

CHEMOKINE REGULATION OF IMMUNE CELL RECRUITMENT ACROSS THE
BLOOD-BRAIN BARRIER IN INFLAMMATION

by

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ABSTRACT

A key element of the inflammatory response in the central nervous system (CNS) is the recruitment of leukocytes across the blood-brain barrier (BBB) that normally restricts their passage from blood to brain. Endothelial cell (EC) adhesion molecules and chemokines have emerged as important mediators of leukocyte recruitment to the CNS. The objective of this study was to characterize the kinetics of expression and release of the chemokine CXCL12 by human brain microvessel EC (HBMEC) and to investigate the effects of CXCL12 and the β -chemokines CCL2 and CCL3 on the adhesion and migration of T cell subsets and monocytes across an in vitro model of the BBB. Cytokines and LPS downregulated the constitutive CXCL12 and CXCR4 expression and CXCL12 ligation induced internalization of CXCR4. Monocyte and T cell adhesion and migration in response to these chemokines were studied in a double chamber chemotaxis system in the presence of chemokine concentration gradients. The minimal adhesion and migration of CD4⁺ and CD8⁺ T lymphocytes were significantly increased after cytokine treatment of HBMEC. CXCL12 gradients further enhanced adhesion of both T cell subsets to activated HBMEC and migration across resting monolayers. Concentration gradients of CCL2 increased adhesion of activated CD4⁺ T cells to cytokine treated HBMEC, whereas CCL3 increased adhesion of memory CD4⁺ T cells to resting HBMEC. Migration of resting, activated, naïve and memory CD4⁺ T cells across resting and cytokine treated HBMEC was augmented in the presence of CCL2 or CCL3 in the subendothelial compartment. The number of migrated resting and naïve CD4⁺ T cells across resting HBMEC was slightly decreased by CCL3. Neither CCL2 nor CCL3 had any significant effect on monocyte adhesion, however, both chemokines significantly increased monocyte migration across resting HBMEC. Monocyte adhesion and migration across

cytokine treated HBMEC were dependent upon integrins and EC adhesion molecules, since blocking ICAM-1, β 2- or α 4-integrins significantly reduced transendothelial migration. These studies emphasize the importance of cerebral EC activation and the role of CXCL12, CCL2 and CCL3 in regulating the directional migration of T cell subsets and monocytes across the BBB, thus contributing to the specificity of immune responses in neuroinflammation.

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LIST OF ABBREVIATIONS

| | |
|-------|---|
| ABC | ATP-binding cassette |
| APC | antigen presenting cells |
| BBB | blood-brain barrier |
| CD | cluster of differentiation |
| CNS | central nervous system |
| CSF | cerebral spinal fluid |
| EAE | experimental allergic encephalomyelitis |
| EC | endothelial cells |
| ECM | extracellular matrix |
| ELISA | enzyme linked immunosorbent assay |
| GABA | gamma-aminobutyric acid |
| GAG | glycosaminoglycan |
| GFAP | glial fibrillary acidic protein |
| GPCR | G protein-coupled receptor |
| h | hour(s) |
| HBMEC | human brain microvessel endothelial cells |
| HSPG | heparan sulfate proteoglycans |
| HUVEC | human umbilical vein endothelial cells |
| ICAM | intercellular cell adhesion molecule |
| IFN | interferon |
| IGSS | immunogold silver staining |
| IL | interleukin |
| ISF | interstitial fluid |
| LCA | leukocyte common antigen |
| LFA | leukocyte function antigen |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| MCP | monocyte chemotactic protein |
| MHC | major histocompatibility complex |
| min | minute(s) |

| | |
|-------------------|---|
| MIP | macrophage inflammatory protein |
| MS | multiple sclerosis |
| PECAM | platelet endothelial cell adhesion molecule |
| P-gp | P-glycoprotein |
| PSGL | P-selectin glycoprotein ligand |
| PVC | perivascular cells |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SDF | stromal cell-derived factor |
| SEM | scanning electron microscopy |
| siRNA | small interfering RNA |
| s-Le ^x | sialyl-Lewis ^x |
| TEM | transmission electron microscopy |
| TGF | transforming growth factor |
| Th | helper T cell |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| TRAIL | TNF-related apoptosis inducing ligand |
| UEA | Ulex europaeus agglutinin |
| VCAM | vascular cell adhesion molecule |
| VLA | very late antigen |
| ZO | zonula occludens |

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CHAPTER 1 INTRODUCTION

1.1 Inflammation in the Central Nervous System (CNS)

Inflammation is essential for the survival of an organism by providing protection against infection/invasion by microorganisms. The immune system of vertebrates has evolved to maintain a balance between elimination of pathogen while remaining tolerant to the host. Thus, the immune system is often described as a double-edge sword due to its potential destructive nature.

The brain, eye and endocrine tissues have traditionally been considered immune privileged sites and they are particularly sensitive to damage by inflammation (Mrass and Weninger 2006). The brain also lacks conventional lymphatic drainage present in other organs.

Maintenance of relative isolation from the constituents in the blood is important for the proper function of the neural environment which is rich in electrical (ions) and chemical (neurotransmitters) signals. However, under pathological conditions such as inflammatory diseases, infections, ischemia and stroke, leukocyte infiltration into the CNS occurs, resulting in varying degrees of CNS inflammation (Arshavsky 2006). Furthermore, break-down of tolerance to CNS autoantigens and can also lead to CNS inflammation (Pashenkov et al. 2003).

The question of how leukocyte trafficking across the CNS is regulated in health and disease and which molecules are involved in this process is an area of significant importance and active investigation. Several mechanisms are responsible for maintaining the immune privilege status of the CNS: (i) isolation of the CNS behind the BBB and the epithelial blood-cerebrospinal fluid (CSF) barrier; (ii) absence of resident professional antigen presenting cells (APC); (iii) suppression of immune responses by the production of immunosuppressive

factors; (iv) lack of MHC class I and II expression on parenchymal cells; (v) lack or low expression of endothelial cell adhesion molecules; and (vi) absence of conventional draining lymphatic vessels in the brain parenchyma (Mrass and Weninger 2006). However, interstitial fluid (ISF) in the brain drains to the cerebrospinal fluid (CSF) via the perivascular Virchow-Robin spaces. From the cranial subarachnoid space, ISF and CSF can either drain into dural sinus blood, via arachnoid villi, or escape to extracranial tissue spaces, via olfactory, optic, trigeminal, and acoustic nerves (Keane and Hickey 1997).

However, recent evidence suggests that immune privilege may be an active process rather than passive isolation. Dendritic cells (DC) isolated from mice with experimental allergic encephalomyelitis (EAE) exhibit immature DC phenotype characterized by intermediate surface expression of MHC class II and low expression of the costimulatory molecule CD80. Not only are they unable to prime naïve T cells, but they also inhibit T cell proliferation induced by mature bone marrow-derived DC mediated by TGF- β , IL-10 and TRAIL (Suter et al. 2003). Furthermore, memory T lymphocytes continuously survey immune-privileged sites for the presence of pathogens at a relatively low rate compared to other organs (Mrass and Weninger 2006). A recent review by Kleine and Benes summarized some of the molecules such as: ICAM-1, VCAM-1, PECAM-1 and selectins expressed on brain endothelial cells (EC) and their corresponding ligands on T cells, NK cells and B cells in the entry of lymphocytes into the CNS across the BBB or blood-CSF barrier in the healthy human CNS (Kleine and Benes 2006).

1.2 Immunologically Pertinent Cells of the Central Nervous System

1.2.1 *Microglia*

Microglia belong to the monocyte/macrophage lineage and exert innate immune function in the CNS (Mrass and Weninger 2006). Although the origin of microglia is still somewhat debatable, current evidence supports the derivation of microglia from monocytes, and to a lesser extent from mesodermal progenitor cells (Kaur et al. 2001; Guillemin and Brew 2004; Kim and de Vellis 2005; Vilhardt 2005). Morphologically by light microscopy, microglia are small elongated cells with short irregular processes; greater than 99% of microglial cells appear spurred with spikes on scanning electron microscopy (Guillemin and Brew 2004). Under normal conditions, microglial cells exist in a resting, ramified state. Resting microglia are characterized by the surface expression of CD68, CD4^{low}, CD11b, CD11c and absence of CD14 expression. Pattern recognition receptors such as TLR 2 and TLR 6 have been detected in microglia *in vivo* (Hauwel et al. 2005). When activated, microglial cells express CD14, class II MHC, scavenger receptors class A and B type and acquire an ameboid phenotype along with phagocytic and cytokine production abilities (Hauwel et al. 2005). Recent studies have shown that microglial cells are very active under normal conditions. Thus, they constantly screen the CNS microenvironment for 'danger signals' (Mrass and Weninger 2006) and have the potential of being neuroprotective and proregenerative, as well as proinflammatory and neurotoxic (Vilhardt 2005). It is widely believed that substances released from damaged CNS cells such as β -amyloid, extracellular ATP, serum factors or microbial products can trigger microglial activation. The responses of activated microglia include: migration, proliferation, nitric oxide production, phagocytosis, antigen presentation and secretion of diffusible factors. It has been shown that cultured human microglial cells

express mRNA for the cytokines IL-1 α/β , IL-6, IL-10, IL-12, IL-15, and TNF- α . Microglia has also been shown to express a large repertoire of chemokines such as CCL2, CCL3, CCL4, CCL5, CCL19, CCL22, CXCL1, CXCL8, CXCL 10 and CX3CL1 (Garden and Moller 2006).

1.2.2 *Astrocytes*

Astrocytes are star-shaped cells that express glial fibrillary acidic protein (GFAP). They outnumber neurons in the human brain and constitute up to 20-50% of the brain volume. Astrocytes serve many functions in the brain including continuous tight control of extracellular ion concentration, maintenance of pH homeostasis and glucose levels and provision of metabolic substrates, in addition to the secretion of neuroactive substances. They also scavenge neuronal waste products, including metabolic byproducts and neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) released during synaptic transmission at the synaptic cleft through active uptake by means of specific receptors. They have high K⁺ permeability and are important in regulating extracellular K⁺ necessary for maintaining neuronal excitability. Differentiated astrocytes play a prominent role in the regulation of glutamate homeostasis through the uptake of extracellular glutamate, its conversion to glutamine by the enzyme glutamine synthetase and the release of glutamine which is returned to local neurons. (Volterra and Meldolesi 2005; Koehler et al. 2006; Seth and Koul 2008).

Astrocytes also play an important role in cerebrovascular regulation as part of the neurovascular unit (Koehler et al. 2006) and are important in the control of synapse formation and function. Oscillations of intracellular calcium ions in astrocytes appear to play a dual role in the modulation of synaptic transmission and regulation of cerebral blood flow (Volterra and Meldolesi 2005). Under inflammatory conditions, they are major producers of cytokines (e.g.

IL-1, IL-6, TNF- α , IL-12, IL-17 and IL-23) and chemokines in the CNS. Importantly, secretion of IL-12 and IL-23 by astrocytes plays a role in the activation of both Th1 and Th17 T cells, thereby promoting demyelination (Nair et al. 2008). Astrocytes may also play a role in immune regulation and leukocyte recruitment in the CNS due to their expression of Toll-like receptors (TLR) and chemokines such as CXCL12, CCL2 and CCL5 (Owens 2005; Calderon et al. 2006; Seth and Koul 2008).

1.2.3 *Perivascular Cells*

Perivascular cells (PVC) are found within the CNS in the perivascular or Virchow-Robin space adjacent to CNS vessels. They are bone marrow derived cells that continuously turn over in the CNS approximately every 3-4 months (Keane and Hickey 1997; Bechmann et al. 2001; Williams et al. 2001). Although the ED2 antigen is a universally accepted marker of rat perivascular cells, the identification of human PVC *in vivo* is based on CD14, CD45, MHC II and CD4 expression. In addition, the chemokine receptors CCR3, CCR5 and CXCR4 have been detected on human perivascular cells (Williams et al. 2001). PVC may be involved in antigen presentation due to their ability to phagocytose molecules from the CSF and express class II MHC and B7 molecules (Bechmann et al. 2001).

1.3 The Blood-Brain Barrier (BBB)

Early observations made by Paul Ehrlich in 1885 demonstrated that intravenously administered water soluble dyes stained all the organs except the brain. In 1901, his student, Edwin Goldman showed that the dye trypan blue injected directly into the cerebrospinal fluid of rabbits and dogs, rapidly stained the entire brain, but did not enter the blood to stain other organs (Grant et al. 1998; Wells and Bonetta 2005), suggesting the presence of a blood-brain barrier. By the 1970s, it was established that the CNS was protected by barrier layers at three key sites, namely, 1) the endothelium of brain parenchymal blood vessels [the BBB]; 2) the choroid plexus epithelium; and 3) the arachnoid epithelium of the meninges (Abbott 2005). The BBB is present throughout the CNS with the exception of the circumventricular structures that include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), median eminence, pituitary intermediate lobe, pituitary neural lobe, subcommissural organ (SCO), pineal gland (PI) and area postrema (AP) (Johnson and Gross 1993; Nolte 1999), where the capillary endothelium is permeable to allow communication between specialized neurons and the blood (Abbott 2005).

1.3.1 Endothelial cells

1.3.1.1 Structure

The anatomical basis of the BBB is the cerebral endothelium. The cerebral EC serve at the gateway between the systemic circulation and the brain. A polarity exists between the luminal and abluminal membrane surfaces of the endothelium contributing to the barrier function (Persidsky et al. 2006). Tight junctions that seal the paracellular clefts between adjacent ECs, paucity of cytoplasmic vesicles, reflecting a very low rate of vesicular transport across the endothelium, abundance of mitochondria, presence of specialized transporters for uptake of

nutrients, and polarized ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp) are characteristics of the BBB. Furthermore, over 95% of the abluminal surface of the microvascular basement membrane is covered by astrocytic endfeet (Reese and Karnovsky 1967; Vorbrodt and Dobrogowska 2003). A confocal study of perivascular astrocytic endfeet in the rat brain showed well defined rosette-like structures that lie against the vessel wall (Kacem et al. 1998). Endothelial cells of brain microvessels are surrounded by basal lamina that consists of type IV collagen, fibronectin, laminin and heparan sulfate proteoglycans (HSPG) and separates them from the astrocytic end feet (Tilling et al. 1998; Iivanainen et al. 2003; Hawkins and Davis 2005).

The brain capillaries are approximately 50 to 100 times tighter than peripheral microvessels (Abbott 2002). In frog brain microvessels, the average resistance was determined to be 1870 $\Omega\text{-cm}^2$ (Crone and Olesen 1982). Tight junctions are formed by fusion of the outer leaflets of

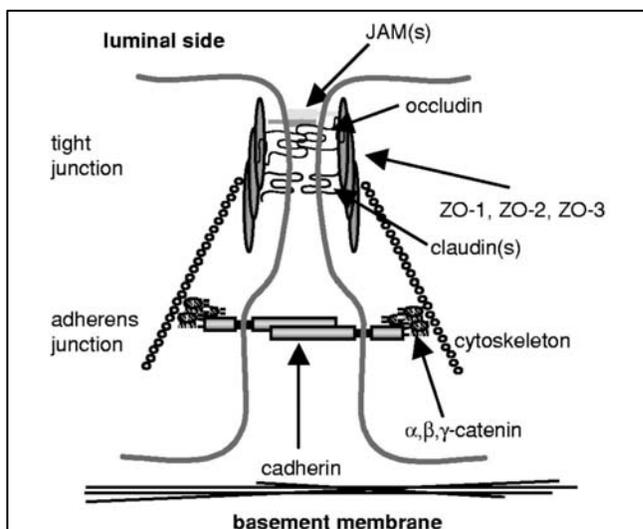


Figure 1 Molecular organization of inter-endothelial tight junctions at the BBB.

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of the plasma membranes of two adjacent EC at focal points along cell-cell contacts resulting in a pentalaminar structure that extends circumferentially around EC.

These inter-endothelial tight junctions are composed of three transmembrane proteins (occludin, claudins 1, 3 and 5 and junctional adhesion molecules (JAM)

1-3) and several cytoplasmic proteins (zonula occludens (ZO)-1, ZO-2, ZO-3,

singulin, AF6 and 7H6) linked to the actin cytoskeleton. The molecular organization of the

BBB tight junction is illustrated in Figure 1. Claudins are exclusively localized at TJ, bind homotypically to claudins of adjacent EC and form the primary seal of the TJ. Occludin is a regulatory TJ protein confined to neural vessel TJ and associated with high electrical resistance and low permeability. JAM 1-3 are members of the IgG superfamily. JAM-1 is also a counter-receptor for the β 2 integrin LFA-1 and, in association with platelet-endothelial cell adhesion molecule (PECAM)-1, regulates leukocyte migration. ZO 1-3 are multidomain cytoplasmic molecules of the MAGUK (membrane-associated guanylate kinase) family of molecules that couple junctional proteins to the actin cytoskeleton (Hawkins and Davis 2005; Persidsky et al. 2006). Cerebral EC differ from extra-cerebral EC in many respects. Tight junctions are strongly developed and functionally very “tight” in EC of the BBB, but only moderately formed between EC of peripheral vasculature (Forster 2008). Brain endothelium expresses lower level of the adhesion molecules, ICAM-1 and VCAM-1, than aortic endothelium following cytokine stimulation. In addition, after cytokine stimulation the distribution and intensity of anionic glycosaminoglycan (GAG) sites on brain EC remains unchanged while a more fibrillar distribution of anion sites are apparent in aortic EC (dos Santos et al. 1996).

1.3.1.2 Function

The BBB has evolved to act as a physical barrier buffering the brain ISF from fluctuations in composition that occur in plasma (eg. caused by respiration, digestion/absorption and exercise).

It is equipped with specific transport systems that regulate molecular traffic to supply nutrients and remove toxic and waste products (Figure 2) (Abbott 2005). ISF is similar in composition to blood plasma, but has a much lower protein content, and lower K^+ and Ca^{2+} concentrations but higher levels of Mg^{2+} (Abbott et al. 2006).

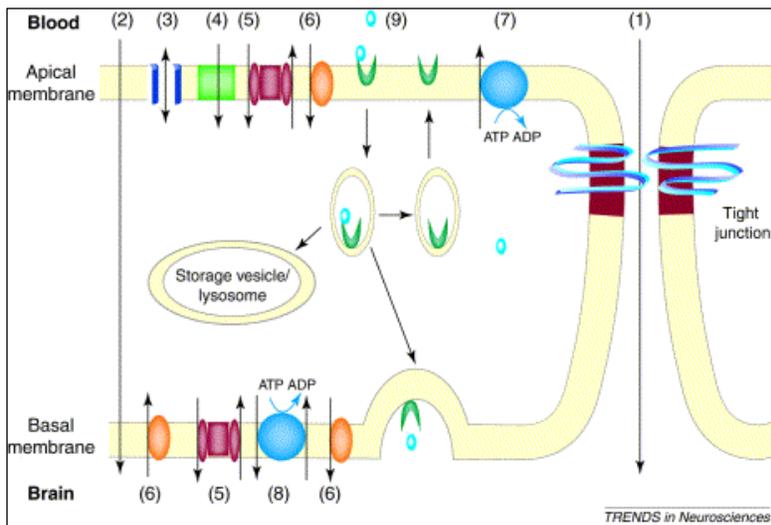


Figure 2 Types of transport mechanisms and structures at the BBB that help maintain brain homeostasis. These include (1) paracellular diffusion, (2) transcellular diffusion, (3) cation channels, (4) ion symports, (5) ion antiports, (6) facilitated diffusion active transport, (7) active transport, (8) active antiport transport and (9) endocytosis (receptor-adsorptive mediated).

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Although small lipid soluble molecules can diffuse passively across the BBB, receptor-molecules active transport systems are mainly responsible for the uptake and efflux of molecules across the BBB. ABC efflux transporters such as P-gp and ABCG2 protect the brain from a broad range of lipophilic agents. P-gp is expressed by brain EC, astrocytes and microglia. P-gp and ABCG2 are located at the luminal side of the cerebral endothelium and prevent drugs from entering the brain. Together with efflux transporters, drug metabolizing enzymes such as cytochrome P450 and uridine diphosphate glucuronosyltransferases serve as an enzymatic barrier at the BBB (Begley and Brightman 2003; Persidsky et al. 2006).

Furthermore, low permeability to most neurotransmitters allows the separation of the CNS and peripheral nervous system (PNS) transmitter pools (Abbott 2005). Due to its large surface area ($\sim 20\text{m}^2$ per 1.3 kg of brain) and the short diffusion distance between neurons and capillaries, the endothelium has a dominant role in regulating the brain microenvironment (Abbott et al. 2006).

1.3.1.3 Role in Inflammation

The cerebral endothelium plays an important role in homeostasis by supporting vasodilatation and inhibiting platelet activation and plasma coagulation (Becker et al. 2000). The BBB endothelium plays a crucial role in regulating the entry of immune cells into the brain. The *de novo* expression and/or upregulation of the EC adhesion molecules ICAM-1, VCAM-1, PECAM-1, P-selectin and E-selectin on human brain EC by cytokines or LPS serve to regulate the adhesion and migration of leukocytes into the CNS (Wong and Dorovini-Zis 1992; Wong and Dorovini-Zis 1995; Wong and Dorovini-Zis 1996a; Wong et al. 1999; Easton and Dorovini-Zis 2001). Expression of class II MHC molecules, CD40 and the costimulatory molecules, CD80, CD86 and LFA-3 suggest that cerebral EC may function as an antigen presenting cell in the CNS (Huynh and Dorovini-Zis 1993b; Omari and Dorovini-Zis 1999; Omari and Dorovini-Zis 2001; Omari and Dorovini-Zis 2003). Since ECs lining the BBB are the first CNS cells to contact circulating leukocytes, molecular changes at the BBB play a critical role in the entry of leukocytes to initiate an inflammatory response in the CNS. TNF- α is believed to play an important role in the increased permeability and disruption of the blood-brain barrier by increasing cyclooxygenase products, nitric oxide and expression of adhesion molecules (Mayhan 2002).

During inflammation, inflammatory cytokines such as TNF- α induce the expression of adhesion molecules on brain microvessel endothelium which act as important ligands to counter-receptors on the surface of leukocytes and mediate both adhesion and subsequent migration (Fabry et al. 1995; Pober 2002). Inflammatory mediators such as bradykinin and histamine, matrix metalloproteinases (MMP), as well as angiogenic factors such as vascular endothelial growth factor (VEGF) have been shown to disrupt tight junctions and be associated with increased barrier permeability (Petty and Lo 2002). Nitric oxide, on the other hand, has been shown to lower endothelial permeability and reduce leukocyte adhesion to human brain EC via a cGMP-dependent pathway (Wong et al. 2004; Wong et al. 2005). BBB leakage as a result of disrupted tight junctions has been observed in cerebral malaria, AIDS encephalitis and multiple sclerosis (MS) (Kirk et al. 2003).

1.3.2 Pericytes

Pericytes are of mesodermal, myeloid origin and are enclosed within the basal lamina of brain capillaries and venules in direct contact with the abluminal surface of EC (Guillemin and Brew 2004). Although their function has not yet been elucidated, pericytes may play an important role in cerebrovascular autoregulation and blood flow distribution as they express α - smooth muscle actin and receptors for several vasoactive mediators such as catecholamines, endothelin-1, vasopressin and angiotensin II (Balabanov and Dore-Duffy 1998; Lai and Kuo 2005). Platelet-derived growth factor (PDGF)- β appears to be important in recruiting pericytes to new blood vessels during angiogenesis (Lai and Kuo 2005). Furthermore, the tightness of interendothelial tight junctions appears to be correlated with the degree of pericyte coverage (Lai and Kuo 2005).

1.4 Inflammatory Cell Entry and Initiation of CNS Inflammation

1.4.1 *Four Key Steps of Leukocyte Transendothelial Migration*

The transendothelial migration of leukocytes is a coordinated and highly regulated multi-step process with four distinct key steps: 1) rolling/tethering, 2) activation of integrins by chemokines, 3) firm adhesion, and 4) diapedesis. Recent studies have provided more detailed and elaborated description of additional steps involved in the adhesion cascade (Figure 3). P-selectin on EC binds to the high affinity ligand P-selectin glycoprotein ligand-1 (PSGL-1), as well as the lower affinity ligand sialyl-Lewis^x on leukocytes. E-selectin on EC interacts with sialyl-Lewis^x. Both E- and P-selectin on EC are involved in initiating the rolling/tethering process to slow down leukocytes. Chemokine receptors expressed on leukocytes respond to chemokines bound to proteoglycans at the apical side of EC and activate leukocyte integrins such as LFA-1 and VLA-4 via inside-out signaling. Activated integrins bind to endothelial cell adhesion molecules ICAM-1 and VCAM-1 with high affinity and spreading of leukocytes over the luminal surface of EC occurs as a result of firm adhesion. The leukocytes can then migrate across the endothelium via the paracellular or transcellular route [see below]. Abluminal chemokines deposited at the site of leukocyte extravasation provide haptotactic and chemotactic signals that upregulate both leukocyte adhesion and motility through the underlying basal lamina. Subsequently, gradients of locally secreted chemokines may navigate the migrating leukocyte through the ECM to its final destination in a chemotactic manner (Cinamon et al. 2001). The length of time required for each step varies depending on the leukocyte subset and the type of the endothelium.

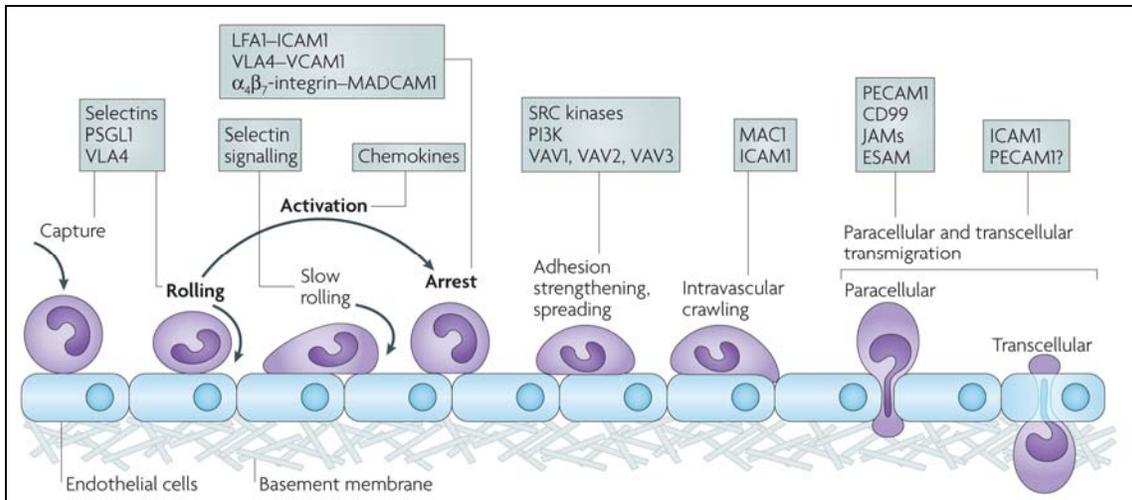


Figure 3 The updated leukocyte adhesion cascade
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1.4.2 Paracellular versus Transcellular Route of Leukocyte Migration

The route of transendothelial migration has been a much debated topic. Large discrepancies exist in the literature possibly due to differences between EC from different organs and species, as well as the subset of leukocytes investigated. Pawlowski (Pawlowski et al. 1988) found that human monocytes selectively bind to intercellular borders of human umbilical vein endothelial cells (HUVEC) and transmigrate only at intercellular margins between EC. Furthermore, a review by Kvietys (Kvietys and Sandig 2001) summarized numerous studies in favor of either paracellular or transcellular route of polymorphonuclear leukocyte (PMN) migration across EC. It is interesting to note that most paracellular and transcellular diapedesis is observed *in vitro* and *in vivo*, respectively. *In vitro*, PMN migration takes place preferentially at intercellular junctions, especially at tricellular corners due to the fact that cell-cell junctions are not as well formed and developed compared to their *in vivo* counterparts. Therefore, the transcellular route might be more prevalent *in vivo* (Hoshi and Ushiki 1999).

Paracellular transmigration involves homophilic interactions between PECAM-1, JAM-1 and CD99 expressed on both leukocytes and interendothelial junctions. Transcellular transmigration, on the other hand, is mediated by leukocytes inserting filopodia-like processes into forming endothelial vesiculo-vascular organelles (VVO) rich in ICAM-1 or transmigratory cup rich in ICAM-1 and VCAM-1 (Muller and Randolph 1999; Kvietys and Sandig 2001; Dejana 2006; Carman and Springer 2008). Nevertheless, under most experimental conditions, neither the transcellular nor the intercellular diapedesis of leukocytes across the endothelium disrupts the integrity of the endothelial barrier.

1.5 Cytokines and LPS in CNS Inflammation

Many inflammatory cytokines and chemokines are not expressed at detectable levels in the normal CNS, but are rapidly induced following insult, injury, or immune triggering (Owens et al. 2006). These cytokines and chemokines are implicated in the pathogenesis of neuroinflammation (Table 1). In EAE, the Th1-associated cytokines IFN- γ , TNF- α , lymphotoxin- α (LT- α), IL-6 and the Th1-inducing cytokine IL-12 are associated with active disease or relapse, whereas the Th2 cytokines IL-4, IL-10 and IL-13 are associated either with remission or with suppression of disease (Owens et al. 2001). LT- α and TNF- α mRNA expressing mononuclear cells have been detected in the CSF of MS patients (Matusevicius et al. 1996). Microglia secrete proinflammatory mediators such as prostaglandins, TNF- α , IL-1 β , chemokines, proteases, reactive oxygen species and nitrogen intermediates. However, microglia also have the potential to produce anti-inflammatory mediators such as IL-10, TGF- β and neurotrophins (Vilhardt 2005).

TNF- α is a prototypic inflammatory cytokine long implicated in autoimmune diseases. It is strongly produced by activated macrophages, but is also expressed by T cells, NK cells, mast

cells, fibroblasts, microglia, and astrocytes. TNF- α can also potently promote inflammation by increasing antigen presentation, inducing dendritic cell (DC) maturation, promoting astrocyte proliferation, and regulating cell trafficking through its dramatic inducing effects on chemokine and adhesion molecule expression. TNF- α and its receptors are upregulated in the peripheral immune organs and the CNS during EAE, as well as in active MS lesions and in antigen presenting cells (APCs) and T cells isolated from the CNS during EAE. TNF- α has been shown to upregulate ICAM-1, VCAM-1, E-selectin and the chemokines CCL4 and CCL5 on cerebral EC (Wong and Dorovini-Zis 1992; Wong and Dorovini-Zis 1995; Wong and Dorovini-Zis 1996b; Shukaliak and Dorovini-Zis 2000).

IFN- γ is present in MS lesions and MS patients have increased numbers of myelin-reactive cells secreting IFN- γ in their blood and CSF. It is produced by activated T cells and NK cells, and primarily affects macrophage and DC function by upregulating antigen presentation capabilities through increasing MHC Class II, costimulatory and adhesion molecules (Begolka et al. 2006). IFN- γ has been shown to upregulate MHC class II, ICAM-1, the costimulatory molecules CD80, CD86 and CD40, as well as the chemokines CCL4 and CCL5 on cerebral EC (Wong and Dorovini-Zis 1992; Huynh et al. 1995; Wong and Dorovini-Zis 1995; Shukaliak and Dorovini-Zis 2000).

IL-1 is expressed at low or undetectable levels in healthy brain. Its expression is rapidly up-regulated by various experimental brain insults including ischemia, trauma, hypoxia and neurotoxic or inflammatory stimuli. Early expression of IL-1 occurs predominantly in activated microglia and perivascular macrophages, whereas later expression occurs in astrocytes. All resident brain cells including neurons and vascular EC can express IL-1. IL-1 exists as two isoforms: IL-1 α and IL-1 β . IL-1 β is the major form released by cells. Most IL-1

effects have been described in astrocytes. It promotes astrocyte proliferation and activation, contributing to astrogliosis. IL-1 also acts on the EC of the brain vasculature to up-regulate the expression of adhesion and chemoattractant molecules such as ICAM-1, VCAM-1, E- and P-selectin, CCL2, CCL4 and CCL5 and even increases the permeability of the BBB, events that are involved in leukocyte recruitment (Wong and Dorovini-Zis 1992; Wong and Dorovini-Zis 1995; Wong and Dorovini-Zis 1996b; Shukaliak and Dorovini-Zis 2000; Simi et al. 2007).

In addition to their roles in MS and EAE, cytokines have been implicated in a variety of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), prion diseases, and others (Owens et al. 2006).

Lipopolysaccharide (LPS) or endotoxin, is a component of the outer membrane of gram-negative bacteria, has been implicated in the pathogenesis of endothelial cell injury and/or dysfunction. LPS is released from the surface of replicating and dying gram-negative bacteria into the circulation, where it interacts with the endothelial lining of the vessel wall. It is composed of three structural elements: a core oligosaccharide, an O-specific chain made up of repeating sequences of polysaccharides and a lipid A component, which is responsible for the proinflammatory properties of LPS. LPS binds to soluble or membrane CD14 and LPS-binding protein (LBP) forming a LPS-CD14-LBP complex which then binds to Toll-like receptor 4 (TLR4) on endothelial cells. LPS is a highly proinflammatory molecule that elicits a wide array of endothelial responses, including the upregulation of cytokines, adhesion molecules, and tissue factor via the activation of NF- κ B. In addition to activation, LPS induces endothelial cell death by apoptosis. LPS also activates monocytes and macrophages

to stimulate the production of proinflammatory mediators, which in turn modulate endothelial function (Bannerman and Goldblum 2003; Dauphinee and Karsan 2006).

Table 1 Cytokine actions in CNS inflammation

Adapted from Owens T et al. (2006) in Cytokines and the CNS. Ransohoff RM and Benveniste EN Eds.

| Critical points in inflammation | Cytokines that are implicated |
|--|---|
| Leukocyte entry to CNS | TNF- α , CCL1, CCL2, CCL5, CCL21, CXCL2, CXCL10 |
| Reactivation of lymphocytes in CNS | IL-12 family, IL-18, IL-10, type I interferons, osteopontin |
| Immune effector function | IFN- γ (human), TNF- α , lymphotoxin, TRAIL, LIGHT, NO, IL-17 |
| Immune regulation | IFN- γ (rodent), TGF- β , IFN γ , IL-10, NO |

1.6 Chemokines and Chemokine Receptors

1.6.1 Structure and Function

Chemokines are a family of small (5-20kDa) secreted chemoattractant cytokines.

Chemokines can be classified by structure according to the number and spacing of the conserved cysteines into 4 groups: CXC [alpha], CC [beta], CX3C [delta] and XC [gamma] (Murphy 2000; Rossi 2000). Aside from the large subfamily of CXC and CC chemokines, the CX3CL1 or fractalkine is a membrane bound chemokine. XCL1 and XCL2 or lymphotactin α and lymphotactin β , respectively, belong to the rather small but unique chemokine subclasses. Lymphotactin is expressed in activated CD8⁺ T cells and CD4-CD8⁻ T cell receptor alpha beta + thymocytes. It has chemotactic activity for lymphocytes but not for monocytes or neutrophils (Kelner et al. 1994). Chemokines exert their action by signaling through chemokine receptors that belong to the family of G-protein coupled, pertussis toxin sensitive seven transmembrane receptors. To date, at least 46 chemokine ligands and 18 functional signaling chemokine receptors have been discovered (Zlotnik et al. 2006).

