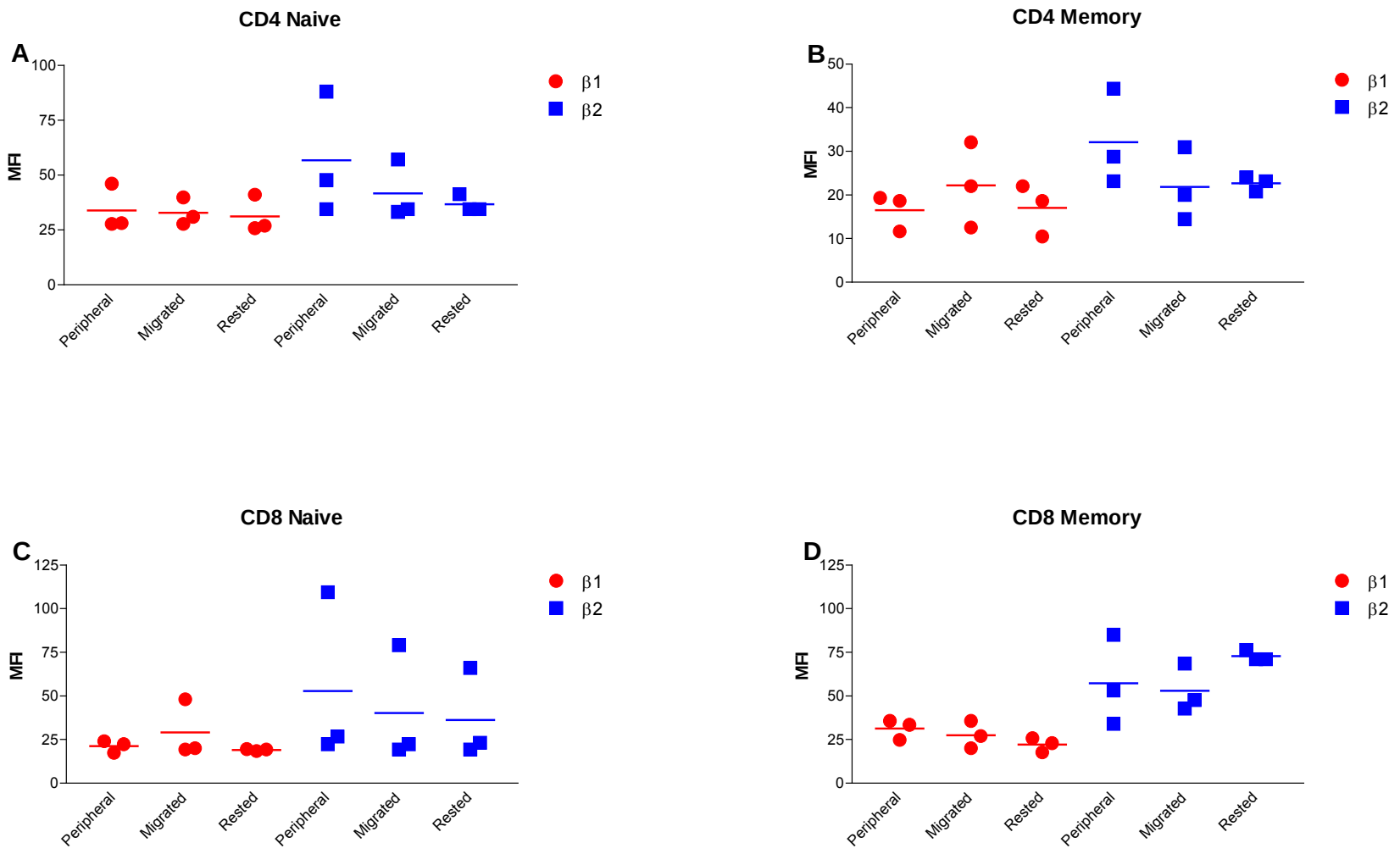


Figure 4.2 Integrin expression of lymphocytes following isolation and migration through HUVEC and lymphocytes rested on plastic. The β1 and β2 integrin expression profile of CD4 naïve lymphocytes (A). (B) β1 and β2 integrin expression profile of CD4 memory lymphocytes. (B) β1 and β2 integrin expression profile of CD8 naive lymphocytes. (C). β1 and β2 integrin expression profile of CD8 memory lymphocytes (D). These data show the mean (horizontal line) from 3 independent experiments.

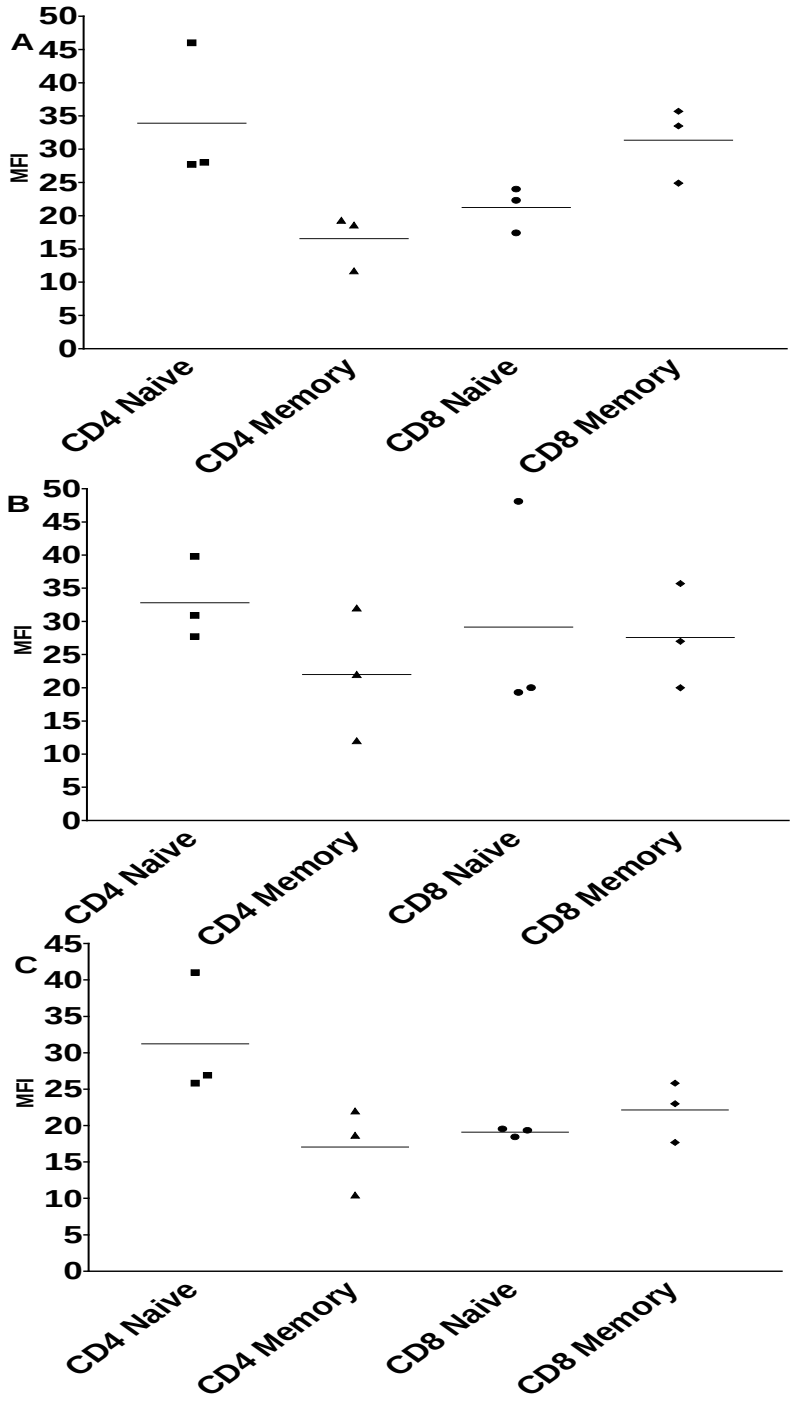


Figure 4.3 β1 integrin expression of lymphocyte subsets following migration through HUVEC or rested on plastic. Lymphocytes were stained for β1 integrin expression following isolation (A), Migration through a stimulated HUVEC monolayer (B) or rested on plastic (C). These data show the mean (horizontal line) from 3 independent experiments.

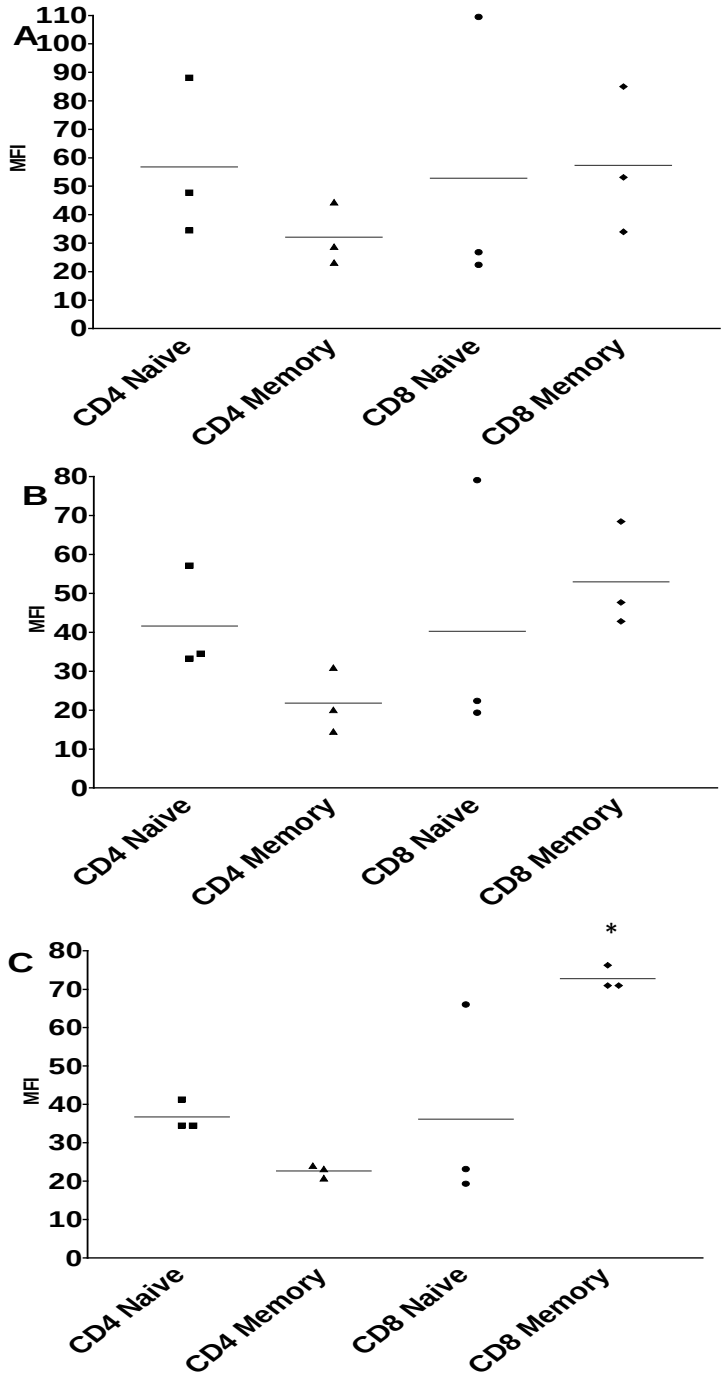


Figure 4.4 β2 integrin expression on lymphocyte subsets following migration through HUVEC or rested on plastic. Lymphocytes were stained for β2 integrin expression following isolation (A), Migration through a stimulated HUVEC monolayer (B) or rested on plastic (C) (*=p<0.05 compared to CD4 memory). These data show the mean (horizontal line) from 3 independent experiments.

4.2.3 Chemokine receptor blockade reduces LEC transmigration

As lymphocyte chemokine receptor expression is not fixed, it is possible that the chemokine receptors may have down regulated before they were assayed for their receptor expression. As we have previously shown that CXCR3 plays critical roles in the attachment of lymphocytes to vascular endothelium under flow conditions, we wanted to investigate if CXCR3 would also play a role in migration across lymphatic endothelium. As we observed a significant change in the expression of CXCR4 and CCR8, we also wanted to investigate the possibility of a role for these receptors in transmigration of lymphocytes across LEC. In addition, as the chemokines CCL19 and CCL21 have been implicated in the constitutive recruitment of lymphocytes to the lymphatic system, we wanted to investigate the potential role of these chemokine and their receptor (CCR7) in the enhanced recruitment across LEC.

An antibody against CXCR3 was no more effective at blocking the migration of mixed lymphocytes across LEC than a control antibody, while a selective antagonist and antibody that blocked CXCR4 or CCR8 respectively, had no consistent effect on migration levels across LEC (Fig 4.5a). Interestingly lymphocytes added onto LEC in the presence of CCR7 neutralising antibody migrated significantly less efficiently through a stimulated LEC monolayer. As CCR7 blockade causes such a significant retardation of transmigration through the LEC we wanted to investigate which lymphocyte subset was most affected by this blockade. Independent experiments were then set up and we found that there did seem to be an effect on CD4 memory cells, although not significant between donors (Fig 4.5b). Although it must be noted that the lymphocytes which were added to the LEC with either an isotype control antibody or

anti-CCR7 had previously migrated through a HUVEC monolayer, we did not observe the strong enrichment of memory cells as we had previously shown, and thus there was a significant CD8 naïve cell population being added to the LEC. However the CD4 memory lymphocytes were still enriched, as we observed previously.

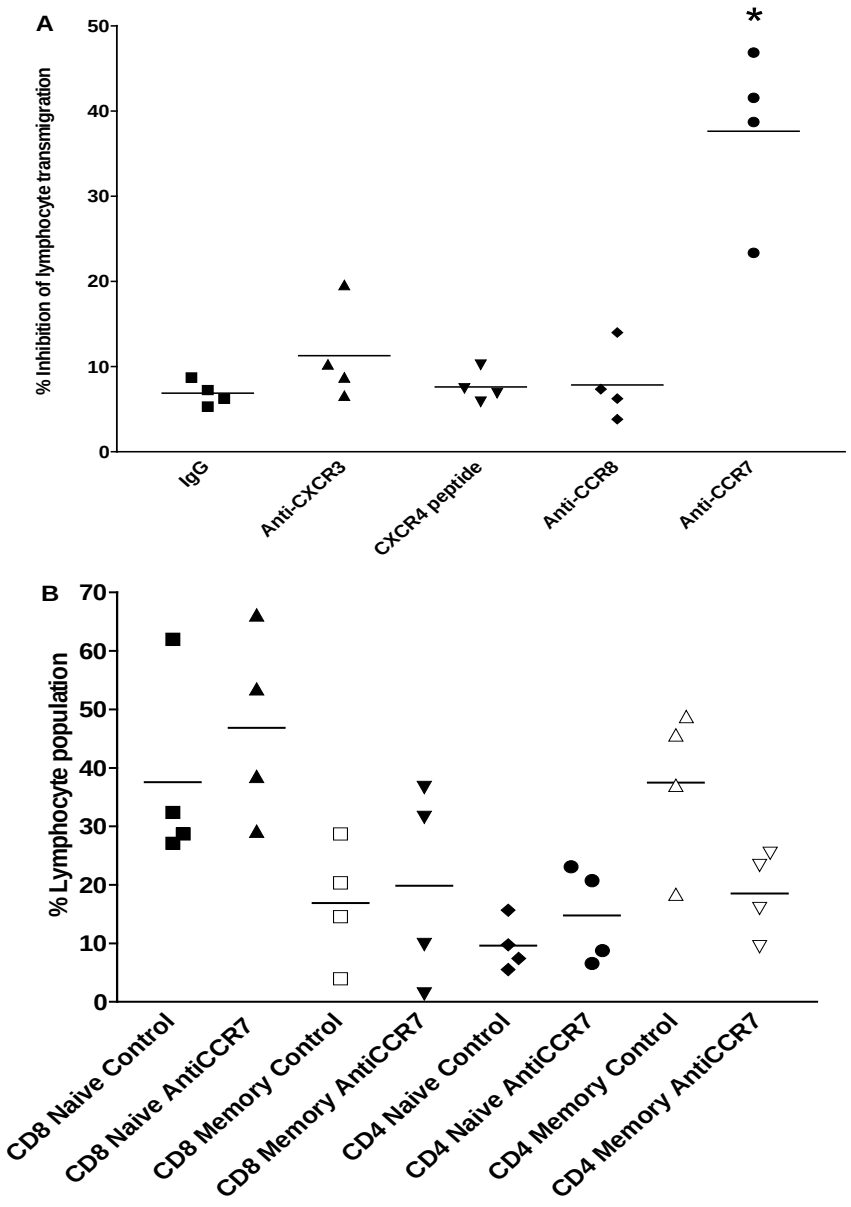


Figure 4.5 CCR7 blockade inhibits lymphocyte transmigration through LEC and specifically reduces CD4 memory subsets. Lymphocytes which had previously migrated through a HUVEC monolayer were incubated with neutralising antibodies or peptide directed towards chemokine receptors and the level of inhibition assessed (A), Lymphocytes able to transmigrate through LEC ± CCR7 neutralising antibodies stained for their phenotype (B). These data show the mean (horizontal line) from 4 independent experiments. p=<0.05 compared to control by Mann-Whitney test.

4.2.4 Integrin blockade reduces migration across LEC

As others and we have previously demonstrated the importance of integrins on lymphocyte adhesion to, and transmigration across vascular endothelium, we wanted to investigate the roles these integrins have on lymphocyte transmigration through a stimulated LEC monolayer. To assess this, we set up an adhesion assay similar to that described above, but in the presence or absence of neutralising antibodies to β1, β2 or β3 integrins.

Lymphocytes which had been migrated across a stimulated HUVEC monolayer were collected and added to the top of a stimulated LEC monolayer in the presence of a function neutralising antibody towards β1, β2, and β3 integrins, or an isotype control. We found that the percentage inhibition was less than 10% (Fig 4.6) in the presence of an isotype control. In the presence of a β1 neutralising antibody we observed a significant inhibition of lymphocyte transmigration (40%). The presence of a neutralising β2 antibody was even more effective and inhibited approximately 75% of migration. A β3 antibody had no effect. In the presence of both β1 and β2 neutralising antibodies we observed no further significant enhancement of inhibition to that seen with the β2 alone.

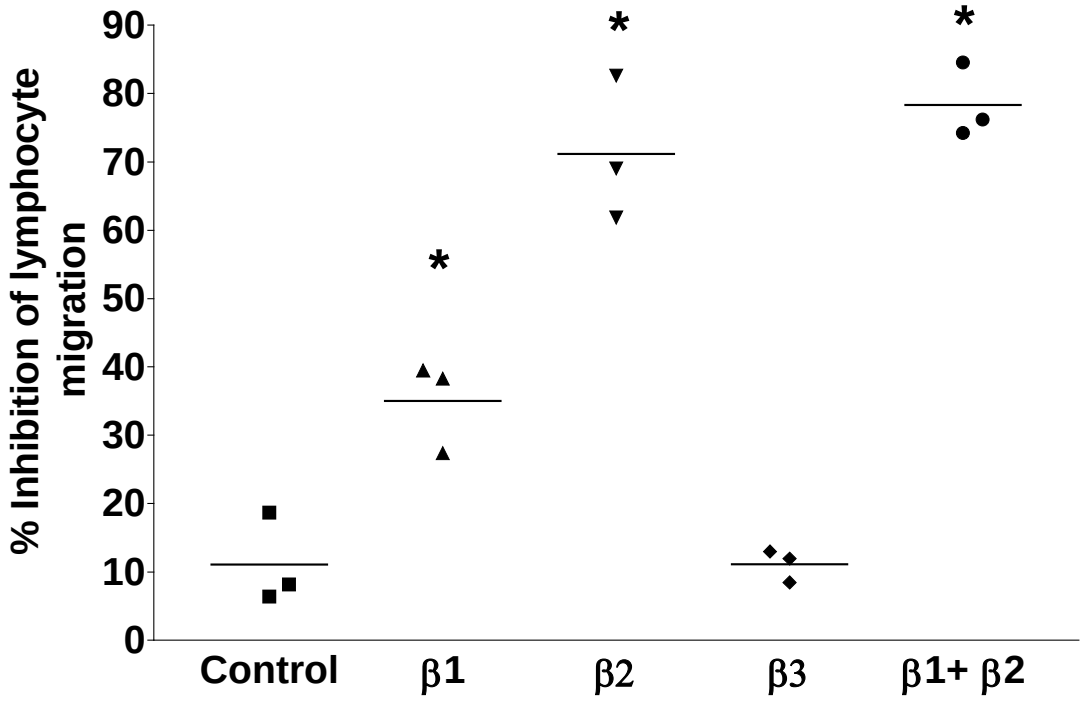


Figure 4.6 The blockade of β1 and β2 dramatically reduces the transmigration of lymphocytes across stimulated lymphatic endothelium. Lymphocytes which have previously migrated across HUVEC and then added on to a stimulated LEC monolayer in the presences of blocking antibodies towards β1 and/or β2 Integrins and the number of lymphocytes counted. These data show the mean (horizontal line) from 3 independent experiments. *P=<0.05 compared to control by Mann-Whitney test.

4.2.5 Blockade of β1 and β2 integrins does not affect a specific lymphocyte sub set

Following lymphocyte blockade with β1 and β2 antibodies, we consistently observed that we were unable to substantially block lymphocyte transmigration through a stimulated LEC monolayer. Maximal inhibition was 75% which indicates that 25% of the lymphocytes were able to transmigrate even in the presence of the neutralising antibodies. We wanted to investigate if the integrin blockade was inhibiting a specific lymphocyte population.

When lymphocytes were added to the top of a stimulated LEC monolayer in the presence of a β1 neutralising antibody we found again that the percentage inhibition was, again, around 40% and when the lymphocytes collected from the bottom of the well were stained for the lymphocyte subsets, we found no significant change in the proportion of the subsets that were able to transmigrate when compared to the control antibody (Fig 4.7a). A similar pattern was observed in the presence of a β2 neutralising antibody (Fig 4.7b). In addition the combination of both β1 and β2 integrin blockade did not cause any changes in the pattern of recruitment compared to a relevant control (Fig 4.7c). Thus, it appears that integrins are utilised by all migrating cells, and inhibitors do not favor the migration of any particular subset.

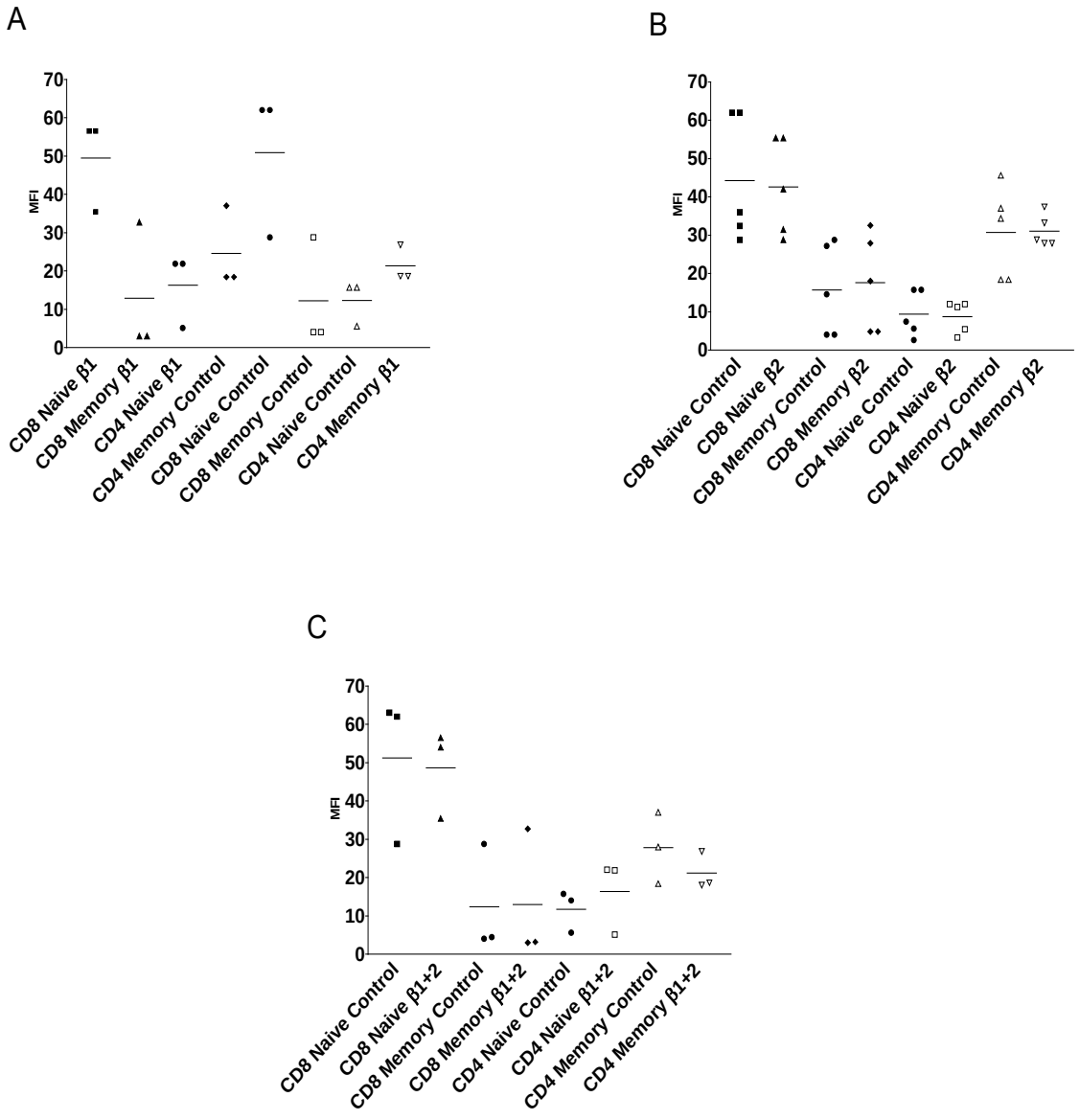


Figure 4.7 β1 and β2 integrin blockade does not specifically reduce a specific lymphocyte population. Lymphocytes were migrated through HUVEC and added to a stimulated LEC monolayer in the presence of a β1 neutralising antibody (A), β2 neutralising antibody (B) and both β1 and β2 neutralising antibodies (C) for 24 hours. Data are the mean of 3 independent experiments.

4.2.6 Stimulation of the endothelium causes up-regulation of some adhesion molecules on HUVEC and LEC monolayers

We wanted to investigate the relative expression levels of key adhesion molecules which are known to be important in the recruitment of leukocytes and also the transmigration process in HUVEC and in LEC prior to stimulation and following stimulation. To address this, HUVEC and LEC were assayed for the expression of adhesion molecules before stimulation by TNF and IFN and then following a 24 hour cytokine treatment by ELISA.

When HUVEC was stimulated with cytokines for 24 hours we saw a small increase in the level of expression of ICAM-1. Interestingly, LEC also showed a marked increase in expression following cytokine treatment although this was not statistically significant (Fig 4.8). VCAM-1 was also increased on HUVEC after cytokine stimulation, however LEC did not express VCAM-1 constitutively and it was not up-regulated by inflammatory cytokines. Cytokines induced expression of E-Selectin in HUVEC but not in LEC, although there was a trend towards higher expression levels in these cells. The levels of CD31 in endothelial cells from either vascular bed in response to cytokine stimulation did not change significantly.

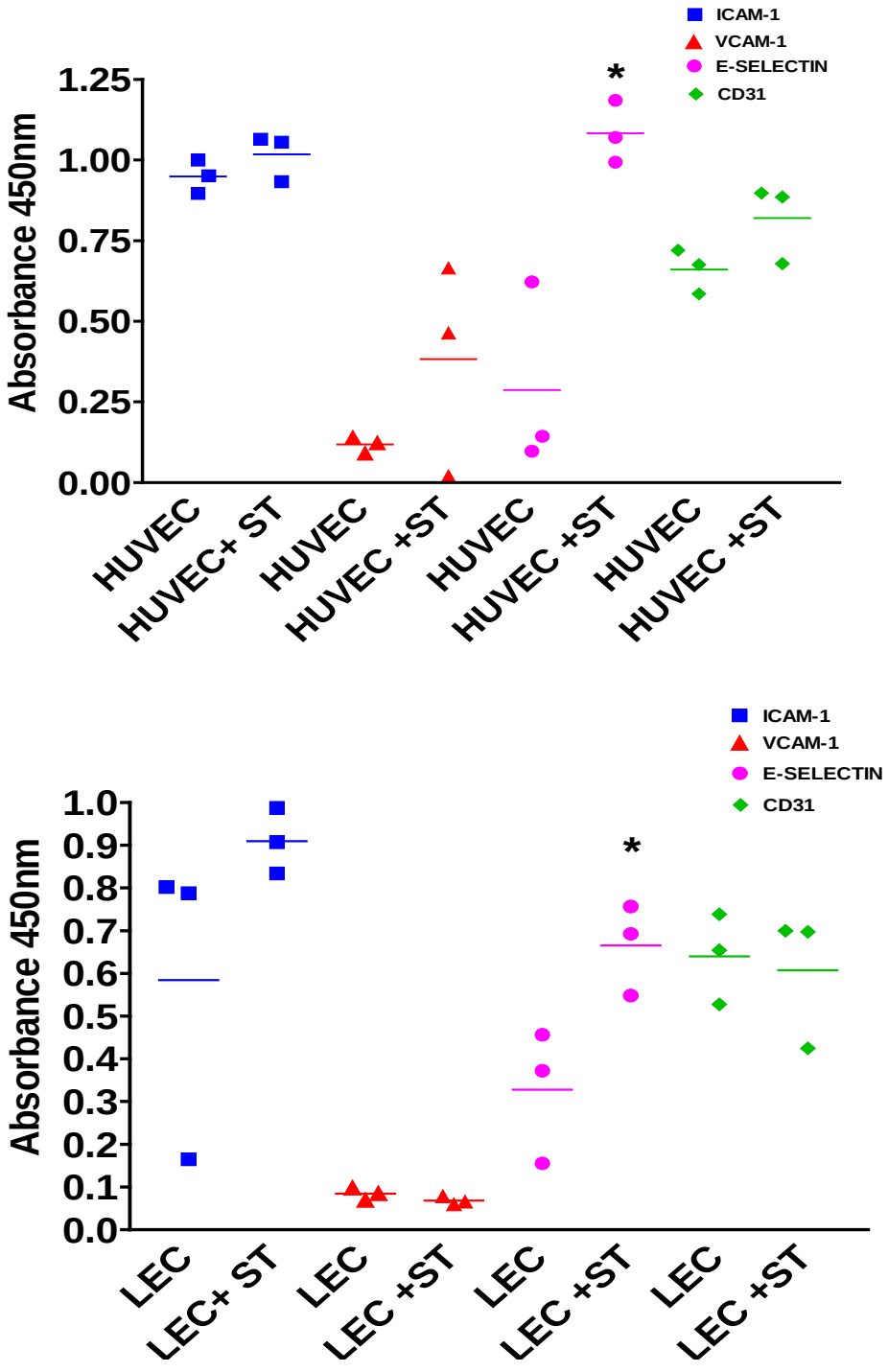


Figure 4.8: The expression of adhesion molecules on HUVEC and LEC following cytokine stimulation. HUVEC or LEC were either treated with 100U TNF and 10ng/ml IFN for 24 hours (ST) or unstimulated, and then assayed for ICAM-1, VCAM-1, E –Selectin and CD31. The data is the mean ± SEM of 3 independent experiments. *=P<0.05 compared to unstimulated by t-test.

4.3 Discussion

In this chapter we wanted to investigate the specific effect the vascular endothelium was having on the lymphocytes to allow them to migrate through the LEC. We hypothesised that the migratory process was causing an up-regulation of specific chemokine receptors that then allowed lymphocytes to migrate through LEC with greater efficiency. Using an *in vitro* transwell filter assay, we have demonstrated that the migration of PBLs across HUVEC causes a significant increase in the level of expression of CXCR4; this increased expression is sustained when the lymphocytes are migrated through the LEC. We also demonstrated that CX3CR1 expression was significantly reduced following migration through HUVEC and was further reduced following migration through LEC. When lymphocytes were stained for β1 or β2 integrins, we observed no significant change in the level of expression when the lymphocytes were rested on plastic or migrated through the HUVEC. Thus changes in the integrin expression are unlikely to be the basis of expedited migration across LEC. When migrated lymphocytes were incubated with a neutralising antibody for CCR7 we observed a dramatic decrease in the number of lymphocytes able to traverse the LEC. When we then looked to see if the blockade was causing a reduction in migration of a specific subset, we found that there was a significant reduction in the proportion of memory cells migrating and this was predominantly due to inhibition of CD4 memory cells. In addition, we demonstrated the previously undocumented observation that lymphocyte transmigration through LEC uses both β1 and β2 integrins.

As we have shown previously that the main lymphocyte subset to migrate through the HUVEC is of the memory phenotype we would have expected that the chemokine receptors that are

associated with memory cells CCR1, CCR3 and CCR5 [350, 351] would be expected to be expressed at higher levels than observed in our chemokine screen. This was not the case, but this can be explained by the fact that we did not conduct analysis on a subset specific basis and such analysis may well have provided a different pattern of expression on a restricted repertoire of lymphocytes.