There is a high level of redundancy in the system since some chemokines can bind multiple chemokine receptors and vice versa. However, there are also non-promiscuous

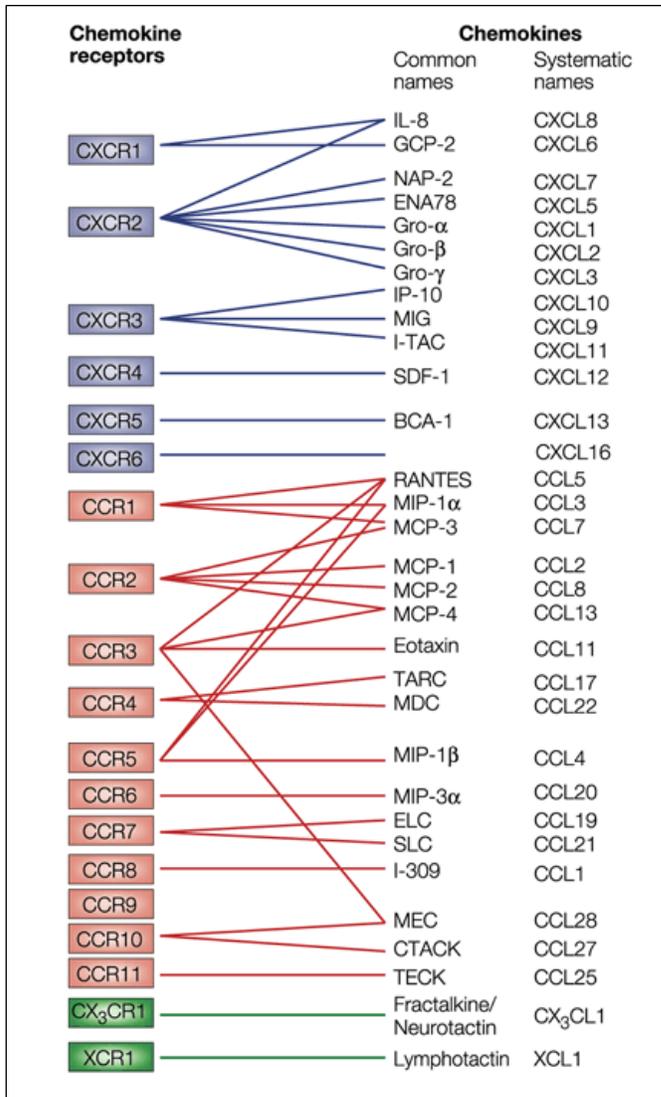


Figure 4 Common and systematic names of four classes of chemokines and their receptors.

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chemokine/chemokine receptor pairs in that one chemokine can only bind with one chemokine receptor. A summary of the chemokine/chemokine receptor pairing is illustrated in Figure 4.

Chemokines can also be classified into

a) homeostatic – constitutively produced and secreted or b)

inflammatory – only produced by cells during infection or a pro-inflammatory stimulus (Fernandez 2002).

Inflammatory chemokines are critical for attracting a diverse set of effector leukocytes to inflammatory sites and as such they are thought to play a key role in the innate immune response by recruiting neutrophils, monocytes/macrophages, DC and NK cells.

Homeostatic chemokines are important for migration of APC and lymphocytes into the lymph nodes, where antigen education and immune surveillance occur. They are also important for effector T cells to reach tissues that contain their cognate antigen (Esche et al. 2005).

Furthermore, chemokines perform diverse functions in wound healing, Th1/Th2 development, angiogenesis, cancer metastasis, cell recruitment, inflammation and leukocyte trafficking

(Rossi and Zlotnik 2000). Interestingly, due to the special distribution of positively charged residues, chemokines possess defensin-like antimicrobial activities (Esche et al. 2005).

1.6.2 CXC Chemokines

CXC chemokines can further be subdivided into ELR+ and ELR- based on the presence or absence of the glutamic acid-leucine-arginine (ELR) tripeptide motif N-terminal to the first cysteine (Murphy et al. 2000). ELR+ CXC chemokines are angiogenic and mainly attract neutrophils, while ELR- CXC chemokines have angiostatic properties and attract mononuclear leukocytes (Menten et al. 2002).

Stromal cell-derived factor-1 (SDF-1 or CXCL12) is a unique ELR- CXC chemokine that has both angiogenic and angiostatic properties. It is a 7.8kDa member of the CXC family, encoded on chromosome 10q. It occurs in two splice variants, SDF-1 α and SDF-1 β , which have similar function and regulated expression (Fedyk et al. 2001). CXCL12 is constitutively expressed by a variety of mesenchymal cells including bone marrow stromal cells, fibroblasts and EC and is involved in the embryonic development of muscle, angiogenesis and bone marrow myelopoiesis (Mirshahi et al. 2000; Nagasawa 2001; Odemis et al. 2002; Salcedo and Oppenheim 2003). The receptor for CXCL12 is CXCR4, a seven transmembrane spanning, G-protein coupled receptor specific for CXCL12. In addition, CXCR4 is a co-receptor for the T-cell tropic human immunodeficiency virus-1 (HIV-1). CXCR4 is constitutively expressed by T cells, monocytes, B cells, hematopoietic progenitor cells and EC (Bleul et al. 1997; Ostrowski et al. 1998; Gupta et al. 2001). The expression of CXCR4 has been shown to vary between EC from different vascular beds indicating tissue-specific immune responses (Hillyer et al. 2003). Binding of CXCL12 to its receptor mediates important processes in the immune system, including B cell proliferation and migration in lymphoid tissues (Feil and Augustin

1998; Blades et al. 2002; Lopez-Giral et al. 2004), homing and retention of leukocytes (Lapidot et al. 2005), chemoattraction of naïve, memory T cells and monocytes (Bleul et al. 1996), costimulation of anti-CD3 activated CD4⁺ T cells by up-regulating CD69, CD25 and CD154 (Nanki and Lipsky 2000) and upregulation of CCL2/MCP-1 and CXCL8/IL-8 in EC via PI-3K and p38-dependent mechanisms (Calderon et al. 2006). Recently, CXCL12 was shown to bind and signal through the CXCR7 chemokine receptor, in addition to CXCR4 (Balabanian et al. 2005; Burns et al. 2006). In the CNS, low constitutive expression of CXCL12 has been demonstrated in neurons, oligodendroglial cells, astrocytes, microglia and EC (Lavi et al. 1997; Ohtani et al. 1998; Gleichmann et al. 2000; Bajetto et al. 2001; Krumbholz et al. 2006). CXCL12/CXCR4 ligation mediates neuronal migration, proliferation and survival in the developing CNS, stimulates proliferation of astrocytes, glutamate release and production of chemokines and cytokines (Bacon and Harrison 2000; Bezzi et al. 2001; Cardona et al. 2008) and induces activation and chemoattraction of microglia (Tanabe et al. 1997). Experimental deletion of either the CXCL12 or CXCR4 genes in mice is associated with fetal death, hematopoietic and cardiac defects and abnormal development of the hippocampal dentate gyrus and cerebellum (Andre et al. 1998; Zou et al. 1998). Recent studies have documented increased expression of CXCL12 and CXCR4 under pathological conditions in the CNS and suggest an active role of CXCL12 /CXCR4 in several diverse disease entities including HIV encephalitis, autoimmune inflammatory diseases, cerebral ischemia and primary and metastatic brain tumors (Rempel et al. 2000; Hoffmann et al. 2001; Zhang et al. 2003; Hill et al. 2004; Krumbholz et al. 2006). Expression of CXCL12 is reportedly increased in microvessel EC and astrocytes in active and chronic MS lesions suggesting a possible role in inflammatory cell recruitment (Calderon et al. 2006; Krumbholz

et al. 2006). At present, the regulation of CXCL12 and CXCR4 expression on cerebral endothelium under inflammatory conditions and the role of CXCL12/CXCR4 ligation on the trafficking of T lymphocytes across the BBB have not been investigated.

1.6.3 CC Chemokines

The β (or C-C) chemokine subfamily, which includes monocyte chemoattractant protein 1 (MCP-1/CCL2), human macrophage inflammatory protein-1 α (MIP-1 α /CCL3), MIP-1 β /CCL4 and regulated on activation, normal T cell expressed and secreted (RANTES/CCL5) have no intervening amino acid between their first two cysteine residues. Each of these chemokines has been shown to induce the directional migration of a number of inflammatory cell types including neutrophils, macrophages, T and B lymphocytes, eosinophils, basophils, and mast cells (Taub et al. 1996).

MIP-1 α encodes for 3 different genes: LD78 α , LD78 β and LD78 γ located at chromosome 17. LD78 α and LD78 β are fully transcribed while LD78 γ is a pseudogene. LD78 α is systemically named CCL3, and is a ligand for the receptors CCR1 and CCR5 on leukocytes. Previous studies have shown that CCL3 secretion is enhanced by monocyte-endothelial cell interactions upon ICAM-1 binding. IL-1 β treated astrocytes and LPS, TNF- α or IL-1 β stimulated microglia have been shown to express CCL3. CCL3 is a potent chemoattractant for monocytes and immature DC (Menten et al. 2002) and promotes Th1 responses by enhancing IFN- γ production by activated T lymphocytes. In resting monocytes and macrophages, only low levels of CCL3 mRNA have been detected. However, upon stimulation of human peripheral blood monocytes with one of several inducers, including LPS, IL-1 β , phytohemagglutinin (PHA), IFN- γ or lipoteichoic acid from Gram-positive bacteria, a significant CCL3 secretion was observed. In addition to the CCL3 production by

peripheral blood leukocytes, CCL3 secretion has also been observed by cells in other tissues or organs, including the brain. Following treatment with IL-1 β , astrocytes released increased levels of CCL3 protein (Lukacs et al. 1994; Menten et al. 2002).

CCL2 was the first CC chemokine to be characterized biologically and shown to attract monocytes, but not neutrophils (Baggiolini 1997). CCL2 was found to be the major chemoattractant made by PHA-stimulated PBMC and attracts activated memory T cell subsets in transendothelial lymphocyte chemotaxis assays (Carr et al. 1994). CCL2 is expressed by various cell types in the brain. In rat brain EC, CCL2 secretion was upregulated following activation with the pro-inflammatory cytokines TNF- α , IL-1 β and IFN- γ , and was reduced following dexamethasone treatment (Harkness et al. 2003). In MS and EAE, CCL2 has been shown to be expressed by astrocytes and microglia. It has also been shown that CCL2 can be transported in the abluminal to luminal direction across brain microvessel EC by transcytosis (Ge et al. 2008). CCL2 binds to CCR2 with high affinity and chemoattracts CCR2+ leukocytes such as monocytes, memory T lymphocytes and natural killer (NK) cells. In humans, CCR2 is expressed by virtually all monocytes and approximately 15% of CD4+ T cells in the circulation, that also express markers of chronic activation such as CD26. Brain endothelium has also been shown to express CCR2 (Berger et al. 1999; Ge et al. 2008). Unexpectedly, significant decrease in CSF CCL2 levels was observed in patients with MS, compared to controls, with further decreases in patients during clinically or radiographically active disease (Franciotta et al. 2001; Sorensen et al. 2004). A recent study by Mahad et al. (Mahad et al. 2006) showed that CCL2 is “consumed” by T cells and monocytes as they migrate across brain EC monolayers *in vitro*, resulting in CCR2 downregulation in response to CCL2, which likely accounts for the decreased levels of CCL2 in the CSF.

Although the expression of chemokines by glial cells has been relatively well documented (Weiss et al. 1998; Bajetto et al. 2002), the expression of chemokines by human brain EC has not been fully characterized. Previous studies from our laboratory have shown that untreated human brain microvessel EC (HBMEC) express low levels of CCL4 and CCL5 that were significantly upregulated following cytokine treatment (Shukaliak and Dorovini-Zis 2000). More recent studies investigated the kinetics of expression and cytokine-induced upregulation and release of CCL2 and CCL3 by HBMEC *in vitro* and showed that under unstimulated conditions HBMEC constitutively synthesized and release low levels of CCL2, whereas CCL3 is minimally expressed and not released. Incubation with TNF- α , IL-1 β or LPS results in significant upregulation of expression and release of both chemokines in a time-dependent manner.

1.6.4 Chemokine Expression in the CNS

Chemokines are expressed in the CNS under both physiological and pathological conditions. CXCL12 and its receptor CXCR4 are essential for CNS development (Asensio and Campbell 1999). The chemokines CCL2, CCL21, CXCL10, CXCL12, CX3CL1 and the receptors CCR2, CXCR3, CXCR4, CX3CR1 are expressed by astrocytes, microglia and neurons (de Haas et al. 2007). Recent studies suggest that chemokines might also function as neurotransmitters and/or neuromodulators in the brain (Adler and Rogers 2005; Callewaere et al. 2007; Rostene et al. 2007). CCL2, CCL3, CCL5, CXCL10, CXCL12 and CX3CL1, are found in the hypothalamus, nucleus accumbens, limbic system, hippocampus, thalamus, cortex and cerebellum (Adler and Rogers 2005). Various chemokines have been shown to be involved in neuroendocrine functions such as stress, body temperature regulation, water balance and control of food intake (Callewaere et al. 2007). The chemokines CCL2, CCL5,

CCL3, CCL4, and CXCL10 are produced by glia and infiltrating leukocytes during MS and EAE. However, CCL2 and its receptor, CCR2, have been implicated as key mediators of leukocyte entry to the CNS, whereas CCL5-CCR5 interactions do not appear to be necessary for this response (Babcock et al. 2003).

1.6.5 Proteoglycans

Proteoglycans are proteins containing glycosaminoglycans (GAGs). GAGs are linear polysaccharides consisting of a repeating disaccharide, generally of an acetylated amino sugar alternating with uronic acid. Classes of GAG chains found on proteoglycans include hyaluronan (HA), chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS) and heparin sulfate (HS) (Perrimon and Bernfield 2001). In general, HS-GAG chains present on brain ECs are bound to core proteins such as syndecans or glypicans. Of the syndecan family, only syndecan-2 is highly expressed on virtually all brain vessels. Syndecans-1 and -3 are predominantly found on larger vessels, and to a lesser extent on brain capillaries. HSPG core proteins are not only expressed on brain endothelium, but also in the basal lamina. A clear structural change in the basal lamina was observed in cerebral vessels during EAE. A perivascular space is formed, surrounded by the endothelial basement membrane and the glial limitans in which infiltrating leukocytes are entrapped (Floris et al. 2003).

The GAG chains are negatively charged and bind avidly to various positively charged molecules such as growth factors, cytokines and chemokines (Hirose et al. 2001). Heparan sulphate can modulate the function of chemokines in at least three ways: 1) at the physicochemical level, the interaction of chemokines with heparan sulphate can protect them from proteolysis and induce them to oligomerize; 2) immobilization of chemokines on the luminal surface of EC and establishment of chemokine gradients across the vascular

endothelium; and 3) transcytosis of chemokines across the endothelial-cell layer (Parish 2006). In addition, heparan sulphate might play a role in leukocyte recruitment by serving as an L-selectin ligand.

1.7 Leukocyte Entry into the CNS in Inflammation

Inflammatory diseases of the CNS are characterized by increased leukocyte trafficking across the BBB and infiltration of the brain parenchyma. The type and quantity of inflammatory cell infiltrates varies depending on the stimulus attracting them and their inherent ability to attack CNS antigens. Neutrophils and macrophages are rapidly recruited in areas of infarcts, neutrophil, lymphocyte, monocyte and plasma cell infiltrates are evident in bacterial meningitis, T cells and macrophages predominate in tuberculosis and viral encephalitides (Keane and Hickey 1997).

The entry of T cells across the BBB is primarily dependent upon the activation state of the lymphocytes. Only activated, but not resting T cells readily cross the intact BBB. Antigen specificity, MHC compatibility, T-cell phenotype, and T-cell receptor gene usage do not appear related to the ability of cells to enter the CNS (Hickey et al. 1991). Recent studies have shown that increased numbers of dendritic cells (DC) are found in the CNS during infection, injury, or autoimmune disease of the CNS (McMahon et al. 2006). In addition, recent study from our laboratory indicate that both immature and mature monocyte-derived DC adhere to cerebral EC through specific interactions between EC adhesion molecules and their corresponding ligands on DC (Arjmandi et al. 2009). They may play an important role in presentation of CNS antigens and activation of auto-reactive T cell.

1.7.1 CD4+ and CD8+ T Cells

CD 4+ or helper T cells have the primary role of producing cytokines. CD4+ T cells respond to antigens presented in association with MHC class II molecules on antigen presenting cells by proliferation and cytokine production. Activated CD4+ T cells are polarized into Th1 or Th2 subsets based on their cytokine production profiles and are generally considered pro-inflammatory or anti-inflammatory, respectively. Th1 cells produce IFN- γ , IL-2, lymphotoxin and TNF- α while Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (Becher et al. 2000). Two chemokine receptors do display very close associations with either IFN- γ (Th1) or IL-4 (Th2) production by effector CD4+ T cells *in vivo*. Nearly all IFN- γ producing cells express CXCR3, while the vast majority of IL-4 producers express CCR4 (Campbell et al. 2003). Recently, a distinct IL-17 producing CD4+ T cells subset, dependent on IL-23 for differentiation, was discovered and named Th17 (Harrington et al. 2006). It has been the paradigm that Th1 cells are the pathogenic cells driving autoimmune tissue damage. However, the recent observations that Th17 cells were more potent than Th1 cells in transferring EAE to naive wild-type host animals suggested that Th17 cells might be responsible for the induction of tissue-specific autoimmunity (Oukka 2007).

CD8+ or cytotoxic T cells play a major role in the destruction of virally infected or cancerous cells. The T cell receptor (TCR) on CD8+ T cells binds to and recognizes short peptides of 8-10 amino acids presented on class I MHC on all nucleated cells. Activation of naïve CD8+ T cells results in the generation of primary armed effector cells and memory cells (Yewdell and Bennink 1999). Effector CD8+ T cells can induce apoptosis of targeted cells by secreting perforin and granzyme B. In EAE, a myelin-derived antigen, pMOG35-55, increased

activation of CD8+ encephalitogenic T cells and inflammation, as well as a more progressive, destructive type of CNS lesions (Sun et al. 2001).

1.7.2 CD4+CD45RO- (Naïve) and CD4+CD45RA- (Memory) T Cells

Naïve T cells are known to re-circulate throughout the body by shuttling between the blood and lymphoid tissue, where they encounter antigens presented by APC. Evidence suggests that naïve T cells are incapable of entering nonlymphoid tissues (Mackay and von Andrian 2001). The activation of naïve T cells requires co-stimulatory signals presented by APC in addition to cognate TCR-MHC complex recognition. In contrast, the activation of memory T cells does not require co-stimulation by APC. The difference in the need for co-stimulation is due to the relative level of rafts and the kinase Lck at the cell surface. Naïve T cells have low levels of rafts and Lck on their surface and therefore depend on costimulation via CD28 to amplify the signaling process by recruitment of rafts and exclusion of phosphatases. In contrast to naïve T cells, effector and memory T cells express high levels of rafts and Lck on their surface. Therefore, they do not need costimulation to amplify signaling (Lanzavecchia and Sallusto 2000). When naïve T cells become activated in response to antigens and switch into memory T cells, the expression of CD62L (L-selectin) and CCR7 is decreased whereas that of CD44 is increased. These changes result in directing the migration of memory T cells from the blood into the tissue and away from lymphoid tissues (Globerson and Effros 2000; Mackay and von Andrian 2001). Differentiation of naïve CD4+ T cells into Th1, Th2 or Th17 effector cells is controlled by TCR activation, costimulatory molecules on the APC and polarizing cytokines in the vicinity of the T cell (Rautajoki et al. 2008). In contrast to naïve T cells, activated memory T cells express high levels of β 1 and β 2 integrins and different chemokine receptors that allow them to enter nonlymphoid tissues (Sprent and Surh 2002).

Memory T cells are heterogeneous and are comprised of 2 main subsets: CCR7⁺ central memory and CCR7⁻ effector memory T cells (Lanzavecchia and Sallusto 2002). Recent evidence suggests that effector memory T cells are replenished continuously by central memory T cells, which proliferate and differentiate under homeostatic conditions (Lanzavecchia and Sallusto 2002). Tissue localization and the migratory capacity of naïve and memory T cells are different. It has been shown that the chemokines CCL2, CCL3 and CCL5 induce transendothelial migration of memory T cells, while CXCL12 induces transendothelial migration of both naïve and memory T cells across cytokine-activated endothelium (Ding et al. 2000).

1.7.3 Regulatory T Cells

Human CD4⁺CD25^{hi} regulatory T cells (Treg) contribute to the maintenance of peripheral tolerance by active suppression (Viglietta et al. 2004). Approximately 1-2% of peripheral blood CD4⁺ T cells have a CD25^{hi} phenotype. In addition, Treg express high levels of the Foxp3 transcription factor (Beissert et al. 2006). Treg exert their suppressive effect primarily by direct cell-cell contact with CD4⁺ cells which is largely mediated by B7/CTLA-4 signaling and partially by TGF- β and IL-10 release (Beissert et al. 2006). Impaired Treg function has been observed in MS patients despite the frequency of Treg in both healthy and MS patients being comparable (Viglietta et al. 2004).

1.7.4 Monocytes

Monocytes are circulating progenitor cells which give rise to tissue macrophages or dendritic cells (Kumar and Jack 2006). Monocytes continuously emigrate from the blood into peripheral tissues with a half-life of 8 to 70 hours in the circulation. It is estimated that 9.4×10^8 monocytes are produced per day in an average adult (Ganz and Robert). Normal CD14⁺

peripheral blood monocytes express the chemokine receptors CCR1 and CCR2, but not CCRs 3 to 5. The expression of the CCR2b isoform is ten times more abundant than CCR2a in monocytes (Tanaka et al. 2002). Furthermore, about 10% of the circulating CD14+ monocytes also express CD16. However, this subset of monocytes expresses CCR1 and CCR5, but virtually no CCR2 (Weber et al. 2000; Katschke et al. 2001; Kumar and Jack 2006). Interestingly, the expression of CCR2 and CD14 on monocytes was markedly decreased in MS patients (Nakajima et al. 2004b). Infiltration of cells of monocytic lineage into the CNS is evident in stroke, traumatic brain injury, infections, MS and EAE as perivascular cells and recently recruited macrophages based on the expression of CD14 and CD45 (Beschoner et al. 2002; Williams and Hickey 2002). Monocyte recruitment into the CNS in HIV infection is also well documented (Boven et al. 2000; Wu et al. 2000; Williams and Hickey 2002).

1.8 Adhesion Molecules

1.8.1 *Endothelial Cell Adhesion Molecules*

Endothelial cell adhesion molecules can be classified into selectins and the immunoglobulin (Ig) superfamily. The selectins belong to the CD62 family (Fabry et al. 1995). The endothelial selectins, E- and P- selectin, are absent on non-activated EC. De novo synthesis and expression of E-selectin is induced by cytokines and LPS within 4 to 6 hours of stimulation and returns to basal levels after 16-24 hours. P-selectin is stored in Weibel-Palade bodies in EC and is rapidly mobilized to the cell surface within minutes after stimulation with histamine, thrombin, calcium ionophore or phorbol esters (Vestweber and Blanks 1999). E-selectin interacts with sialyl-Lewis^x, while P-selectin interacts with sialyl-Lewis^x and PSGL-1 on leukocytes.

The immunoglobulin superfamily of cell adhesion molecules (CAMs) includes ICAM-1 (CD54), VCAM-1 (CD106) and PECAM-1 (CD31). ICAM-1 is constitutively expressed on EC at low levels. Activation of EC by inflammatory cytokines upregulates its expression. VCAM-1 expression is virtually absent on non-activated EC. PECAM-1 is constitutively expressed by EC, particularly at cell-cell junctions, as well as on the cell surface. Its expression is relatively unchanged after endothelial cell activation by cytokines or LPS. While ICAM-1 and VCAM-1 bind to LFA-1 and VLA-4, respectively, PECAM-1 on EC binds to PECAM-1 on leukocytes by homophilic interaction.

1.8.2 Leukocyte Integrins

Integrins are heterodimeric glycoproteins composed of an α and β subunit. These cell surface receptors mediate numerous cell-cell, cell-matrix and cell-pathogen interactions, thus participating in a wide range of biological processes such as development, tissue repair, angiogenesis, inflammation and haemostasis (Curley et al. 1999; Shimaoka and Springer 2003). The cytoplasmic domain of the α and β subunits have been shown to interact with actin-binding proteins and signaling proteins which function in both outside-in and inside-out signaling (Liu et al. 2000). Integrin ligands are usually members of the immunoglobulin superfamily or large modular extracellular matrix (ECM) molecules (Curley et al. 1999). Examples of leukocyte integrins that are important for interaction with the endothelium include α L β 2 (LFA-1), α M β 2 (Mac-1) and α 4 β 1 (VLA-4). Integrins are crucial in cellular immunity. A mutation in the CD18 (β 2 integrin) gene results in the clinical condition leukocyte adhesion deficiency type I (LADI) and the patients often die at a young age (Gahmberg 1997).

1.8.3 Adhesion Molecules at the BBB in CNS Inflammation

Elevated levels of adhesion molecules in both cell-bound and soluble forms are found during CNS inflammation, such as MS and EAE (Lee and Benveniste 1999). Previous work from our laboratory has shown that inflammatory cytokines significantly upregulate adhesion molecule expression on cerebral ECs and increase trafficking of T cells and PMN across human brain endothelial cell monolayers. Activation of EC by inflammatory cytokines upregulates ICAM-1 expression with highest levels observed after 24 hours of cytokine treatment. Treatment with cytokines or LPS induces VCAM-1 expression on brain EC with maximal levels at 18 hours post-stimulation and returns to pre-stimulation levels after 48 hours (Wong et al. 1999; Wong et al. 2007). Recent clinical trials in relapsing–remitting MS using natalizumab, a humanized antibody directed against the α 4 integrin, demonstrated the potential therapeutic effect of interfering with the binding of adhesion molecules on EC to their cognate ligands on leukocytes (Man et al. 2007).

1.9 In vitro Model of the Human Blood-Brain Barrier

Several in vitro models of the BBB have been developed to date: human, rodent, bovine and porcine brain microvessel endothelial cells cocultured with or without astrocytes or astrocyte conditioned media, and HUVEC cocultured with astrocytes or astrocyte conditioned media. Cerebral EC can be isolated by 1) the use of enzymatic digestion of isolated microvessel using collagenase, collagenase-dispase or trypsin or 2) the cloning of endothelial cell islands emerging from identified capillaries plated *in vitro* (Deli and Joo 1996). Endothelial cells isolated by enzymatic digestion are heterogeneous consisting of EC of capillary, arteriolar and venular origin. In vitro models of the BBB can be evaluated by four major criteria:

transmonolayer resistance, transmonolayer permeability to nontransported molecules, tight-junction complexity and endothelial-specific markers (Greenwood 1991).

The in vitro model of the human blood-brain barrier used in our laboratory was established in the early 1990s (Dorovini-Zis et al. 1991) from primary cultures of human cerebral EC obtained from cortical microvessel following enzymatic digestion of cortical fragments. This model has been used to study the physiology and pathophysiology of human brain microvessel EC, the responses of these cells to cytokines, the expression of adhesion, costimulatory molecules and MHC molecules, endothelial antigen presentation, monolayer permeability and leukocyte adhesion and transendothelial migration.

1.10 Objective and Specific Aims

1.10.1 Overall Hypothesis

A key event in the initiation and evolution of inflammatory diseases in the CNS is the timely recruitment of circulating peripheral blood leukocyte subsets to sites of antigenic challenge across the BBB that, under normal conditions, restricts their entry into the brain.

Transendothelial passage of leukocytes at the BBB is an active multistep process orchestrated by interactions between adhesion molecules induced or upregulated on EC by inflammatory mediators and their respective ligands on leukocytes, as well as chemokines and chemokine receptors. The overall objective of this study is to test the hypothesis that chemokines and adhesion molecules regulate the adhesion and migration of T cell subsets and monocytes across human cerebral EC in normal or inflammatory conditions.

1.10.2 Specific Aims

Using a well characterized in vitro model of the human BBB, the following are the specific aims of the project:

1. To study the expression and function of CXCL12 in unstimulated and cytokine- or LPS- stimulated HBMEC.
2. To study the role of CXCL12 in the adhesion and migration CD4+ and CD8+ T cells across confluent HBMEC monolayers.
3. To study the role of CCL2 and CCL3 in the adhesion and migration T cell subsets across confluent HBMEC monolayers.
4. To investigate the involvement of adhesion molecules and integrins in monocyte adhesion and migration across confluent monolayers of HBMEC
5. To study the role CCL2 and CCL3 in monocyte adhesion and migration across confluent monolayers of HBMEC.

CHAPTER 2 MATERIALS AND METHODS

2.1 Isolation and Culture of Endothelial Cells

Primary cultures of HBMEC were established from normal brains obtained at autopsy as previously described (Dorovini-Zis et al. 1991). The endothelial origin of the cells was determined by their strong positive staining for Factor VIII-related antigen, binding of *Ulex europeus* agglutinin-1 (UEA-1), uptake of acetylated low-density lipoprotein, and high alkaline phosphatase activity. The isolated clumps of HBMEC were plated onto fibronectin-coated 96 well plates (Corning Plastics, Corning, NY) or Cellagen discs CD-24 (MP Biomedicals, Solon, OH) and cultured in medium 199 (M199) (Invitrogen) supplemented with 10% horse plasma-derived serum (PDS) (Cocalico Biologicals, Reamstown, PA), 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B (all from Gibco), 20µg/ml endothelial cell growth supplement and 100µg/ml heparin (both from Sigma, St. Louis, MO) at 37°C in a humidified 5% CO₂/95% air incubator. Culture media were changed every 2 days and HBMEC cultures were used upon reaching confluence, 8-10 days after plating.

2.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood of healthy donors by centrifugation in Histopaque-1077 density gradients (density = 1.077g/ml, Sigma) according to manufacturer's instructions.

2.3 Isolation and Characterization of T Cell Subsets

Human CD4⁺, CD4⁺CD45RO⁻ and CD4⁺CD45RA⁻ and CD8⁺ T cells were isolated from other mononuclear cells by negative selection using T cell recovery columns (Cedarlane Laboratories, Burlington, ON).

The purity of the isolated T lymphocytes was greater than 90% as determined by flow cytometry using the following panel of antibodies: CD14 PE, CD45 FITC, CD4 PE, CD8 FITC, CD3 PerCP, CD19 APC, CD3 FITC, CD16/56 PE, CD20 PE (all from Becton Dickinson, San Jose, CA) on FACS Calibur using CELLQuest software (Becton Dickinson).

Viability was greater than 97% by the trypan blue exclusion test.

2.4 Isolation and Characterization of Monocytes

Human monocytes were isolated from heparinized venous blood by negative selection using RosetteSep monocyte enrichment cocktail (StemCell Technologies, Vancouver, BC) according to manufacturer's instructions. The purity of the isolated monocytes was greater than 75% as determined by flow cytometry using the same panel of antibodies (Abs) described in Section 2.3. Viability was greater than 97% by the trypan blue exclusion test.

2.5 CD4⁺ T Cell Activation

T cell activation was carried out by incubating CD4⁺ T cells for 24 hours in 4-well plates precoated with mouse anti-human CD3 mAb (clone HIT3a, BD Pharmingen, San Diego CA) at 10 µg/ml in 10% AB serum in RPMI supplemented with 30U/ml IL-2 (NIH AIDS Research and Reference Reagent Program). Flow cytometric analysis showed greater than 60% and 45% of the activated CD4⁺ T cells expressing CD25 and CD69 markers, respectively, as compared to less than 25% and 1% of resting CD4⁺ T cells.

2.6 Antibodies

Monoclonal mouse anti-human CXCL12/SDF-1, MAB310 (R&D Systems, Minneapolis, MN), mouse anti-human CXCR4, clone 12G5 (NIH AIDS Research and Reference Reagent Program: from Dr. James Hoxie) were used as primary antibodies (Abs). Goat anti-mouse IgG LM conjugated to 5nm gold particles and goat anti-mouse IgG EM conjugated to 10 nm gold particles (British Biocell International, Cardiff, UK) were used as secondary Abs.

Mouse anti-human leukocyte common antigen (LCA/CD45) (DAKO, Glostrup, Denmark) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or the DAKO ARKTM (Animal Research Kit), Peroxidase in combination with anti-LCA/CD45 were used for staining adherent lymphocytes in adhesion experiments in the absence or presence of blocking antibodies, respectively. Normal mouse IgG was from Calteg Laboratories, San Francisco, CA. Isotype control Abs were from Dako, Biogenix or Cedarlane Laboratories, Burlington, ON.

For blocking studies, mouse anti-human ICAM-1 (84H11, IgG1), anti-VCAM-1 (1G11, IgG1) Abs (Immunotech, Marseille, France) were used.

To characterize chemokine receptor expression on monocytes and lymphocyte subsets, Phycoerythrin (PE)-conjugated anti-CCR1 (FAB145P) and anti-CCR2 (FAB151P), Fluorescein (FITC)-conjugated anti-CCR5 (FAB181F) and anti-CXCR4 (MAB172) were used (all from R&D systems). Secondary PE-conjugated Goat F(ab')₂ anti-mouse IgG was purchased from Cedarlane Laboratories.

2.7 Cytokines, LPS and Chemokines

Human CXCL12, CCL2 and CCL3 were a kind gift from Dr. Ian Clark-Lewis, UBC Biotechnology Laboratory. Recombinant human TNF- α and lipopolysaccharide (LPS) were

obtained from Sigma (St. Louis, MO). Recombinant human IFN- γ was obtained from the NIH AIDS Research and Reference Reagent Program. Recombinant human IL-1 β was obtained from Boehringer Mannheim.

2.8 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Confluent HBMEC monolayers grown on collagen coated 35mm plates were used resting or after activation with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 hours. Total RNA from HBMEC was isolated using TRIZOL Reagent (Gibco) according to the manufacturer's instructions. Two μ g of RNA per reaction was used to prepare cDNA. Reverse transcription was performed at 42°C for 90 min in 50 μ l of the following mixture: 1 \times reverse transcriptase buffer (50mM Tris-HCL pH 8.3, 75mM KCl, 3mM MgCl₂) containing 2 μ g of RNA, 5mM dithiothreitol (DTT), 0.2 μ g random hexamer primers (Pharmacia Biotech, Baie d'Urfé, PQ, Canada), 1mM dNTP (Gibco), 40 units of RNase inhibitor (Pharmacia Biotech) and 400 units of M-MLV reverse transcriptase (Gibco). At the end of the incubation period, the enzyme was inactivated by heating at 65°C for 10 min. For PCR, 2 μ l of the synthesized cDNA was amplified using specific forward and reverse primers for CXCL12/SDF-1 and GAPDH (Table 2). PCR was carried out in a 25 μ l reaction mixture containing GeneAmp PCR buffer (10mM Tris-HCl, 50mM KCl), 2mM MgCl₂ (Perkin-Elmer, Roche Molecular System, Branchburg, NJ), 200 μ M of each dNTP, 0.5 μ M of each primer and 2.5U Taq DNA polymerase (AmpliTaq Gold, Perkin-Elmer). A 10 μ l sample of each PCR reaction product was analyzed on 6% polyacrylamide gel with 1kb plus DNA ladder as molecular weight standards. As a negative control, total cellular RNA without reverse transcription was amplified in order to confirm the

absence of contaminating genomic DNA. Gels were photographed using a FB-PDC-34 polaroid camera. Photographs were scanned and analyzed by NIH ImageJ.

Table 2 PCR amplification primer sequences.

| Primer | Sequence (5' to 3') | Product size (bp) | Annealing temperature | Source |
|----------------|--------------------------------------|-------------------|-----------------------|-----------------|
| CXCL12 forward | ATG AAC GCC AAG GTC GTG | 231 | 55°C | Gibco |
| CXCL12 reverse | CTT TAG CTT CGG GTC AAT GC | | | |
| GAPDH forward | CCA TGT TCG TCA TGG GTG TGA ACC A | 272 | 55°C | ID Laboratories |
| GAPDH reverse | GCC AGT ACA GGC AGG GAT GAT GTT C | | | |

Primers were designed according to GenBank sequences, accession numbers L36034 (CXCL12) and J04038 (GAPDH).

2.9 Intracellular Immunogold Silver Staining

Intracellular localization of CXCL12/SDF-1 was performed by immunogold silver staining (IGSS) as previously described (Shukaliak and Dorovini-Zis 2000). Confluent HBMEC monolayers were treated for 24, 48 or 72 hours at 37 °C with TNF- α (10-100U/ml), IFN- γ (200-500U/ml), TNF- α (100U/ml) + IFN- γ (200U/ml), IL-1 β (10U/ml) or LPS (5 μ g/ml). At the end of the incubation period, the cells were washed with PBS, fixed in buffered formaldehyde/acetone (PFA) containing 0.03% Triton X-100 for 10 minutes, washed with wash buffer [PBS containing 1% bovine serum albumin (BSA) and 1% normal goat serum (NGS)] and incubated with anti-CXCL12 monoclonal antibody at 5 μ g/ml in carrier buffer (PBS with 5% BSA and 4% NGS) for 60 minutes at room temperature. After a brief wash, the monolayers were incubated with secondary Ab (goat anti-mouse IgG) linked to 5nm gold particles at 1:50 dilution in carrier buffer for 60 minutes, washed, incubated with silver enhancement solution (Amersham Biosciences, Buckinghamshire, England), and

counterstained with Giemsa. Controls included unstimulated HBMEC and monolayers incubated with normal mouse IgG, carrier buffer or irrelevant Ab instead of primary Ab.

2.10 Surface Immunogold Silver Staining

Surface expression of CXCR4 on HBMEC was quantitated by immunogold silver staining. Confluent HBMEC monolayers were treated with cytokines or LPS as described above for 24, 48 or 72 hours at 37 °C. In separate experiments HBMEC cultures were incubated with exogenous CXCL12 (0.05, 1 or 10µg/ml) for 15, 30 or 60 minutes. Following these treatments, monolayers were stained with the immunogold silver staining technique for surface localization of CXCR4 as previously described (Omari and Dorovini-Zis 2003). Briefly, cultures were washed and incubated with primary Ab (mouse anti-human CXCR4 IgG) at 5µg/ml for 60 minutes at room temperature. Following washing, cells were incubated with secondary Ab (goat anti-mouse IgG) linked to 5nm gold particles at 1:40 dilution for 60 minutes. Sodium azide was added to both the wash and carrier buffers to prevent receptor internalization. Cells were then fixed in buffered formaldehyde/acetone for 30 seconds, washed, incubated with silver enhancement solution, and counterstained with Giemsa. Controls included unstimulated HBMEC and monolayers incubated with normal mouse IgG, irrelevant Ab or carrier buffer instead of primary Ab.

2.11 Cell surface Enzyme Linked Immunosorbent Assay (ELISA)

Confluent HBMEC monolayers grown in replicate wells were incubated with cytokines or LPS as described above for 24, 48 or 72 hours at 37 °C. In separate experiments HBMEC cultures were treated with CXCL12 for 15, 30 or 60 minutes. At the end of the incubation period, cells were fixed in 0.025% glutaraldehyde, washed and incubated with 10µg/ml of anti-CXCR4 Ab for 60 minutes at room temperature, followed by incubation with 0.16µg/ml

goat anti-mouse HRP conjugated secondary Ab for 60 min. 100 μ l of 0.8mg/ml O-phenylenediamine (CalBiochem, La Jolla, CA) in citrate phosphate buffer/0.03% H₂O₂ was added for 30min. The color development was stopped by adding 50 μ l of 2M H₂SO₄. Absorbance was read at 492nm. Controls included incubation with isotype matched irrelevant antibodies, normal mouse IgG, or carrier buffer without the primary antibody.

2.12 Sandwich ELISA of HBMEC Culture Supernatants

The release of CXCL12 in supernatants of resting and cytokine or LPS treated cultures was investigated with sandwich ELISA. Confluent HBMEC monolayers were used unstimulated or following treatment with cytokines or LPS as described above for 24, 48 or 72 hours at 37°C. CXCL12 sandwich ELISA was performed using the R&D Systems (Minneapolis, MN) Human SDF-1 α Immunoassay (DSA00) as per the manufacturer's instructions. Chemokine concentrations were extrapolated from a standard curve reading the absorbance at 450nm over a serial dilution of SDF-1 α standard included in the assay kit ranging from 156 - 100,000 pg/ml, as well as the zero standard (0 pg/ml). The sensitivity of the assay was 18 pg/ml according to the manufacturer.

2.13 Chemokine Diffusion Assays

Chemokine diffusion assays were performed as previously described (Quandt and Dorovini-Zis 2004). HBMEC were grown to confluence on permeable collagen membranes (Cellagen discs) mounted on plastic supports and placed inside individual wells of 4 or 24 -well plates. This double chamber system allows the diffusion of small molecular weight substances across the collagen membrane from the basal-to-apical or apical-to-basal direction. Confluent monolayers with a transendothelial electrical resistance (TEER) greater than 100 Ω ·cm² were

used untreated or following coincubation with TNF- α (100U/ml) and IFN- γ (200U/ml). HBMEC cultures grown in 100 μ l of 10% PDS in M199 in the upper chamber were placed over 350 μ l of 10% horse serum in M199 in the lower chamber of a 24-well plate containing 125 I-labelled CXCL12 (100ng/ml), CCL3 (100ng/ml) or CCL2 (500ng/ml) and incubated for up to 3.5 hours at 37°C. Radioactivity in the supernatants from the upper and lower chambers was measured at 0.5, 1.5 and 3.5 hours using a Beckmann Gamma 5500. Results were expressed as % equilibrium, i.e. the concentration of radioactive counts (cpm/ μ l) in the upper chamber divided by the concentration of radioactive counts in the lower chamber. Each experiment was performed twice using duplicate wells to test for chemokine diffusion. Controls consist of chemokine diffusion across Cellagen discs in the absence of EC.

2.14 Immunoelectron Microscopic Localization of Chemokines

Surface localization of CXCL12, CCL2 or CCL3 following establishment of chemokine gradients across HBMEC was carried out by immunoelectron microscopy. 400 μ l of CXCL12 (100ng/ml), CCL3 (100ng/ml) or CCL2 (500ng/ml) were added to the lower chamber of a 4-well plate and allowed to diffuse for 3 hours across confluent resting or cytokine-treated (TNF- α and IFN- γ) HBMEC monolayers grown on Cellagen discs. The monolayers were then washed with wash buffer (1% BSA and 1% NGS in PBS) and incubated with 15 μ g/ml of primary Ab (mouse anti-human CXCL12) for 40 minutes. Secondary goat anti-mouse IgG Ab conjugated to 10nm gold particles was added for 60 minutes at room temperature. Cells were then washed and fixed in $\frac{1}{2}$ strength Karnovsky's fixative (2.5% glutaraldehyde and 2% PFA) in 0.2M Cacodylate buffer for 1 hour at 4°C, washed with cacodylate buffer, post-fixed in 1% OsO₄ for 1 hour at 4°C, washed with cacodylate buffer and block stained overnight in

uranyl Mg acetate. Following dehydration in a graded series of methanol, the cultures were embedded in Epon-Araldite. Thin cross sections were viewed under a Zeiss 10 electron microscope following lead citrate staining.

2.15 T Cell and Monocyte Adhesion Assay

Confluent HBMEC monolayers grown on Cellagen discs in duplicate wells were used unstimulated or stimulated with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 hours to upregulate endothelial cell adhesion molecules and to mimic inflammatory conditions. The HBMEC monolayers were washed twice with 10% horse serum in M199 and chemokine gradients were established by adding chemically synthesized CXCL12, CCL2 or CCL3 in the lower chamber for 30 minutes to allow diffusion of the chemokines through the Cellagen membrane and binding to the EC. As a result, both soluble (chemotactic) and bound (haptotactic) chemokine gradients were obtained. The media in the upper chamber were removed and replaced with 2×10^5 resting or activated CD4⁺ T cells, 2×10^5 resting CD8⁺ T cells or 1×10^5 monocytes in 100 μ l of 10% horse serum in M199. Following one hour incubation with HBMEC at 37 °C in a 5% CO₂/95% air incubator, the non-adherent cells were removed by gently pipetting up and down at each of the lateral walls of the upper chamber. The HBMEC along with the adherent leukocytes were fixed with ice cold 1:1 acetone/ethanol for 7 minutes at 4 °C. Mouse anti-human leukocyte common antigen (LCA/CD45) (DAKO, Glostrup, Denmark) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or the DAKO ARKTM (Animal Research Kit), Peroxidase in combination with anti-LCA/CD45 were used for staining adherent lymphocytes in adhesion experiments in the absence or presence of blocking antibodies, respectively. The number of adherent T cells or monocytes was determined by

counting 1 central and 8 peripheral fields using a 20× objective lens and a 1-cm² grid as previously described (Santambrogio et al. 1999). All counts were performed blindly. Results are expressed as the number of adherent leukocytes per mm² of the HBMEC monolayer. In addition to immunoperoxidase staining, adhesion of monocytes to HBMEC was also examined by scanning electron microscopy as previously described (Wong et al. 1999). Briefly, adherent monocytes were fixed with 2.5% glutaraldehyde in 0.05M sodium cacodylate for 1 h at 4 °C and processed for SEM. Specimens were critical dried with “Jumbo” critical point dryer E3100, sputter coated with 20 to 25nm gold particles and observed with Cambridge Steroscan 260.

2.16 T Cell and Monocyte Migration Assay

Confluent unstimulated or cytokine treated monolayers cultivated on Cellagen discs in a double chamber chemotaxis system were incubated with resting or activated CD4⁺ T cells (2×10^5 cells/well), CD8⁺ T cells (2×10^5 cells/well) or monocytes (1×10^5 cells/well) in the presence or absence of CXCL12, CCL2 or CCL3 concentration gradients for 3 hours at 37 °C. At the end of the incubation period, non-adherent leukocytes were washed, and the monolayers with migrated T cells or monocytes were fixed with 2.5% glutaraldehyde/2% paraformaldehyde in 0.2M sodium cacodylate buffer for 1 hour, post-fixed with OsO₄ for 1 hour, stained en bloc with uranyl magnesium acetate, dehydrated and embedded in Epon-Araldite. One hundred 1µm thick cross plastic sections taken 20µm apart were cut from each Cellagen disc and stained with toluidine blue. The length of the monolayer in each section was measured and the number of migrated leukocytes counted. The results are expressed as number of migrated T cells or monocytes per mm of monolayer length.

2.17 Statistics

Statistical analyses were performed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA). Student's t-test was used to compare means between two groups. For 3 or more groups, one way analysis of variance (ANOVA) was performed to determine differences between treatment groups, followed by Dunnett's post test to analyze individual differences against control groups. Non-normally distributed data was analyzed by Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparison test. p values less than 0.05 were considered statistically significant.

CHAPTER 3 RESULTS

3.1 Human Brain Microvessel Endothelial Cells (HBMEC)

Primary cultures of HBMEC grown on 96-well tissue culture plates or on Cellagen discs in a double chamber chemotaxis system form confluent, contact inhibiting monolayers made up of elongated EC. The cells express Factor VIII related antigen and bind Ulex europeous lectin. By electron microscopy, adjacent EC are bound together by tight junctions (Figure 5).

3.2 CXCL12 Expression by HBMEC in Primary Culture

Under resting conditions, HBMEC constitutively expressed CXCL12 RNA (Figure 6A, B). Following 24 h co-incubation with TNF- α (100U/ml) and IFN- γ (200U/ml), CXCL12 RNA levels decreased by $42 \pm 20\%$ as shown by densitometric analysis using NIH Image J (Figure 6A, B). Intracellular immunogold silver staining showed positive cytoplasmic staining in the form of fine black granular deposits indicating the presence of CXCL12 in untreated HBMEC cultures (Figure 7A). Untreated cultures in which the primary antibody was omitted showed no cytoplasmic staining (Figure 7B). Incubation of HBMEC with IFN- γ (500U/ml) for 24 h (Figure 7C) or combination of TNF- α (100U/ml) and IFN- γ (200U/ml) for 24h (Figure 7E) markedly decreased the intensity of the intracellular staining. However, 72 h following IFN- γ (500U/ml) (Figure 7D) or combination of TNF- α (100U/ml) and IFN- γ (200U/ml) stimulation (Figure 7F), the intensity of staining increased to near pre-stimulation levels suggesting increased synthesis of CXCL12. Treatment of HBMEC with IL-1 β (10U/ml) or LPS (5 μ g/ml) resulted in a similar but less striking decrease in intracellular staining, which returned to normal after 72 h (data not shown).

Since the decrease of intracellular CXCL12 staining after cytokine treatment was greater than the decrease of RNA levels (Figs. 6, 7), and in view of the fact that CXCL12 is released by

various cell types and exerts its effect on CXCR4 expressing target cells, we next investigated whether the decreased levels of intracellular protein are due to increased release of CXCL12 into the culture supernatants. Supernatants from cytokine or LPS treated HBMEC were collected at 24 and 72 h and chemokine concentrations were analyzed by sandwich ELISA. Approximately 480 pg/ml of CXCL12 were detected in untreated HBMEC cultures (Figure 8). There was no evidence of accumulation of the protein in culture supernatants, since the concentration of CXCL12 did not increase over a 3-day period. Cytokine or LPS treatment for 24 h significantly decreased the release of CXCL12 down to 350-260pg/ml. This was followed 72 h later by an increase of CXCL12 release that reached higher than pre-stimulation levels (640 ± 51 pg/ml) in cultures treated with 100U/ml of TNF- α for 72 h.

3.3 CXCR4 Expression and Regulation by HBMEC

The expression of CXCR4 by HBMEC was detected by light microscopy using surface immunogold silver staining (Figures 9 A-C, E, G and H). Untreated HBMEC constitutively expressed CXCR4 as indicated by the presence of fine black granular deposits distributed throughout the cell surface (Figure 9A). The intensity of staining varied slightly among the cells in culture. Treatment with 100U/ml of TNF- α (Figure 9B), 200U/ml of IFN- γ (Figures 9C, D) or combination of 100U/ml TNF- α and 200U/ml IFN- γ (Figures 9E, F) for 24 h resulted in almost complete attenuation of staining indicating decreased surface expression of CXCR4. IL-1 β at 10U/ml and LPS at 5 μ g/ml were less effective in decreasing CXCR4 expression on HBMEC (Figures 9G, H).

Surface ELISA was then performed to quantitate the overall expression of CXCR4 on HBMEC monolayers. Unstimulated HBMEC constitutively expressed CXCR4. Treatment with cytokines or LPS for 24 or 48 h significantly decreased the surface expression of CXCR4

(Figure 9I). The TNF- α -induced decrease was dose dependent between 10U/ml and 100U/ml, whereas different concentrations of IFN- γ had similar results. Co-incubation with TNF- α and IFN- γ was most effective in reducing CXCR4 surface expression. The effect of IL-1 β on CXCR4 expression was transient compared to LPS and the other cytokines tested. Although there was significant downregulation at 24 h, CXCR4 expression returned to pre-stimulation levels at 48 h. LPS significantly reduced CXCR4 surface expression at 24 and 48 h. Since CXCR4 is the cognate receptor for CXCL12, it is important to examine the effect of exogenously added CXCL12 on surface CXCR4 expression on HBMEC. Exogenously added CXCL12 at 0.05 to 10 μ g/ml induced a rapid and significant decrease of surface CXCR4 expression in HBMEC after 15 and 30 min in a concentration-dependent manner indicating that CXCR4 expressed on HBMEC is functional (Figures 10A-E). The levels of CXCR4 remained low at 60 min, but were not statistically significant from the untreated controls. An irrelevant chemokine, CCL3, used as negative control, had no effect on CXCR4 expression, thus confirming the specificity of the CXCL12/CXCR4 interaction. Similar to CXCR4, CXCR7 is constitutively expressed by unstimulated HBMEC. However, unlike the effect of CXCL12 on CXCR4, treatment of HBMEC with 10 μ g/ml for 15 min and 1 μ g/ml for 15, 30 or 60 min did not have an effect on the surface expression of CXCR7. Treatment of HBMEC with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 hours resulted in a slight decrease in CXCR7 expression by ELISA. Similar to resting HBMEC, incubation of cytokine-treated cultures with 10 μ g/ml CXCL12 for 15 min had no effect on CXCR7 expression (unpublished observations).

3.4 Chemokine Diffusion across HBMEC *in vitro*

The rate of diffusion of CXCL12, CCL2 and CCL3 across Cellagen discs and confluent HBMEC monolayers in the presence of chemokine gradients was determined in the double chamber chemotaxis system using radiolabeled chemokines. The diffusion assay was performed using confluent cultures with trans-endothelial electrical resistance (TEER) greater than $100 \Omega \cdot \text{cm}^2$ (Figure 11A). Confluent resting monolayers restricted the passage of CXCL12, CCL2 and CCL3 to the upper chamber as compared to Cellagen discs alone during the entire 3.5 h of the assay (Figure 11B-D). Stimulation of confluent HBMEC monolayers with 100U/ml TNF- α and 200U/ml IFN- γ for 24 h decreased the TEER to less than $50 \Omega \cdot \text{cm}^2$ and led to a rapid increase of chemokine diffusion at 1.5 and 3.5 h compared to resting HBMEC. In the absence of HBMEC monolayers, there was a steep increase in % equilibrium between 0.5 and 1.5 h. The rate of equilibrium slowed down after 1.5 h for CXCL12 while CCL2 and CCL3 reached a plateau after 1.5 h.

3.5 Localization and Binding of Chemokines to HBMEC *in vitro*

Immunoelectron microscopy was performed to quantitate the binding of CXCL12 (Figure 12), CCL2 (Figure 13), and CCL3 (Figure 14) to the endothelial surface and the subendothelial matrix. For CXCL12, the total number of gold particles bound per unit length of both the apical and basal cell surfaces was similar in unstimulated and stimulated HBMEC. However, the distribution of bound CXCL12 was different under resting and stimulated conditions. Thus, in resting HBMEC, a greater number of gold particles was associated with the basal surface (Figure 12A, D) and the subendothelial basal lamina-like material (Figure 12B) as compared to the apical surface. Following cytokine treatment, the number of gold particles bound to the basal surface decreased significantly and there was a significant increase in the

apical binding of CXCL12, so that the apical/basal polarization of CXCL12 was no longer present after cytokine stimulation of HBMEC (Figures 12C, D).

CCL2 bound mostly to the basal cell surface and subendothelial matrix in both unstimulated and cytokine stimulated HBMEC (Figure 13B). Very few gold particles were found on the apical surface (Figure 13A). There was a moderate, but not significant increase in the number of gold particles bound to the basal surface of stimulated HBMEC (Figure 13C), whereas the apical density remained unchanged. Similarly, the binding of CCL3 was mostly basal under both stimulated and unstimulated condition (Figure 14A). However, there was a mild increase in both apical and basal binding following cytokine stimulation (Figure 14B). Thus, the binding of CXCL12, CCL2 and CCL3 to the endothelium and the extracellular matrix in the presence of chemokine gradients was different among the three chemokines under resting and cytokine-stimulated conditions.

3.6 Chemokine Receptor Expression on Leukocyte Subsets

3.6.1 *Monocytes*

The alpha chemokine receptor CXCR4 was present on 76% of the isolated monocytes. The beta-chemokine receptors CCR1, 2 and 5 were present at a much lower levels ranging from 20 to 35% of the monocytes (Figure 15). However, the peak fluorescence of CCR1, 2 and 5 was very distinct relative to the isotype control. These definitive peaks were absent in both CD4+ and CD8+ T cell populations (see below).

3.6.2 *Resting and Activated CD4+ T Cells*

Greater than 90% of resting and activated CD4+ T cells stained brightly for CXCR4. Up to 15% of resting CD4+ cells expressed the CCR1, 2 or 5 receptors. Anti-CD3 activation of CD4+ T cells for 24 hours increased the surface expression of all three beta-chemokine

receptors. Both resting and activated CD4⁺ T cells stained brightly for CCR5 compared to CCR1 and CCR2 (Figure 16).

3.6.3 CD4⁺CD45RO⁻ (Naïve) and CD4⁺CD45RA⁻ (Memory) T Cells

Further separation of the resting CD4⁺ T cells population into naïve and memory phenotypes based on their different CD45 isoforms, showed that memory T cells are the main cell type that expressing CCR1, 2 and 5. Less than 10% of naïve CD4⁺ T cells expressed these three beta-chemokine receptors (Figure 17). In contrast to the resting CD4 population, CCR5^{bright} cells were absent in both resting naïve and memory CD4⁺ pools (Figure 16, 17). This suggests that CCR5^{bright} cells belong to the CD45RA⁺/45RO⁺ double positive CD4⁺ T cell subset, which was removed during the isolation of naïve or memory CD4⁺ T cells. Consistent with the resting CD4⁺ T cell population, CXCR4 was constitutively expressed by naïve and memory T cells.

3.6.4 CD8⁺ T cells

While CXCR4 was constitutively and highly expressed on CD8⁺ cells, less than 10% of the cells showed low constitutive expression of CCR1, 2 and 5 (Figure 18). The expression of these 3 beta-chemokine receptors was lower compared to resting CD4⁺ T cells (Figure 16, 18).

3.7 Adhesion and Migration of CD4⁺ and CD8⁺ T Cells across HBMEC in Response to CXCL12

Leukocyte adhesion and migration experiments were carried out in a double chamber chemotaxis system as illustrated in Figure 19.

3.7.1 Adhesion of CD4⁺ and CD8⁺ T Cells to HBMEC

Under unstimulated conditions, only 22% of the CD4⁺ T cells added to the upper chamber were bound to the monolayers at the end of the 1h adhesion assay (Figures 20A, G). Addition

of CXCL12 to the lower (Figures 20C, G) or both upper and lower chambers (Figures 20E, G) had no effect on adhesion to the unstimulated endothelium. Treatment of HBMEC with TNF- α and IFN- γ for 24 h resulted in a 3.5 fold increase in adhesion with 61% of CD4⁺ T cells bound to the monolayers after 60 min incubation with HBMEC in the absence of chemokine gradients (Figures 20B, G). Addition of CXCL12 to the lower chamber at 100ng/ml (Figures 20D, G), or to the upper and lower chambers at 10ng/ml (Figures 20F, G) further enhanced adhesion up to 5 fold compared to the unstimulated endothelium with nearly 100% of T cells adhering to the monolayers at the end of the 60 min incubation period. Lower concentrations of CXCL12 in the lower chamber had no effect on adhesion. Pretreatment of HBMEC with an anti-ICAM-1 mAb significantly reduced CD4⁺ T cell adhesion to activated HBMEC in the absence or presence of chemokine gradients by 49% and 54% respectively (Figure 20H). Blocking of VCAM-1 on HBMEC similarly reduced adhesion in the absence or presence of CXCL12 gradients by 48% and 40% respectively (Figure 20H). In the presence of 10ng/ml CXCL12 in both chambers, VCAM-1 blocking downregulated adhesion by 33%.

Adhesion of CD8⁺ T cells to resting HBMEC was minimal with only 11% of T cells bound to the monolayers after 60 min incubation (Figures 21A, G). The presence of CXCL12 gradients or addition of CXCL12 to both chambers had no effect on adhesion to the unstimulated endothelium (Figures 21C, E, G). Cytokine activation of HBMEC significantly augmented adhesion up to 4 fold resulting in 41% of CD8⁺ T cells binding to HBMEC (Figures 21B, G). However, unlike the CD4⁺ T cell population, the presence of 100ng/ml of CXCL12 in the lower chamber (Figures 21D, G) or 10ng/ml in both chambers (Figures 21F, G) resulted in slight, but not significant upregulation of adhesion (55% and 47% respectively). Adhesion to cytokine treated HBMEC was significantly reduced with mAb to ICAM-1 and

VCAM-1 (Figure 21H). Blocking of ICAM-1 was most effective in the absence of CXCL12 gradients and when CXCL12 was present in both chambers, whereas the greatest effect of VCAM-1 blocking was observed when CXCL12 was added to both chambers (Figure 21H). The use of HBMEC and lymphocytes from different donors resulted in some variability in the overall numbers of adherent cells between experiments, however, the trend remained the same.

3.7.2 *Migration of CD4+ and CD8+ T Cells across HBMEC*

Only a very small number of CD4+ T cells migrated across unstimulated HBMEC monolayers (approximately 0.2 T cells/mm length of monolayer or 15% of the cells added to the upper chamber) after 180 min incubation with HBMEC (Figure 22A, E). Occasional T cells were still attached to the endothelium at the end of the 3h migration assay (Figure 22A). Addition of 100ng/ml CXCL12 to the lower chamber 30 min prior to the addition of CD4+ T cells to the upper chamber significantly increased migration across unstimulated EC (Figures 22B, E). Cytokine activation of HBMEC increased transendothelial migration by 2.7 fold (Figures 22C, E). Migration across cytokine stimulated HBMEC was not affected by the presence of CXCL12 gradients (Figures 22D, E).

The number of CD8+ T cells that migrated across resting HBMEC cultures during the 3 h of incubation with HBMEC was minimal (Figures 23A, E) and smaller than the number of migrated CD4+ T cells (0.03 versus 0.18 cells/mm length of monolayer). The addition of 100ng/ml CXCL12 to the lower chamber increased migration across unstimulated monolayers by 3.5-fold (Figures 23B, E). Cytokine pretreatment of HBMEC significantly enhanced transendothelial migration by 30-fold (Figures 23C, E), so that the number of CD8+ T cells crossing cytokine-activated monolayers was 2 times greater than the number of CD4+ T cells

transmigrating across similarly activated HBMEC during the same length of time. In the presence of CXCL12 gradients, migration across cytokine-treated HBMEC was increased by 10-fold (Figures 23D, E). CD4⁺ and CD8⁺ T lymphocytes often migrated in groups of two or more cells at several points across the monolayers. Upon completion of the migration process, T cells became flattened and remained between the overlying HBMEC and the underlying collagen membrane. The monolayers resumed their continuity at the end of the migration period.

3.8 Adhesion and Migration of CD4⁺ Subsets and CD8⁺ T Cells to HBMEC in Response to CCL2 and CCL3

3.8.1 Adhesion of CD4⁺ T Cell Subsets to HBMEC

Adhesion of resting CD4⁺ T cells to unstimulated HBMEC was low (< 100 cells/mm²).

Treatment of HBMEC with TNF- α and IFN- γ for 24 hours significantly increased binding to HBMEC (up to 400 cells/mm²) (Figure 24, 25). The presence of CCL2 or CCL3 gradients did not increase adhesion of resting CD4⁺ T cells to either resting or cytokine-stimulated HBMEC.

Anti-CD3 activated CD4⁺ T cells adhered to unstimulated HBMEC more readily than resting CD4⁺ T cells (>200 cells/mm²) (Figure 26, 27). Adhesion was further augmented when HBMEC were stimulated with cytokines. In the presence of CCL2 gradients (50 or 500 ng/ml of CCL2 placed in the lower chamber), a significantly greater number of activated CD4⁺ T cells adhered to stimulated HBMEC (Figure 26). However, when CCL2 was present in both upper and lower chambers (ie. no gradients), adhesion was not increased. In contrast, CCL3 gradients had no effect on activated CD4⁺ T cell adhesion to either resting or cytokine stimulated HBMEC (Figure 27).

Naïve CD4⁺ T cells minimally adhered to resting HBMEC monolayers (< 50 cells/mm²). Cytokine stimulation of HBMEC increased naïve CD4⁺ T cell binding to HBMEC (>100 cells/mm²). The presence of CCL2 or CCL3 gradients did not further increase adhesion to unstimulated or cytokine stimulated HBMEC (Figure 28). Unexpectedly, the presence of CCL3 in both upper and lower chambers decreased the adhesion of naïve T cells to both unstimulated and stimulated cerebral endothelium (Figure 28B).

Memory T cells adhered more readily than naïve T cells to both resting and stimulated HBMEC monolayers (>50 cells/mm²). CCL2 had no effect on memory T cells adhesion (Figure 29A), whereas CCL3 increased adhesion of memory T cells to unstimulated endothelium (Figure 29B). The opposite effect was observed when memory T cells were incubated with stimulated HBMEC: adhesion was increased when 100ng/ml of CCL3 was added to the lower chamber. However, the addition of 10ng/ml of CCL3 to both chambers resulted in decreased adhesion (Figure 29B).

3.8.2 *Transendothelial Migration of CD4⁺ T Cell Subsets and CD8⁺ T Cells*

Only occasional resting CD4⁺ T cells migrated across unstimulated HBMEC cultures. Stimulation of HBMEC with combined TNF- α and IFN- γ for 24 hours increased resting T cell migration by 2-fold (Figure 30). The response of resting CD4⁺ T cells to the presence of CCL2 or CCL3 on the “brain side” of the BBB was dependent upon the activation status of the endothelium. Thus, CCL2 gradients increased migration of CD4⁺ T cells across unstimulated but not cytokine-stimulated HBMEC (Figure 30). CCL3 had a directly opposite effect in that it decreased migration across unstimulated HBMEC, but increased migration across stimulated HBMEC monolayers.

Activated CD4⁺ T cells readily migrated across resting endothelium and migration was further enhanced following treatment of HBMEC with TNF- α and IFN- γ for 24 h. CCL2 increased activated CD4⁺ T cell migration independent of the activation status of HBMEC. However, the relative increase in activated T cell migration in response to CCL2 was greater across unstimulated EC (Figure 31G). CCL3 moderately, but not significantly increased the migration of activated CD4⁺ T cells across unstimulated monolayers while downregulated migration across stimulated HBMEC. Following transendothelial migration, both resting and activated T cells remained as elongated cells between the endothelial monolayers and the collagen substrate. The HBMEC retained their continuity over the migrated lymphocytes (Figure 31A-F).

Remarkably fewer naïve T cells migrated across resting HBMEC monolayers compared to the resting CD4⁺ T cell population (Figure 32). Stimulation of HBMEC with cytokines only marginally increased migration. CCL3 significantly reduced the already low frequency of naïve T cell migration by a further 67%. In contrast, both CCL2 and CCL3 significantly increased naïve T cell migration across cytokine activated HBMEC by more than 2-fold (Figure 32G). Most naïve T cells remained adherent to resting HBMEC but did not migrate across the endothelial monolayers after 3 hours (Figure 32 A, C, E).

Memory CD4⁺ T cells migrated at a similar frequency as resting CD4⁺ T cells. In the absence of chemokines, cytokine stimulation of the monolayers increased memory T cell migration more noticeably than resting CD4⁺ T cell population (4.3 versus 3 fold) (Figures 30G, 33G). Migration across resting or TNF- α and IFN- γ stimulated endothelium was not increased in response to CCL2 or CCL3 (Figure 33).

CD8⁺ T cell migration across unstimulated endothelium was negligible (Figure 34). Upon cytokine stimulation, CD8⁺ T cells readily crossed the endothelial barrier. CCL2 did not have a significant effect on CD8⁺ T cell migration across resting or stimulated HBMEC. Addition of CCL3 in the lower chamber dramatically upregulated CD8⁺ T cell migration across resting and stimulated HBMEC by 7.5 and 1.7 fold, respectively. Taken together, CCL3 most consistently augment CD8⁺ T cell migration regardless of the activation state of endothelium.

3.9 Adhesion and Migration of Monocytes across HBMEC Monolayers in Response to CCL2 and CCL3

3.9.1 *Monocyte Adhesion to HBMEC*

Monocyte adhesion to HBMEC was time dependent and influenced by the activation status of the endothelium. Only a few monocytes adhered to unstimulated HBMEC in the first 30 min and the number of adherent cells gradually increased thereafter (Figure 35). Monocytes readily adhered to cytokine-stimulated monolayers within 15 min and adhesion increased further overtime (Figure 35).

By scanning EM, monocytes adhered to unstimulated HBMEC by 30 min (Figure 36A) and the number of adherent cells increased after a 60 min (Figure 36B) incubation. Cytokine stimulated HBMEC became spindly and overlapped (Figure 36C to F). Significantly more monocytes adhered to stimulated HBMEC at 30 min (Figure 36C) and 60 min (Figure 36D) compared to unstimulated HBMEC. Adherent monocytes positioned themselves at or near interendothelial junctions by 30 min (Figure 36E) and began migrating between EC at 60 min (Figure 36F).

The minimal adhesion of monocytes to unstimulated HBMEC was increased significantly after treatment of HBMEC with TNF- α and IFN- γ for 24 h (Figure 37). Preincubation of HBMEC with blocking antibodies to CD62E (E-selectin), CD62P (P-selectin), CD54 (ICAM-

1), CD106 (VCAM-1) and CD31 (PECAM-1) had negligible effect on monocyte binding to both stimulated and unstimulated monolayers. Blocking of CD49d ($\alpha 4$ integrin) on monocytes significantly reduced monocyte adhesion to endothelial monolayers. A combination of VCAM-1 and CD49d blocking antibodies did not further reduce monocyte adhesion (Figure 37). The effectiveness of anti- $\alpha 4$ blocking was most apparent in cytokine stimulated HBMEC where the number of adherent monocytes was reduced to baseline levels (Figure 38).

The presence of CCL2 or CCL3 in the lower chamber increased the frequency of uropod-like structures on monocytes and induced monocyte aggregation (Figure 39A-C). However, the presence of CCL2 or CCL3 concentration gradients only marginally increased adhesion of monocytes to unstimulated or cytokine-stimulated HBMEC (Figure 39D, E).

3.9.2 Transendothelial Migration of Monocytes

Migration of monocytes was directly related to the length of the incubation time and the activation status of the HBMEC (Figure 40). Under unstimulated conditions, only a small number of the adherent monocytes migrated across the endothelial monolayers after 1 hour. By 3 hours, twice as many monocytes had transmigrated indicating a continuous movement of the previously adherent cells at a constant rate across the monolayers. Pretreatment of HBMEC with cytokines significantly augmented migration by greater than 4-fold after 3 hours (Figures 40-42). At the end of the migration period, migrated monocytes with elongated shape remained under the monolayers which resumed their continuity and appeared intact (Figures 40-42). Both CCL2 and CCL3 significantly increased monocyte migration across unstimulated endothelium (Figure 41G). In contrast, neither chemokine further increase monocyte migration across stimulated HBMEC. Monoclonal blocking antibodies

against CD18 and CD49d significantly decreased monocyte migration across cytokine-stimulated HBMEC, in the order: anti-CD49d > anti-CD18 > anti-PECAM-1 (Figure 42D). In the presence of CD18 or CD49d blocking antibodies, many of the non-migrated monocytes remain adherent to the HBMEC monolayers (Figure 42B, C).