When we migrated lymphocytes through the HUVEC, the only chemokine receptor we found to be up-regulated was the CXCR4, which is generally regarded as more of a homeostatic chemokine receptor and has been shown to be critical to the development of embryos, and the stromal microenvironment in the bone marrow [352]. CXCR4 has also been shown to play roles in the organisation of lymphoid structures, and there are reports that there is increased expression of CXCL12 in the joints of patients with RA and in some skin inflammatory diseases [353, 354]. The CXCR4 receptor does, therefore, exhibit both homeostatic and pro-inflammatory functions.CXCL12 has been extensively studied, in terms of its role in migration by Alon, R, found that lymphocytes require this ligand to be able to traverse vascular endothelium and specifically required it when lymphocyte migrated under the influence of fluid shear stress. We do not agree with these observations and have not been able to reproduce them (unpublished data). The main cellular sources of CXCL12 are resident stromal cells and epithelial cells which are found in high numbers in the tissues. One explanation for the up-regulation we observed could be that migration across EC primes lymphocytes for interaction with stromal environment. An interesting example of this may be found in RA. Here CXCL12 has been shown to be highly expressed on the surface of the EC lining the synovial joint and in joint tissue. It plays a role in recruiting lymphocytes but also in retaining the patrolling lymphocytes close to

the recruiting vessel. In this inflammatory setting the high concentration of the CXCL12 leads to ectopic localisation of recruited cells, but in a non-inflamed tissue the levels of CXCL12 are much lower so that it may serve to aid temporary retention thereby allowing immune surveillance [276].One of the drawbacks of investigating the chemokine expression profiles in our system is the length of time elapsed following the transmigration through the HUVEC. It is probable that observations at earlier or later time points may have yielded a different pattern of expression. Nonetheless, we did observe a decrease in the level of CX3CR1 expression following migration through the HUVEC and this decreased further following migration through the LEC. Imai *et al* (1997) have shown T lymphocytes express CX3CR1 [329] and Foussat *et al* (2000) have reported that they found a two-fold lower expression of CX3CR1 on the CD4+ T helper fraction compared to the CD8+ CD45RO+ lymphocytes [355]. This highlights the necessity of repeating our own analysis on a sub-set basis. Interestingly, it has been shown that venous endothelium express fractalkine following TNFα and IFNγ stimulation [356], while others show expression on arterial and capillary EC [357]. Thus, fractalkine receptor could be shed or internalized after ligation during passage across endothelial cells. This makes sense if one subscribes to the hypothesis that highly expressed receptors are down regulated upon migration and once they are no longer needed for this process. This would avoid inappropriate activation of cells within the tissue compartment [358].

When we investigated the expression of β1 and β2 integrins we found no difference in the expression following either migration through HUVEC or after culture on plastic. This indicates that the expression level of these receptors does not play a role in expedited migration across LEC. In fact, it has been shown that upon activation, many adhesion molecules can become

functionally activated and this could provide enhanced activity without the requirement for increased expression [359, 360]. *In vitro* lymphocytes activated by PMA do exhibit increased levels of β1 integrins (personal communication Prof G E Rainger). In addition, memory lymphocytes isolated from inflamed tissues, show increased levels of β1, as demonstrated by Bank [361, 362]. It is possible that additional stromal signals are required to induce these changes, signals that are lacking in our monocultures of EC. It must also be noted that the reported expression of β2 integrin on memory cells was not seen in our experiments, although due to the inherent variability of donors, increased numbers of donors may be required to confirm the true expression of the integrins investigated.

When lymphocytes were migrated across HUVEC and then placed onto a LEC monolayer in the presence of neutralising antibodies to CCR7 we found a significant inhibition of transmigration through the LEC. This suggests that one of the main signals for allowing lymphocytes migration across LEC is through the CCR7 ligands. Moreover, this occurs in the absence of up-regulation of expression of this receptor. The requirement for CCR7 signalling for lymphocyte migration across LEC in our experiments was expected, as it has been well documented that mobilisation of DCs from the periphery to lymph nodes is regulated by this chemokine receptor. For example, knockout mouse studies have shown a significant defect in DC migration from the skin to the lymph nodes in the absence of CCR7 [112]. CCR7 was thought to be expressed on naive lymphocytes and it was required to allow entry into lymphoid tissue. The majority of the lymphocytes recruited to the HUVEC on our system are of the memory phenotype. This is not consistent with the known expression of CCR7, as central memory lymphocytes are known to express this receptor. Other reports have shown that CCR7 is expressed on T lymphocytes that

exert immediate effector functions [363-365] after infiltration of extra-lymphoid tissues such as skin, synovium and lung [326, 364, 366]. Of course, a ligand for CCR7, CCL21 is constitutively expressed by LEC [144, 367], where it has been demonstrated to support migration of mature antigen presenting cells into the afferent lymphatics [111, 112, 367]. In addition, CCL21 expressing lymphatic vessels can be seen histologically in the skin, mainly adjacent to venules involved in lymphocyte recruitment or immediately underlying the epithelium [368, 369]; thus these vessels would be ideally situated to mediate CCR7 dependant exit of the T lymphocytes from inflamed tissues. Indeed, observations in our *in vitro* system may mirror this paradigm. Interestingly, in non-inflamed tissue, CCL21 expression by LEC is relatively low [368, 369] perhaps indicating, that trafficking of cells using this receptor occurs predominantly during inflammation, or even during resolution of an immune response.

It has been shown that the CCR7 molecule undergoes conformational shape change to an 'activation state' in order to carry out its functions. Thus, it is obvious that enhanced CCR7 function can be achieved in the absence of increased expression. Indeed, we believe that such a paradigm could account for the lack of change in expression that we saw after transmigration of lymphocytes across VEC [370]. The signals that have been shown to cause the CCR7 receptor to function with greater efficiency is Prostaglandin E_2 [370] and leukotriene C4 [371]. These signals have been shown to be produced by EC in inflamed conditions and to expedite the migration of DCs across lymphatic EC in the absence of an increase in CCR7 expression on these cells. Thus, we believe that following migration through HUVEC, lymphocytes in our system are exposed to a signal appropriate for enhanced CCR7 function without up-regulation of expression. Indeed, the lack of such signal for enhanced CCR7 function in our control cells

incubated on plastic would explain the lack of enhanced migration across LEC that was evident in this population.

β1 integrins play major roles in T lymphocyte firm adhesion and transmigration in VEC. The activation of β1 integrins on rolling T lymphocytes induced their firm adhesion *in vitro* [372],-*in vivo*, the inhibition of the α4 or β1 integrin components completely inhibited lymphocyte firm adhesion to rat mesenteric post capillary venules[373, 374]. Interestingly α4 integrin has been reported to be highly expressed on most memory T lymphocytes [375] and appears to play important roles during inflammation. Whilst the β1 integrins have been demonstrated to play important roles for T lymphocyte recruitment and homing, β2 integrins appear less important, and their roles in recruitment of lymphocytes are still poorly defined. For instance, inhibition of β2 in VEC only partially reduced T lymphocyte firm adhesion , whereas inhibition of both β1 and β2 integrins completely blocked T lymphocyte adhesion and transmigration through TNFα stimulated VEC [190]. Others have reported that β2 integrins are required in the transmigration of lymphocytes [190, 376]. However, lymphocytes isolated from patients with LAD-1 (deficient in expression of β2) are recruited normally and transmigrate in response to inflammation *in vivo* [377]. Thus, it would seem that, transmigration in the context of inflammation requires β1 integrins while a role for β2 integrins remains to be clearly defined.

The data on the integrins involved in lymphocyte migration across LEC is very limited, indeed it is only available for migrating DCs. These studies have shown that transmigration of DCs across a LEC monolayer, involves CD31, while DC migration into draining lymph nodes in mice deficient in β2 integrins [378] and $\alpha_6$integrins [379] was markedly reduced. Our studies into the integrins

involved in lymphocyte migration across LEC, offers the first insight into the integrins used during this process. We showed that β1 integrin blockade caused a partial inhibition and β2 integrins showed a substantial block of the lymphocyte transmigration across LEC. Interestingly, it has been demonstrated that CCL19 and CCL21 can trigger the higher affinity state of the β2 integrin (LFA-1) and induce a rapid, but transient increase in lymphocyte adhesion to ICAM-1, a ligand for the β2 integrins *in vitro* [144, 380, 381].

Thus to summarise, it would seem lymphocytes utilise β1 integrins to cross VEC and then switch to predominantly β2 integrins to cross the LEC. As stated above, this is consistent with other reports that demonstrate that DCs use β2 integrins to cross LEC [382].

We investigated adhesion molecule expression in lymphatic endothelium after cytokine stimulation and found many similarities to the responses of blood vascular endothelium. Constitutive ICAM-1 expression was found on both HUVEC and LEC and following cytokine stimulation, there was an increase in expression in the LEC. It was, however, surprising that ICAM-1 did not increase more on HUVEC following stimulation. We have previously reported changes in response to TNF, and it is possible that the combination of cytokines used here elicits a distinct response in HUVEC. In addition VCAM-1 expression increased in HUVEC after cytokine stimulation, however we did not observe any concurrent inducible expression of this molecule in LEC. This observation is in contrast to Johnson et al (2006), who did not report inducible VCAM-1 expression on LEC. We also found that E-selectin was inducible on both HUVEC and LEC. It might be assumed that E-selectin expression on LEC is unusual, as it is considered to be a molecule that captures leukocytes from flowing blood. In the context of the

stromal environment it is not necessary to invoke such a paradigm. However, E-selectin has previously been described on LEC and the observation that engagement of selectins has been shown to provide signals regulating integrin function (β2 integrins) indicates that capture from flow may not be the only function of selectin molecules [383, 384].

CHAPTER 5

PGD2 is essential for transmigration of lymphocytes through endothelium

5 Introduction

The swift induction of an inflammatory response is essential for effective host defense. A wide range of pro-inflammatory agents including members of the prostaglandin and leukotriene family derived from arachidonic acid ensure that this response occurs [385]. Cyclooxygenase (COX) metabolises phospholipase A_2-derived arachidonic acid to prostaglandin (PGH_2) which is further metabolised by a series of downstream synthetases to prostanoids, and thromboxanes. Alternatively, the lipoxygenase enzymes generate leukotrienes (LTs) and lipoxins [385]. These eicosanoids induce and assist in the recruitment of immune cells, and some such as the lipoxins may facilitate resolution of inflammation.

Until recently, the resolution of inflammation was thought to occur mainly as a result of the passive removal of pro-inflammatory mediators. It is now evident that inflammatory pathways have check-points as well as temporal and spatial switches that regulate its onset, maintenance, and finally resolution [386]. Interestingly, the pathways required for inflammation to resolve, often need prior production of lipid mediators generated during the initial phases of inflammation [387]. For example PGE_2 and PGD_2, have a wide variety of roles *in vivo* [388], including complicated and often opposing effects in the immune system. PGD_2 has been shown to induce inflammation in a number of different settings. In humans, intradermal injection of PGD_2 causes erythema [230], and intratracheal PGD_2 administration induces lung eosinophilia in dogs [389] and rats [230]. Furthermore, mice over-expressing lipocalin-type PGD_2 synthase, one of the enzymes that produces PGD_2, display increased eosinophil and T lymphocyte recruitment to the lung [231]. PGD_2 is a relatively unstable molecule, which is readily degraded by a series of spontaneous dehydration and

isomerisation reactions *in vitro* and also by enzymatically catalysed reactions *in vivo* to a wide variety of metabolites [390, 391]. Several of these metabolites are now known to be bioactive and the production of J series metabolites, PGJ_2, Δ^{12}-PGJ_2 and 15d-PGJ_2 production is critically involved during the resolution of carrageenan-induced pleural inflammation, and when this PG production is blocked with COX inhibitors, the immune reaction is exacerbated [392]. It has been reported that 15d-PGJ_2 induces apoptosis of infiltrating neutrophils and aid recruitment of the macrophages to clear the apoptotic neutrophils [393]. Furthermore, evidence for the potential anti-inflammatory roles of PGD_2 and its metabolites has been provided in animal knockout studies, where mice lacking the enzymes required for the production of PGD_2 (hematopoietic PGD_2 synthase) displayed a more severe inflammatory response and failed to resolve the inflammation [394]. It is difficult from the current *in vivo* models to definitively understand the apparent dual nature of PGD_2 during the inflammatory process. Studies demonstrating the *in vitro* effects of PGD_2 on leukocytes are now beginning to explain how PG is able to induce such diverse *in vivo* responses. We have recently demonstrated that PGD_2 signalling is crucial for the transmigration of neutrophils through VEC [62]. The addition of a DP1 receptor (receptor for PGD_2 on neutrophils) antagonist inhibited the ability of the captured neutrophils to transmigrate through the endothelium. Interestingly, it has been demonstrated that lymphocytes express a functional receptor for PGD_2 termed CRTH2 or DP2 receptor, potentially expressed on Th2 helper subsets of lymphocytes [395], although DP2 may not be on all Th2 cells [395]. It has been suggested that these cells represent a subset of Th2 lymphocytes, which are more committed to the lineage than the $CRTH2^-$ Th2 cells [396], and are possibly central memory T lymphocytes [397]. Selective agonists of the DP2 have been shown to induce a chemotactic

response in cultured Th2 but not Th1 cells [398], and also cause the up-regulation of CD11b on these cells [399]. Th2 cells typically produce IL-4, IL-5 and IL-13, and DP2 mediated signaling in Th2 cells induces the expression of these cytokines *in vitro* [399]. The same study also showed that production of the Th1 cytokine IFNγ was inhibited by DP2 signaling which may skew the immune system to a more Th2-type response [399]. It would seem that PGD_2 signalling on T lymphocytes provides an important mechanism linking the recruitment of Th2 cells, the expression of Th2 cytokines [398], and the inhibition of Th1 cytokine production. The current literature suggests that PGD2 has both pro and anti-inflammatory effects. Due to the uncertainty of the location of PGD2 expression and its function on various cell types, and given that we have demonstrated the essential role it plays in neutrophil transmigration, we wanted to investigate the role it plays in lymphocyte migration.

5.1 Materials and Methods

5.1.1 Isolation of lymphocytes

Lymphocytes were isolated from whole blood as detailed in section 2.1.1

5.1.2 Isolation of HUVEC

Endothelial cells were isolated as detailed in section 2.1.2

5.1.3 Sub-culturing endothelium

EC was sub-cultured on to transwell filters as detailed in 2.1.5

5.1.4 Assaying the migration of lymphocytes across HUVEC in the presence of DP2 receptor antagonist

The assay was performed as described in section 2.1.6, with the following modifications:

The HUVEC was cultured on the inside of a transwell filter for 24 hours until a confluent monolayer was formed. The monolayer was stimulated with the cytokines as described previously. Lymphocytes were isolated as described and incubated with either vehicle, or Bay u 345 (DP2 antagonist) (Cayman chemicals, USA) or BW A868C (DP1 antagonist) (Cayman chemicals, USA) for 30 minutes before being added to the top of the HUVEC monolayer. The plate was then incubated and the number of cells transmigrated counted as previously described.

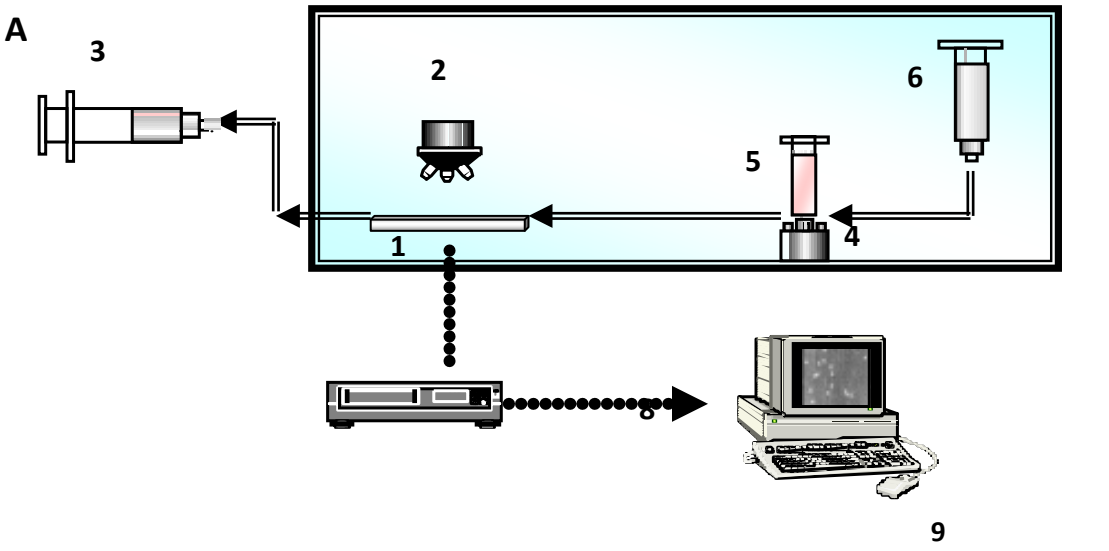
5.1.5 Flow based adhesion assay in the presence of DP2 antagonist

The flow based adhesion assay consists of an ibidi slide mounted on the stage of a microscope. One end of the ibidi slide is connected, *via* silicone tubing, to an electronic

switching valve (Lee Products, UK) which allows a switch between the lymphocyte cell suspension and the 0.15% PBSA wash buffer. The other end of the ibidi slide is attached to a Harvard syringe pump (Model 906, Harvard Apparatus, South Natwick, MA) which controls flow through the microslide. All experiments were carried out at a wall shear stress of 0.1Pa; this is comparable to the wall shear stress experienced in post capillary venules *in vivo*. The assay was maintained at 37°C inside a heated Perspex box. A video camera connected to the microscope enabled recording of the lymphocyte-HUVEC interactions which were analysed off line using Image Pro software (Image-Pro Plus, USA.) (Fig 5.1).