Since CCL2 and CCL3 generally increased monocyte migration and blocking Abs reduced migration, it would be of interest to determine if blocking Abs could overcome or diminish the effect of chemokines, thus reducing monocyte migration. In the presence of CCL2 gradient, blocking β 2-intergrin (CD18) on monocytes and to a lesser extent, ICAM-1 (CD54) on the endothelial side effectively reduced migration of monocytes across unstimulated HBMEC. Blocking anti-CD49d on monocytes had little or no effect on migration. When HBMEC were treated with the proinflammatory cytokines TNF- α and IFN- γ , blocking antibodies to ICAM-1, CD18 and CD49d significantly reduced the transendothelial migration of monocytes by more than 35%.

CHAPTER 4 DISCUSSION

4.1 In vitro Models of the BBB

Progress in isolating and culturing brain EC over the last 30 years has provided a unique opportunity for studying the biology, function and pathophysiology of cerebral EC, their responses under pathological conditions including hypoxia and inflammation and their interactions with circulating white blood cells. There have been several reported methods for isolation and culture of EC from rat, mouse, bovine and porcine brain and a small number of in vitro models of the human BBB. There are differences in both the method of isolation and subsequent culture of EC among the various investigators, the principal aim being the establishment of a reliable and reproducible in vitro model that retains important in vivo properties of the BBB. Astrocytes are sometimes co-cultured with EC in some in vitro models of the BBB since the work of Stewart and Wiley showed that extracerebral EC transplanted to the brain acquire BBB properties, whereas cerebral EC transplanted to other organs rapidly become leaky (Rubin 1991). Later on Janzer and Raff injected highly purified astrocyte preparation into the anterior chamber of adult rat eyes and showed that newly formed vessels in the astrocyte aggregate were impermeable to the Evans Blue dye (Arthur et al. 1987). Therefore, the microenvironment of the brain rather than the brain EC themselves seem to be responsible for the differentiation of the BBB phenotype. Structurally, astrocytic endfeet cover much of the capillary's basal surface *in vivo* (Kacem et al. 1998; Rubin and Staddon 1999; Abbott 2002). Upon co-culture with astrocytes, brain EC appear to retain some phenotypic properties that are typically lost in mono-culture systems (Rubin et al. 1991; Rubin and Staddon 1999). However, the astrocytic mediators, such as direct cell-cell contact or soluble factors in conditioned media that regulate some of the phenotypic properties of

brain EC are not presently known (Reinhardt and Gloor 1997). In a bovine model of the BBB, the removal of serum or addition of C6 glioma cells to the basolateral side greatly enhanced the TEER and decreased the permeability of the monolayer. In addition, occludin expression, occludin/ZO-1 association and ZO-1 membrane localization were influenced by the presence or absence of basolateral astrocytes and/or serum (Colgan et al. 2008).

Monoculture of EC offers a simple in vitro model of the BBB that permits the study of the biology, physiology and immune function of the cerebral endothelium and the trafficking of leukocytes across the BBB. One of the greatest challenges in primary cultures of brain EC is the presence of contaminating cells such as pericytes and smooth muscles cells. Several recent studies have reported that the antibiotic puromycin can greatly improve the purity of rat BMEC by inhibiting the growth of pericytes. Brain EC express the efflux transport P-gp which effectively effluxes the drug from the cells (Perriere et al. 2005; Calabria et al. 2006).

In cells lacking P-gp, such as pericytes, puromycin is metabolized in the cytoplasm into puromycin aminonucleoside which is known to be cytotoxic due to the production of reactive oxygen species (Perriere et al. 2005). Culturing BMEC with plasma-derived serum (PDS) also improves purity due to the lack of platelet-derived growth factor (PDGF), which reduces the growth rate of contaminating pericytes and smooth muscles cells (Calabria et al. 2006).

Due to the limited availability of human brain tissue for isolation of HBMEC, some researchers have attempted to create in vitro models of the human BBB using non-brain derived endothelium such as HUVEC cultured in the presence of astrocyte conditioned media. However, as stated by Bennet et al. in 1959 (Baldwin 2000) “The variations in structure between capillaries of different organs are so fundamental that one must caution against using measurements made on one capillary bed to support a permeability hypothesis with respect to

a capillary bed of a different type.” Thus, primary cultures of EC derived from human brain most closely resemble the human BBB *in vivo* since they maintain important morphological and functional properties of their *in vivo* counterparts.

An *in vitro* model of the blood-brain barrier developed in this laboratory (Dorovini-Zis et al. 1991) using primary cultures of HBMEC does not rely on the presence of astrocytes and yet the HBMEC retain much of the *in vivo* BBB properties. Culturing HBMEC with PDS in the initial presence of puromycin effectively minimizes the presence of contaminating pericytes and smooth muscle cells. Confluent HBMEC monolayers consist of elongated, contact inhibiting EC that are bound together by tight junctions of high electrical resistance ($> 100\Omega\text{-cm}^2$), have a paucity of cytoplasmic vesicles, express Factor VIII related-Ag and bind UEA-1.

4.2 Effects of Chemokines on the Permeability and Function of Endothelial Cell Monolayers

The measurement of monolayer permeability is a useful quantitative assessment of endothelial integrity and barrier function *in vitro*. Paracellular permeability to ions is measured by the TEER across confluent monolayers, whereas permeability to larger molecules can be measured by fluorescently or radioactively labeled substrates. Permeability data from various *in vitro* BBB models were shown to correlate well with *in vivo* cerebrovascular permeability coefficients (Deli et al. 2005).

In a recent study, using mouse brain microvessel EC, Pachter’s group reported that exogenous unlabeled CCL2 depressed the release of endogenous CCL2 as well as its mRNA levels (Ge et al. 2008). They also found that CCL2 is transported transcellularly from the abluminal to the luminal side via binding to its receptor, CCR2 and subsequently being carried to caveolar vesicles for transcytosis. The question of endothelial activation by chemokines has been

addressed in two studies in which incubation of HUVEC with 20ng/ml of CCL2 for 4 hours did not induce upregulation in the expression of the EC adhesion molecules ICAM-1, ICAM-2, VCAM-1, E-selectin or P-selectin (Carr et al. 1994; Roth et al. 1995). In our double chamber chemotaxis assay system, incubation of HBMEC with CCL2, CCL3 or CXCL12 for 30 minutes did not have any significant effect on the TEER across HBMEC monolayers. These findings are consistent with a previous report from our laboratory indicating that incubation with two other β -chemokines, CCL4 or CCL5, did not alter the permeability of the HBMEC monolayers over a 24-hour period (Quandt and Dorovini-Zis 2004). In contrast, Andejkovic et al. reported that CCL2 increases the permeability of the BBB and causes redistribution of the tight junctional proteins occludin, claudin-5, ZO-1 and ZO-2 in co-cultures of mouse brain EC with astrocytes and *in vivo* after intracerebral and intracerebroventricular administration in mice (Stamatovic et al. 2005; Dimitrijevic et al. 2006). In the present study, radio-labeled CCL2, CCL3 or CXCL12 added to the lower chamber (basolateral side) gradually diffused across confluent HBMEC from the lower to the upper chamber at different rates. This might be due to transcytosis since confluent unstimulated HBMEC display high electrical resistance and low permeability. The rate difference in chemokine transport across resting sephenous vein, dermal microvascular and lung microvascular EC has been described (Mordelet et al. 2007), and this difference is independent of paracellular permeability.

4.3 Leukocyte Adhesion and Migration across Endothelium under Static or Flow Conditions

Leukocyte-endothelial interactions *in vivo* occur under physiological flow. Nevertheless, most *in vitro* leukocyte adhesion and migration assays are performed under static conditions due to the simple setup and relatively low cost. The use of a flow system is generally low

throughput and requires the use of a temperature and CO₂ controlled chamber attached to microscope equipped with time-lapse video camera. In contrast, the static system does not rely on expensive laboratory set-up and has the advantage that at the end of the experiment, the cells can be fixed and stained for further analysis. However, several studies published over the years have indicated that shear flow is important for transmigration of leukocytes across the endothelium (Cinamon et al. 2001). It is becoming clear that shear flow modulates the cytoskeleton and promotes leukocyte-endothelial interactions. Chemokine-activated lymphocyte integrins require shear forces to optimally adhere to their ligand (Woolf et al. 2007). In experimental designs where soluble chemokines are used to study the trafficking of leukocytes, non-flow, double chamber systems such as Transwell or Boyden chambers, are widely used by several investigators.

4.4 Expression of CXCL12 and CXCR4 by HBMEC

Under unstimulated conditions, HBMEC constitutively express CXCL12 RNA and protein and release modest amounts of CXCL12 into the culture supernatants with significant downregulation 24 h following treatment with cytokines and LPS. The receptor for CXCL12, CXCR4, is strongly expressed on HBMEC under standard culture conditions and is rapidly downregulated upon binding to CXCL12 and after incubation of the monolayers with cytokines and LPS. Expression of CXCL12 has been reported in several human small and large vessel EC including HUVEC, coronary, iliac artery, dermal and bone marrow endothelium (Volin et al. 1998; Pablos et al. 1999; Molino et al. 2000; Yun and Jo 2003). CXCL12 is constitutively expressed by EC in the CNS. Increased expression has been reported in active and chronic inactive MS lesions (Krumbholz et al. 2006) and in the penumbra of focal cerebral ischemia in mice in association with concomitant infiltration by

CXCR4 expressing leukocytes (Stumm et al. 2002). Beyond these observations, little is known about the regulation and function of CXCL12 and CXCR4 at the BBB. Cytokines and LPS had a time-dependent biphasic effect on the expression and release of CXCL12 by HBMEC characterized by a significant decrease after 24 h of treatment followed by a subsequent reversal and increase to steady state or higher levels of expression in the continuous presence of cytokines or LPS. The combination of TNF- α with IFN- γ and LPS alone were most potent in downregulating CXCL12, followed by IL-1 β , IFN- γ and TNF- α . With the exception of one other study showing inhibition of CXCL12 expression in human fibroblasts by IL-1 β and TNF- α (Fedyk et al. 2001), the effect of cytokines or LPS on CXCL12 expression and release by EC has not been previously investigated. At present, the mechanisms by which cytokines and LPS regulate CXCL12 expression remain unknown. Internalization of chemokine receptors is enhanced following binding to their ligands and is the most likely cause of chemokine receptor downregulation. Recent studies suggest that internalization of CXCR4 occurs primarily through the clathrin-mediated pathway and possibly via lipid rafts and caveolae (Neel et al. 2005). The internalized CXCR4 is directed to the endosomal compartment from where it is subsequently recycled to the surface. Internalization of CXCR4 following CXCL12 ligation has been reported in rat basophilic leukemia cells (Haribabu et al. 1997), HELA cells (Tarasova et al. 1998; Venkatesan et al. 2003) and T cells (Signoret et al. 1997; Forster et al. 1998) with different rates of internalization and recycling. In the present study, the addition of CXCL12 at 10, 1 and 0.5 $\mu\text{g/ml}$ to HBMEC cultures resulted in receptor internalization as indicated by a 59% and 47% decrease in CXCR4 surface staining respectively after 15 and 30 min with return to near normal baseline levels after 60 min, which suggests recycling to the cell surface. Since The

pathways of internalization and recycling of CXCR4 in cerebral EC have not been investigated. Since EC of the BBB have clathrin-coated pits but lack vesicular transport, it is possible that CXCR4 internalization is predominately clathrin-mediated at the BBB. The effect of cytokines and LPS on CXCR4 receptor expression by cerebral EC has not been previously addressed. Downregulation of CXCR4 mRNA has been reported in mouse astrocytes treated with TNF- α (Han et al. 2001) and in HUVEC treated with TNF- α (Feil and Augustin 1998) or IFN- γ , TNF- α , IL-1 β and LPS (Gupta et al. 1998). Of the cytokines used in the present study, IFN- γ in combination with TNF- α induced the most pronounced downregulation of CXCR4 followed by IFN- γ alone, TNF- α , LPS and IL-1 β . The levels of CXCR4 surface expression remained low for 48 h in the presence of cytokines and LPS. At present, the mechanisms of cytokine or LPS- induced downregulation of CXCR4 expression are not known.

4.5 Diffusion and Binding of CCL2, CCL3 and CXCL12 to HBMEC Monolayers

It is now well established that binding of chemokines to glycosaminoglycans (GAGs) on the EC surface and subendothelial matrix is required for efficient leukocyte integrin activation and directional migration of leukocytes by enhancing binding to high affinity G protein coupled receptors and protecting chemokines from proteolytic cleavage.

The distribution of chemokines at the apical/luminal and basal/abluminal surfaces is of physiological significance since chemokines bound to the luminal endothelial surface can trigger integrin-dependent adhesion, while abluminally bound chemokines direct arresting leukocytes to transmigrate along chemotactic gradients (Man et al. 2007). Treatment of HBMEC monolayers with TNF- α and IFN- γ decreased the TEER of the cultures and significantly enhanced the diffusion of CCL2, CCL3 and CXCL12 across the monolayers.

Since cytokines increase the permeability of HBMEC monolayers by increasing the permeability of interendothelial tight junctions (Huynh and Dorovini-Zis 1993a; Wong et al. 2004), the rapid chemokine diffusion across the monolayers most likely reflects paracellular diffusion rather than active transcellular transport.

Although the total number of CXCL12 molecules bound per mm length of both apical and basal cell surfaces was not different between resting and cytokine-activated HBMEC, a much greater number of gold particles were distributed along the basal/basolateral surface and the subendothelial matrix versus the apical cell surface of resting HBMEC. This polarized binding was no longer present after TNF- α treatment resulting in the redistribution of CXCL12 towards the apical surface and the presence of similar numbers of gold particles along the apical and basal cell surfaces. Increased diffusion of CXCL12 present on the “tissue side” of the BBB across activated HBMEC monolayers and cytokine-induced changes in the distribution of GAGs may contribute to the redistribution of CXCL12 under inflammatory conditions (Klein et al. 1992). These findings are consistent with recent observations in EAE and MS showing that the basolateral expression of CXCL12 on EC of the normal CNS is altered in EAE and active MS lesions resulting in the redistribution of CXCL12 toward the luminal side of the endothelium. This altered pattern of expression correlated with increased inflammatory cell infiltration and demyelination suggesting that the normal basolateral expression functions to retain infiltrating leukocytes within the perivascular space, whereas change in polarity facilitates trafficking and inflammatory cell infiltration (McCandless et al. 2006; McCandless et al. 2008). In contrast to the non-polarized distribution of bound CXCL12 on cytokine-treated HBMEC, the distribution of the β -chemokines CCL4 and CCL5 on the surface of cytokine activated HBMEC in the presence of chemokine gradients is

predominately basolateral (Quandt and Dorovini-Zis 2004). In this study, the binding patterns of the two other β -chemokines, CCL2 and CCL3, are also predominately basolateral in resting and cytokine-treated HBMEC. A similar distribution along the luminal surface of dermal microvascular EC has been reported for IL-8 (Hub and Rot 1998). These differences in the distribution and presentation among different members of the chemokine family may be of functional significance with mostly apically-bound chemokines involved in integrin activation and firm adhesion, and chemokines distributed along the basal surface and subendothelial matrix establishing the haptotactic gradients necessary for transendothelial migration. In this respect, a recent study showed that, under shear stress, CXCL12 applied to the apical surface of HUVEC cultures at low concentrations that do not promote T cell migration, induces transendothelial migration of T cells in response to CCL5 added to the subendothelial compartment (Schreiber et al. 2007). However, it is important to note that the binding of chemokines to EC derived from different organs can differ greatly (Crola Da Silva et al. 2008).

4.6 Functional Role of CCL2, CCL3 and CXCL12 in Mediating Adhesion and Migration of Monocytes and T Cell Subsets across Human Brain Microvessel Endothelial Cells.

4.6.1 *Adhesion and Migration of CD4⁺ and CD8⁺ T Cell Subsets across HBMEC Monolayers in Response to CXCL12*

Accumulating evidence indicates that CXCL12 is a potent chemoattractant for lymphocytes, monocytes, CD34⁺ hematopoietic progenitor cells and cutaneous dendritic cells (Bleul et al. 1996; Ding et al. 2000; Netelenbos et al. 2002; Kabashima et al. 2007). Furthermore, immobilization of CXCL12 on cytokine-treated human bone marrow EC induced

redistribution of CXCR4 to the leading edge of migrating hematopoietic progenitor cells and co-localization with lipid rafts at sites of contact with the endothelium (van Buul et al. 2003).

In vitro studies have shown that CXCL12 induces migration of both naïve and memory T cells across HUVEC cultures (Ding et al. 2000). Interestingly, CXCL12 appears to have a concentration-dependent bi-directional effect on T cell migration, with maximal chemotactic activity at 100ng/ml, whereas higher concentrations up to 1µg/ml cause the movement of T cells away from CXCL12 (Poznansky et al. 2000).

In the present study, the adhesion of resting CD4⁺ and CD8⁺ T cells to unstimulated HBMEC was minimal, consistent with the very low numbers of non-activated T cells adhering to the BBB endothelium under normal physiological conditions and the lack of or low constitutive expression of adhesion molecules by unstimulated cerebral EC (Wong and Dorovini-Zis 1992; Wong and Dorovini-Zis 1995; Wong and Dorovini-Zis 1996a; Wong and Dorovini-Zis 1996b; Easton and Dorovini-Zis 2001). Following treatment with TNF- α and IFN- γ for 24h, adhesion of CD4⁺ and CD8⁺ T cells increased by 3.5 and 5 fold respectively consistent with maximal upregulation of ICAM-1 and VCAM-1 on HBMEC (Wong et al. 1999). The upregulated adhesion of both T cell subsets to cytokine-activated HBMEC was significantly reduced by blocking antibodies to ICAM-1 and VCAM-1. These observations are consistent with previous studies from this laboratory showing that adhesion of resting T cells to HBMEC is dependent upon the activation status of cerebral EC and is mediated by ICAM-1/LFA-1 and VCAM-1/VLA-4 interactions (Wong et al. 1999). The results of our adhesion studies indicate that slightly more CD4⁺ than CD8⁺ T cells adhere to unstimulated HBMEC, however, CD8⁺ T cells respond to endothelial activation by adhering more avidly to HBMEC monolayers compared to CD4⁺ T cells. Consistent with these findings are previous observations showing

that CD8⁺ T cells bind to IFN- γ activated rat brain EC more efficiently than CD4⁺ T cells (Pryce et al. 1991). The greater adhesive and migratory response of CD8⁺ T cells to activated HBMEC, compared to CD4⁺ T cells, raises the question of additional, presently unknown, receptor-ligand interactions between activated HBMEC and CD8⁺ T cells during T lymphocyte extravasation. Both CD4⁺ and CD8⁺ T lymphocytes enter the CNS and participate in the inflammatory infiltrates of MS lesions. It has been previously shown that migration of resting T cells across TNF- α activated HBMEC monolayers is mediated by ICAM-1 and to a lesser extent by PECAM-1 and E-selectin (Wong et al. 1999). Furthermore, Ding et al. have shown that CXCL12 increased a β 1-integrin activation epitope on T cells and enhanced VLA-4-mediated adhesion (Ding et al. 2001).

Previous studies have shown that CXCL12 directs the transendothelial migration of both naïve and memory T cells, whereas CCL2, CCL3 and CCL5 attract memory T cells only (Carr et al. 1994; Ding et al. 2000). This selective recruitment of memory T cells may be due in part to higher levels of expression or to a higher affinity state of the α 4 integrins expressed on memory T cells as compared to naïve T cells (Ding et al. 2000).

Binding of CXCL12 to HBMEC in the presence of concentration gradients significantly upregulated CD4⁺ and to a lesser extent CD8⁺ T cell adhesion to activated HBMEC suggesting that CD4⁺ T cells are more responsive to CXCL12 than CD8⁺ cells. A similar effect on adhesion was observed when CXCL12 was added to the upper and lower chambers at lower concentrations. In contrast, CXCL12 in the subendothelial compartment had no effect on adhesion to unstimulated HBMEC monolayers. These findings are in keeping with our previous observations showing that increased T cell adhesion to cerebral EC in response

to CCL4 and CCL5 occurs only in the presence of activated EC with no effect on adhesion to unstimulated HBMEC (Quandt and Dorovini-Zis 2004).

4.6.2 Adhesion and Migration of T Cell Subsets across HBMEC Monolayers in Response to CCL2 and CCL3

4.6.2.1 Resting and Activated CD4+ T Cell Subsets

In the present study, the expression of the chemokine receptors CCR1 and 5 which bind to CCL3, and CCR2 which binds to CCL2 was higher in CD4+ T cells after anti-CD3 activation. Neither CCL2 nor CCL3 increased the adhesion of resting CD4+ T cells to unstimulated or cytokine-stimulated HBMEC. However, CCL2 increased the migration of resting CD4+ T cells across unstimulated HBMEC after a 3 hour migration assay. CCL2 augmented the adhesion of activated CD4+ T cells to cytokine-stimulated but not unstimulated HBMEC. This is in agreement with previous work from this laboratory reporting that CCL4 and CCL5 increase the adhesion of activated CD4+ T cells to cytokine-treated, but not resting HBMEC monolayers (Quandt and Dorovini-Zis 2004). Accordingly, CCL2 augmented migration of activated CD4+ T cells across both unstimulated and cytokine-stimulated HBMEC. The observation that CCL2 is a T-lymphocyte chemoattractant is rather surprising, since CCL2, originally described as monocyte chemoattractant protein-1 was previously thought to be specific for monocytes (Carr et al. 1994). Only when the investigators purified the lymphocyte chemoattractant from supernatants of mitogen-stimulated peripheral blood mononuclear cells, they found that it was indeed CCL2. Our findings are in agreement with a previous study showing that T cells that migrate in response to CCL2 express high levels of the T cell differentiation and activation marker CD26 (dipeptidyl peptidase IV), while T cells that migrate in response to CCL3 have a more resting phenotype as they have much lower expression of CD26 (Qin et al. 1996).

CCL3 is an important chemokine in the pathogenesis of neuroinflammation since the Th1 associated chemokines CXCL10, CCL3 and CCL5 were significantly elevated in CSF samples collected from MS patients as compared to control groups (Nakajima et al. 2004a). CCL3 is a more potent T lymphocyte chemoattractant than CCL4 *in vitro* with a biphasic lymphocyte response at concentrations of 100pg/ml and 10ng/ml (Schall et al. 1993). At 100pg/ml, CCL3 strongly attracts B cells and all T cells subsets (CD4+, CD8+, naïve and memory). However, at a higher concentration (10ng/ml), there was a reduction of B cell and CD8+ T cell migration, whereas the migration of CD4+ T cells was further increased. Therefore, the response of T cell subsets to CCL3 is highly concentration dependent. In the present study, although CCL3 decreased the migration of resting CD4+ T cells across unstimulated HBMEC monolayers, it increased their migration of across cytokine-stimulated HBMEC. The observed decrease is probably due to the few numbers (less than 0.1cell/mm) of migrated CD4+ T cells counted. Furthermore, CCL3 did not have any effect on the adhesion and migration of activated CD4+ T cells regardless of the activation status of the endothelium. Our results are consistent with a previous study reporting that CCL4/MIP-1 β and CCL5/RANTES, but not CCL3/MIP-1 α increased the adhesion of anti-CD3 activated CD4+ T cells to HUVEC stimulated with IL-1 α (Taub et al. 1993).

4.6.2.2 *CD45RO-* (naïve) and *CD45RA-* (memory) CD4+ T Cells Subsets

The maturation and differentiation of T cells correlates with the expression of different isoforms of the leukocyte common antigen (CD45). In the absence of chemokines, memory (CD4+ CD45RA-) T cells uniformly adhere and migrate more readily than naïve (CD4+CD45RO-) T cells across resting and cytokine activated HBMEC monolayers. Similar to our findings, it has been previously reported that up to 97% of the migrated CD4+ T cell

population expresses CD45RO (Callahan et al. 2004). Furthermore, the activation marker CD69 is also enriched in the migrated population, suggesting that activated memory T cells, which express CD69, constitutively traffic across the blood-brain barrier.

When the differences in adhesion and migration across resting and cytokine-activated HBMEC are closely examined, the increase in naïve T cell adhesion to activated versus resting HBMEC is more dramatic than the increase in migration. In contrast, both adhesion and migration of memory T cells are augmented by at least 4-fold after cytokine-activation of the endothelial monolayers.

While the presence of CCL2 and CCL3 gradients do not have any effect on naïve CD4⁺ T cell adhesion, the presence of CCL3 in both upper and lower chambers (no gradients) significantly decreases the adhesion of naïve CD4⁺ T cells to both unstimulated and cytokine-stimulated HBMEC. The hyporesponsiveness naïve CD4⁺ T cells to CCL3 has been shown thru the binding of CCR1 on naïve T cells CD4⁺ T cells (Sato et al. 2002). In addition, the presence of a relatively high concentration of CCL3 in the upper chamber might prematurely trigger internalization of CCR5 upon ligand binding, thereby decreasing the ability of naïve CD4⁺ T cell to firmly adhere due to lack of integrin activation (Longden et al. 2008).

In this study, cytokine-stimulation of HBMEC in the absence of chemokines or the presence of chemokine gradient across resting HBMEC failed to augment naïve CD4⁺ T cell migration across the monolayers. Moreover, CCL3 slightly reduces migration of naïve CD4⁺ T cells across resting HBMEC. The low number of transmigrated cells (fewer than 0.1cell/mm) may account for this decrease. However, the migration of naïve CD4⁺ T cells across cytokine-activated HBMEC is augmented in the presence of CCL2 or CCL3. Therefore, the recruitment of naïve CD4⁺ T cells to sites of neuroinflammation appears to require both

cytokine activation and upregulation of adhesion molecules on cerebral EC, as well as the presence of chemokines on the tissue side of the BBB.

The presence of CCL3 in the subendothelial region increases the adhesion of memory CD4⁺ T cells to resting HBMEC. When higher concentrations of CCL3 (100ng/ml) are added to the lower chamber, memory CD4⁺ T cell adhesion to cytokine-treated HBMEC is also upregulated. This is in agreement with the FACS data showing that memory T cells express higher levels of the chemokine receptors CCR1 and CCR5 on their surface as compared to naïve T cells. Interestingly, the presence of CCL2 or CCL3 gradients in the lower chamber do not have any effect on the migration of memory CD4⁺ T cells across resting or cytokine-treated HBMEC. The lack of further increase in memory CD4⁺ T cell migration across resting HBMEC in response to CCL2 or CCL3 is consistent with previous unpublished observation in our laboratory that CCL4 or CCL5 does not increase memory CD4⁺ T cell migration across resting HBMEC.

4.6.2.3 *CD8⁺ T Cell Subsets*

CD8⁺ T cell migration across both resting and cytokine-treated HBMEC is augmented in the presence of CCL3, but not CCL2 in the lower chamber. Although CCL2 does not seem to influence CD8⁺ T cells migration in our study, recent evidence suggests that CCL2 is important for the generation, survival and function of memory CD8⁺ T cell subsets (Wang et al. 2008). In addition, it has been shown that CCL3 is required for cytokine production and cytolytic activity of CD8⁺ T cells in mice (Trifilo et al. 2003). In a murine experimental cerebral malaria study, it was shown that CCL3, CCL5 and CXCL10 are necessary for the migration of pathogenic CD8⁺ T cells to the brain (Belnoue et al. 2008).

4.6.3 Adhesion and Migration of Monocytes across HBMEC Monolayers in Response to CCL2 and CCL3

The adhesion and migration of monocytes across EC derived from different vascular beds have been investigated extensively. Previous studies by William A. Muller's laboratory indicate that the monocytes readily transmigrate across resting HUVEC and this process largely involves the adhesion molecule PECAM-1, which is expressed on both leukocytes and at the interendothelial junctions (Muller and Weigl 1992; Liao et al. 1995). Expression of VCAM-1 on EC has been shown to play an important role in monocyte adhesion to EC (Kalogeris et al. 1999). The basal adhesion of peripheral monocytes to EC is relatively high compared with neutrophils and lymphocytes. Cytokine activation of endothelium typically resulted in 2 to 5 fold increase in monocyte adhesion (Luscinskas et al. 1994). A recent study by Helga E. de Vries' group showed that migration of monocytes across rat brain EC utilizes the paracellular route and that the redistribution of the tight junctional protein, occluding, is MMP dependent (Reijerkerk et al. 2006). CCL3 has been shown to attract monocytes *in vitro* and *in vivo*. The binding of CCL3 to CCR1, but not CCR5 is thought to upregulate the expression of CD11b on monocytes (Lee et al. 2000).

A previous study indicates that the adhesion of monocytes to TNF- α activated human pulmonary artery EC (HPAEC) under flow conditions is dependent on the β 2-integrins LFA-1 and Mac-1 and the β 1-integrin VLA-4 (Maus et al. 2002). Furthermore, they showed that the CCL2-mediated increase of monocyte adhesion to inflamed HPAEC is mostly dependent on β 2, but not β 1-integrin.

Monocyte adhesion to unstimulated EC is not enhanced in the presence of CCL2 or CCL3 gradients. However, both chemokines significantly increase monocyte migration across resting HBMEC monolayers. Previous kinetic studies of cell migration indicate that

monocytes respond significantly to CCL2 in as little as 1 h while T lymphocytes do not respond until 4 h (Carr et al. 1994). A possible mechanism by which chemokines can increase migration of monocytes is by inducing secretion of matrix metalloproteinases. The regulation of monocyte chemotaxis and the signaling pathways involved are of special interest due to their therapeutic implications in atherosclerosis, MS and HIV infection. Chemotaxis and polarization of monocytes in response to CCL2 and CCL3 require interaction with a Ras superfamily protein, Cdc42. Patients with Wiskott-Aldrich Syndrome (WAS) that have a mutation in the WAS protein have impaired responses to CCL2 and CCL3 (Badolato et al. 1998). Furthermore, monocyte chemotaxis is negatively regulated by activated cAMP-dependent protein kinase A (Fine et al. 2001).

In addition to the chemotactic mediators, the endothelium appears to play a determining role in the transmigration of monocytes. Preincubation of rat brain endothelium with heparin, enzymatic removal of heparan sulfate side chains with heparitinase-I or treatment of EC with a sulfate synthesis inhibitor sodium chlorate dramatically reduce monocyte migration (Floris et al. 2003). These treatments most likely reduce monocyte migration by decreasing the presentation of GAG bound chemokines on the apical (luminal) surface of EC.

Previous *in vivo* experimental studies have implicated the involvement of CCL3 in monocyte recruitment in the CNS. Thus macrophage accumulation within the CNS was significantly decreased in CCL3 knockout mice, correlating with reduced demyelination (Trifilo et al. 2003). CCL5 and CXCL12 have been shown to induce MMP-9 (Gelatinase B) secretion by primary human monocytes (Klier et al. 2001), which may facilitate infiltration of tissues by transmigrated monocytes. However, cleaved CCL7 (MCP-3), which is closely related to CCL2, by Gelatinase A (MMP-2) is a potent antagonist of CCR-1, 2 and 3, suggesting that

matrix metalloproteinases are both effectors and regulators of the inflammatory response (McQuibban et al. 2000). In our studies, cytokine treatment of HBMEC results in greater than 10 fold increase in monocyte migration in the absence of chemokine gradients, and neither chemokine further enhances monocyte migration across cytokine-treated HBMEC. Blocking antibodies against α 4-integrin (CD49d) on monocytes significantly reduces monocyte adhesion to resting and cytokine-treated HBMEC. Interestingly, Natalizumab, a humanized anti- α 4-integrin antibody, was recently approved as a treatment for MS (Rose et al. 2009). Blocking antibodies against β 2 and α 4-integrins are effective in reducing the number of migrating monocytes across cytokine-treated HBMEC. This is in agreement with earlier studies reporting that blocking of β 2 and α 4-integrins could significantly reduce transendothelial migration of monocytes across HUVEC *in vitro* (Muller and Weigl 1992; Weber et al. 1996; Weber and Springer 1998). The transendothelial migration of monocytes requires the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMP) and is regulated by tissue inhibitors of MMPs (TIMP) (Kintscher et al. 2001). A recent report emphasized the importance of ICAM-1/CD18 and MMP interactions in monocyte migration across extracerebral EC (Sithu et al. 2007). The authors have shown that the cytoplasmic tail of ICAM-1 regulates monocyte transmigration through membrane-type 1-MMP interactions and that anti- β 2 (CD18) mAb completely abolishes monocyte migration. In the presence of CCL2 gradients, β 2 but not α 4-integrin blocking is effective in decreasing monocyte migration across unstimulated HBMEC. Blocking of monocyte β 2 or α 4-integrins, or endothelial ICAM-1 is similarly effective in reducing the number of transmigrated monocytes across cytokine-treated HBMEC in the presence of CCL2 gradients. Since the alpha chain of the α 4 β 1 (VLA-4) integrin is involved in binding to VCAM-1 on endothelium,

it has been shown that blocking of this molecule decreases the infarct size in transient focal cerebral ischemia in rats by limiting lymphocyte and monocyte infiltration (Becker et al. 2001). It was also found that IFN- β downregulates both basal and IFN- γ -induced expression of VLA-4 on monocytes (Soilu-Hanninen et al. 1995). IFN- β also reduces monocyte infiltration in EAE by decreasing the expression of the adhesion molecules ICAM-1 and VCAM-1 on brain capillaries (Floris et al. 2002). The involvement of ICAM-1 and VCAM-1 in monocyte adhesion is not limited to cerebral endothelium. Anti- β 2 or anti- α 4 integrins significantly reduce monocyte adhesion to human saphenous vein (Crook et al. 2002). Although we did not detect any apparent loss of tight junctions after monocyte migration by light microscopy and TEER studies, it has been reported that monocyte infiltration in HIV-associated dementia is associated with loss of tight junctions (Boven et al. 2000).

4.7 Routes and Efficiency of Leukocyte Transendothelial Migration *in vitro*

Adhesion and migration are sequential steps in the leukocyte adhesion cascade leading to leukocyte extravasation. It is generally accepted that leukocyte rolling and firm adhesion precede the final step of transendothelial migration, even though not all adherent cells migrate. In our studies, among the adherent monocytes, CD4⁺ and CD8⁺ T cells only a small fraction migrate across unstimulated HBMEC monolayers. In the absence of chemokine gradients, the baseline migration of T cells across HUVEC is between 6 to 8%, and, depending on the type of leukocytes and the vascular bed of origin of the endothelium, both para- and trans-cellular pathways may coexist (Dejana 2006). T cell migration studies across an *in vitro* model of the blood-brain barrier performed in our laboratory also indicate that T cells may utilize both pathways to move across confluent HBMEC monolayers (Wong et al. 1999). In an elegant high resolution confocal microscopy study using TNF- α activated

HUVEC, Carman and Springer demonstrated that transendothelial migration of monocytes, neutrophils and lymphocytes can occur through both a paracellular and transcellular route utilizing a “cuplike” transmigratory structure highly enriched in ICAM-1 and VCAM-1 (Carman and Springer 2004).

CHAPTER 5 CONCLUSIONS

5.1 Summary and Significance

Transendothelial passage of leukocytes is an active multistep process orchestrated by interactions between adhesion molecules induced or upregulated on EC by inflammatory mediators and their respective ligands on leukocytes, as well as chemokines and chemokine receptors. During inflammation, endothelial-displayed chemokines bind to specific G protein-coupled receptors on leukocytes and trigger inside-out signaling that increases the avidity of integrins and induces rapid conformational alterations at the leukocyte-EC contact site resulting in firm adhesion to EC. The directional transendothelial migration of leukocytes is further guided by chemokines present in the subendothelial tissue. The differential expression patterns of integrins and their ligands and chemokines and their receptors largely define the subset of recruited leukocytes, thus contributing to the specificity of the immune response. A key event in the initiation and evolution of inflammatory diseases in the CNS is the timely recruitment of circulating peripheral blood leukocyte subsets to sites of antigenic challenge across the BBB that, under normal conditions, restricts their entry into the brain, thus maintaining the CNS in an immunologically privileged state. At present, the molecular mechanisms that regulate the processes of leukocyte adhesion and transmigration across the BBB are not well defined.

The studies reported herein provide new insights into the regulation of CXCL12 and its receptor, CXCR4 expression at the BBB and the role of the endothelial-bound CXCL12 and the β -chemokines CCL2 and CCL3 on the recruitment of T cell subsets and monocytes across HBMEC in an in vitro model of the BBB. Resting HBMEC constitutively express CXCL12 and CXCR4. Treatment with TNF- α , IFN- γ , IL-1 β and LPS downregulate CXCL12 and

CXCR4 expression and CXCL12 ligation induces internalization of CXCR4. The reversible downregulation of the constitutive CXCL12 expression in cytokine or LPS activated HBMEC is intriguing and suggests a complex regulatory process and a possible homeostatic role in the CNS. The cytokine and LPS-induced downregulation of CXCR4 may have important implications in autoimmune and infectious CNS diseases. Since CXCR4 is a coreceptor for the T-cell tropic HIV-1 strain, the protective effect of CXCL12 against HIV infection may be related not only to competition for binding, but also to downregulation of CXCR4 surface expression. Furthermore, internalization of CXCR4 after CXCL12 ligation in HBMEC would result in more chemokine available for binding to inflammatory cells, thus enhancing the immune response. The associated increase of the apical presentation of this chemokine in an inflammatory milieu, together with its presence in the subendothelial compartment, would further contribute to the amplification of CD4⁺ and CD8⁺ T cell recruitment. The minimal adhesion and migration of CD4⁺ and CD8⁺ T lymphocytes across resting HBMEC is increased after cytokine treatment of HBMEC. The presence of CXCL12 gradients upregulates CD4⁺ T cell adhesion to cytokine-treated, but not resting monolayers, and migration across unstimulated, but not cytokine-activated HBMEC. Adhesion of CD8⁺ T cells to resting and activated HBMEC is not affected by CXCL12, whereas migration across both unstimulated and activated HBMEC is significantly enhanced in the presence of CXCL12 gradients. Interestingly, CD8⁺ T cells have a greater migratory response to activated HBMEC monolayers as compared to CD4⁺ T cells.

Figure 44 summarizes some of the key events and molecules involved in the transmigration of monocytes and T cell subsets across the BBB in the presence of CCL2, CCL3 and CXCL12.

Further investigations into the role of the β -chemokine CCL2 in T cell subset recruitment across the BBB showed that, whereas the adhesion of resting CD4⁺ T cells to resting or cytokine-activated HBMEC monolayers is not influenced by CCL2, the adhesion of activated CD4⁺ T cells to TNF- α and IFN- γ treated HBMEC is significantly increased in the presence of CCL2 gradients, but not when CCL2 is present in both upper and lower chambers (no gradients). Further separation of resting CD4⁺ T cells into naïve and memory subsets showed upregulated adhesion of all CD4⁺ T cell subsets to cytokine-activated endothelium with no further increase in adhesion in the presence of CCL2 gradients. The transendothelial migration of all T cell subsets is significantly greater across cytokine-treated versus resting HBMEC. The migration of activated CD4⁺ T cells across resting or cytokine-activated HBMEC monolayers is significantly augmented by CCL2. Naïve CD4⁺ T cells readily migrate across cytokine-activated, but not resting HBMEC monolayers in response to CCL2 gradients. The migration of CD8⁺ T cells across resting or cytokine-treated HBMEC is not affected by the presence of CCL2 in the subendothelial compartment.

The adhesion of resting and activated CD4⁺ T cells to resting or cytokine-treated HBMEC is not affected by CCL3. Interesting results were obtained regarding the adhesion of naïve and memory CD4⁺ T cells to HBMEC in response CCL3. Although the presence of CCL3 gradients in the lower chamber does not affect naïve CD4⁺ T cell adhesion to either untreated or cytokine-treated HBMEC monolayers, low concentrations of CCL3 in both upper and lower chambers (absence of gradients) significantly decrease the adhesion of naïve CD4⁺ T cells to both untreated and cytokine-treated endothelium. On the other hand, the presence of CCL3, in the lower chamber or both upper and lower chambers increases the adhesion of memory CD4⁺ T cells to untreated HBMEC. The migration of resting CD4⁺ T cells across

cytokine-activated HBMEC is noticeably increased in the presence of CCL3 gradients. Furthermore, naïve CD4⁺ T cells readily migrate across cytokine-activated, but not resting HBMEC monolayers in response to CCL3 gradients. The migration of CD8⁺ T cells across resting or cytokine-treated HBMEC is significantly augmented by CCL3. These results strongly suggest that each chemokine plays a distinct role in the adhesion and/or migration of T cell subsets across resting or cytokine-treated monolayers.

In addition to T cell subsets, a series of studies were conducted to investigate the adhesion and migration of peripheral blood monocytes across resting or cytokine-treated HBMEC monolayers and the role of CCL2 and CCL3, as well as endothelial cell adhesion molecules and monocyte integrins in these processes. Our data indicate that monocytes adhere to resting HBMEC to the same extent as resting and memory CD4⁺ T cells, less avidly than activated CD4⁺ T cells and more readily than naïve CD4⁺ T cells. The minimal adhesion of monocytes to unstimulated HBMEC is significantly upregulated after 24 hour treatment of the monolayers with TNF- α and IFN- γ . Kinetic studies showed that monocytes readily adhere to HBMEC monolayers as early as 15 minutes with maximal binding after 60 minutes. The adhesion of monocytes to resting or cytokine-treated HBMEC is not influenced by the presence of CCL2 or CCL3 in the subendothelial region. Adhesion to resting or cytokine-treated HBMEC is effectively reduced by blocking the α_4 -integrin (CD49d) on monocytes, indicating that VLA-4 is important for monocyte adhesion.

Only a small number of adherent monocytes migrate across resting HBMEC monolayers. Cytokine treatment of HBMEC significantly increases transendothelial migration. The movement of monocytes across the monolayers starts at 60 minutes and reaches maximal levels after 3 hours. Both chemokines significantly increase monocyte migration across

resting, but not cytokine-treated monolayers. Monocyte migration across cytokine-treated HBMEC is significantly decreased by blocking the β_2 -integrin (CD18) or α_4 -integrin (CD49d) on monocytes, suggesting that both LFA-1 and VLA-4 are involved in monocyte transendothelial migration. Interestingly, the increased migration of monocytes across resting HBMEC in response to CCL2 gradient is reduced by CD18, but not CD49d blocking antibodies. This is most likely related to the minimal expression of ICAM-1 and lack of VCAM-1 expression by resting HBMEC. Both CD18 and CD49d blocking antibodies effectively inhibit monocyte migration across cytokine-treated HBMEC in the presence of CCL2 gradient, consistent with the upregulated expression of ICAM-1 and VCAM-1 in HBMEC after cytokine activation. These results indicate that CD49d is not utilized during the minimal trafficking of peripheral blood monocytes across the non-inflamed BBB in response to chemokines, whereas it appears to be involved in the transendothelial migration of monocytes across inflamed endothelium. Similar to adhesion, the number of migrated monocytes across resting HBMEC is comparable to resting CD4⁺ T cell. However, monocyte migration across cytokine-activated HBMEC greatly exceeds that of all T cells subsets after 3 hours. Taken together, these studies emphasize important and differential roles for CXCL12, CCL2 and CCL3 in the trafficking of leukocytes across the BBB in CNS in inflammation.

5.2 Future Directions

To further characterize the effect of chemokines on adhesion and migration of T cell subsets and monocytes, a combination of chemokines may be used to determine possible additive or synergistic effects. Addition of chemokines that augment adhesion to the upper chamber and migration to the lower chamber could determine whether the combination of these chemokines can selectively recruit monocytes and different T cell subsets across resting and activated cerebral endothelium. These experiments will provide insight into the fine tuning of leukocyte recruitment across the BBB in physiological and pathological conditions.

Exogenously added chemokines together with leukocytes or chemokine receptors could be fluorescently tagged by different fluorochromes to determine the direct effect of these chemokines in the recruitment of leukocytes across resting or cytokine activated endothelium. Labeling of monocytes and T cells with different fluorescent dyes and comparing the relative adhesion and migration rates of these cells can provide further information on how these cells interact with the endothelium.

In addition to the use of blocking antibodies, future investigations on the role of each endothelial cell adhesion molecule and leukocyte integrins on the adhesion and migration of leukocytes across HBMEC monolayers can utilize small molecule antagonists and/or small interfering RNA to downregulate the expression of adhesion molecule or integrin of interest to minimize the potential cross reactivity or steric hindrance due to the relatively large size of antibodies. In addition to the traditional one hour adhesion and three hour migration assays, recent advances in microscopy and digital imaging enable the use of confocal and live cell microscopy techniques to track the adhesion and migration of each type of leukocytes in real

time. With the combination of powerful computing hardwares and softwares, the kinetics of leukocyte trafficking could be tracked and quantitated at a single cell level.

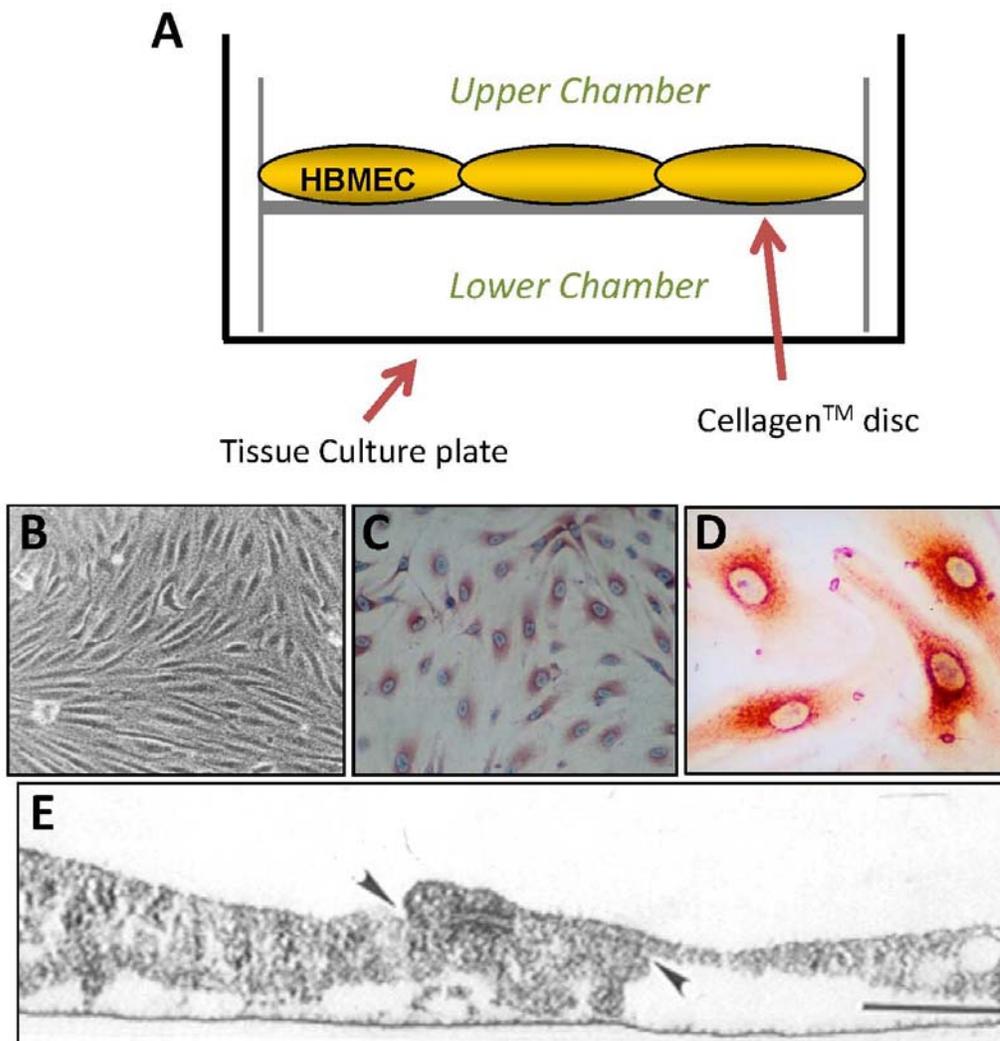


Figure 5 Primary cultures of human brain microvessel endothelial cells (HBMEC) as an in vitro model of the blood-brain barrier (BBB).

(A) HBMEC grown on Cellagen inserts are placed in wells of 4 or 24 well plates to form a double chamber chemotaxis assay system. Confluent monolayers of HBMEC grown on Cellagen inserts (B) or 96 well plates to form contact inhibited monolayers of elongated cells as shown by phase contrast microscopy. (C) The endothelial origin of these cells is demonstrated by the positive, granular cytoplasmic staining for Factor VIII-related antigen, and (D) binding of *Ulex europeaeus* agglutinin by the indirect immunoperoxidase technique. (E) The presence of tight junctions formed along intercellular contacts (arrow heads) between endothelial cells is shown by electron microscopy.

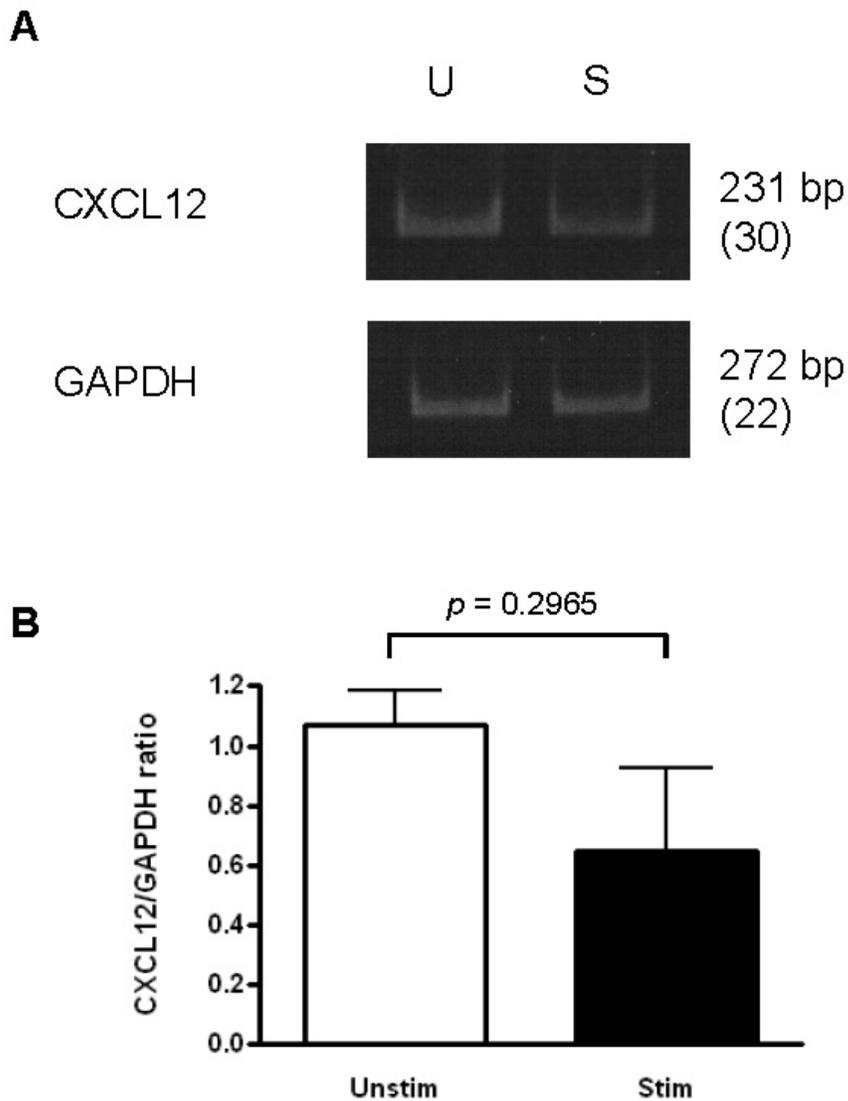


Figure 6 RT-PCR of CXCL12 RNA expression.

(A) RT-PCR analysis of primary HBMEC cultures shows constitutive expression of CXCL12 RNA in unstimulated HBMEC with slight downregulation after TNF- α (100U/ml) and IFN- γ (200U/ml) treatment for 24 h. GAPDH was used as a control to normalize the amount of DNA loaded in each lane. U- Unstimulated; S- Stimulated HBMEC.

Representative data from one of two experiments are shown. Results were obtained using 30 cycles for CXCL12 and 22 cycles for GAPDH. (B) Quantification of CXCL12 RNA levels by densitometry using NIH Image J shows a 42 \pm 20% decrease following cytokine treatment of HBMEC. Data from two experiments are shown.

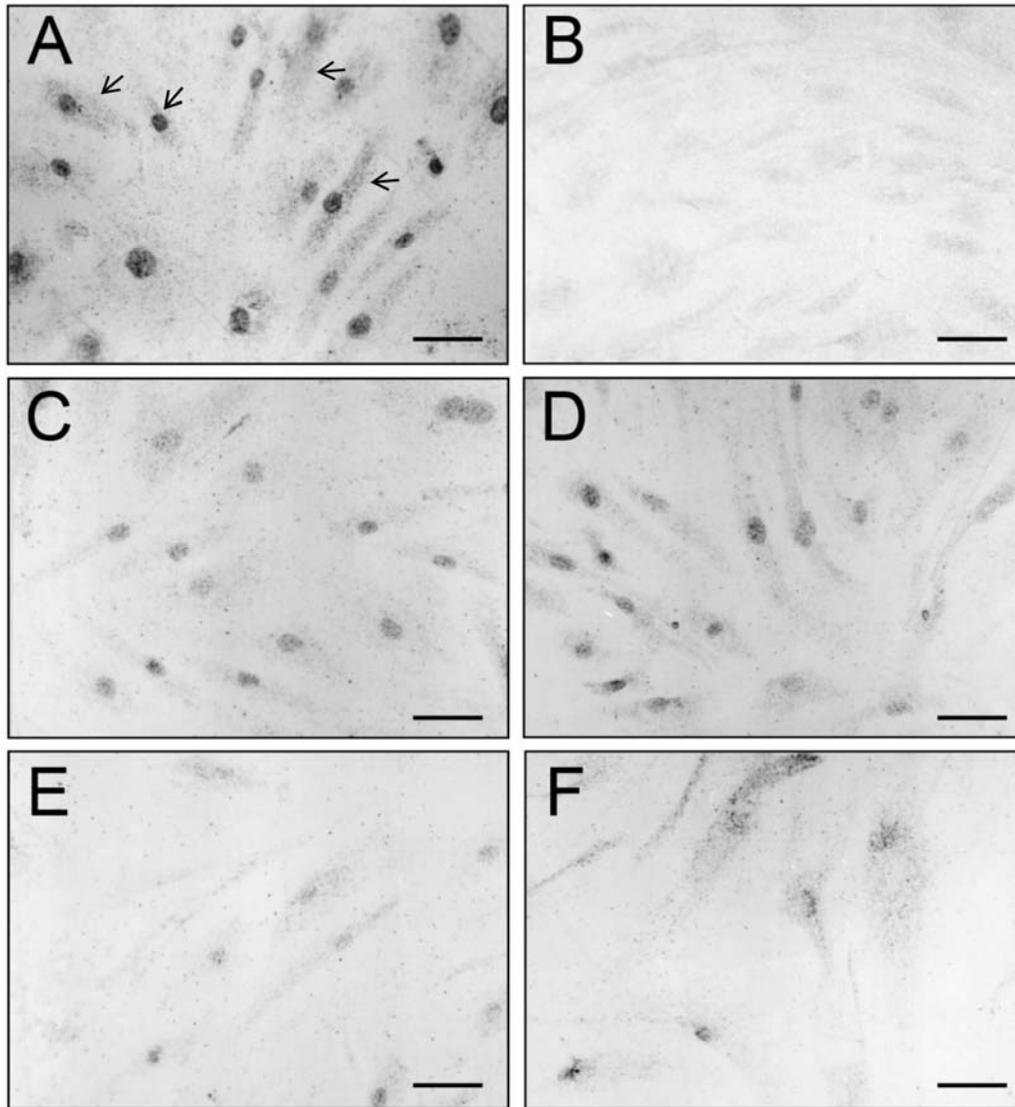


Figure 7 Intracellular localization of CXCL12 in HBMEC by immunogold silver staining.

(A) Untreated HBMEC show positive staining in the form of fine, black, granular cytoplasmic deposits of silver-enhanced gold particles (arrows). (B) Untreated cultures in which the primary Ab was omitted show no cytoplasmic staining. The intensity of staining is significantly and uniformly reduced following treatment with (C) IFN- γ (500U/ml) for 24 h and (E) TNF- α (100U/ml) for 48 h. In monolayers treated with (D) IFN- γ (500U/ml) for 72 h and (F) TNF- α (100U/ml) and IFN- γ (200U/ml) for 72 hrs, scattered EC show positive cytoplasmic staining indicating re-appearance of CXCL12 in the cytoplasm. Bars = 50 μ m.

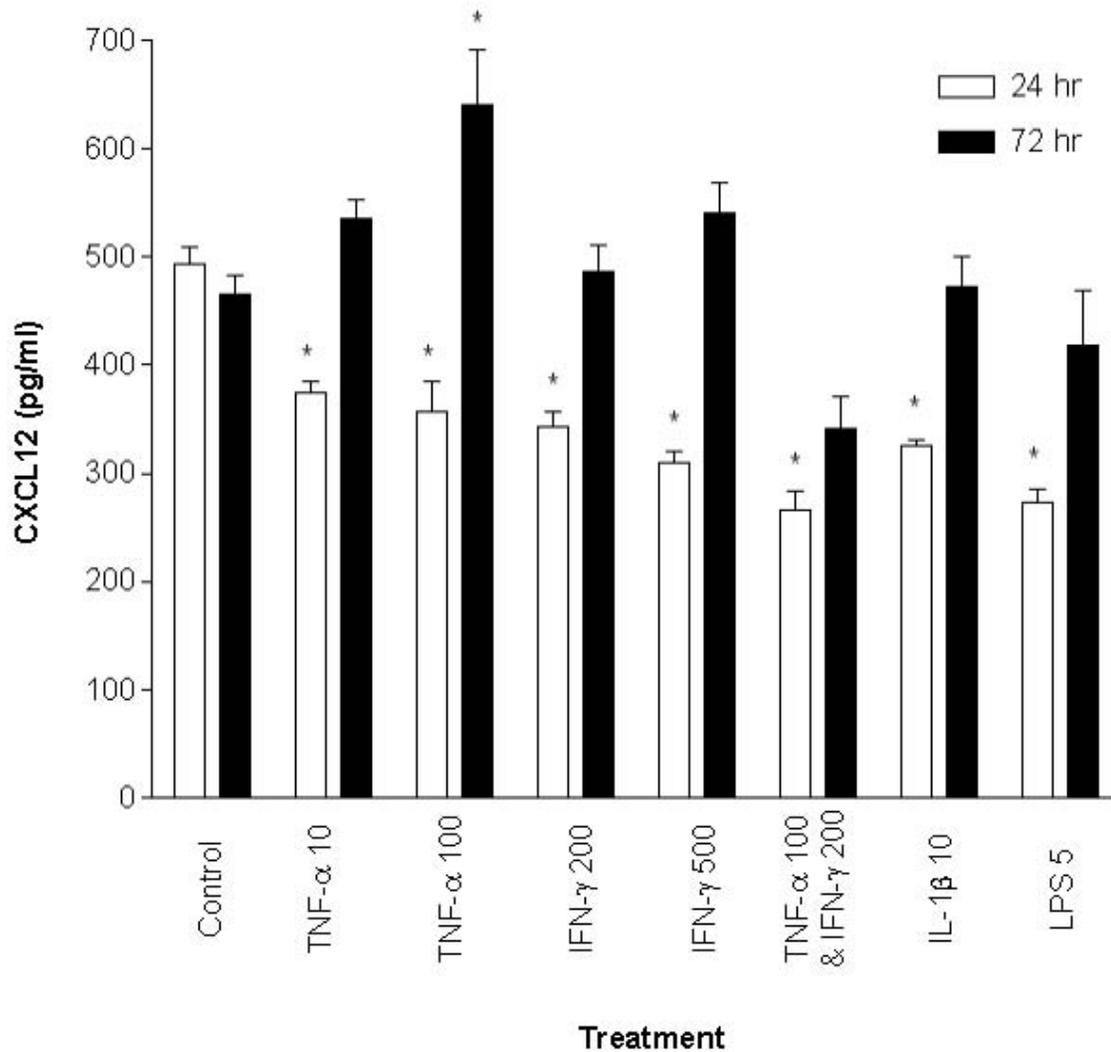
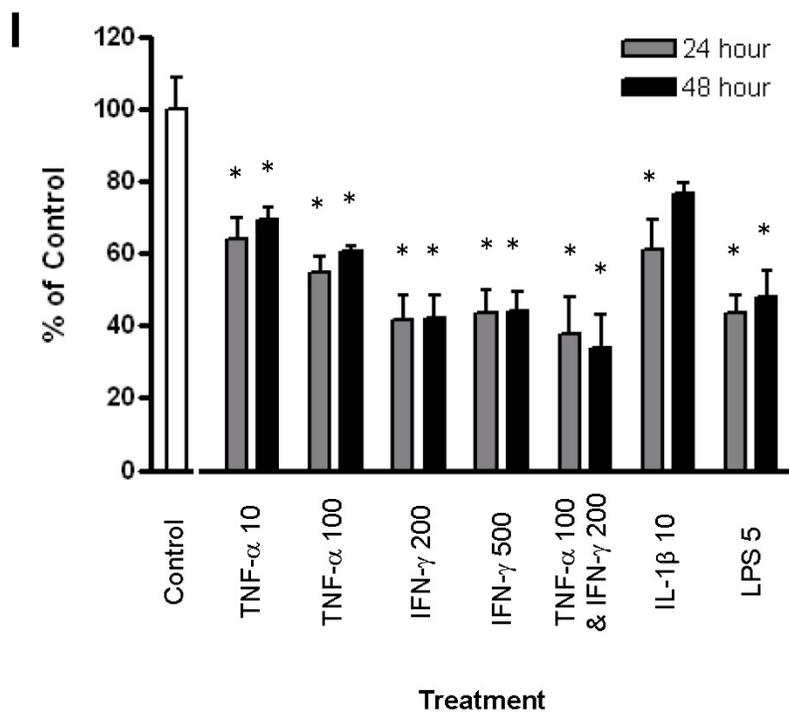
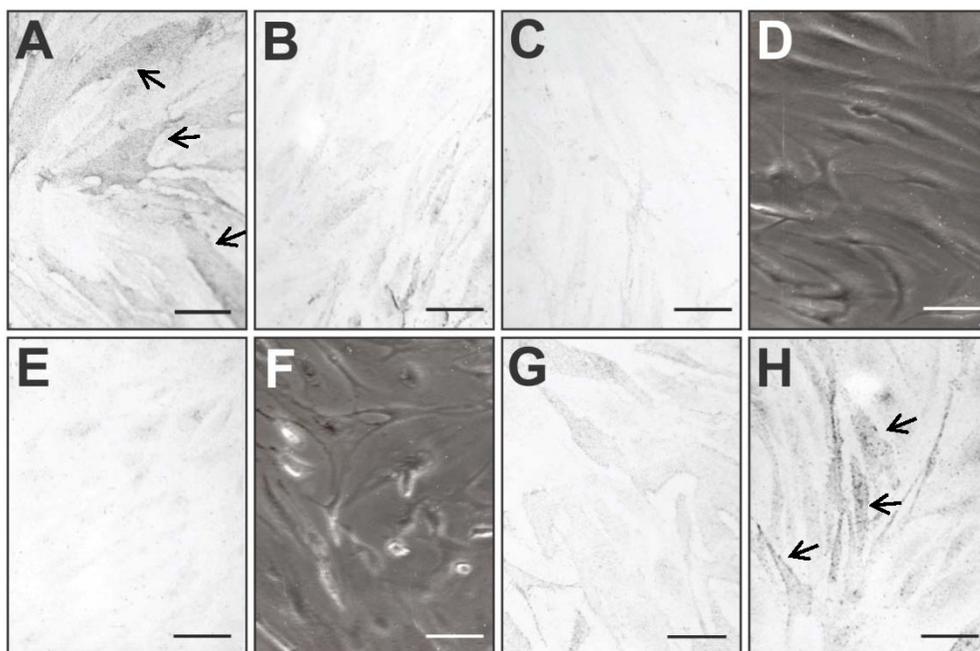


Figure 8 Release of CXCL12 by HBMEC.

Detection and quantification of CXCL12 protein in supernatants of untreated and cytokine or LPS treated HBMEC monolayers by sandwich ELISA at the indicated time intervals. Values represent mean release (pg/ml) \pm SEM of triplicate wells from 1 of 2 representative experiments. * $p < 0.05$ compared to control cultures in the absence of cytokines or LPS.

Figure 9 Regulation of CXCR4 surface expression in HBMEC by cytokines and LPS.

(A-H) Surface expression of CXCR4 in primary HBMEC cultures detected by the immunogold silver staining technique. (A) In resting HBMEC cultures, fine, granular, black deposits of silver-enhanced gold particles distributed on the cell surface indicate the presence of CXCR4 (arrows). There is some variation in the intensity of staining between individual EC. Treatment with TNF- α (100U/ml) for 24 h (B), IFN- γ (500U/ml) for 24 h (C) and combination of TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 h (E) significantly downregulates CXCR4 surface expression as demonstrated by the decrease in the intensity of staining and the number of cells expressing CXCR4. (D, F) Phase contrast images of monolayers treated with IFN- γ (D) and combination of TNF- α and IFN- γ . Incubation with IL-1 β (10U/ml) (G) or LPS (5 μ g/ml) (H) for 24 h has less pronounced effect on CXCR4 expression. Bars = 50 μ m. (I) Quantification of CXCR4 expression on unstimulated HBMEC cultures following 24 to 48 h stimulation with cytokines or LPS by surface ELISA. Values represent mean absorbance \pm SEM of triplicate wells from 2 experiments. * p < 0.05 compared to control cultures in the absence of cytokines or LPS.



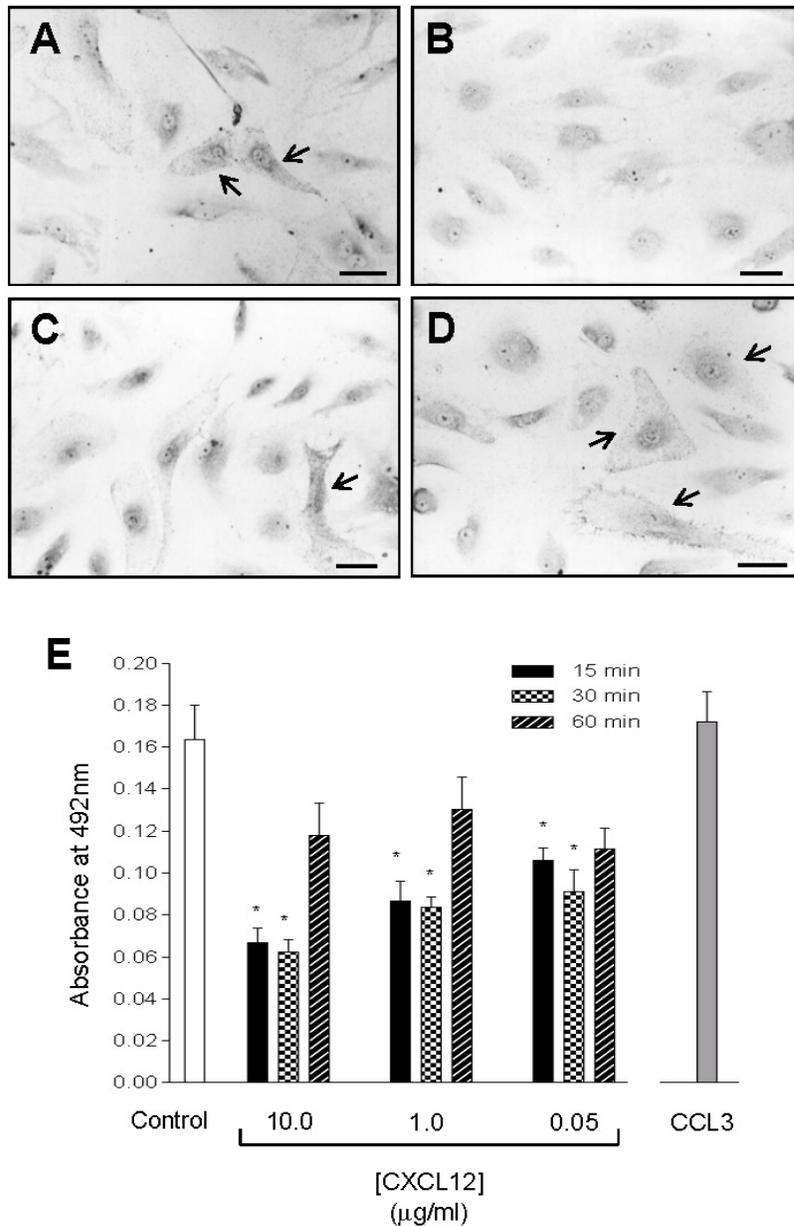


Figure 10 CXCL12 –CXCR4 binding down-regulates CXCR4 on HBMEC.

(A-D) Downregulation of surface expression of CXCR4 on HBMEC following CXCL12 ligation detected by immunogold silver staining. The positive granular surface staining of untreated HBMEC (A) is obliterated within 15 min from the addition of CXCL12 (10μg/ml) (B). At lower CXCL12 concentrations (1μg/ml), surface expression is moderately decreased after 15 min (C). After incubation with 1μg/ml CXCL12 for 1 h, CXCR4 expression returns to near normal levels (D). Bars = 30μm. (E) Quantification of CXCR4 surface expression in untreated HBMEC and following CXCL12 ligation by surface ELISA. Values represent mean absorbance ± SEM of triplicate wells. Representative data from one of two experiments are shown. **p* < 0.05 compared to control untreated cultures.