The protocol for the flow based assay was as follows: HUVEC were washed with buffer for 2minutes, followed by a 4 minute lymphocyte bolus and 15 minutes of wash buffer. Video recordings of 10 seconds duration were made, of 8 randomly selected fields, and these were taken at 10 minutes post lymphocyte bolus. During the last 5 minute wash period the microscope focused on a single field which was continuously recorded for 5 minutes. This was used to measure the migration velocity of lymphocytes which had transmigrated underneath the EC monolayer. Data presented here is from 15 minutes post lymphocyte bolus. Videos of the adhesive behavior of lymphocytes i.e. rolling, firmly adherent or transmigrated cells on EC were analysed using software Image Pro (Image-Pro Plus, USA.). The cell numbers were normalised per unit area and for the number of cells perfused (per $mm^2/10^6$ lymphocytes perfused). There were a number of populations of adherent lymphocytes. Phase-bright cells adherent to the apical surface of the ECs were either rolling, or activated firmly adherent cells. Phase-dark cells were transmigrated under the EC monolayer. Total lymphocyte adhesion was calculated as the total number of phase-bright and phase dark cells. The number of cells observed rolling, firmly adherent or transmigrated

was calculated as percentages of total adhesion. Using the video record of a single frame for 5minutes between 10 and 15 minutes after inflow of lymphocytes, a sequence of 10 digitised images was captured, each separated from the next by a 30 second interval. The analysis software allowed cells to be tracked and their velocity of migration to be measured.



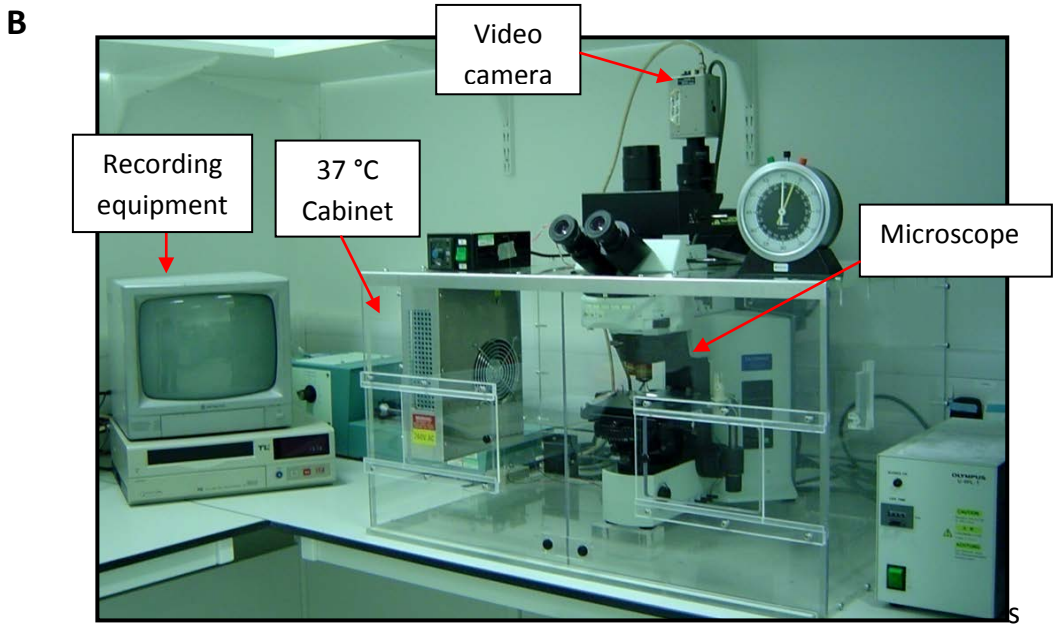


Figure 5.1 the flow-based adhesion assay. ***(A)*** ibidi slides (1) containing EC were mounted on the stage of a video-microscope (2) and attached *via* silicone tubing to a 50 ml glass syringe (3) and an electronic switching valve (4). Isolated lymphocytes (5) or PBSA (6) were perfused through the ibidi slides at a wall shear stress of 0.1 Pa. Experiments were conducted in a 37 ˚C Perspex cabinet (7) and video recordings made (8,9). (**B**) The video-microscope and recording equipment *in situ*.

5.1.6 Direct visual assay of lymphocyte adhesion and migration in static conditions

A direct visual assay was developed to investigate lymphocyte adhesion and transendothelial migration (including migration velocity). HUVEC were cultured until confluent in 6-well plastic plates, at levels that reach confluence within 24 hours. HUVEC were stimulated with 100U/ml TNFα and 10ng/ml IFNγ for 24 hours before assay. The monolayer surfaces were rinsed, prior to the assay, with PBSA to remove any residual cytokines.

The phase contrast video microscope (Inverted Labovert, Leitz) used to visualise lymphocyte adhesion and migration was contained within a Perspex box, the inside of which was maintained at 37^{o}C by a heater and thermostat. A 6-well plate holder, designed and made in house, was fixed to the stage. The following stages were performed inside the incubated box. The M199+BSA used had been pre-warmed to 37^{o}C. At time = 0, 2ml of lymphocytes +/- DP2 antagonist at 10^{6}/ml were added to each well and allowed to settle for 5 minutes. At time = 5 min the lymphocyte suspension was aspirated off and non-adherent lymphocytes were rinsed off with two gentle washes with M199+BSA. The washed surface was covered with 2ml of M199+BSA. The plate was viewed using a phase contrast video-microscope at an objective magnification of x 20.

The first sets of video recordings were made immediately after the wash stage. At least 5 different fields were chosen at random and recorded. Then one field was chosen at random and recorded continuously for 5 minutes, and this was repeated at 1, 2, 4 and 6 hours.

The video microscopic recordings were analysed off-line using a computer-assisted image

analysis system (ImagePro; USA). Lymphocytes in each field were counted, and the average taken for the repeated fields. The average was converted to cells/mm^2 using the known field dimensions, multiplied by the area of the well, divided by the total number of lymphocytes added, and then multiplied by 100 to express lymphocytes adhesion as a percentage of the cells added.

5.1.7 Conditioned medium stimulation of lymphocytes

HUVEC were cultured on 6-well plates and allowed to reach confluence. The HUVEC monolayer was then stimulated with 100U/ml TNFα and 10ng/ml IFNγ for 24 hours. The monolayer was washed with M199+BSA and incubated for 24 hours. At the same time, HUVEC from the same donor were cultured on transwell filters and stimulated with the same cytokine regime as above. Lymphocytes were then isolated and added to the top of the stimulated HUVEC cultured on the transwells, a portion of the lymphocytes was rested on plastic and another portion was incubated with the medium collected from the HUVEC cultured in 6 well plates for 24 hours. Subsequently, the lymphocytes that had been migrated through the HUVEC monolayer were collected as well as the rested lymphocytes and those that had been cultured in the conditioned media. Lymphocytes were washed, resuspended to $1x10^6$/ml and added to the top of a second transwell filter with LEC subcultured on the basal surface that had been previously treated with or without TNFα and IFNγ stimulation for 24 hours. The number of transmigrated lymphocytes were assessed at 24 hours as described previously.

5.1.8 Assaying the migration of lymphocytes across LEC after migration across VEC

The assay was performed as described in section 2.1.8, with the modifications to investigate the effects of lymphocyte migration across HUVEC and LEC of 1) blockade of G-protein coupled receptors 2) exogenous PGD_2 3) lymphocyte stimulation using exogenous adhesion molecules and chemokines 4) unstimulated HUVEC transmigration

1) To investigate G-protein coupled receptor blockade on lymphocyte recruitment, isolated lymphocytes were allowed to migrate through a HUVEC monolayer for 24 hours. These lymphocytes were then collected and resuspended to $500x10^3$/ml of M199+BSA +/- pertussis toxin (Sigma UK) and incubated for 2 hours. 2ml of the cell suspension was then added to the top of a transwell insert which had cytokine-treated LEC cultured on the basal surface. The plate was then incubated for 24 hours at 37°C after which, lymphocytes were collected from the well and counted.

2) An adhesion assay was performed as detailed in 2.1.8, but a portion of the lymphocytes rested on plastic for 24 hours was incubated with 10nM PGD_2 or 100nM DP1 antagonist for 24 hours. The lymphocytes were collected from the wells, washed and then added to the top of a transwell filter with LEC subcultured to the basal surface of the filter. The plate was then incubated for 24 hours at 37°C. The lymphocytes were then collected from the well and counted.

3) An adhesion assay was set up as described previously, but with the following modifications, recombinant human VCAM-1, ICAM-1 and CD31 (R & D Systems) were diluted to 20μg/ml in PBS and added to a six-well plate and incubated at 4°C

overnight. The wells were washed with PBS. Lymphocytes were isolated as usual on day 3 and a proportion were either (1) allowed to migrate through HUVEC, (2) incubated on plastic or (3) were incubated with the recombinant human adhesion molecules or 80ng/ml chemokines, CXCL10, CXCL9 and CXCL11 (Peprotech, USA).

4) An adhesion assay was set up as described 2.1.8, but the HUVEC monolayer was not stimulated with cytokines. The lymphocytes were allowed to migrate through the un-stimulated HUVEC for 24 hours. The lymphocytes were then collected and added to the top of a LEC monolayer which was subcultured on the basal surface +/- TNFα and IFNγ.

5.2 Results

5.2.1 Lymphocyte transmigration is retarded in the presence of a DP_2 antagonist in static conditions

We recently demonstrated that neutrophils require a PGD_2 signal to enable them to migrate through a cytokine stimulated HUVEC monolayer; abrogation of this signal inhibited transmigration but had no effect on neutrophil capture or subsequent stabilisation of these adhesive interactions [62]. Thus, we wanted to determine whether lymphocytes also require a PGD_2 signal to migrate across the HUVEC. Initially, we used a static transwell adhesion assay to investigate this idea.

When lymphocytes were added to the top of a cytokine stimulated HUVEC monolayer in the presence of a specific DP1 antagonist (neutrophil receptor for PGD_2) and allowed to migrate for 24 hours, we found that there was no effect on the level of transmigration observed compared to untreated control (Fig.5.2) When lymphocytes were incubated with a DP2 antagonist we found that the ability of the lymphocytes to transmigrate through the HUVEC decreased in a dose-dependent manner (Fig 5.2). The highest concentration of the DP2 antagonist (1µM) caused a significant reduction in the number of lymphocytes able to migrate through the HUVEC, although approximately 10% of the lymphocytes were able to migrate through the endothelium, even at this dose.

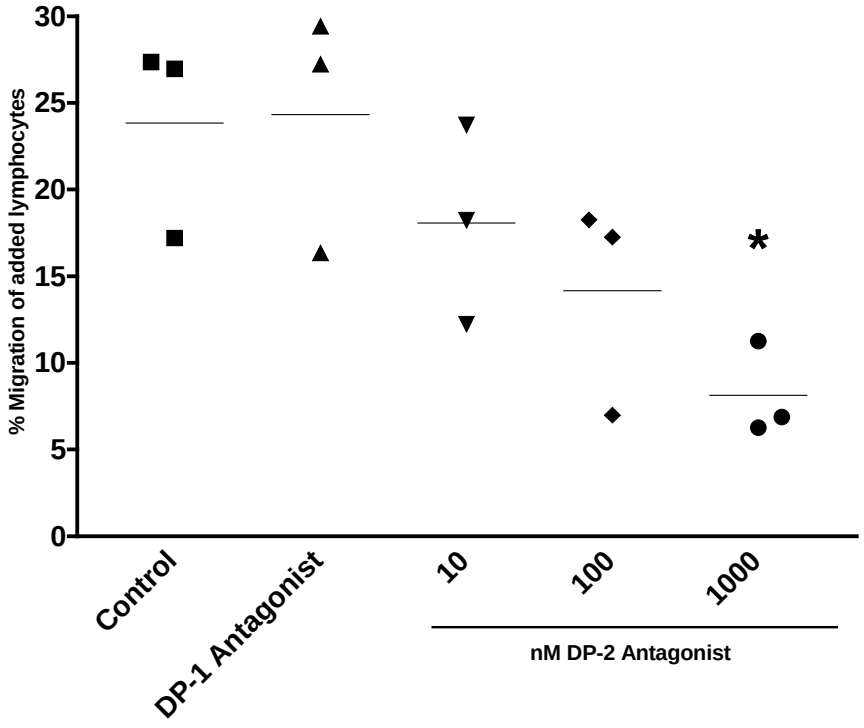


Figure 5.2: DP2 antagonist retards the transmigration of lymphocytes through a stimulated HUVEC monolayer. Lymphocytes were isolated from healthy donors and incubated with 100nM of DP1 antagonist or DP2 antagonist for 20 mins prior to being added to the top of a HUVEC monolayer. These data show the mean (horizontal line) from 3 independent experiments. *=$p<0.05$ by Mann-Whitney test compared to control.

5.2.2 Lymphocyte transmigration is retarded in the presence of a DP2 antagonist under flow conditions

As we demonstrated that antagonism of the DP2 receptor caused a significant reduction in lymphocyte transmigration, we wanted to investigate whether this observation would still be present under flow conditions. In addition, we wanted to elucidate a possible mechanism for the reduction in transmigration. To this end, a flow adhesion assay was performed. When lymphocytes were perfused across an unstimulated HUVEC monolayer, the endothelium captured very low numbers of lymphocytes. When the HUVEC were stimulated with cytokines, we found that the EC was able to capture significantly more flowing lymphocytes compared to an unstimulated EC (Fig 5.3). When lymphocytes were incubated with a DP2 antagonist and flowed over stimulated HUVEC, we found the level of adhesion to be very similar to that of the cytokine treated HUVEC (Fig 5.3a). When the level of transmigration was assessed in the presence of the DP2 antagonist we found transmigration to be significantly reduced when compared to the cytokine treated HUVEC (Fig 5.3b). These observations suggest that antagonism of the DP2 receptor does not affect the capture and firm adhesion of the lymphocyte by the action of chemokines expressed on the surface of the endothelium, but specifically inhibits the transmigration phase.

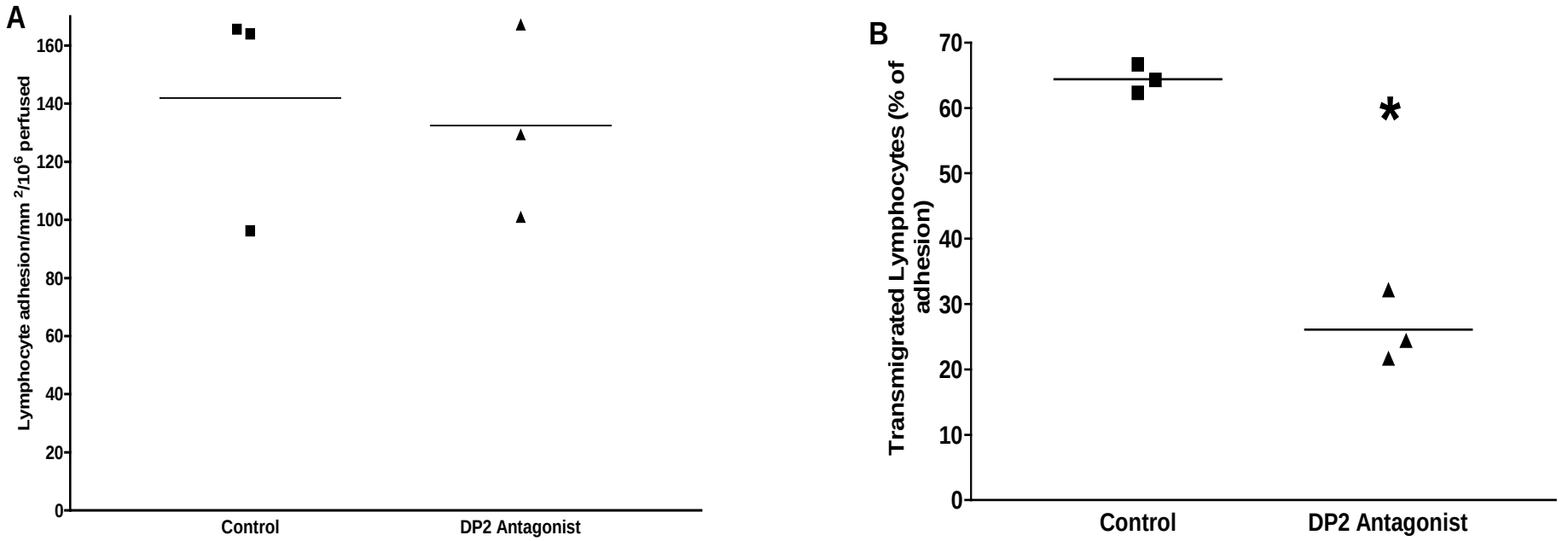


Figure 5.3: Lymphocyte transmigration through a cytokine stimulated HUVEC was inhibited by a DP2 antagonist under flow conditions. A 4-min bolus of lymphocytes was perfused over HUVEC that had been cultured inside ibidi chamber slides and treated with TNFα and IFNγ for 24 hours. (A) Effects of PGD2 antagonist on lymphocyte adhesion. (B) Effect of PGD2 antagonist on lymphocyte transmigration. The lymphocytes were perfused over the HUVEC in the presence of the DP2 antagonist. These data show the mean (horizontal line) from 3 independent experiments. *=P<0.05 compared to control by Mann-Whitney test.

5.2.3 The direct observation of lymphocytes transmigrating through HUVEC in the presence of a PGD2 receptor antagonist

We next wanted to investigate the behavior of the lymphocytes on EC over an extended period of time in the presence and absence of the DP2 antagonist. To this end HUVEC were cultured in multi-well plates and cytokine stimulated for 24 hours before the addition of lymphocyte in the presence or absence of a DP2 antagonist. The adhesion and transmigration of the lymphocytes was assessed at 10 mins and at 1, 2, 3, 4 and 6 hours.

Unstimulated HUVEC supported low levels of adhesion (Fig 5.4a), which was significantly increased following cytokine treatment of the HUVEC, by approximately two-fold (Fig 5.4a). Interestingly, in the presence of the DP2 antagonist we found that the level of adhesion was slightly higher than cytokine treated endothelium, although this was not statistically significant (Fig 5.4a). When the level of transmigration was assessed we found a low level of transmigration on the unstimulated HUVEC (Fig 5.4b). The number of transmigrated lymphocytes observed in the presence of the DP2 antagonist was significantly lower than control when this comparison was made on cytokine treated EC (Fig 5.4b). We observed no effect of time on adhesion or transmigration, over a period of 6 hours. Thus, the effect of antagonising the PGD2 receptor occurs early in the assay, and there is no indication that over the 6 hours of this assay that the lymphocytes adapt to utilise alternative signals to achieve trans-endothelial migration.

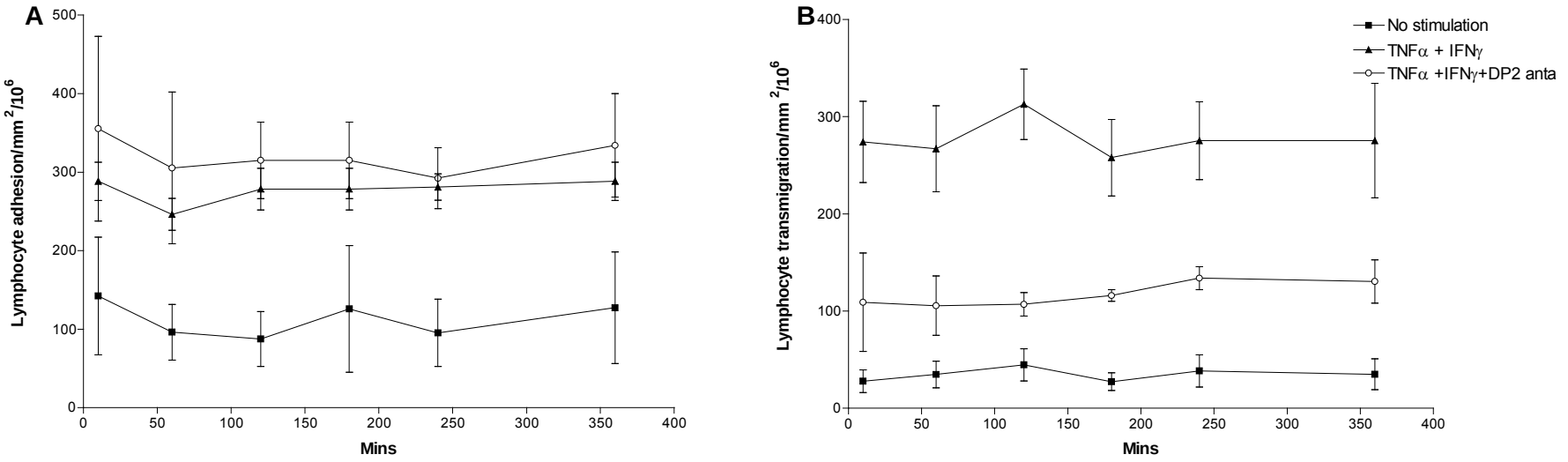


Figure 5.4: Lymphocyte adhesion and transmigration across HUVEC does not change over a period of 6 hours. HUVEC were stimulated with 100 U/ml TNF and 10 ng/ml IFN for 24 h. Lymphocytes were allowed to settle for 5 min, non-adherent cells were washed off, and lymphocyte adhesion and transmigration were analysed by phase-contrast microscopy. (A) Effect of lymphocyte adhesion. (B) Effect of lymphocyte transmigration. Data are mean ± SEM from three independent experiments. ANOVA shows a significant effect of treatment but not of time p=0.0127.

5.2.4 Lymphocyte stimulation using exogenous chemokines and integrin ligands

Thus far, we have demonstrated that inhibition of CCR7 had a significant impact on the ability of lymphocyte to transmigrate across LEC, but still have not elucidated the signal(s) imparted to the lymphocyte by the vascular endothelium which caused the enhanced transmigration. Data from our own lab and other groups have demonstrated the importance of CXCR3 receptor signalling with respect to lymphocyte adhesion [42, 285]. Thus, we wanted to investigate the possibility that lymphocytes incubated with various exogenous chemokines and or integrin ligands would be able to recapitulate the endogenous signals imparted onto the lymphocytes during interactions with vascular endothelium. An adhesion assay was set up where the lymphocytes were incubated with various exogenous chemokines and proteins for 24 hours and then placed onto a cytokine stimulated LEC monolayer.

When lymphocytes were migrated across HUVEC and then added to a stimulated LEC we found the level of transmigration was approximately 35%, and the level of transmigration of plastic cultured lymphocytes was approximately 12%-data in line with observations in other sections of this thesis. When the lymphocytes were incubated with CXCL10, CXCL9 and CXCL11, we found no significant difference in transmigration when compared to plastic rested lymphocytes, although we did see a small but consistent increase in transmigration with chemokines CXCL9 and CXCL11 (Fig 5.5a). Lymphocyte stimulation with CD31, VCAM-1 or ICAM-1 caused a small increase in transmigration through the LEC, although these changes were not significant (Fig 5.5B). Thus, we would conclude that chemokine and

adhesion molecules stimulation of lymphocytes does not enhance transmigration through the LEC.

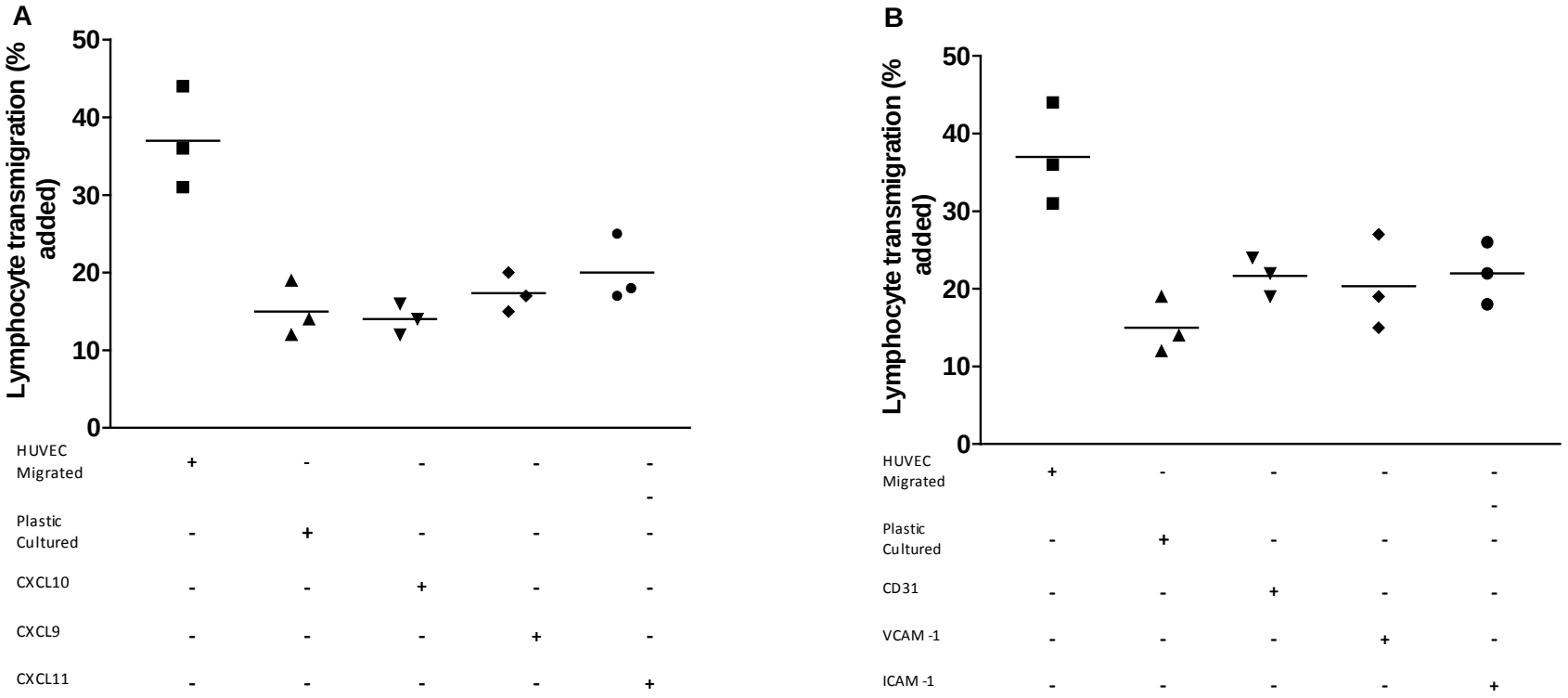


Figure 5.5 Exogenous chemokines or integrin ligands do not restore lymphocyte transmigration through LEC. Lymphocytes were incubated with exogenous chemokines (A) or integrin ligands (B) for 24 hours and added to the top of a stimulated LEC monolayer for 24 hours, lymphocyte were collected from bottom of the well after a period of 24 hours and counted. These data show the mean (horizontal line) from 3 independent experiments.

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5.2.5 The effect of incubating lymphocytes with conditioned medium on their ability to transmigrate through LEC

As we could not show enhanced recruitment through the LEC following exogenous chemokine and adhesion molecule stimulation, we wanted to investigate the possibility that other unknown soluble factors released by the HUVEC could stimulate the lymphocytes to allow the enhanced transmigration. To test this, we cytokine treated HUVEC for 24 hours, washed the HUVEC and added M199+BSA. The HUVEC were incubated for a further 24 hours, and the "conditioned medium" was collected. Lymphocytes were incubated with the conditioned medium for 24 hours and then allowed to migrate across LEC and we assessed the level of transmigration that occurred.

When lymphocytes were incubated with cytokine treated conditioned medium for 24 hours and then added to a stimulated LEC monolayer, we found that the level of transmigration to be similar to that observed when lymphocytes were cultured on plastic (Fig 5.6). Interestingly, when lymphocytes is incubated with the conditioned medium and for 24 hours and allowed to interact with an unstimulated LEC monolayer we found that the level of transmigration was significantly decreased, even below the level of transmigration observed for plastic cultured lymphocytes (Fig 5.6). Taken together, these data would suggest that any soluble factors that could potentiate the migratory ability of the lymphocytes is either not released by the HUVEC into the media, the potential soluble factor is broken down and no longer has an effect on the lymphocytes, or the HUVEC signal imparted on the lymphocytes is not released into the media. Moreover, it appears that HUVEC generate and release an

agent that efficiently inhibits the migration of lymphocytes across LEC in the absence of an inflammatory stimulus, which promotes such transmigration.

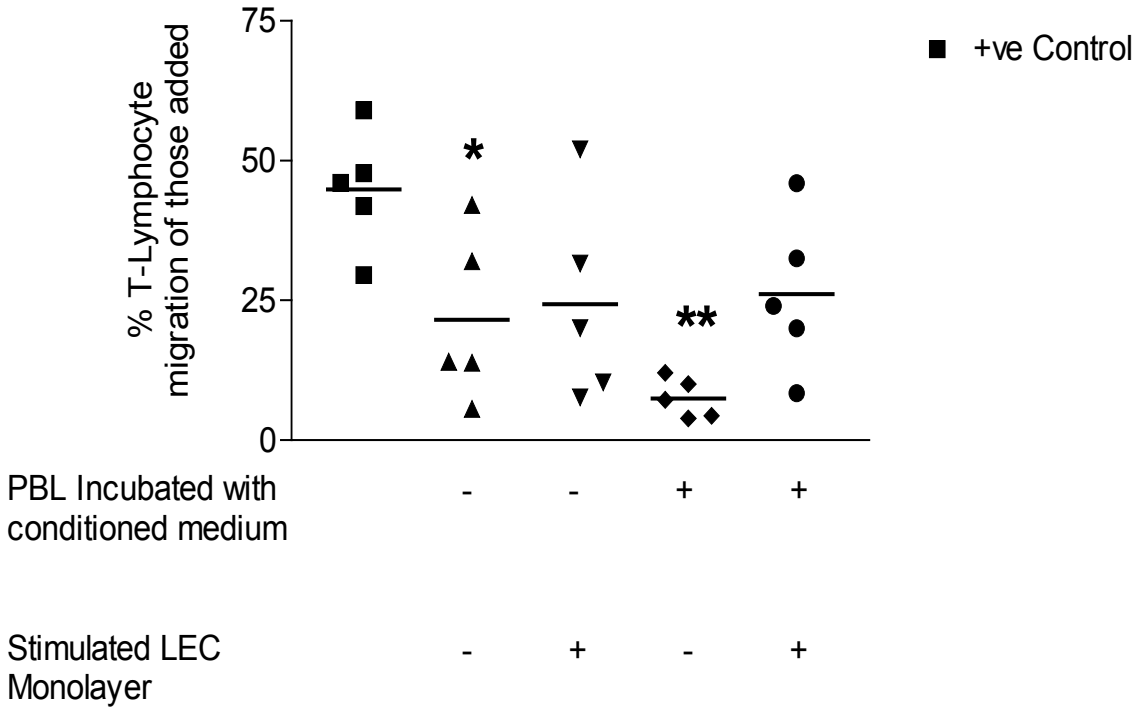

Figure 5.6 HUVEC conditioned medium does not restore the ability of lymphocytes to migrate across the LEC with greater efficiency. Lymphocytes were incubated with or without conditioned medium for 24 hours and added on to the top of either a stimulated or un-stimulated LEC monolayer. +ve Control= lymphocytes previously migrated through a HUVEC monolayer. These data show the mean (horizontal line) from 5 independent experiments. ANOVA showed a significant effect of conditioned medium on transmigration $p=0.0054$. *=$p<0.05$. **=$p<0.001$ compared to positive control Dunnett's multiple comparison test.

5.2.6 Pertussis toxin significantly reduces the transmigration of lymphocytes through a LEC monolayer

We have previously shown that COX inhibition is able to reduce the transmigration of neutrophils through a HUVEC monolayer, so we wanted to investigate whether COX inhibitors are able to reduce the enhanced transmigration of lymphocytes through a LEC monolayer. In addition, we also wanted to investigate whether G protein coupled receptors play a role in the transmigration of lymphocytes through a LEC monolayer. To this end, HUVEC were cytokine stimulated in the presence of COX and/or LOX inhibitors, and lymphocytes were added and allowed to transmigrate through the HUVEC for 24 hours. Migrated lymphocytes were then collected and added to the top of a LEC monolayer. In addition, a proportion of the lymphocytes were incubated with pertussis toxin for 2 hours and then added to the top of a stimulated LEC monolayer.