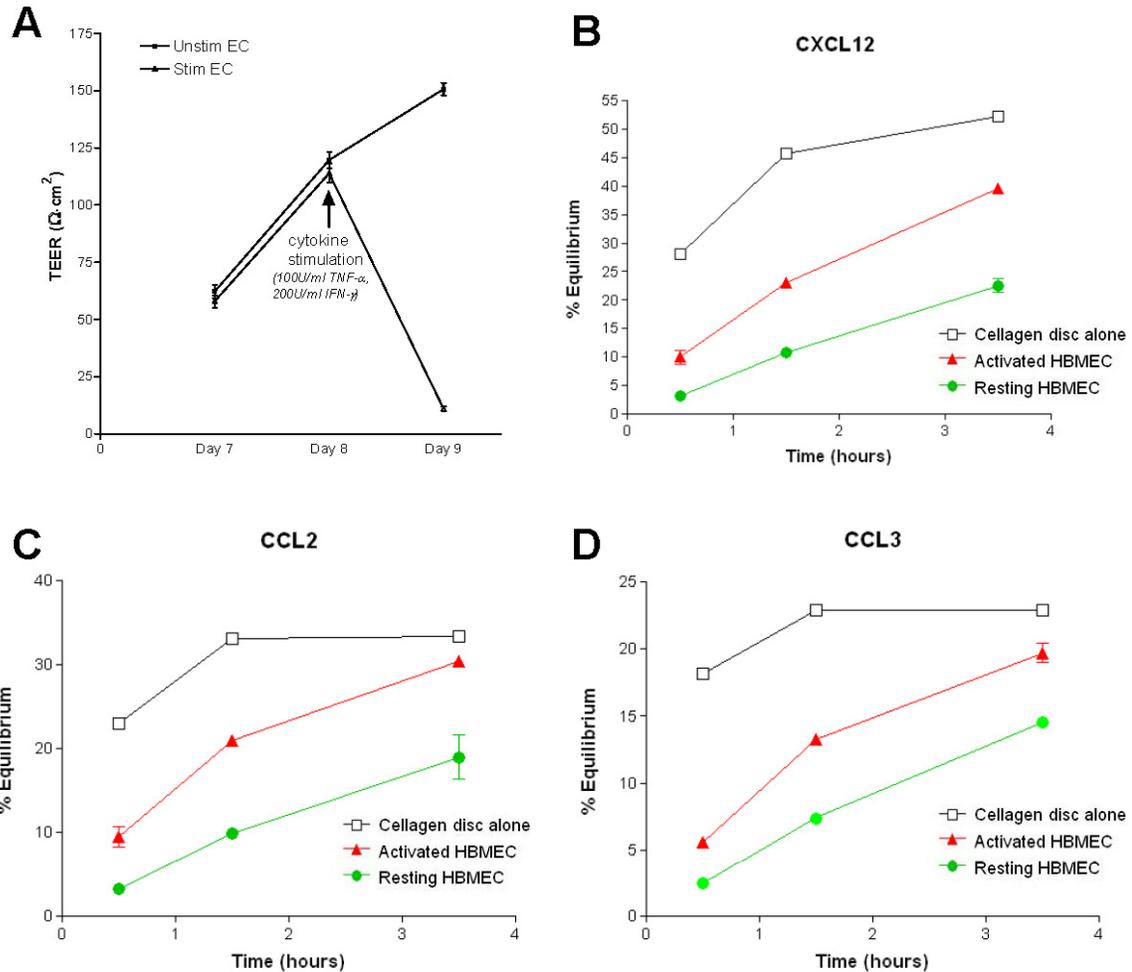


Figure 11 Assessment of HBMEC permeability by TEER and diffusion kinetics of CXCL12, CCL2 and CCL3 across HBMEC monolayers.

Confluent HBMEC monolayers grown on Cellagen® discs were used when the transendothelial electrical resistance (TEER) reached over $100 \Omega \cdot \text{cm}^2$ by Day 8 after plating. Stimulation of HBMEC with 100U/ml TNF- α and 200U/ml IFN- γ for 24 hours resulted in drastic decrease of TEER to less than $25 \Omega \cdot \text{cm}^2$ (A). Radiolabelled CXCL12 (B), CCL2 (C) and CCL3 (D) readily diffuse across the Cellagen® membrane from the lower to the upper chamber in the absence of endothelial monolayers. The diffusion of labeled chemokines across unstimulated HBMEC is slow. The rate of chemokine diffusion across HBMEC was increased after 24 hour cytokine stimulation with 100U/ml TNF- α and 200U/ml IFN- γ and corresponded with the decrease of TEER across the monolayers. Representative data from one of two experiments are shown.

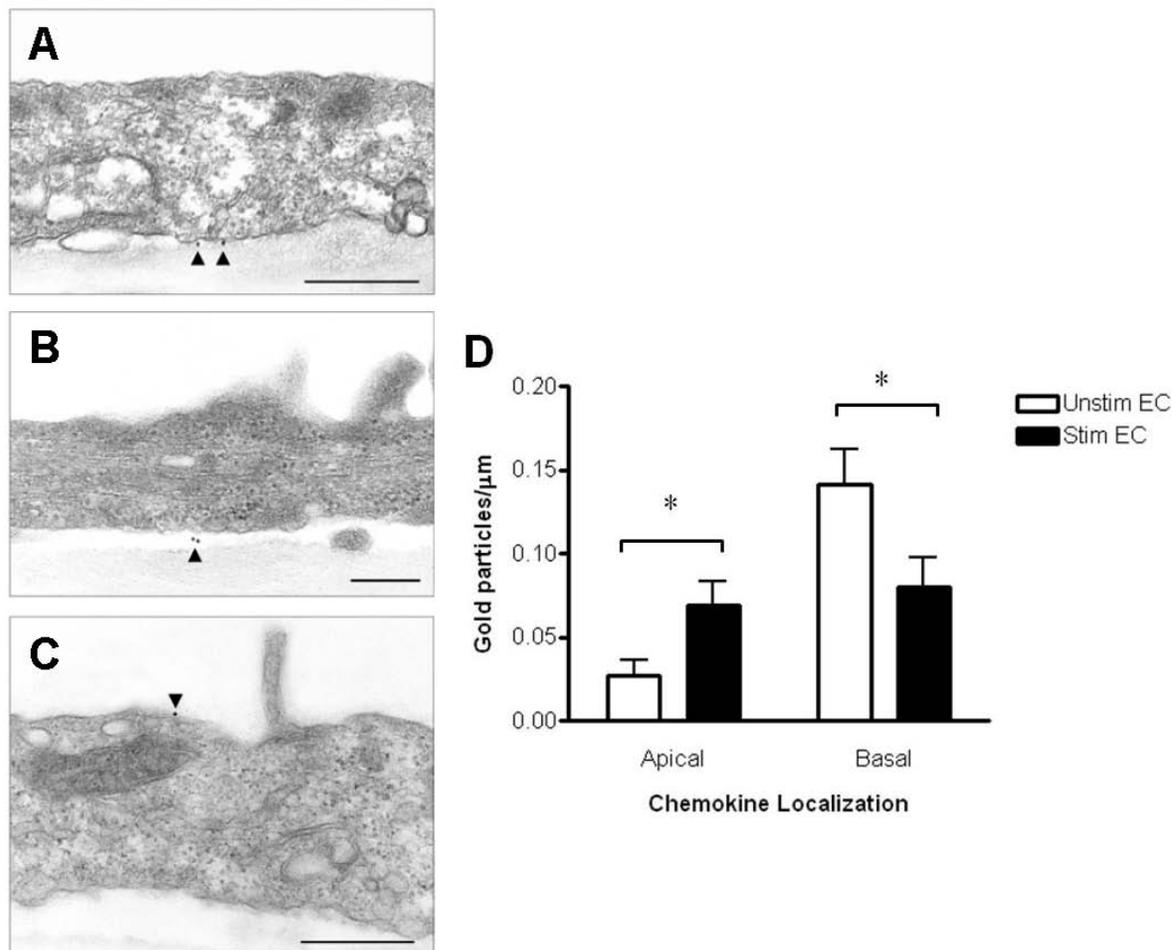


Figure 12 Immunoelectron microscopic localization of CXCL12.

Localization of CXCL12 on HBMEC 1 h after establishment of chemokine gradients by immunogold electron microscopy. In resting monolayers, gold particles indicating the presence of CXCL12 are distributed preferentially along the basal cell surface (A, arrow heads) and the subendothelial matrix (B, arrow head). (C) In cultures treated with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 h, the basal binding decreased and the apical binding increased (arrow head). Bars = 250nm. (D) Quantification of the apical and basal binding of CXCL12 in resting (open bars) and cytokine treated (solid bars) HBMEC in the presence of 100ng/ml CXCL12 in the lower chamber. * $p < 0.05$ compared to resting HBMEC.

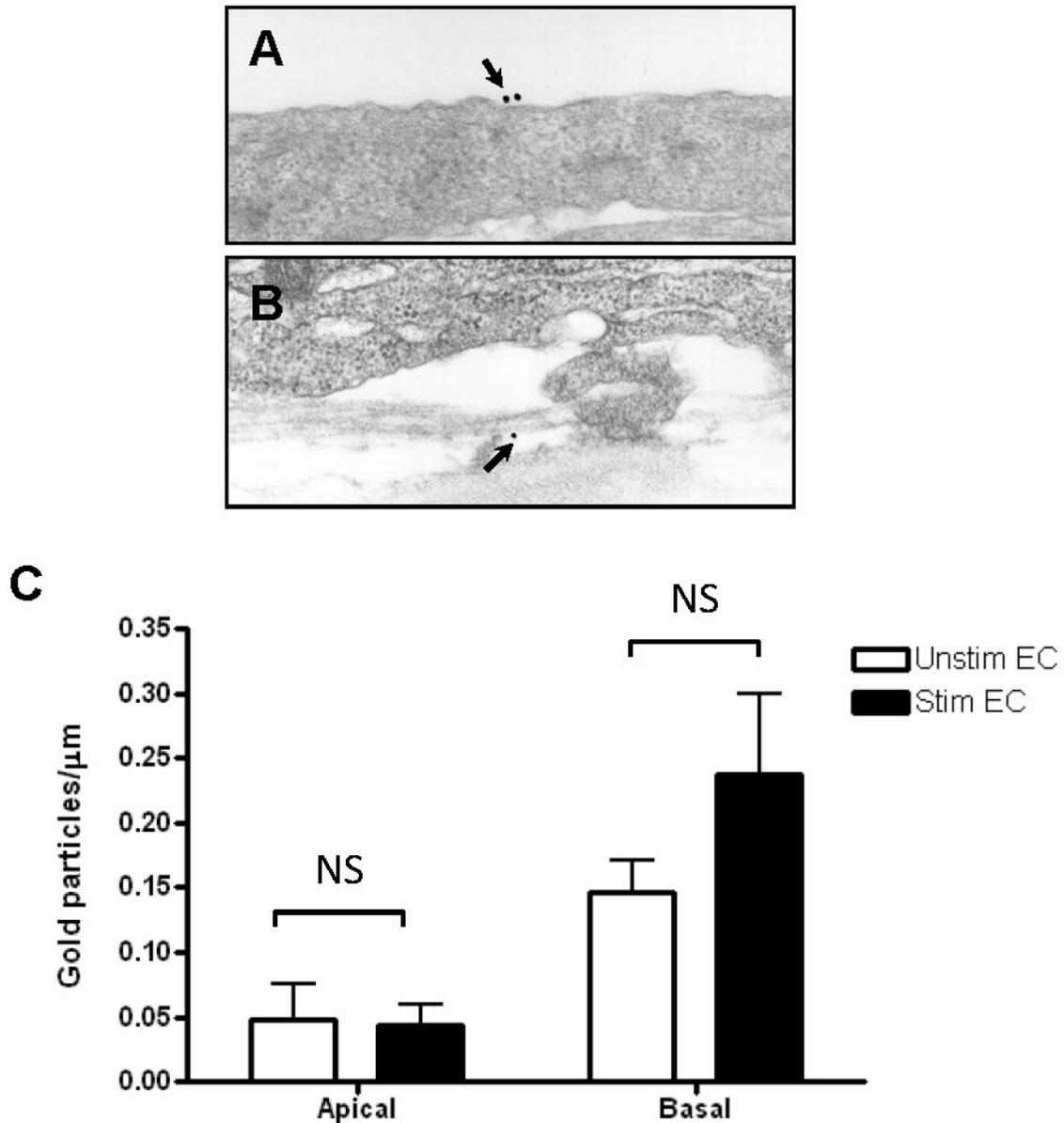


Figure 13 Immunoelectron microscopy localization of CCL2.

CCL2 (arrows) binds to the apical (A) and basal surface (B) of HBMEC as well as the subendothelial matrix. (C) Quantification of the apical and basal binding of CCL2 in unstimulated (open bars) and stimulated (solid bars) HBMEC after the addition of 500ng/ml CCL2 in the lower chamber. The number of gold particles bound to the apical and basal surface of either resting or stimulated HBMEC is similar. However, there is a slight but not statistically significant increase in basal binding of CCL2 after TNF- α and IFN- γ stimulation. No apparent change in apical binding was observed.

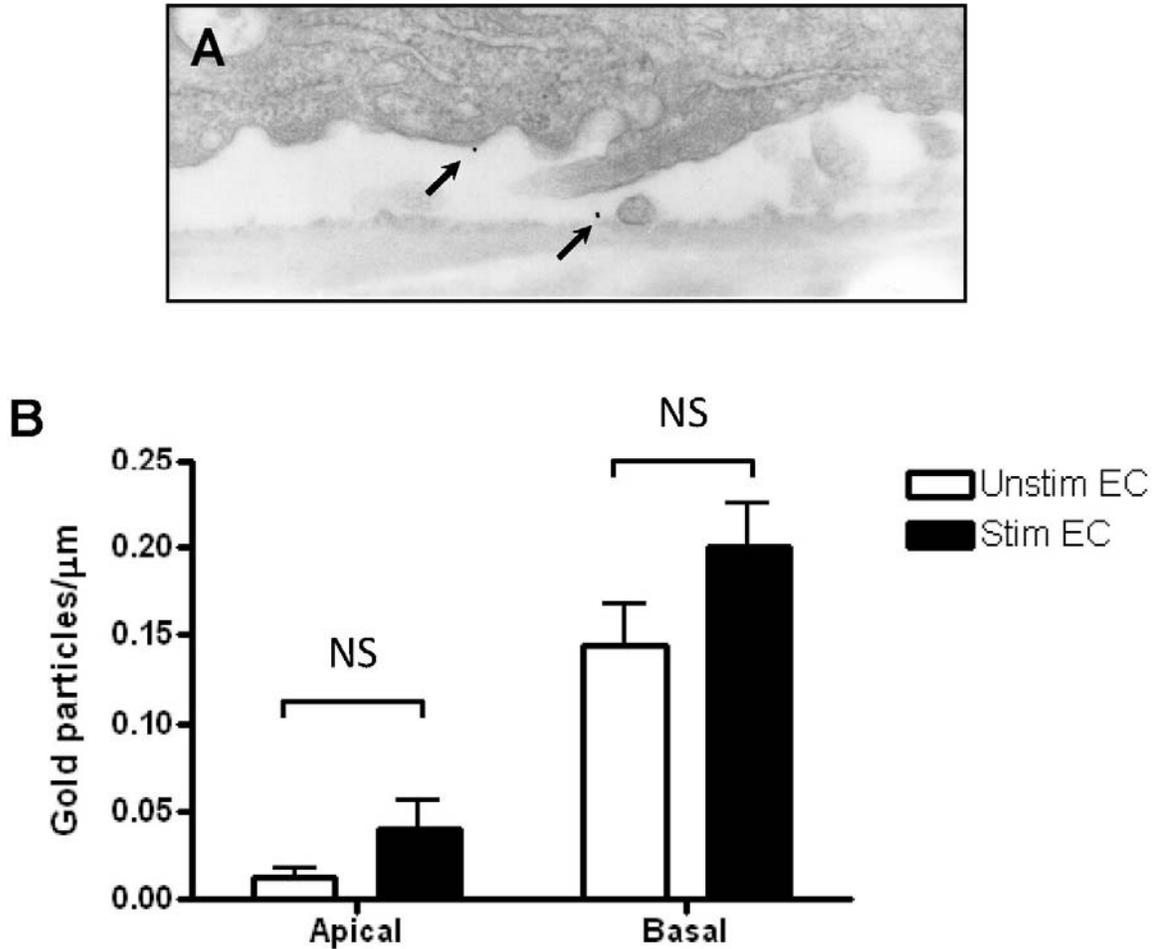


Figure 14 Immunoelectron microscopic localization of CCL3.

(A) CCL3 (arrows) binds to the basal surface of HBMEC and the subendothelial matrix.

(B) Quantification of the apical and basal binding of CCL3 in unstimulated (open bars) and stimulated (solid bars) HBMEC after the addition of 100ng/ml CCL3 in the lower chamber. CCL3 is localized at the basolateral side with very little apical binding in either resting or cytokine-treated HBMEC. However, there is a slight, but not statistically significant increase in apical and basal binding of CCL3 after TNF- α and IFN- γ stimulation.

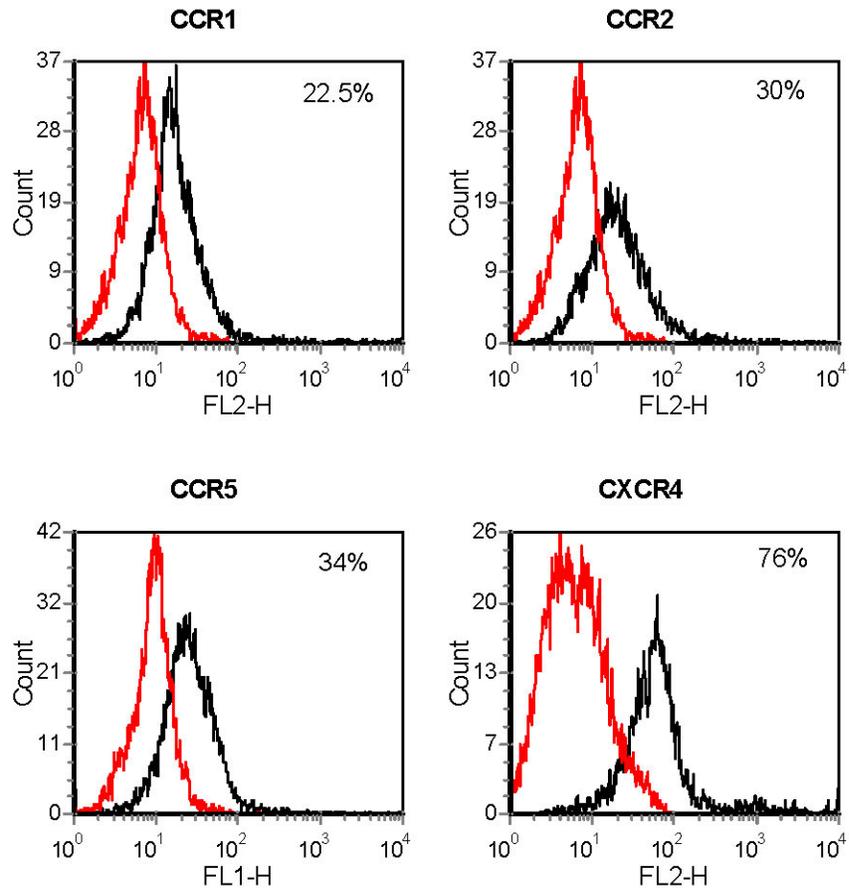


Figure 15 Expression of chemokine receptors by monocytes.

Chemokine receptor expression on freshly isolated peripheral blood monocytes as determined by FACS analysis. The black lines indicate the chemokine receptor tested, and the red lines represent isotype controls. The percentage of cells expressing each chemokine receptor is shown at the top right hand corner.

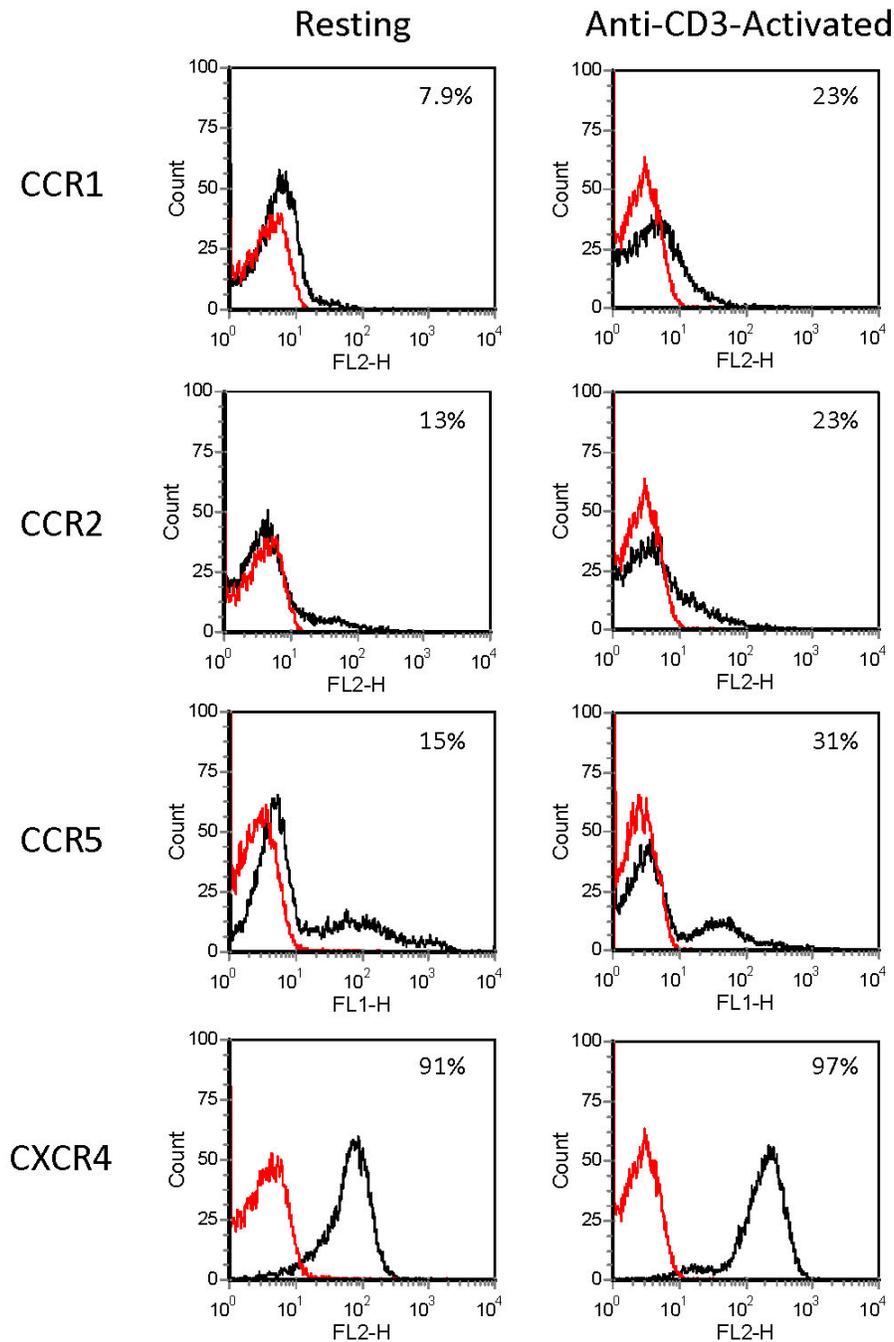


Figure 16 Expression of chemokine receptors by resting and activated CD4+ T cells. Chemokine receptor expression on resting or anti-CD3 activated CD4+ T cells as determined by FACS analysis. The black lines indicate the chemokine receptor tested, and the red lines represent isotype controls. The percentage of cells expressing each chemokine receptor is shown at the top right hand corner. All 4 chemokine receptors are up-regulated in activated CD4+ T cells. CXCR4 is constitutively expressed at high levels in both resting and activated CD4+ T cells.

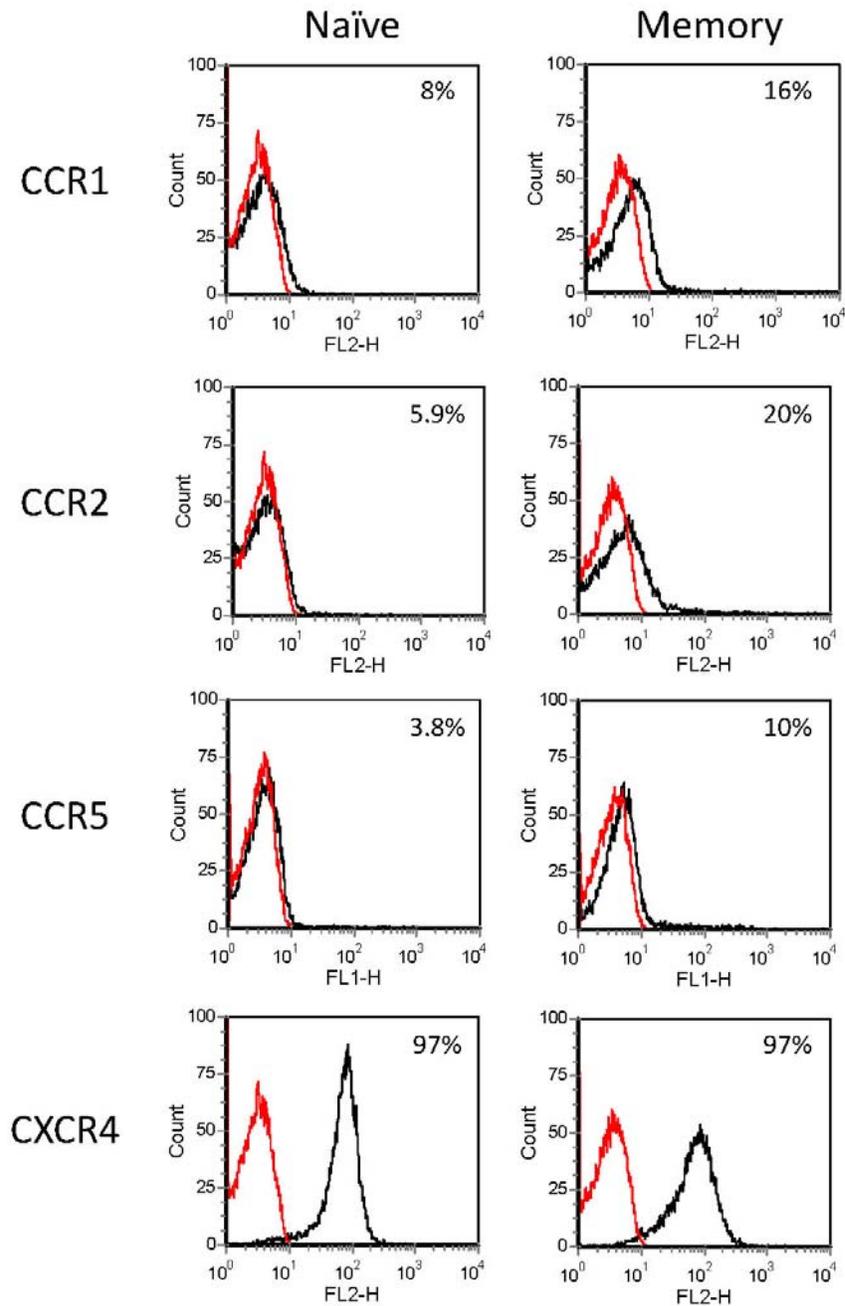


Figure 17 Expression of chemokine receptors by naïve and memory CD4⁺ T cells. Chemokine receptor expression on CD45RO⁻ (naïve) and CD45RA⁻ (memory) CD4⁺ T cells as determined by FACS analysis. The black lines indicate the chemokine receptor tested, and the red lines represent isotype controls. The percentage of cells expressing each chemokine receptor is shown at the top right hand corner. Naïve CD4⁺ T cells minimally express CCR1, 2 and 5. All 3 CCRs are up-regulated in memory CD4⁺ T cells. CXCR4 is constitutively expressed at high levels in both naïve and memory CD4⁺ T cells.

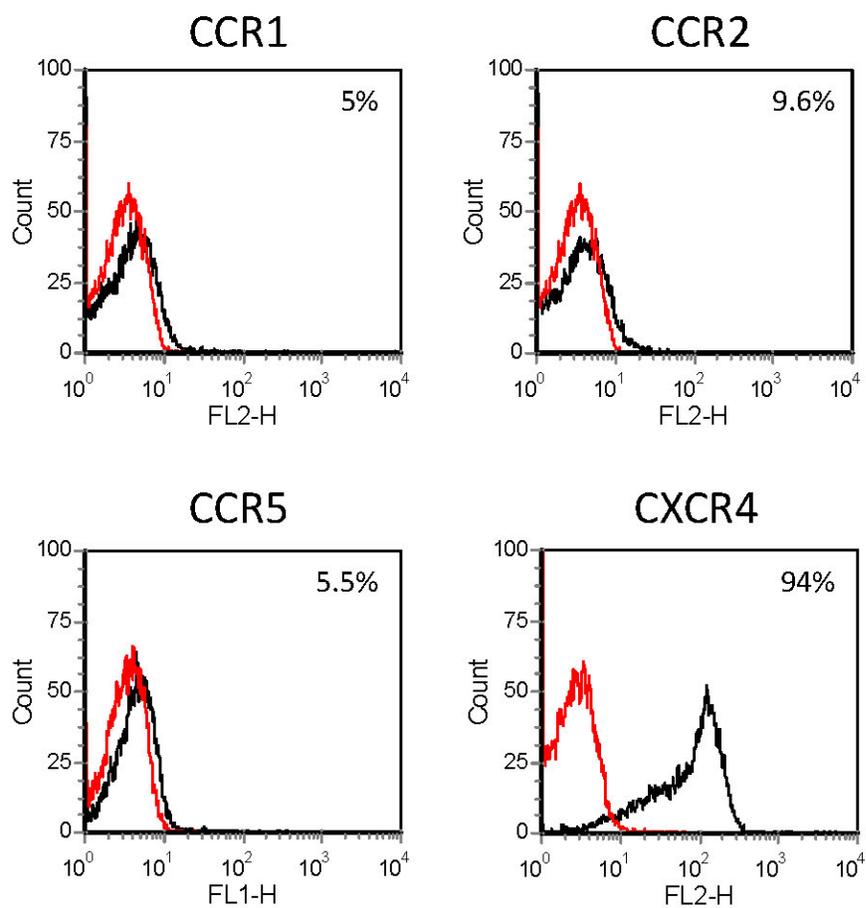


Figure 18 Expression of chemokine receptors by CD8+ T cells.

Chemokine receptor expression on peripheral blood CD8+ T cells as determined by FACS analysis. The black lines indicate the chemokine receptor tested, and the red lines represent isotype controls. The percentage of cells expressing each chemokine receptor is shown at the top right hand corner.

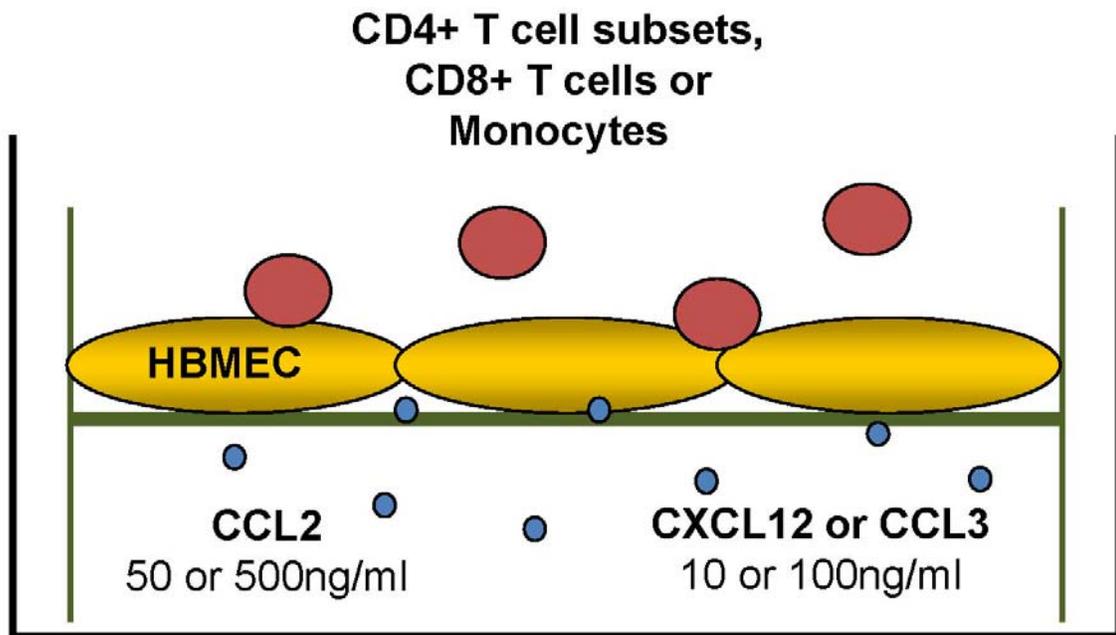


Figure 19 Double chamber chemotaxis system used in adhesion and transendothelial migration assays.

HBMEC are grown to confluence on collagen membranes attached to plastic supports (upper chamber), which are placed inside wells of 24 well plates (lower chamber) to form a double chamber chemotaxis system. Chemokines are added to the lower chamber to obtain concentration gradients.

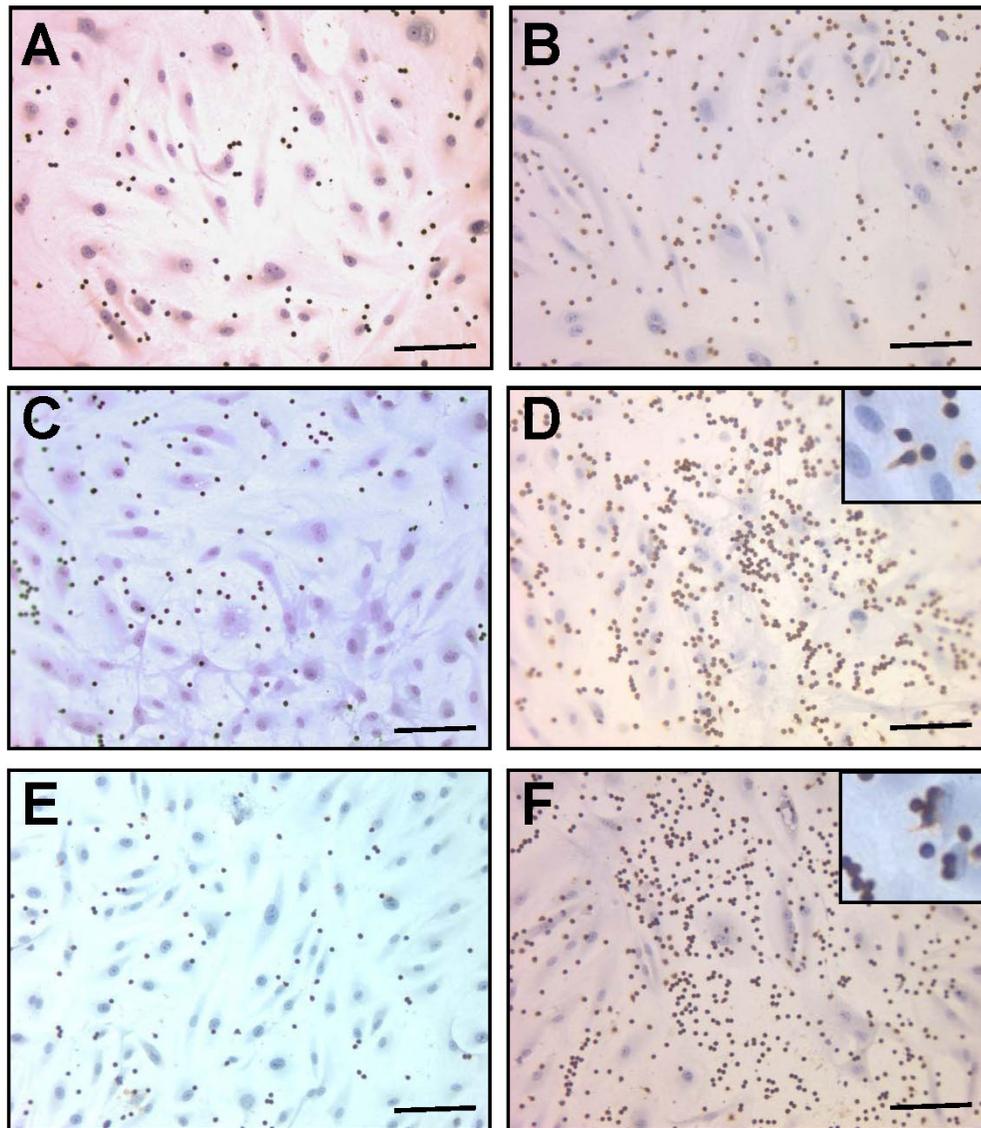


Figure 20 Adhesion of CD4⁺ T cells to HBMEC in response to CXCL12.