When HUVEC are cytokine stimulated in the presence of a COX inhibitor (indothemacin) and lymphocytes are allowed to transmigrate through the HUVEC, and then added to the LEC monolayer we found there is no significant change in the level of transmigration through the LEC (Fig 5.7), i.e. the enhanced transmigration observed is still present. When HUVEC are stimulated in the presence of a LOX inhibitor we found a small but non-significant reduction in the transmigration of the lymphocytes through the LEC. The combination of both COX and LOX inhibitors caused a further reduction of transmigration, although this was not significant. When lymphocytes which have migrated through HUVEC were incubated with pertussis toxin, an inhibitor of receptors coupled to G proteins, we found that it caused a reduction in lymphocyte transmigration through a stimulated LEC monolayer, although not statistically significant. Taken together it would seem that the COX and LOX enzymes do not

play a major role in the signal imparted on the lymphocytes that allow for the enhanced transmigration. Moreover, it would seem that G protein coupled receptors may play a role in the priming of the lymphocytes.

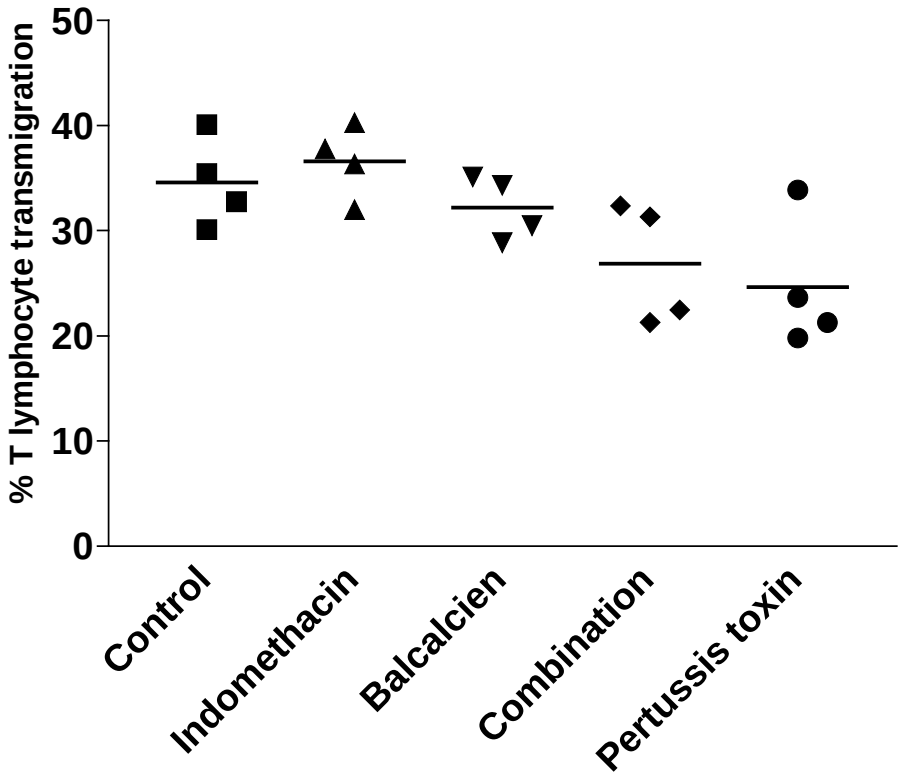

Figure 5.7 COX and LOX inhibitors are unable to retard the enhanced transmigration through the LEC. HUVEC were stimulated with cytokines in the presence of either Indomethacin (10µM) and/or Baicalein (10µM) or both (Combination) for 24 hours, lymphocytes were then added and incubated for 24 hours. Lymphocytes were collected and then added to the top of a stimulated LEC monolayer. Lymphocytes were incubated with 100ng/ml pertussis toxin for 2 hours before being added to the top of a stimulated LEC monolayer.

5.2.7 The signal imparted on to lymphocytes by VEC does not occur in the absence of cytokine stimulation

When lymphocytes are added to an un-stimulated HUVEC monolayer a percentage of the lymphocytes are still able to transmigrate. We wanted to investigate whether the signal imparted on to the lymphocytes during transmigration is stimulation dependent. To answer this, we migrated lymphocytes across an un-stimulated HUVEC monolayer, and then allowed the lymphocytes to migrate across LEC and assessed the transmigration ability of the lymphocytes. When lymphocytes were migrated across an unstimulated HUVEC and then allowed to migrate across the LEC we found that the transmigratory ability of the lymphocytes was lost (Fig 5.8). The level of transmigration was comparable to the levels observed when lymphocytes are rested on plastic (Fig 5.8). The stimulation of the LEC monolayer did not cause an increase in the percentage of the cells able to transmigrate, as we saw previously.

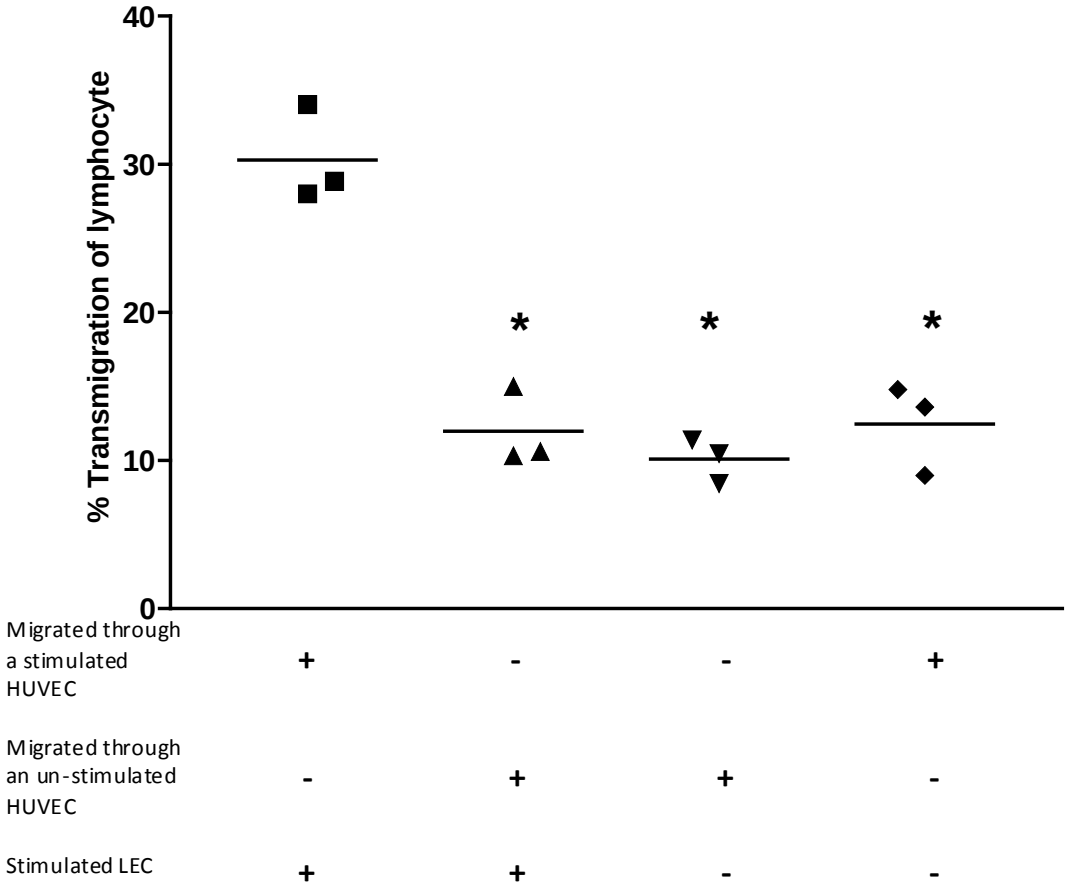

Figure 5.8: The enhanced transmigration across LEC is ablated if the HUVEC is not cytokine treated. Lymphocytes were added on to the top of an unstimulated HUVEC monolayer and allowed to transmigrate; they were collected and allowed to migrate through LEC monolayer +/- stimulation. These data show the mean (horizontal line) from 3 independent experiments. *=$p<0.05$ by Mann-Whitney test.

5.2.8 PGD_2 is able to cause enhanced transmigration through the LEC

We have demonstrated the important role that PGD_2 plays in the transmigration of lymphocytes across a vascular endothelium, but its potential role in priming lymphocytes to migrate across LEC is unknown. To enable us to investigate this, we incubated lymphocytes on plastic with exogenous PGD_2 and then allowed the lymphocytes to transmigrate across a LEC monolayer.

When lymphocytes were incubated with exogenous PGD_2 we found that it was able to partially restore the enhanced transmigration observed when lymphocytes are migrated through a HUVEC monolayer (Fig 5.9), although this was consistent between donors it was not statistically significant. Migration of the lymphocytes significantly increased by 10% (Fig 5.9), although this increase did not quite reach levels observed when migrated lymphocytes are added to the LEC. When the LEC monolayer was unstimulated and lymphocytes incubated with exogenous PGD_2 and allowed to transmigrate, the lymphocytes showed a similar level of transmigration, comparable to plastic cultured lymphocytes added to a stimulated LEC monolayer. In addition, this observation is similar to previous experiments when lymphocytes were migrated through HUVEC and allowed to migrate across an unstimulated LEC. We also found that a 15 minute incubation of the exogenous PGD_2 was sufficient to cause the same level of transmigration as 24 hour incubation. Interestingly however, when lymphocytes were incubated with a specific DP1 antagonist we found that it had no effect on the ability of lymphocytes to traverse the LEC monolayer.

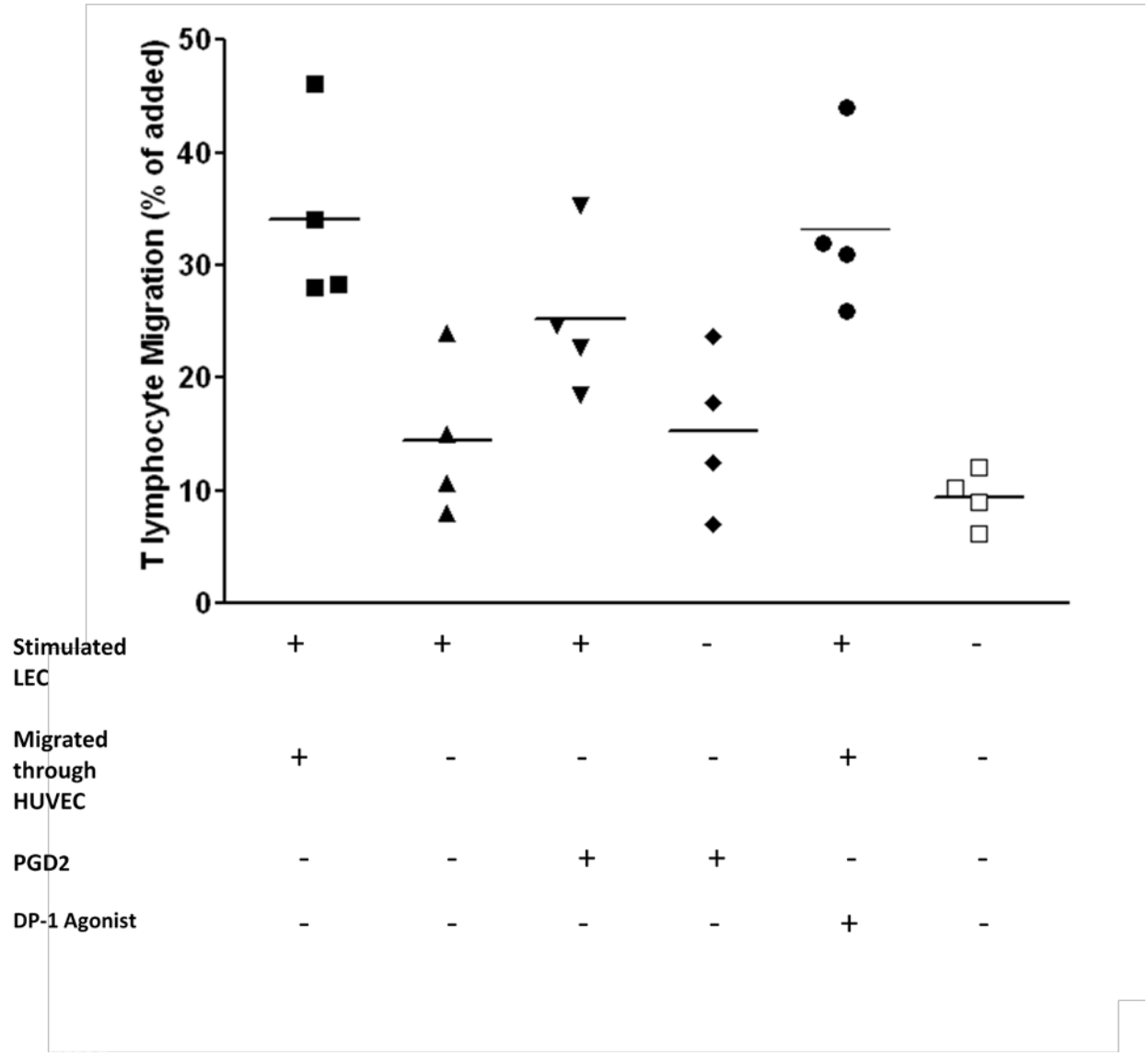

Figure 5.9 Exogenous PGD_2 is able to partially induce the enhanced transmigration of lymphocytes through LEC. Lymphocytes were incubated with exogenous 10nM PGD_2 for 24 hours before being added to the top of a LEC monolayer. Alternatively lymphocytes that were treated with the DP1 antagonist were first migrated through HUVEC and then migrated through LEC in the presence of the DP1 antagonist. These data show the mean (horizontal line) from 4 independent experiments.

5.2.9 PGD_2 enhances the migration of lymphocytes

As we have demonstrated that the incubation of PGD_2 with lymphocytes is able to enhance the transmigration of the lymphocytes through the LEC, we wanted to investigate whether this is due to an increased migration velocity or better response to the chemokine gradients in our system. We set up a Boyden chamber assay, where lymphocytes were either rested on plastic, migrated through HUVEC or incubated with exogenous PGD_2 for 24 hours. The cells were then migrated towards either CXCL12 or CCL21.

When no chemokine was added to the bottom of the chamber we found that there was a basal level of spontaneous migration into the lower chamber of approximately 6% (Fig 5.10). When CXCL12 was added to the bottom chamber we found that it caused a significant increase in the percentage of lymphocytes in the bottom of the well when compared to the chamber which did not contain chemokines (Fig 5.10). We found that the percentage of migrated lymphocytes was significantly higher in the bottom of the CXCL12 wells when compared to the resting cells, this was also the case for the PGD_2 incubated cells; in fact, we observed no difference in the migration between migrated or PGD_2-stimulated lymphocytes in the presence of CXCL12 (Fig 5.10). When the lymphocytes were migrated towards the chemokine CCL21, lymphocytes migrated through HUVEC or those that had been PGD_2-stimulated were present in significantly higher numbers than the plastic cultured lymphocytes (Fig 5.10). We conclude that migration through HUVEC or incubation with PGD2 caused significantly more migration towards CXCL12 and towards CCL21.

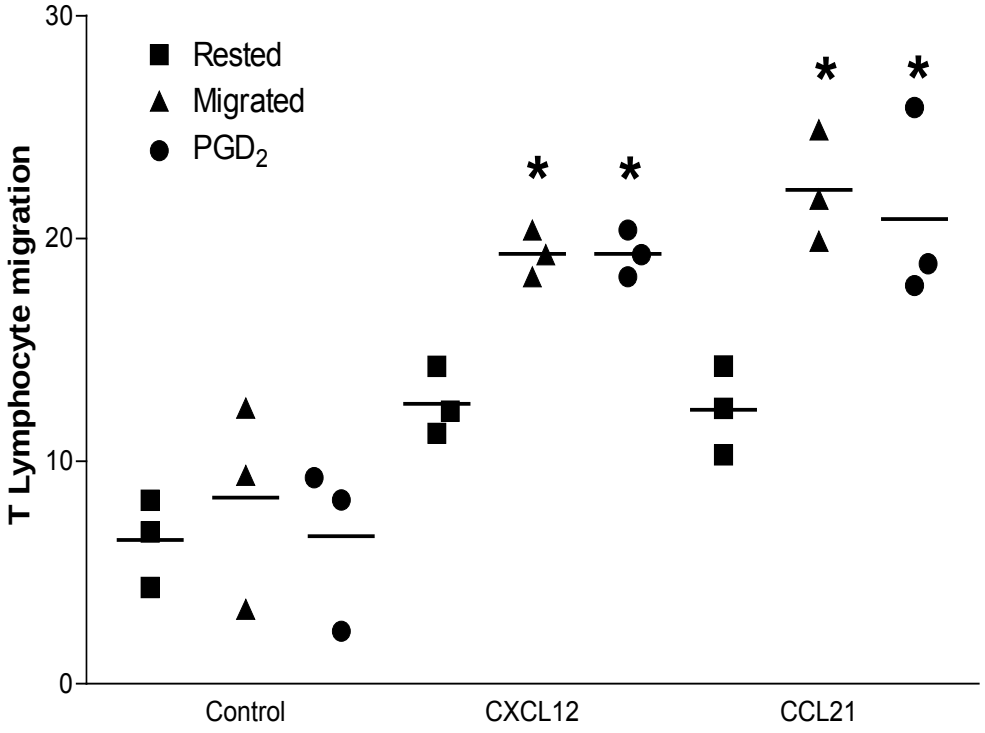

Figure 5.10 Transmigration or PGD_2 stimulation is able to enhance the migration of lymphocytes to both CXCL12 and CCL21. Lymphocytes were either migrated through HUVEC or incubated with PGD_2 (10nM) for an equivalent period of time and migrated towards the chemokines CXCL12 (100ng/ml) or CCL21 (100ng/ml) for 2 hours. These data show the mean (horizontal line) from 4 independent experiments.*=P<0.05 compared to rested lymphocytes by Mann-Whitney test.

5.2.10 The CCR7 expression of lymphocytes does not change with incubation with PGD_2 or transmigration through HUVEC

The migration of either transmigrated lymphocytes or those incubated with PGD_2 showed a greater response to the chemokine CCL21 than CXCL12 in the Boyden chamber assays. As the receptor for this chemokine is CCR7 which we have shown to be involved in lymphocyte migration in our assay, and as it is known that this chemokine is constitutively expressed by LEC, we wanted to investigate if PGD_2 causes an upregulation of the receptor, thereby explaining the enhanced response to the chemokine CCL21.

When CD4 naïve lymphocytes were stained for CCR7 expression and migrated through HUVEC or incubated for 24 hours with PGD_2 we observed no significant changes in the level of CCR7 expression (Fig 5.11). This was also the case for the CD4 memory lymphocytes, although we did observe a small increase in CCR7 expression on transmigrated lymphocytes when compared to their naïve counterparts (Fig 5.11). CD8 lymphocytes showed similar expression patterns as the CD4 lymphocytes but we also observed a small but consistent decrease in the level of CCR7 expression when lymphocytes were migrated across the HUVEC, and this was true for both CD8 subsets (Fig 5.11).

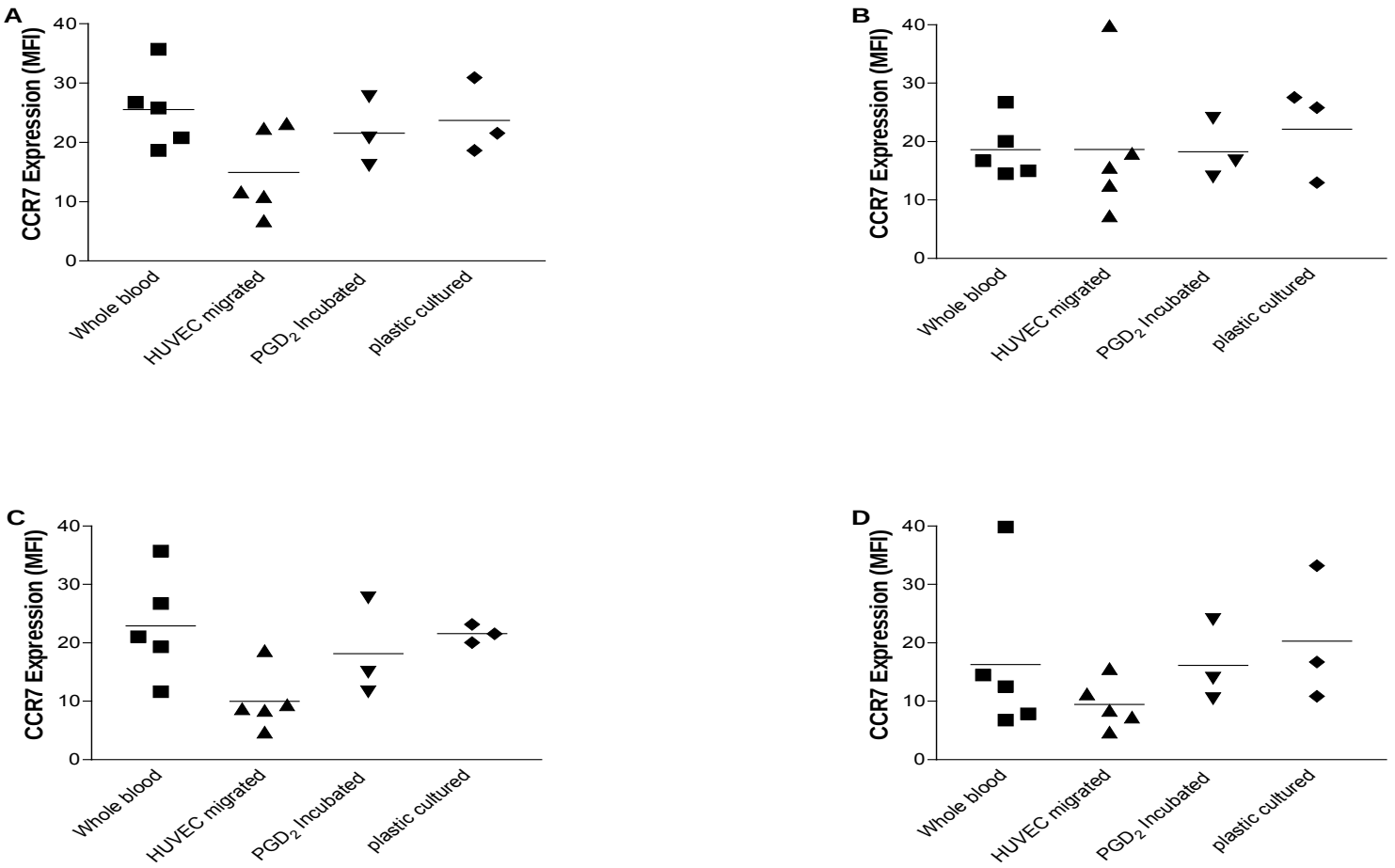

Figure 5.11 Expression of CCR7 does not change following stimulation with PGD_2. The CCR7 expression profile of; (A) CD4 naïve lymphocytes. (B) CD4 memory lymphocytes, (C) CD8 naive lymphocytes, and (D) CD8 memory lymphocytes, following isolation from blood, transmigrated through HUVEC or incubation with PGD_2 for 24 hours. These data show the mean (horizontal line) from 4 independent experiments.

5.3 Discussion

Previously published data from our lab had demonstrated that neutrophils, once captured on stimulated HUVEC, require a PGD_2 signal to migrate through the EC monolayer [62]. We have demonstrated in this chapter that lymphocytes also require this prostanoid signal to migrate through a HUVEC monolayer, both under static and flow conditions. The PGD_2 signal given to the lymphocytes is through a different PGD_2 receptor known as the DP-2 receptor. In addition, we have shown that the incubation of lymphocytes with exogenous PGD_2 is able to partially recapitulate the signal imparted on to lymphocytes during transmigration through vascular endothelium that allows the enhanced migration through the LEC. We also demonstrated that the migration towards exogenous CCL21 is enhanced when lymphocytes are pre-incubated with PGD_2.

When lymphocytes are incubated with a DP-2 antagonist we found a dramatic reduction in the number of lymphocytes able to transmigrate through the HUVEC. This is very similar to our previous observation with neutrophils, where we showed that antagonism of the PGD_2 receptor, DP-1, inhibits transmigration [62]. It has been reported that the PGD_2 receptor on lymphocytes is CRTH2 or DP-2. This, allegedly, is expressed primarily on the Th2 lymphocytes, and is not present on other lymphocyte subsets [395]. Taking this into account, this would suggest that the majority of the lymphocytes migrating through the VEC in our system are of the Th2 phenotype, as antagonism of the DP-2 receptor caused a 60% reduction in migration. Therefore, the reports that state that only Th2 lymphocytes have the DP-2 receptor may be inaccurate, or the PGD_2 in our experiments is acting through another receptor which has similar functions to the DP-2 receptor. Furthermore, it was demonstrated that PGD2 receptors are also

found on B1 cells, which would indicate that the reports which demonstrated DP-2 expression exclusively on Th2 cells are incorrect [387]. An important consideration when assessing the data on DP2 expression on lymphocytes is the fact that most data has been generated from cells obtained after polarisation along the Th1 or Th2 pathways *in vitro*. In this context, DP-2 may be restricted to a 'Th2' population. However, as these artificially polarised cells have only some resemblance to circulatory PBL, it is a safe assumption that the DP-2 receptor has a wider distribution and probably is not relevant to the Th1/Th2 classification, at least within the vascular compartment.

There are problems associated with static (filter based) adhesion assays used in this thesis, one such problem is the long incubation times associated with static assays, which causes high levels of adhesion and migration. In fact, it has been reported that 3-20% of neutrophils migrate through unstimulated EC over a period of one hour [400, 401]. This is likely due to an accumulation of soluble mediators which would normally be washed away under flow conditions. For example, resting cultured EC have been shown to secrete up to 10ng/ml IL-8 over a period of 6 hours [402], which is sufficient to activate neutrophils and induce adhesion [403]. In addition, the capture process seen *in vivo* does not take place. However, it is important to note that the efficiency of leukocyte transmigration is similar under static and flow conditions, but the appearance of leukocytes below the filter is more rapid under flow conditions [404]. Interestingly it has been shown that shear stress experienced by EC causes the upregulation of ICAM-1, which may aid the transmigration of leukocytes through the EC [405] . In addition, it was shown that shear stress is required for CD62L expression on HEV [406].

We did not observe any effect of incubation with the CXCR3 ligands or the recombinant human adhesion proteins with respect to increasing the migration across LEC. The incubation of the lymphocytes with the chemokines and or adhesion molecules for 24 hours was chosen because in our current system, the lymphocytes are exposed to the chemokines and adhesion molecules for 24 hours and the potential downregulation or desensitisation of particular receptors may be crucial to allow expedited migration across LEC. To allow us to ascertain the effect of the specific CXCR3 ligands independant of native integrin ligand interactions on the EC, we added the chemokines and integrin ligands separately. This may have revealed differential effects of the different CXCR3 ligands with respect to the enhanced migration we observe through LEC.

CXCR3 receptor expression is rapidly induced on naïve T cells following activation, and preferentially remains highly expressed on type-1 helper (Th1)-type CD4(+) T cells, effector CD8(+) T cells, and innate-type lymphocytes, such as natural killer (NK) and NKT cells [407]. Differential regulation of the three ligands at specific times in defined anatomically restricted locations *in vivo* likely participates in the fine control of T-cell trafficking over the course of an immune response. Among the differences in regulation, CXCL10 is induced by a variety of innate stimuli that induce IFN-α/β as well as the adaptive immune cell cytokine IFN-γ, whereas CXCL9 induction is restricted to IFN-γ.

Endothelial cells generate COX products constitutively, and COX products have been shown to play roles in vascular tone [408]. These prostanoids have been reported to have half lives of minutes in plasma. PGD_2 is metabolised non-enzymatically to produce PGs of the J series, including PGJ_2 and 15d-PGJ_2 whose relevance in inflammatory responses is controversial [393].

It has been shown that the DP-2 receptor is also able to bind the J series of prostanoids. This could mean that the exogenous PGD_2 we are adding could be broken down and interact with other receptors. In fact it has been shown that one of the metabolites of PGD_2, 15d-PGJ_2 is a ligand for PPARγ [409] and its interaction may inhibit the transcription of inflammatory cytokines such as IFNγ [410], therefore these prostanoids may also be exerting transcriptional changes in the lymphocytes which aid migration across LEC. Indeed, the time elapsed between HUVEC migration and LEC migration (24h) would be permissive of such a paradigm. When we incubated HUVEC with inflammatory cytokines in the presence of COX inhibitors we found no reduction in the ability of the lymphocytes to migrate across a HUVEC or LEC monolayer, and as the prostanoids are generated downstream of the COX enzyme actions it is surprising that we did not see a significant reduction in transmigration, as it has been reported that there is a reduction of neutrophil migration in the presence of COX inhibitors [62]. This would suggest that the source of PGD_2 may not just be from the EC, but produced by the lymphocyte itself. It has been shown that mast cells are able to produce PGD_2very rapidly following allergen driven cross linking of IgE [229]. Furthermore, Th2 cells produce low levels of PGD_2 following anti-CD3/CD28 stimulation [411].

When lymphocytes were migrated through unstimulated HUVEC and then allowed to migrate across a stimulated LEC monolayer we found that the enhanced transmigration normally seen was absent. This would indicate that the signal imparted on to the lymphocytes is dependent on the activation of the EC. In fact it has been shown that following HUVEC stimulation COX-2 activity is increased, leading to more PGE_2 production [408]. The reduced PG production in un-stimulated EC could explain the loss of the enhanced transmigration through LEC. However, this

has to be rationalised with the data indicating that COX inhibitors are ineffective at ablating the EC driven process of migration.

The incubation of lymphocytes with exogenous PGD_2 partially recapitulated the enhanced migration observed when the lymphocytes were migrated through a HUVEC monolayer. This enhancement of the ability of the lymphocytes to migrate through the LEC could be attributed to enhanced downstream signaling pathways which lead to enhanced CCR7 signaling following engagement of PGD_2 with DP-2 receptor. This could also explain the faster migration observed when lymphocytes are migrated towards CCL21, the ligand for CCR7, in a Boyden chamber assay. When the expression of CCR7 was investigated on the lymphocyte subsets we found that there was no change in expression levels following either migration through VEC or following incubation of PGD_2. Thus, it seems probable that prostanoids enable increased function of the CCR7 without a concomitant increase in receptor expression.

CHAPTER 6
GENERAL DISCUSSION

6 General Discussion

Herein we investigated the role vascular and lymphatic EC play in regulating lymphocyte traffic through tissue and the potential signals produced by vascular EC that govern the fate of newly transmigrated lymphocytes. We designed and utilised an *in vitro* assay to mimic the conditions the lymphocytes would be exposed to during an acute inflammatory response. By culturing LEC on the basal surface of a transwell insert, we were able to orientate the LEC so that the lymphocytes migrated in a basal to apical direction, as takes place *in vivo*.

During the onset of inflammation, lymphocytes are recruited and they migrate through the vascular endothelium to get to the site of inflammation [3]. We have shown that following firm adhesion of the lymphocyte to vascular EC, lymphocytes require a prostanoid signal in the form of PGD_2 that then allows them to subsequently migrate through the EC barrier. This PGD_2 signal operates downstream of the initial chemokine signal and is essential for the transendothelial migration; at least in a model of inflammation driven by TNFα and IFNγ [42]. We hypothesise that AA released from EC phospholipids is metabolised to PGD_2, which is released locally and binds DP2 on firmly adherent and chemokine activated lymphocytes. This DP2 signalling is in turn responsible for full integrin activation and the initiation of lymphocyte transmigration (Fig 6).

In the current model of lymphocyte trafficking, we have identified new steps that regulate the migration of T lymphocytes across the EC of both the blood and lymphatic vasculature [42]. Thus, PGD2 is required for the efficient transendothelial migration of memory T cells across BVEC, and this PGD2-driven step of recruitment also primes CD4 memory T cells for efficient migration across LVEC (Fig. 6). During migration across vascular endothelium, the

PGD2 signal is subordinate to a primary activating stimulus delivered by chemokines of the IFN inducible family (CXCL9–11), which promote stable integrin-mediated adhesion *via* the CXCR3 chemokine receptor [286]. However, without receipt of the PGD2 signal, T lymphocytes do not traffic efficiently across vascular or lymphatic vessels [42]. PGD2 mediated priming for efficient migration across LVEC did not cause upregulation of integrin adhesion receptors or chemokine receptors on CD4 memory T cells, but did modify the function of CCR7 so that the efficiency of migration toward CCL21 was significantly increased.

Current paradigms of T cell trafficking during an inflammatory response assume that the delivery of a chemokine signal is sufficient to promote integrin-mediated stable adhesion, followed by reorganisation of the actin cytoskeleton during spreading on the EC surface, eventually leading to transendothelial migration [412]. However, blockade of the PGD2 receptor DP-2 [formally known as chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2)] greatly reduces the transmigration of memory T cells, leaving the great majority attached to the apical surface of the EC monolayer [42]. These observations strongly imply the presence of a hierarchy of activation signals that are required for efficient recruitment. Thus, we propose an initial chemokine signal activates lymphocyte integrins allowing immobilisation on the endothelium. In turn, PGD2 permits downstream functions such as migration and diapedesis. Such a model for the recruitment of lymphocytes during inflammation resembles our recently published observations of the recruitment of neutrophils.