(A) A few T cells (small round cells immunoreactive for CD45) adhere to resting HBMEC. (B) Treatment with TNF- α and IFN- γ significantly increases the number of adherent T cells. CXCL12 present in the subendothelial compartment has no effect on adhesion to resting HBMEC (C), but significantly upregulates adhesion to cytokine treated HBMEC (D). Some of the adherent CD4⁺ T cells display a prominent cytoplasmic process (insert) resembling a uropod in the presence of CXCL12. Adhesion to resting HBMEC is not altered in the presence of CXCL12 in the upper and lower chambers (E), whereas adhesion to activated HBMEC is significantly augmented (F). Prominent clustering of adherent T cells often occurs (insert) in the presence of CXCL12 in both chambers. Bars = 100 μ m.

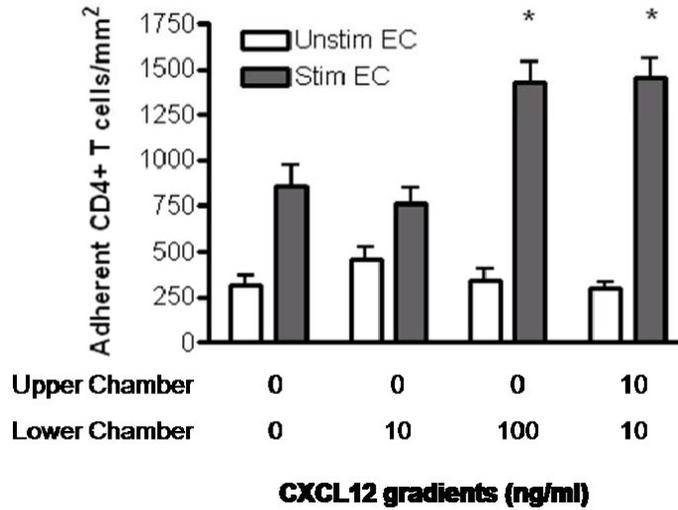
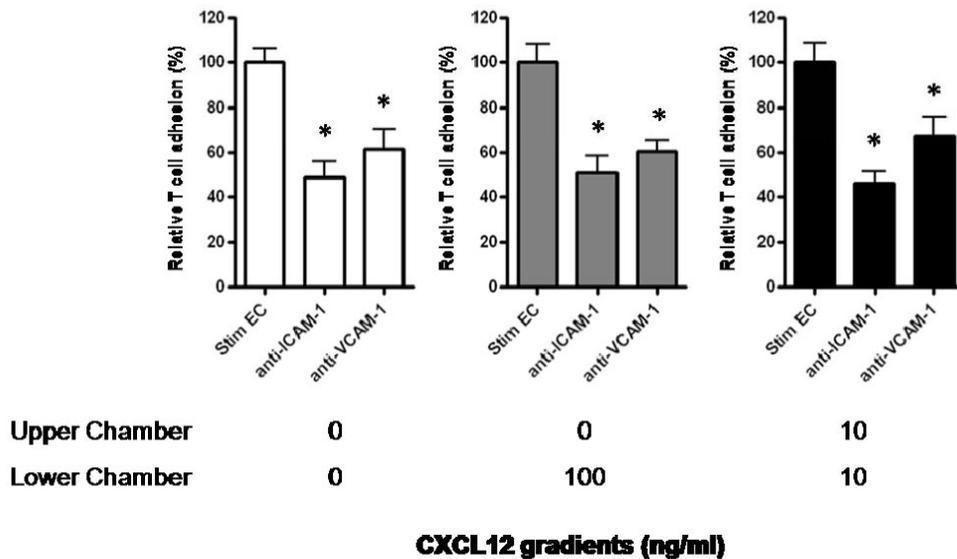
G**H**

Figure 20 (cont.) (G) Quantification of CD4⁺ T cell adhesion to resting and cytokine activated HBMEC. Treatment of confluent HBMEC cultures with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 h increases adhesion to EC in the absence of chemokine gradients. The presence of CXCL12 in the subendothelial compartment or in both chambers results in further significant increase in adhesion to activated EC. Values represent mean \pm SEM of duplicate wells of 1 of 2 representative experiments. * $p < 0.05$ compared to cultures without CXCL12 added. (H) Mab blocking of ICAM-1 and VCAM-1 significantly reduces adhesion to activated HBMEC in the presence or absence of chemokine gradients. The number of adherent T cells per mm² for each treatment is normalized to the level of adhesion in the absence of chemokines to give the relative T cell adhesion. * $p < 0.05$ compared to cytokine stimulated cultures without blocking Abs.

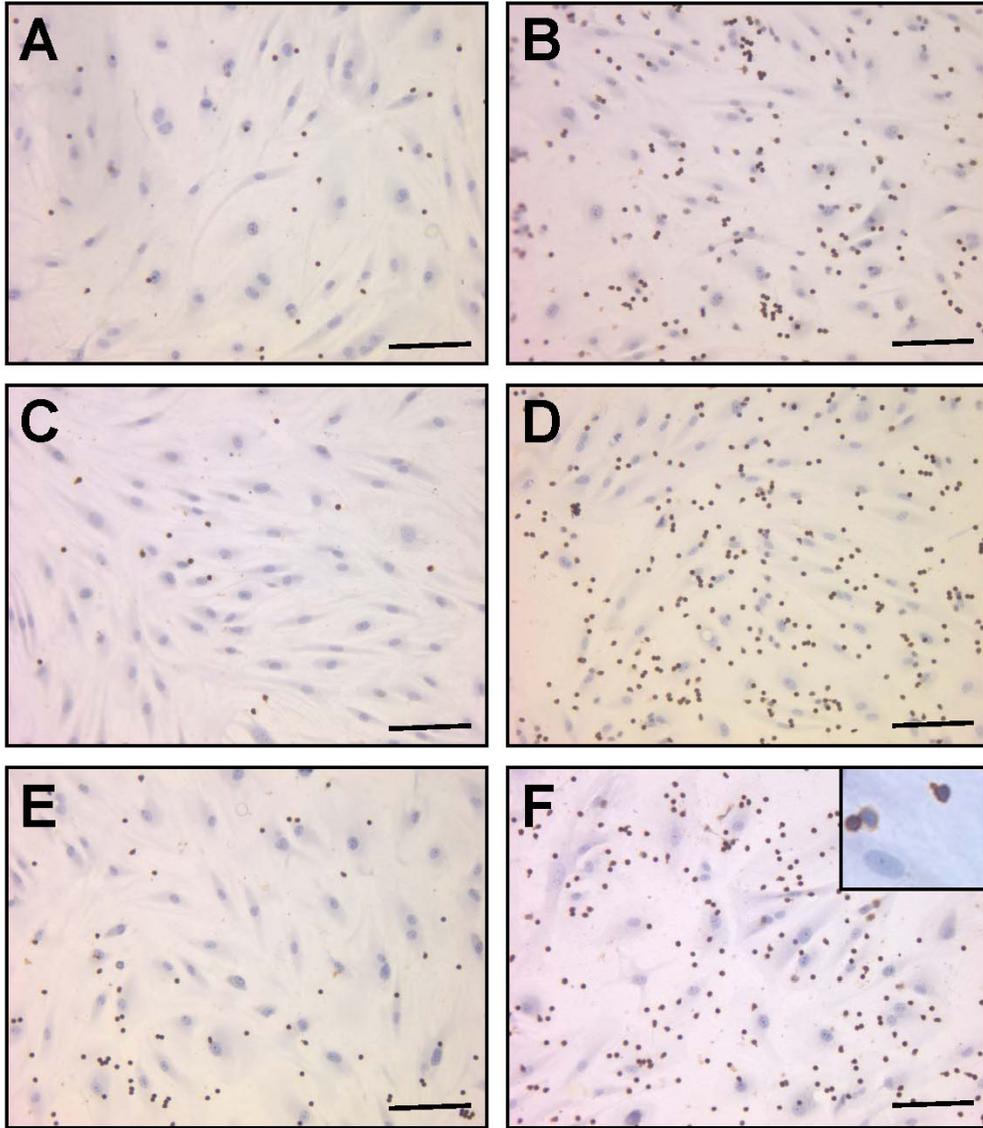


Figure 21 Adhesion of CD8+ T cells to HBMEC in response to CXCL12.

(A) A small number of T cells adhere to resting HBMEC. (B) Cytokine treatment significantly increases the number of adherent T cells. The presence of CXCL12 in the subendothelial region has no effect on binding to resting HBMEC (C), whereas adhesion to activated HBMEC is moderately increased in the presence of CXCL12 (D). In the presence of CXCL12 in both chambers, (F) adhesion to activated HBMEC is modestly, but not significantly upregulated, whereas binding to resting EC is unaffected (E). CD8+ T cells adhering to HBMEC often form small clusters (B, D, F) and extend uropod-like processes (insert) towards the endothelium. Bars = 100 μ m.

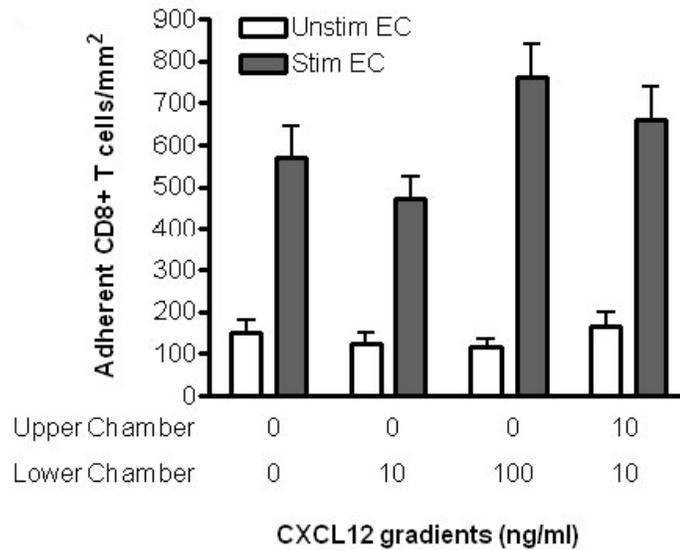
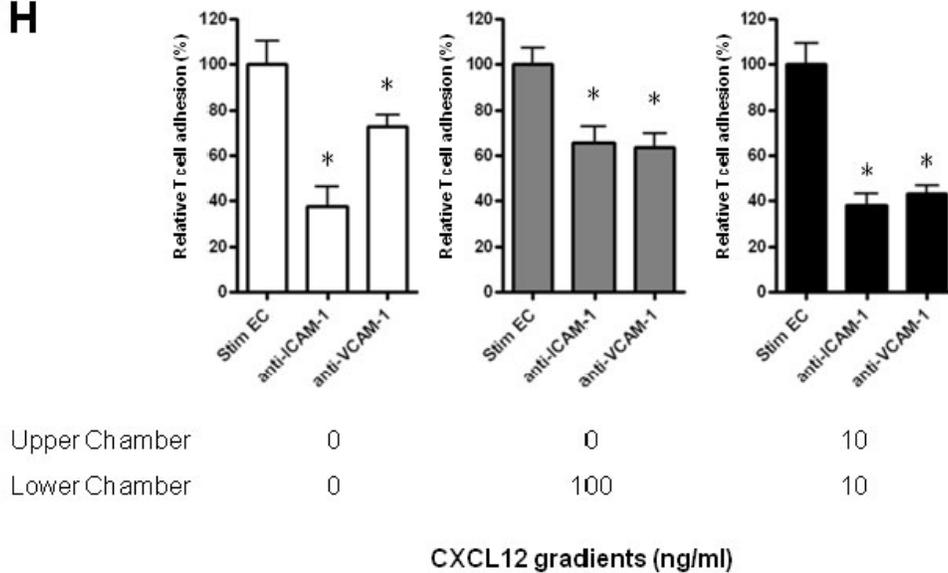
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Figure 21 (cont.) (G) Quantification of CD8+ T cell adhesion to cytokine activated and resting HBMEC. Treatment with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 h significantly upregulates adhesion of CD8+ T cells to activated HBMEC in the absence of chemokine gradients. The presence of 100ng/ml CXCL12 in the subendothelial compartment results in further modest increase in adhesion to activated EC, whereas adhesion is only slightly increased in the presence of chemokines in the upper and lower chambers. Values represent mean \pm SEM of duplicate wells of 1 of 2 representative experiments. (H) Blocking of ICAM-1 and VCAM-1 with mAb significantly downregulates adhesion to cytokine activated HBMEC in the presence or absence of chemokine gradients. The number of adherent T cells per mm² for each treatment is normalized to the level of adhesion in the absence of chemokines to give the relative T cell adhesion. * $p < 0.05$ compared to cytokine stimulated cultures without blocking Abs.

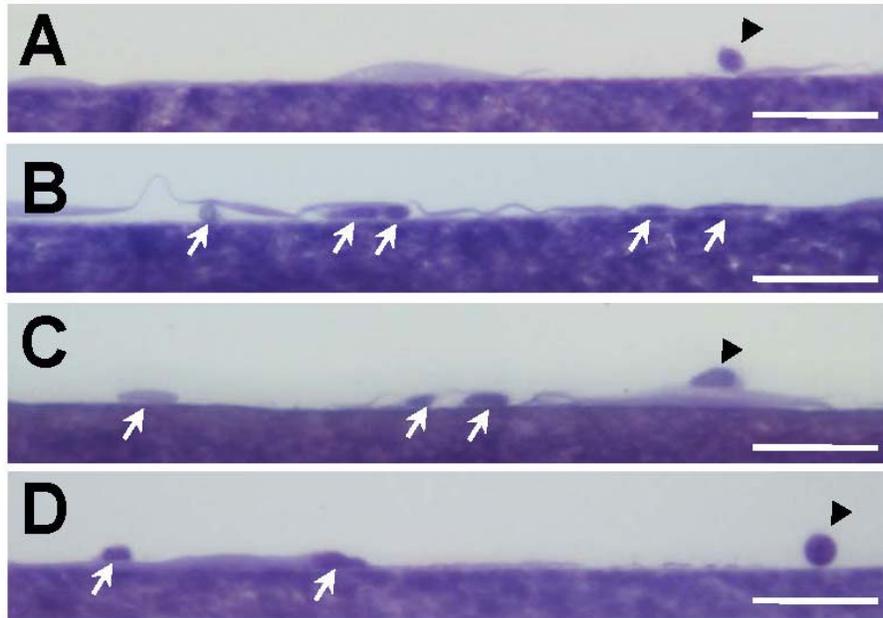


Figure 22 CD4+ T cell migration in response to CXCL12.

(A-D) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers cultured on Cellagen discs with migrating resting CD4+ T cells. (A) In unstimulated cultures, occasional T cells are still attached to the endothelium (arrow head) and only rare cell migrate. (B) In the presence of CXCL12 (100ng/ml) in the lower chamber, T cells readily migrate across the endothelium, most often in clusters of two or three. The migrated cells become flattened and remain in the subendothelial region (arrows). (C) Increased transendothelial migration following endothelial stimulation with TNF- α (100U/ml) and IFN- γ (200U/ml) for 24 h. Three cells have crossed the monolayer (arrows) and another one has become flattened (arrow head) in preparation for diapedesis. (D) Two cells have migrated (arrows) and one is still adherent (arrow head) to the stimulated endothelium in the presence of CXCL12. The continuity of the cultures is retained during the migration process. Bars = 20 μ m.

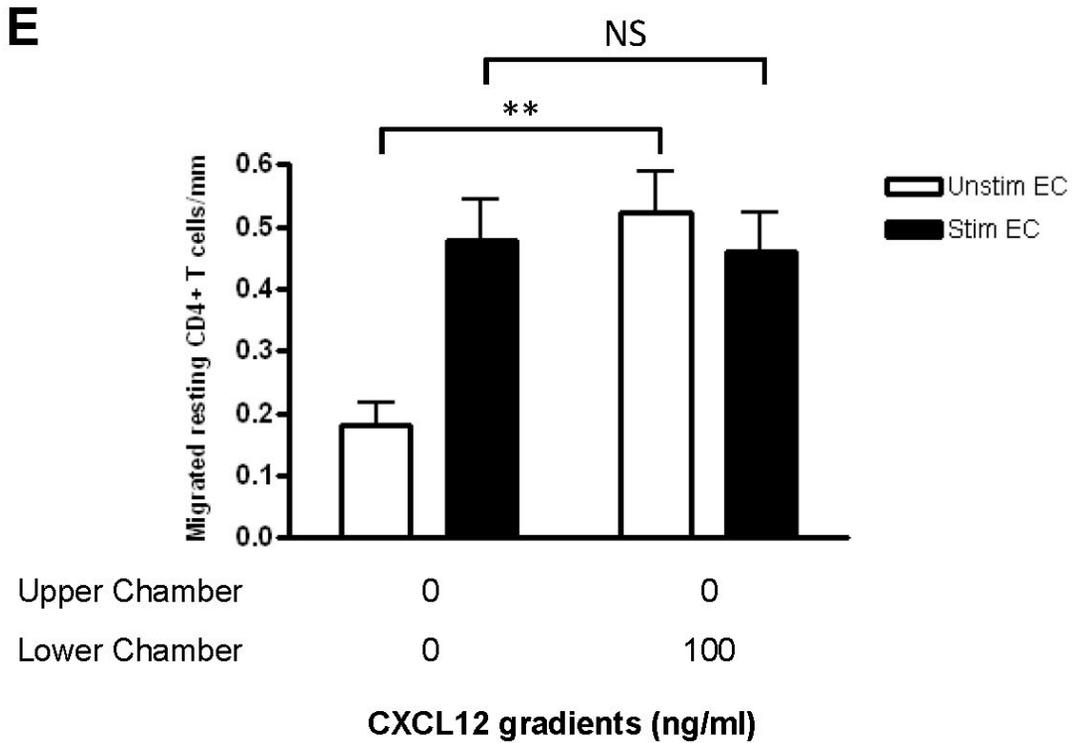


Figure 22 (cont.) CD4+ T cell migration in response to CXCL12.

(E) Quantification of resting CD4+ T cell migration across HBMEC. The minimal migration of T cells across resting monolayers is significantly upregulated following cytokine activation of HBMEC in the absence of chemokine gradients. In the presence of CXCL12, similar numbers of T cells migrate across resting or activated monolayers. CXCL12 significantly increases T cell migration across unstimulated, but not cytokine-stimulated HBMEC so that the rate of migration is similar between resting and activated monolayers. $**p < 0.01$.

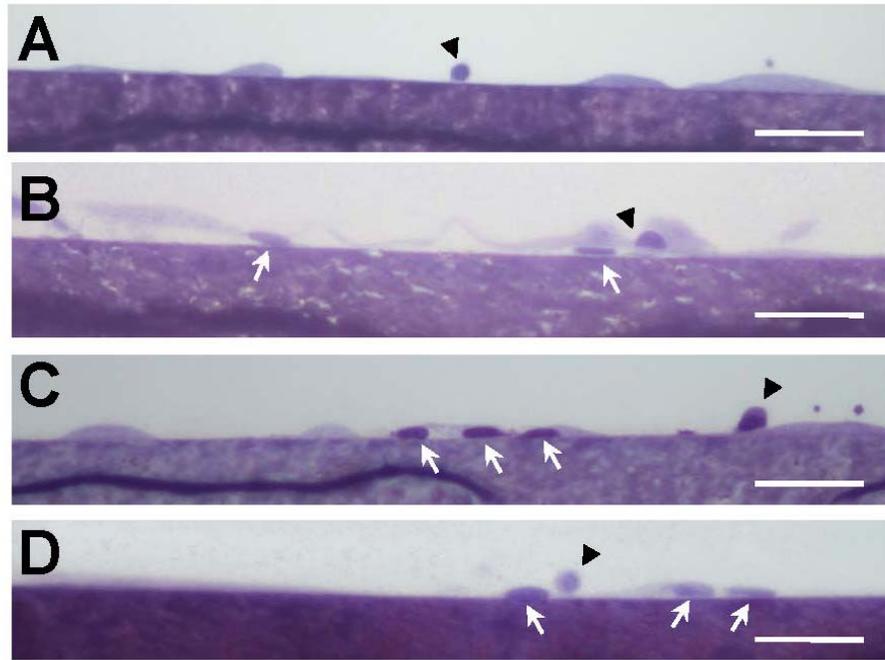


Figure 23 CD8+ T cell migration in response to CXCL12.

(A-D) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated CD8+ T cells. (A) In unstimulated HBMEC cultures, there are no migrated cells, but occasional round T cells (arrow head) are still adherent to EC. (B) In response to CXCL12 gradients, two T cells (arrows) have crossed the monolayer and another one is migrating (arrow head) between two adjacent EC. (C) Migration is significantly increased following cytokine treatment of HBMEC. In addition to the three migrated cells lying under the overlying thin EC (arrows), another T cell is extending a process toward the apical EC surface (arrow head) possibly in preparation for migration. (D) CD8+ T cells readily migrated across cytokine treated HBMEC monolayers (arrows) in response to CXCL12. A single cell (arrow head) is still adherent to EC. Bars = 20 μ m.

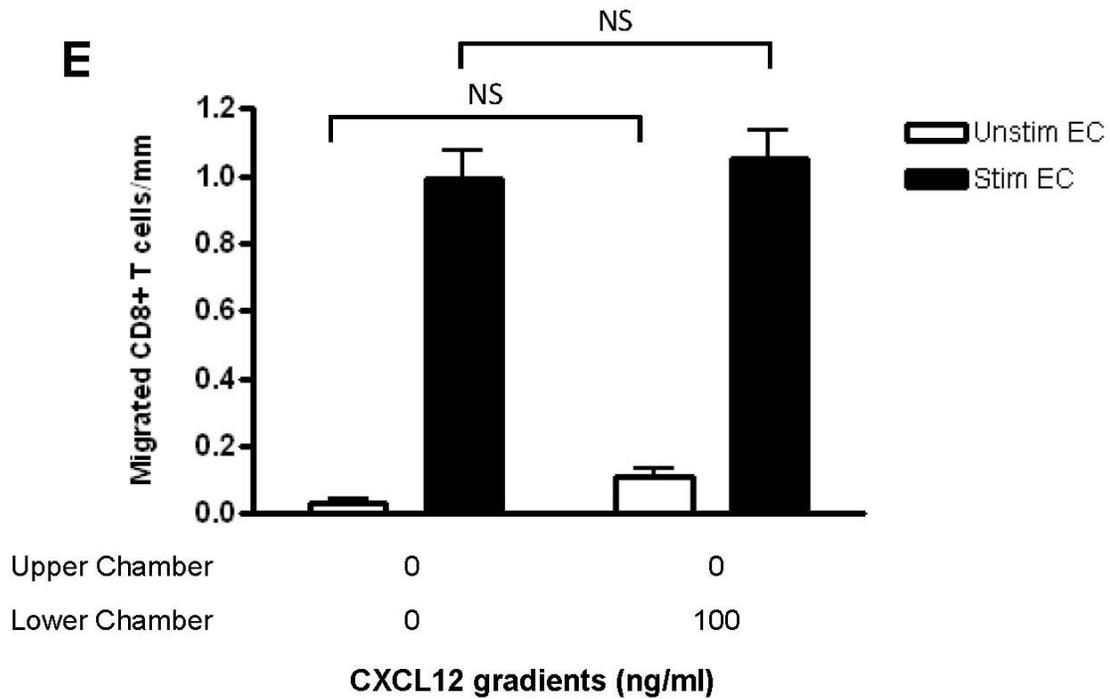


Figure 23 (cont.) CD8+ T cell migration in response to CXCL12.

(E) Quantification of CD8+ T cell migration across HBMEC monolayers. Migration is significantly upregulated following cytokine activation of HBMEC. The presence of CXCL12 gradients has no effect CD8+ T cell migration across resting and activated HBMEC.

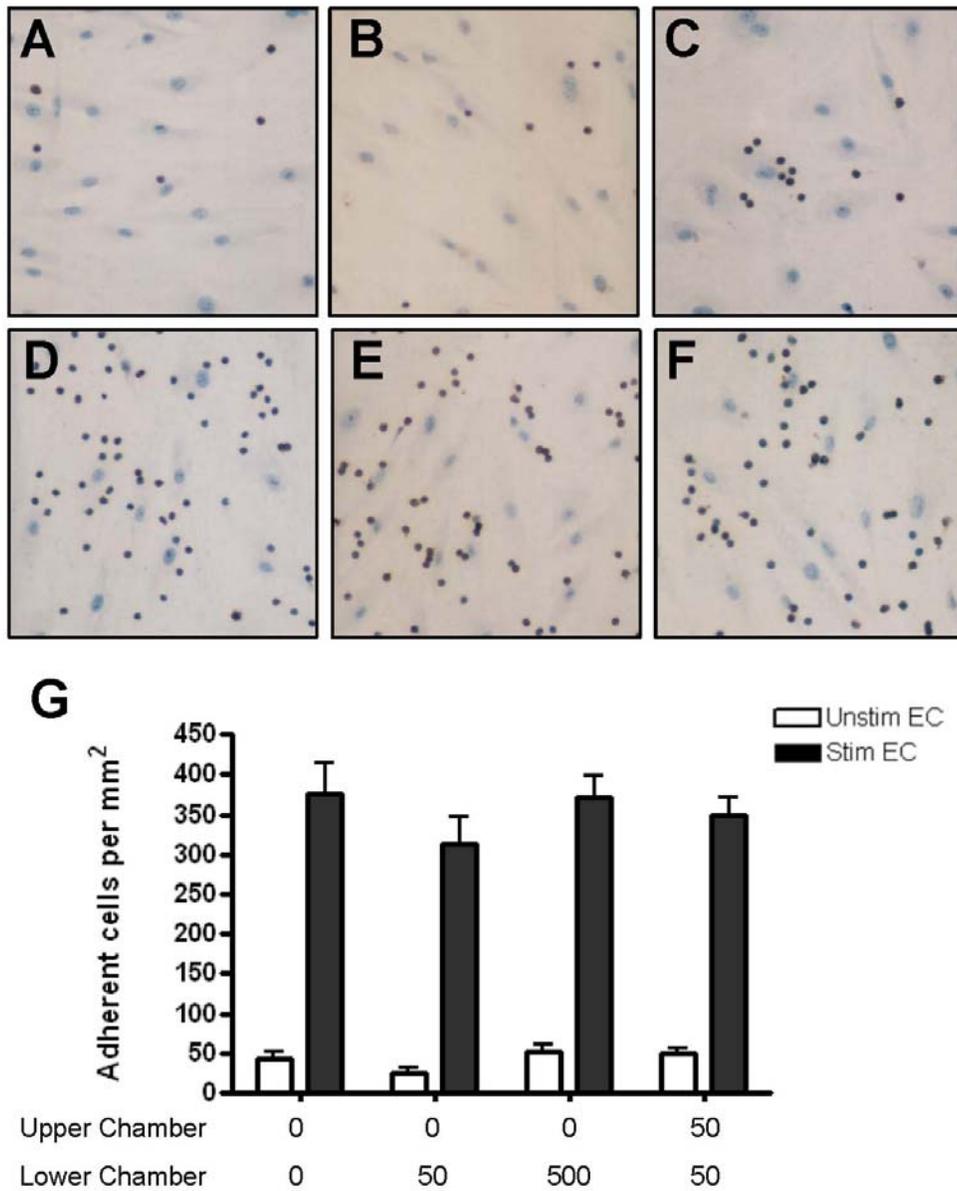


Figure 24 Adhesion of resting CD4+ T cells to unstimulated and cytokine-treated HBMEC in response to CCL2.

(A) A small number of CD4+ T cells adhere to resting HBMEC. (D) Cytokine treatment significantly increases the number of adherent T cells. The presence of CCL2 in the subendothelial region has no effect on binding to unstimulated (B) or cytokine-treated (E) HBMEC. In the presence of CCL2 in both chambers has no effect on T cell adhesion to unstimulated (C) or cytokine-treated HBMEC (F). (G) Quantification of resting CD4+ T cell adhesion to resting and cytokine activated HBMEC. Values represent mean \pm SEM of duplicate wells from 3 experiments.

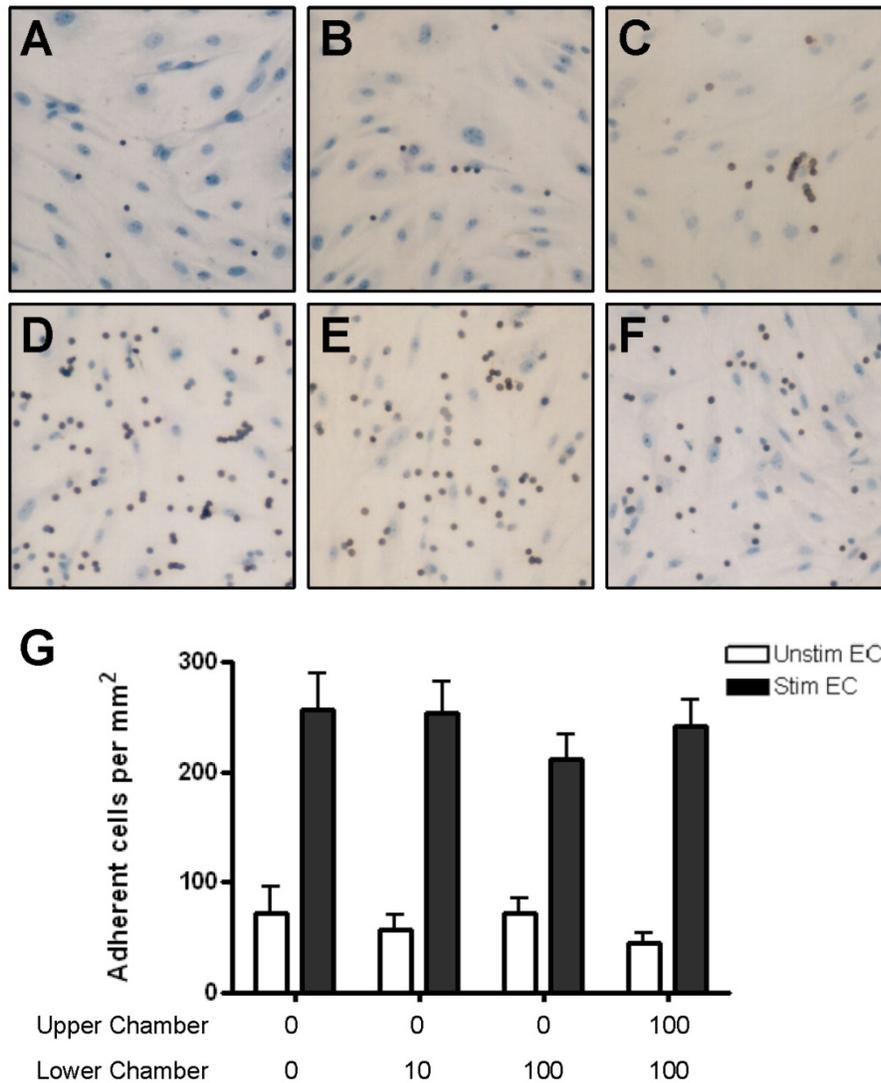


Figure 25 Adhesion of resting CD4+ T cells to resting and cytokine-treated HBMEC in response to CCL3.

(A) A small number of CD4+ T cells adhere to resting HBMEC. (D) Cytokine treatment significantly increases the number of adherent T cells. The presence of CCL3 in the subendothelial region has no effect on binding to unstimulated (B) or cytokine-treated (E) HBMEC. In the presence of CCL3 in both chambers has no effect on T cell adhesion to unstimulated (C) or cytokine-treated HBMEC (F). (G) Quantification of resting CD4+ T cell adhesion to resting and cytokine activated HBMEC. Values represent mean \pm SEM of duplicate wells from 3 experiments.

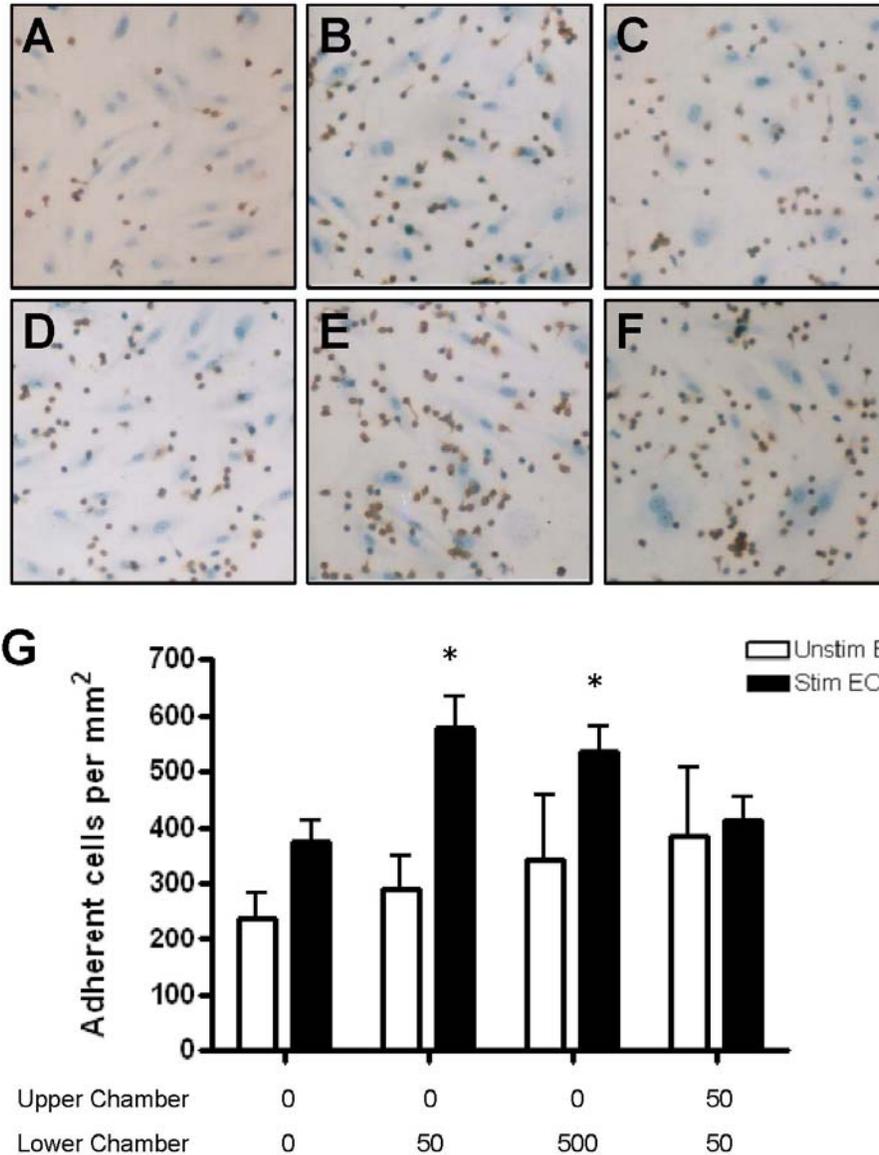


Figure 26 Adhesion of activated CD4+ T cells to resting and cytokine-treated HBMEC in response to CCL2.