The mechanism by which T cell migration across LVEC was expedited was also identified. Thus, either migration across BVEC or exogenous PGD2 could promote the function of the CCR7 without increasing its surface expression. Evidence for this came through the use of a function-neutralising antibody against CCR7 and also blockade of GPCR signaling, both of which significantly retarded CD4 memory T cell migration across LVEC. In addition, CD4 memory T cells, which had migrated across BVEC or received an exogenous PGD2 stimulus, were capable of migrating more efficiently toward the CCR7 ligand CCL21 in a Transwell chemotaxis assay. The use of CCR7 by CD4 T cells for migration across LVEC is in accordance with previous observations made in murine models, in which T cells lacking the receptor were unable to efficiently traffic into the lymphatic vasculature from the skin [113] or the lung [114]. A role for prostanoids in regulating the function of CCR7 in T cells has not previously been demonstrated, although the phenomenon has close parallels with the regulated migration of DCs out of peripheral tissue and into the afferent lymphatic vasculature. In this case, the differentiation and maturation of DCs within peripheral tissue is driven by PGE2 [370, 413, 414].This prostanoid signal promotes the function (but not the expression) of the chemokine receptor CCR7, so that DCs also respond more efficiently to the chemokines CCL19 and CCL21. The striking similarity of the pathways used by DCs and CD4 memory T cells for exit from peripheral tissue may indicate that as the adaptive immune system has evolved, the different populations of effector cells have adopted a similar mechanism for localisation in secondary lymphoid tissue, except that their emigration from tissue is regulated by different prostanoids.

In addition, we show for the first time, to our knowledge, that passage of CD4 memory T cells across LVEC requires both β1 and β2 integrins. Interestingly, the PGD2- dependent

priming of these cells for efficient transit of the lymphatic endothelium did not require upregulation of integrin receptors that were highly expressed on this subset of T cells. However, taken together, these studies indicate that migration of leukocytes across lymphatic endothelium uses adhesion receptors that are remarkably similar to those used for transit of the vascular endothelium.

In conclusion, the current paradigms that postulate an exclusive role for chemokines in the recruitment of T lymphocytes from the blood require updating (see Fig. 6 for details). Although an initial chemokine signal is essential for the activation of T lymphocytes on BVEC, a downstream prostanoid signal, delivered by PGD2 *via* the DP-2 receptor, is required to drive the process of diapedesis. In addition, passage of the blood vasculature and receipt of a PGD2 signal significantly increases the efficiency of passage across lymphatic vasculature.

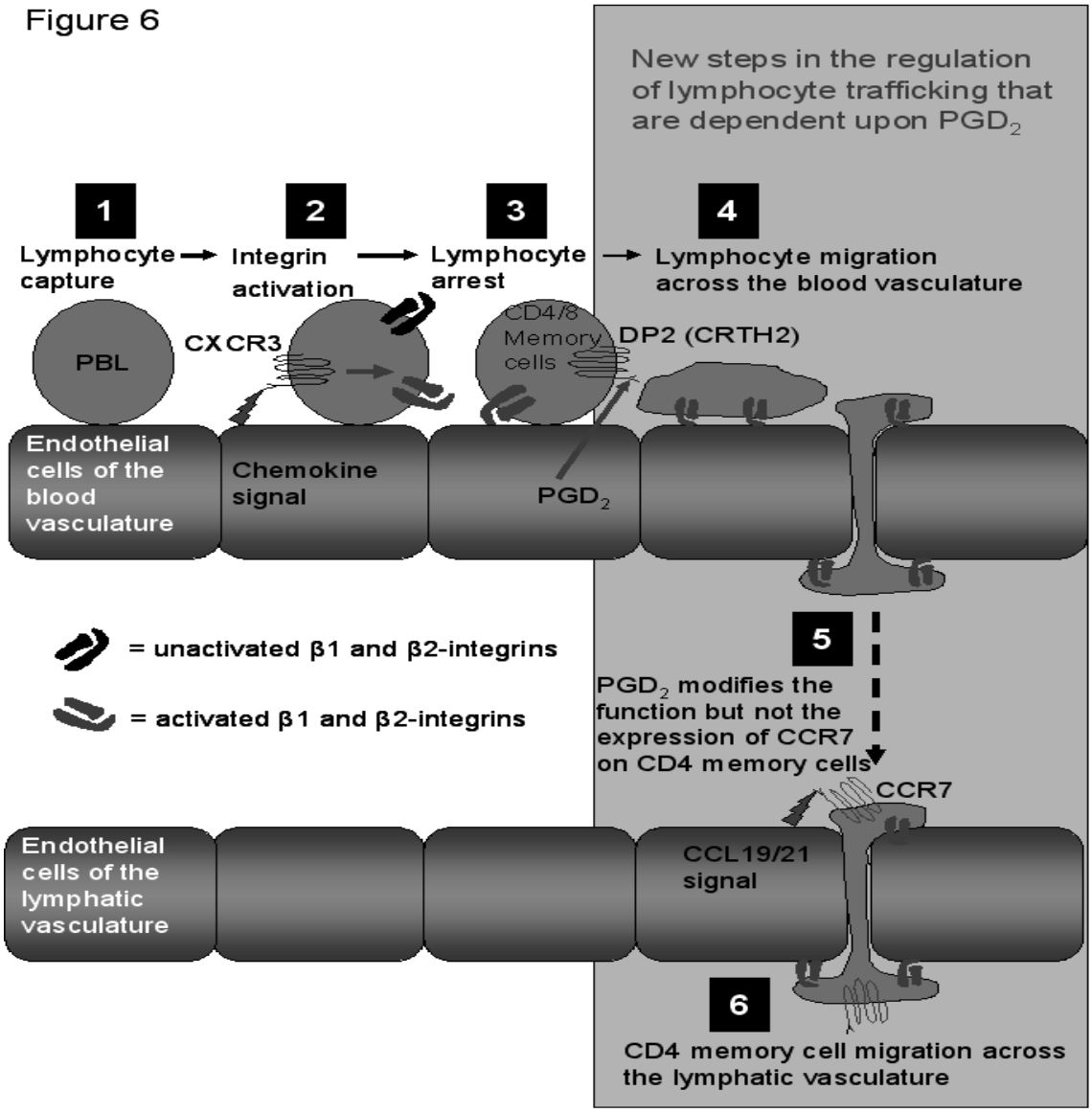

Figure 6: New steps in the regulation of T cell trafficking that are dependent upon PGD2. **1**, BVEC stimulated with cytokines (TNF and IFN) selectively recruit memory T cells from flowing blood. **2**, The T cells receive an activating stimulus from IFNγ –inducible chemokines (CXCL9–11), which operate exclusively through the CXCR3 receptor. **3**, T cell integrins are transiently activated and immobilise the cell on the surface of vascular EC. **4**, PGD2 stimulates the DP-2 receptor, a signal that is essential for efficient passage across the vascular EC monolayer. **5**, Receipt of a PGD2 signal during passage of the vascular EC or stimulation with exogenous PGD2 promotes the function, but not the expression, of the lymphocyte chemokine receptor CCR7. **6**, Memory T lymphocytes migrate across lymphatic EC in response to the chemokines CCL19 and CCL21 with significantly increased efficiency.

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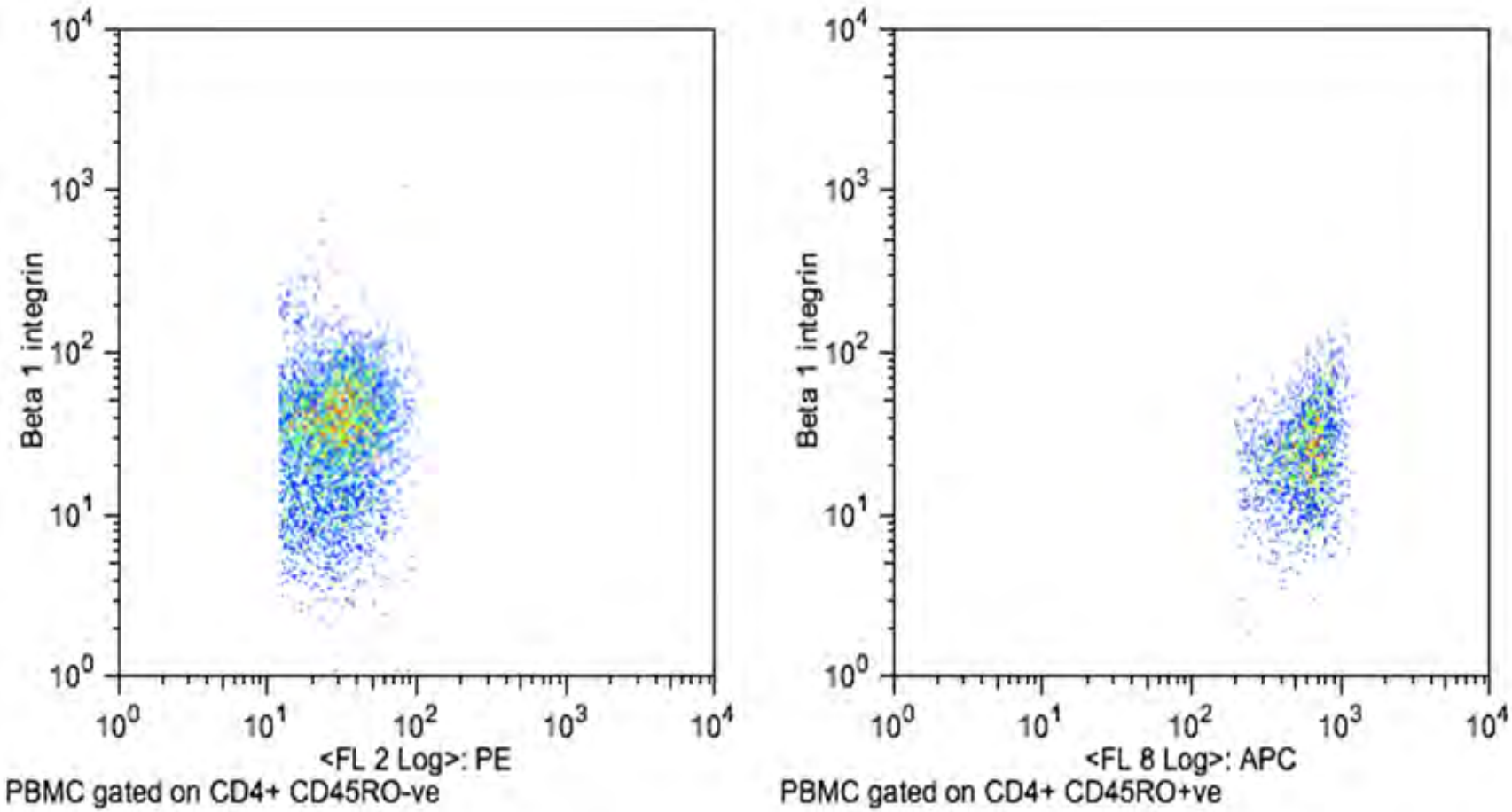
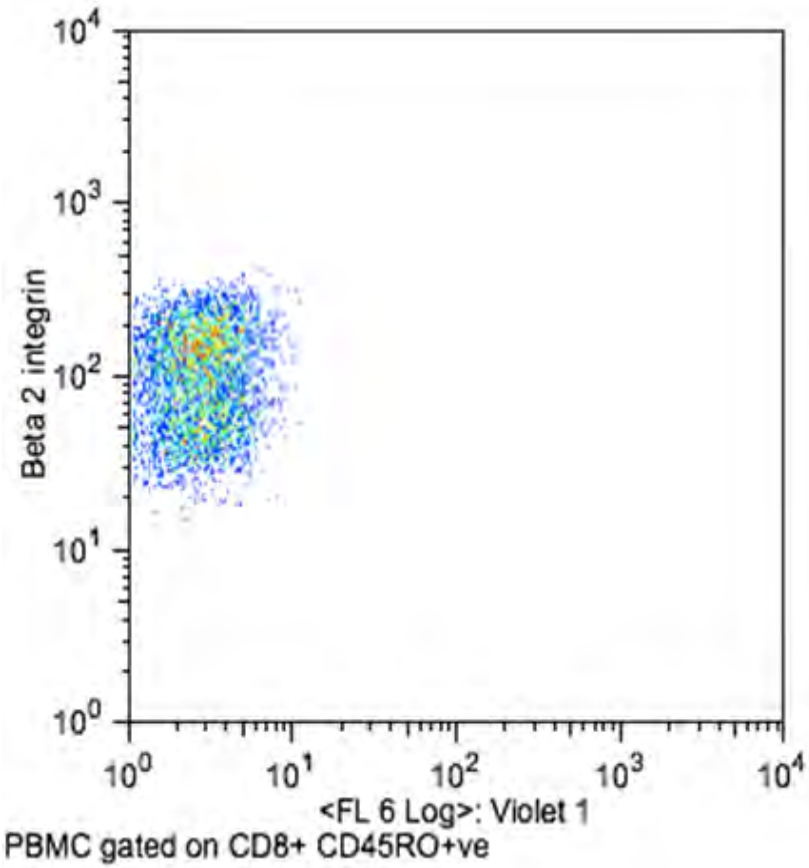
Appendix

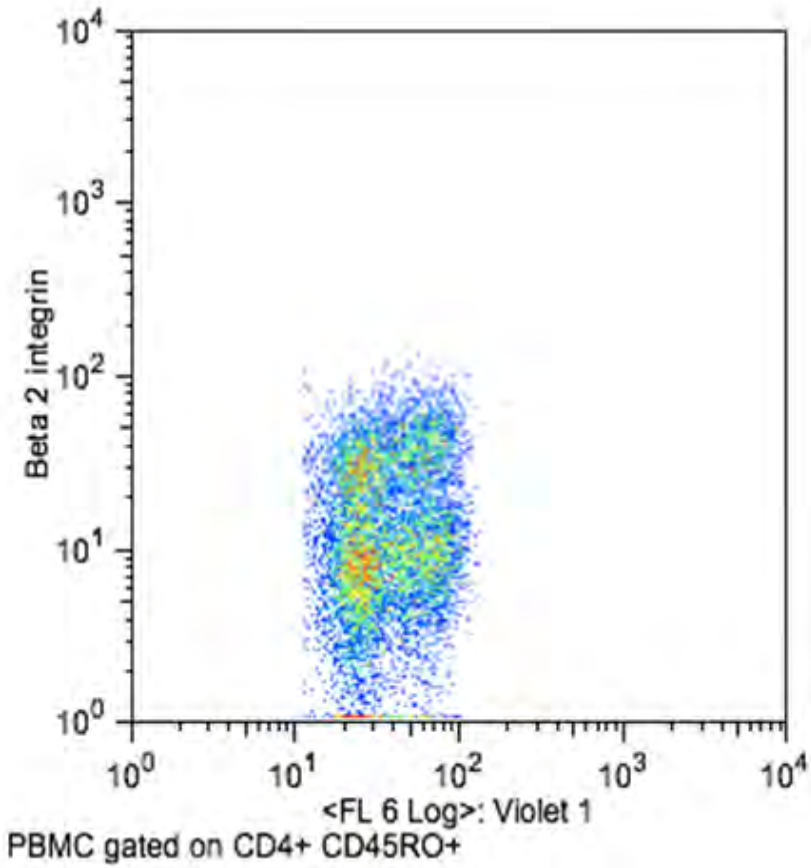
Antibodies used

Target	Conjugate	clone	Supplier
CD4	FITC		BD
CD4	APC		BD
CD8	PE	RPA t-8	BD
CD8	FITC	RPA t8	BD
CCR7	FITC	150503	R N D Systems
B1	FITC	TDM29	Southern Biotech
B2	FITC	MAR4	eBioscience
CD45RO	APC	UCHL1	BD
CD45RA	PE-CY5	BU78	Serotech
CCR1		53504	R n D systems
CCR2		48607	R n D systems
CCR3		61828	R n D systems
CCR4		205410	R n D systems
CCR5		45531	R n D systems
CCR6		53105	R n D systems
CCR7		150503	R n D systems
CCR9		112509	R n D systems
CCR10		314305	R n D systems
CX3CR1			IN HOUSE
CXCR1		42765	R n D systems
CXCR2		242216	R n D systems
CXCR3		49801	R n D systems
CXCR4		12G5	R n D systems
CXCR5		51505	R n D systems
CXCR6		56811	R n D systems



Representative dot plots of β1 integrin expression following isolation from blood. Lymphocytes were isolated and stained for CD4, CD45RO and β1 integrin.





Representative dot plots of β2 integrin expression following 24 hour culture on plastic. Lymphocytes were isolated and stained for CD4, CD8 CD45RO and β1 integrin.