(A) Anti-CD3 activation of CD4+ T cells increases baseline adhesion to unstimulated HBMEC. (D) Stimulation of HBMEC with TNF- α and IFN- γ further increases T cell adhesion to the endothelium. The presence of CCL2 in the lower chamber has no effect on binding to unstimulated HBMEC (B), whereas adhesion to cytokine-treated HBMEC is significantly increased in the presence of CCL2 (E). In the presence of CCL2 in both chambers has no effect on T cell adhesion to unstimulated (C) or cytokine-treated HBMEC (F). Activated CD4+ T cells adhering to HBMEC often form small clusters (E, F) and extend uropod-like processes towards the endothelium. (G) Quantification of activated CD4+ T cell adhesion to resting and cytokine activated HBMEC. CCL2 gradients in the lower chamber significantly increase the adhesion of activated CD4+ T cells to cytokine activated HBMEC. Values represent mean \pm SEM of duplicate wells from 3 experiments. * $p < 0.05$ as compared to controls.

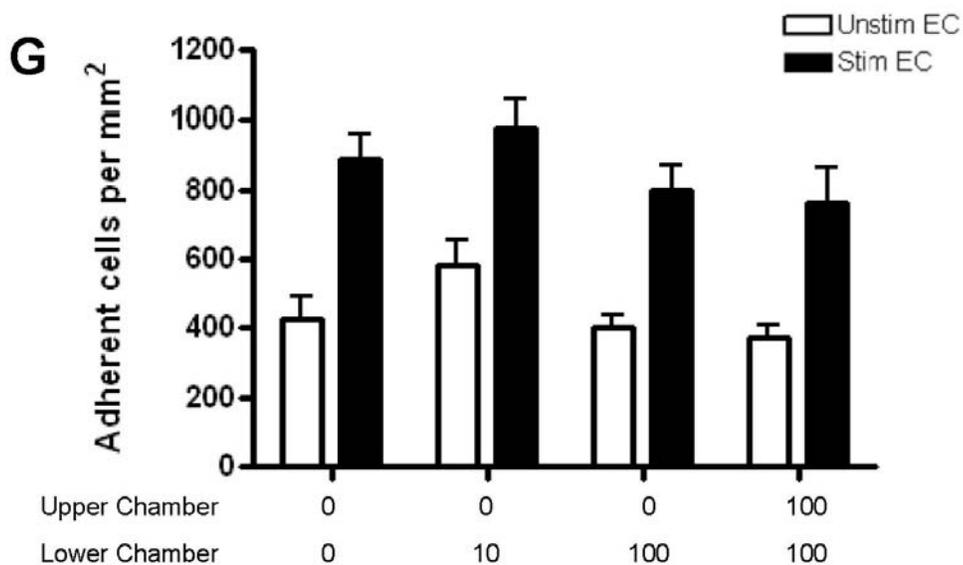
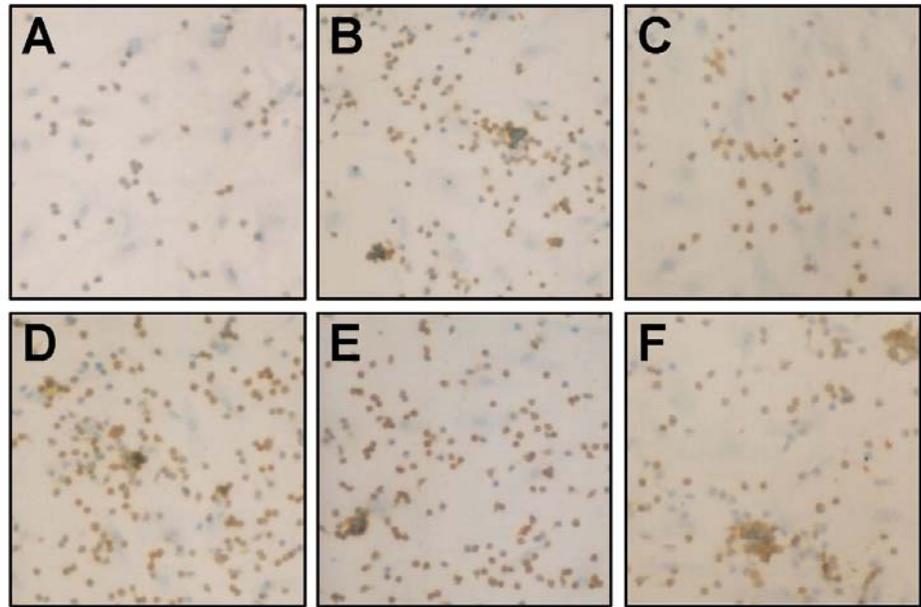


Figure 27 Adhesion of activated CD4+ T cells to resting and cytokine-treated HBMEC in response to CCL3.

(A) Anti-CD3 activation of CD4+ T cells increases baseline adhesion to unstimulated HBMEC. (D) Stimulation of HBMEC with TNF- α and IFN- γ further increases T cell adhesion to the endothelium. The presence of CCL3 in the lower chamber has no effect on binding to unstimulated HBMEC (B) or cytokine-treated (E) HBMEC. In the presence of CCL3 in both chambers has no effect on T cell adhesion to unstimulated (C) or cytokine-treated HBMEC (F). Activated CD4+ T cells adhering to HBMEC often form small clusters (B-F) and extend uropod-like processes towards the endothelium. (G) Quantification of resting CD4+ T cell adhesion to resting and cytokine activated HBMEC. Values represent mean \pm SEM of duplicate wells from 3 experiments.

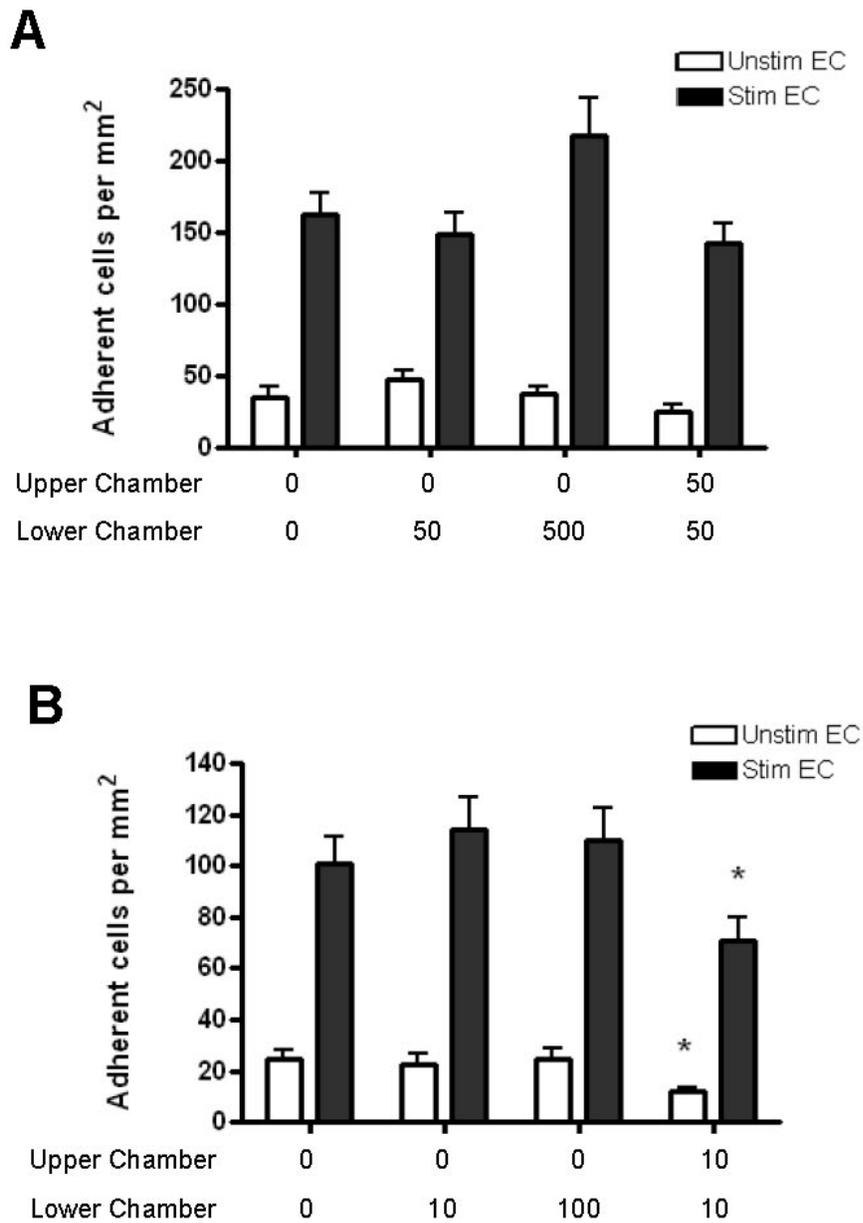


Figure 28 Adhesion of naïve CD4+ T cells to resting or cytokine-treated HBMEC. Quantification of naïve CD4+ T cell adhesion to resting and cytokine activated HBMEC in the presence of CCL2 (A) and CCL3 (B). Neither CCL2 nor CCL3 increased adhesion of naïve CD4+ T cells to resting or cytokine-treated HBMEC. Interestingly, there is a significant decrease in adhesion of naïve T cells when CCL3 is present in both chambers (B). Values represent mean \pm SEM of duplicate wells from 2 experiments. * $p < 0.05$ as compared to controls.

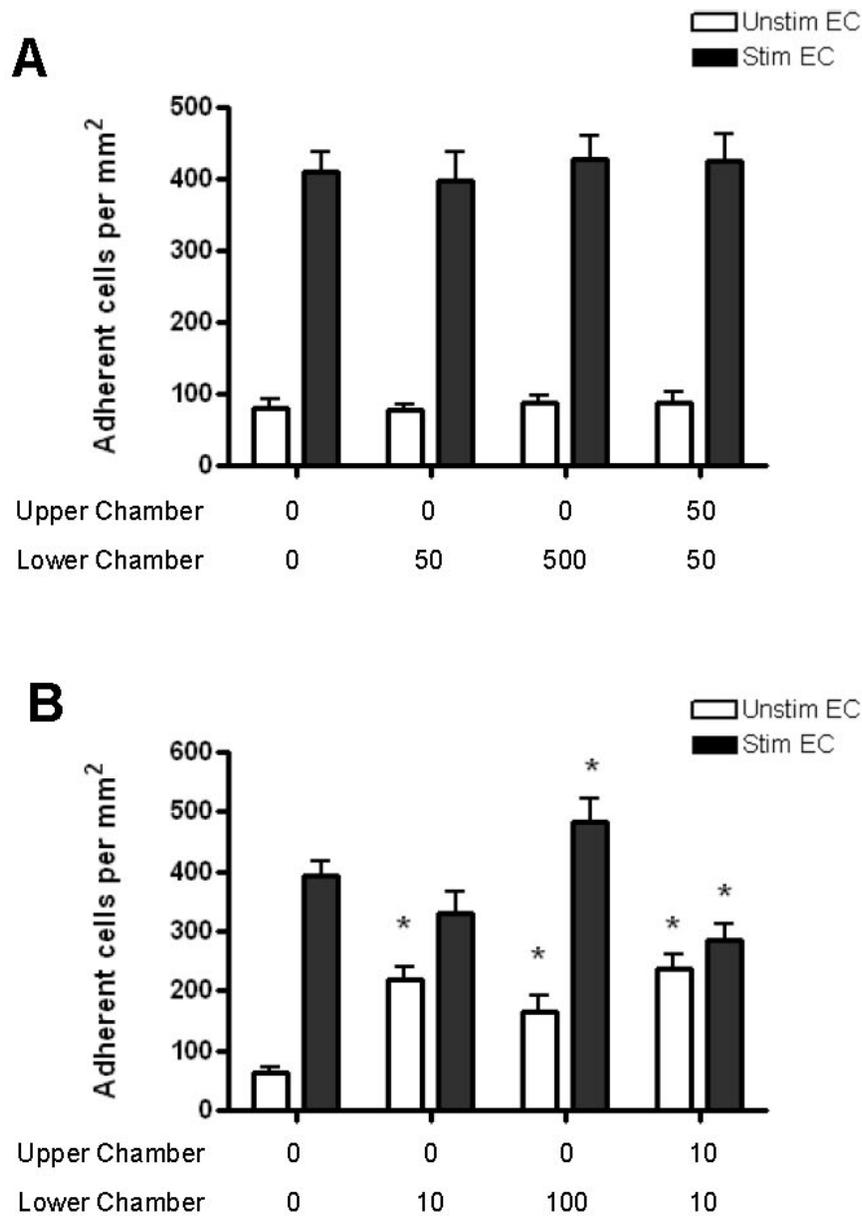


Figure 29 Adhesion of memory CD4+ T cells to resting or cytokine-treated HBMEC. Quantification of memory CD4+ T cell adhesion to resting and cytokine activated HBMEC in the presence of CCL2 (A) and CCL3 (B). CCL2 does not have an effect on the adhesion of memory CD4+ T cell to resting or cytokine activated HBMEC (A). The presence of CCL3 in the lower chamber or both chambers increased memory T cell adhesion to resting HBMEC. The presence of 100ng/ml of CCL3 in the lower chamber increased memory T cell adhesion to cytokine-activated HBMEC, while 10ng/ml of CCL3 in both upper and lower chambers decreased adhesion. Values represent mean \pm SEM of duplicate wells from 2 experiments. * $p < 0.05$ as compared to controls.

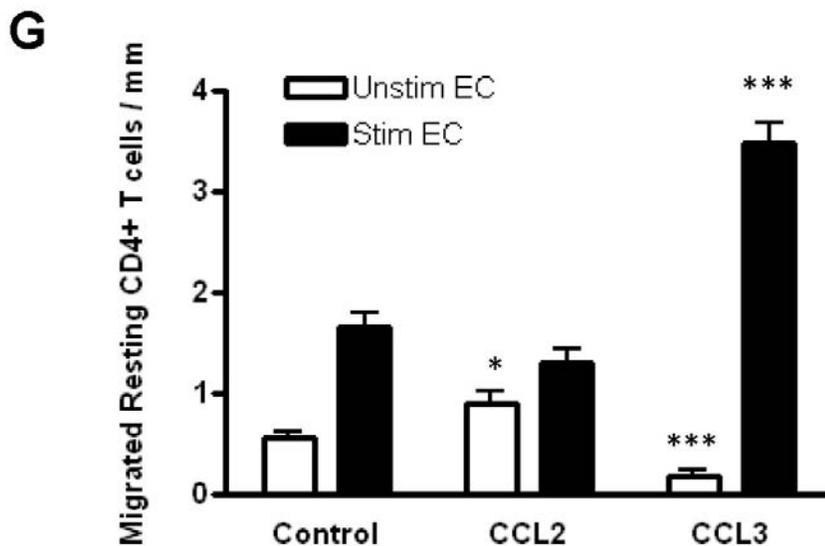
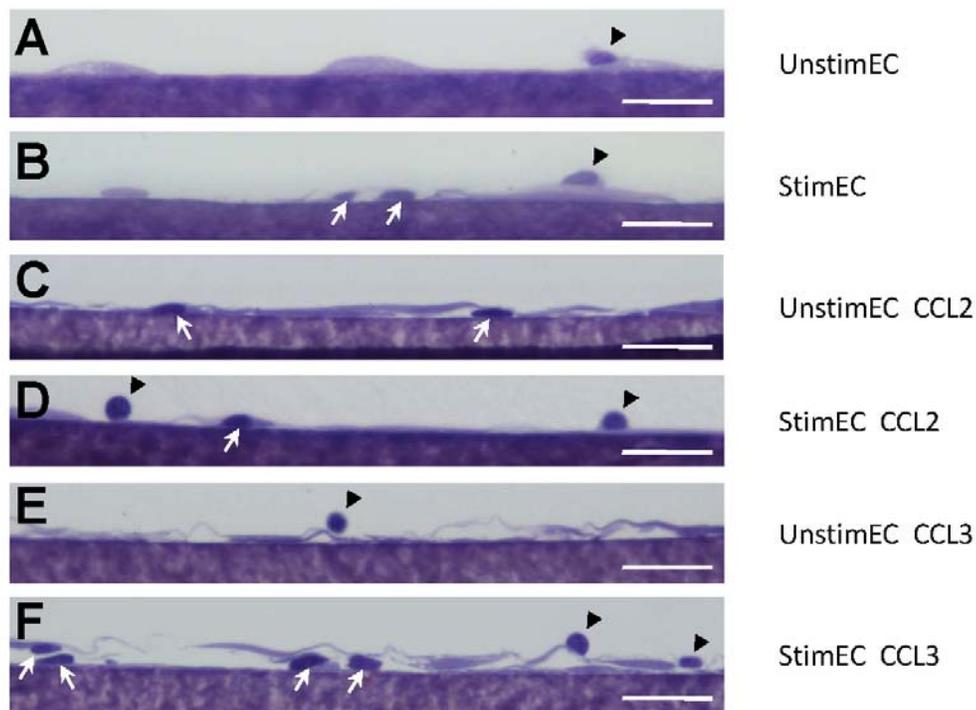


Figure 30 Migration of resting CD4+ T cells across resting and cytokine-treated HBMEC in the presence of CCL2 or CCL3.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated resting CD4+ T cells. Transmigrated CD4+ T cells are indicated by arrows and adherent CD4+ T cells are indicated by arrow heads. Bars = 20 μ m. (G) Quantification of resting CD4+ T cell migration across HBMEC monolayers. Migration is significantly upregulated following cytokine activation of HBMEC. * $p < 0.05$ and *** $p < 0.001$ as compared to controls.

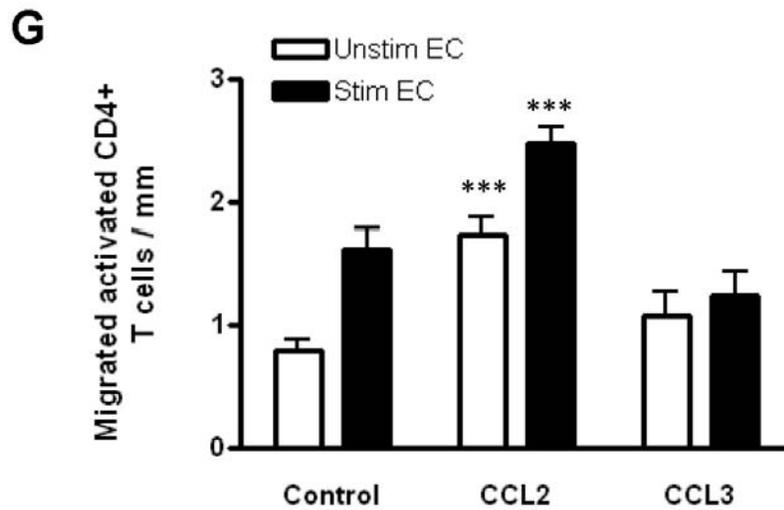
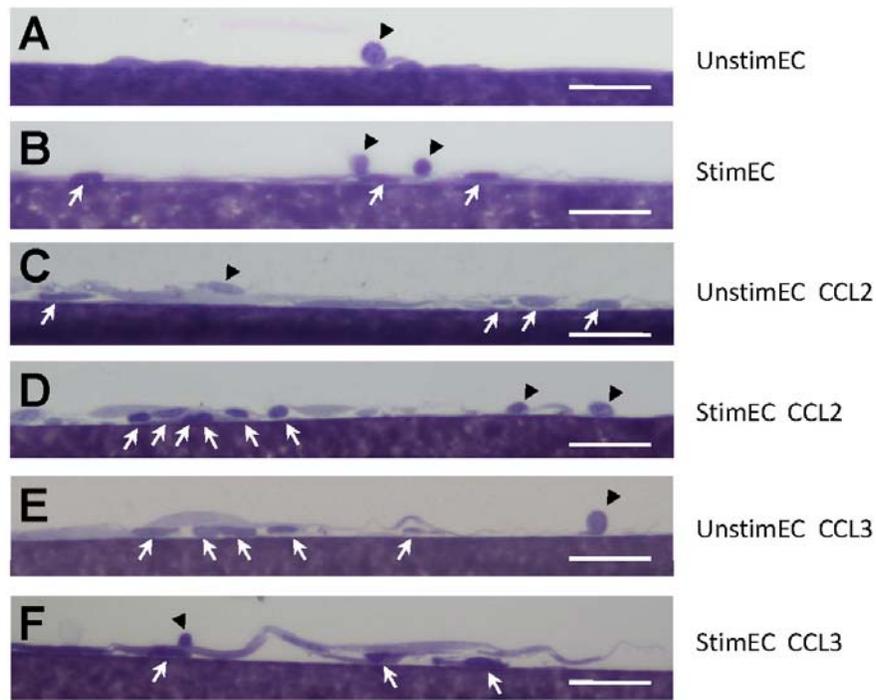


Figure 31 Migration of activated CD4+ T cells across resting and cytokine-treated HBMEC in the presence of CCL2 or CCL3.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated activated CD4+ T cells. Transmigrated CD4+ T cells are indicated by arrows and adherent CD4+ T cells are indicated by arrow heads. Bars = 20 μ m. (G) Quantification of activated CD4+ T cell migration across HBMEC monolayers. Migration is further upregulated following cytokine activation of HBMEC. *** $p < 0.001$ as compared to controls.

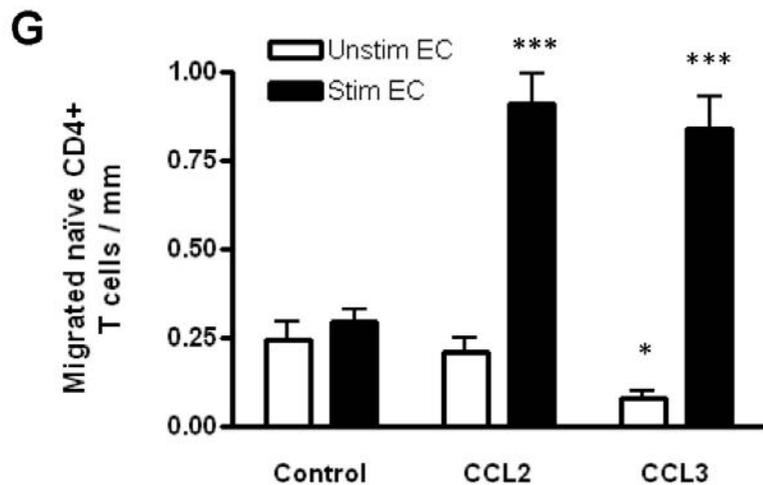
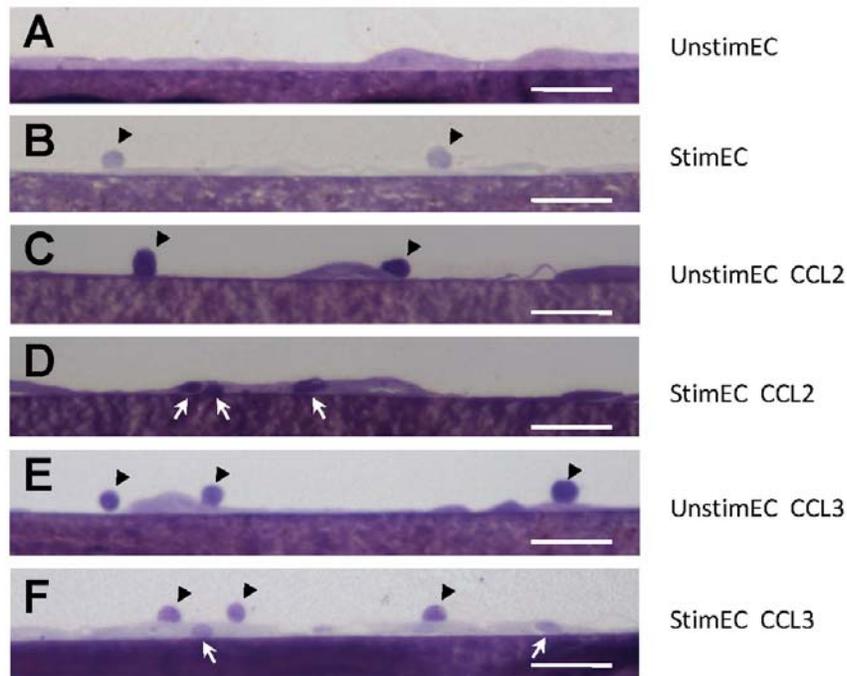


Figure 32 Migration of naïve CD4+ T cells across resting and cytokine-treated HBMEC in the presence of CCL2 or CCL3.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated naïve CD4+ T cells. Transmigrated naïve CD4+ T cells are indicated by arrows and adherent naïve CD4+ T cells are indicated by arrow heads. Bars = 20 μ m. (G) Quantification of naïve CD4+ T cell migration across HBMEC monolayers. Migration is minimally upregulated following cytokine treatment of HBMEC in the absence of chemokines. CCL2 or CCL3 significantly increased naïve CD4+ T cell migration across cytokine-treated HBMEC. * $p < 0.05$ and *** $p < 0.001$ as compared to controls.

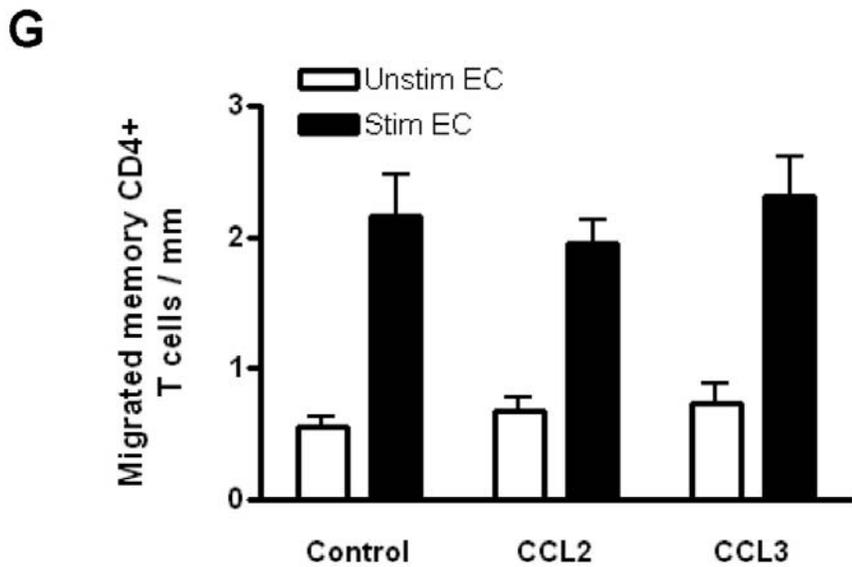
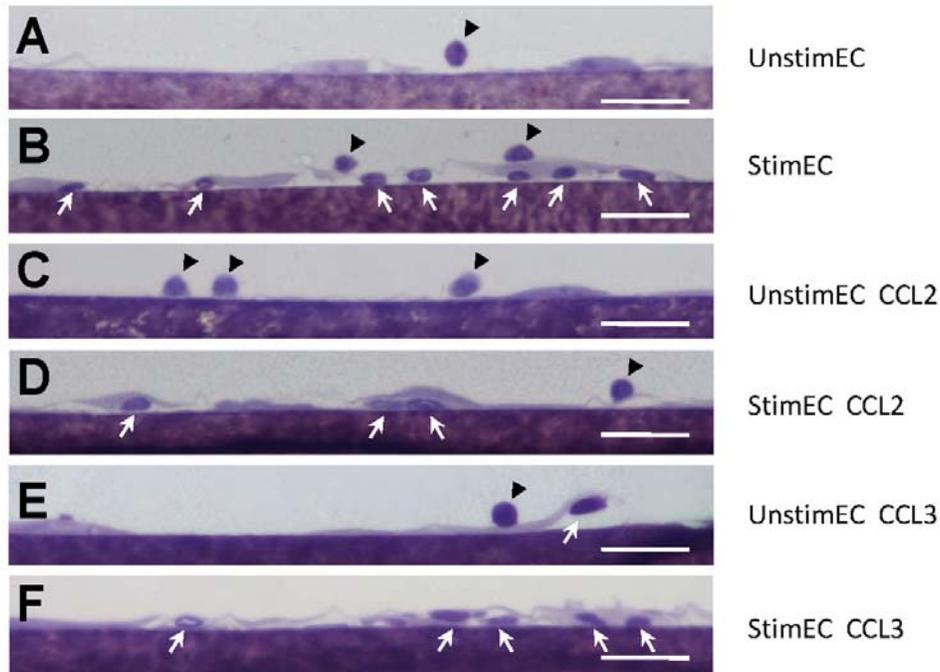


Figure 33 Migration of memory CD4+ T cells across resting and cytokine-treated HBMEC in the presence of CCL2 or CCL3.

(A-F) Toluidine blue stained, 1μm thick plastic cross-sections of HBMEC monolayers with migrated memory CD4+ T cells. Transmigrated memory CD4+ T cells are indicated by arrows and adherent memory CD4+ T cells are indicated by arrow heads. Bars = 20μm. (G) Quantification of memory CD4+ T cell migration across HBMEC monolayers. Migration is upregulated following cytokine treatment of HBMEC. The presence of chemokine gradients had no effect on memory CD4+ T cell migration.

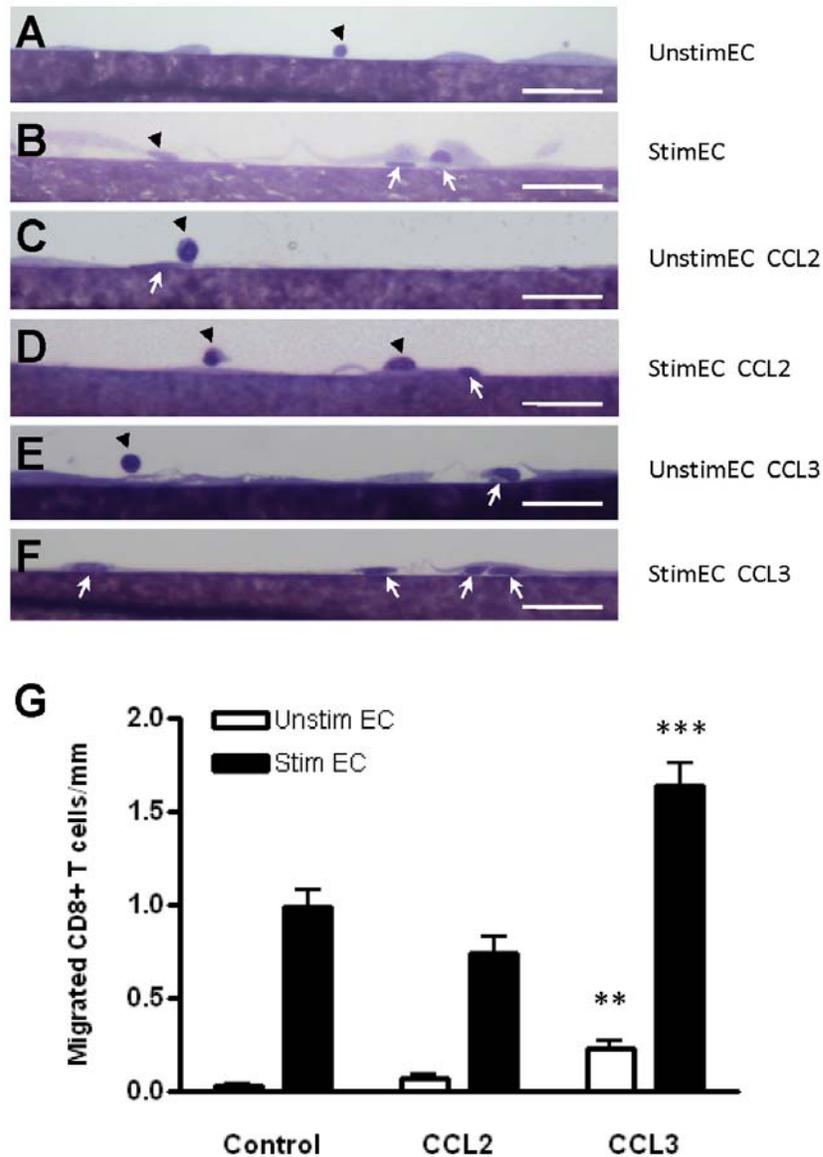


Figure 34 Migration of CD8+ T cells across resting and cytokine-treated HBMEC in the presence of CCL2 or CCL3.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated CD8+ T cells. Transmigrated CD8+ T cells are indicated by arrows and adherent CD8+ T cells are indicated by arrow heads. Bars = 20 μ m. (G) Quantification of CD8+ T cell migration across HBMEC monolayers. Migration is significantly upregulated following cytokine-treated of HBMEC. CCL2 and CCL3 increased basal migration of CD8+ T cells across resting HBMEC. CCL3, but not CCL2 increased migration of CD8+ T cells across cytokine-treated HBMEC. ** $p < 0.01$ and *** $p < 0.001$ as compared to controls.

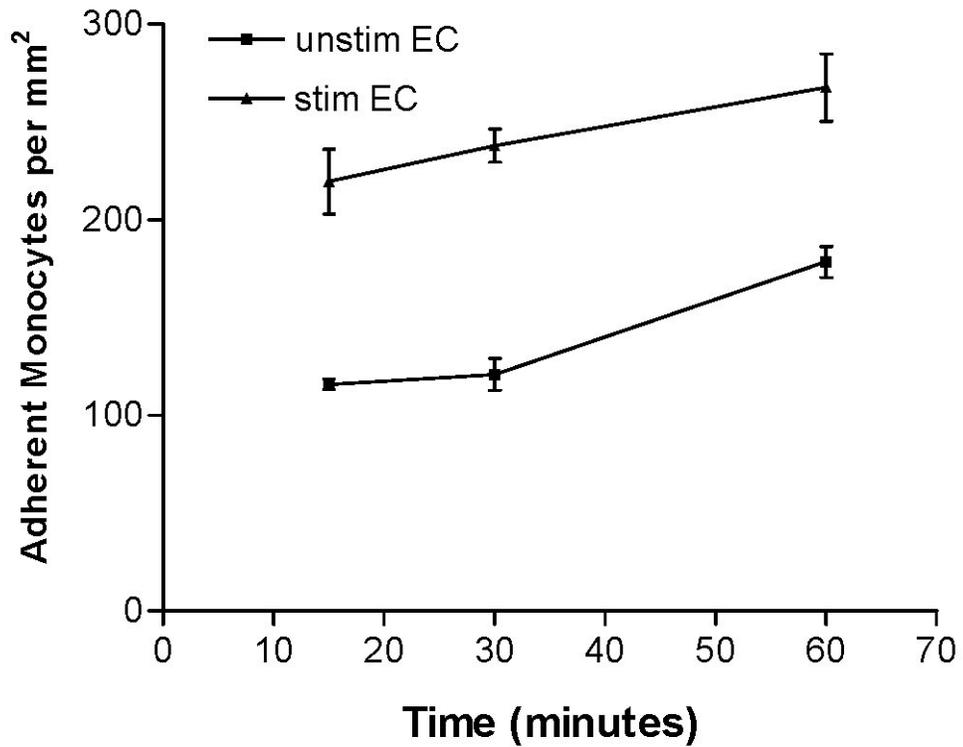


Figure 35 Kinetics of monocyte adhesion to resting or cytokine-treated HBMEC. Monocyte adhesion to HBMEC is time dependent and influenced by the activation status of the endothelium. Only few monocytes adhere to unstimulated HBMEC in the first 30 min and adhesion gradually increases thereafter. When HBMEC monolayers are pretreated with TNF- α and IFN- γ , monocytes readily adhere to the monolayers within 15 min and adhesion increases further overtime. Representative data from one of two experiments are shown.

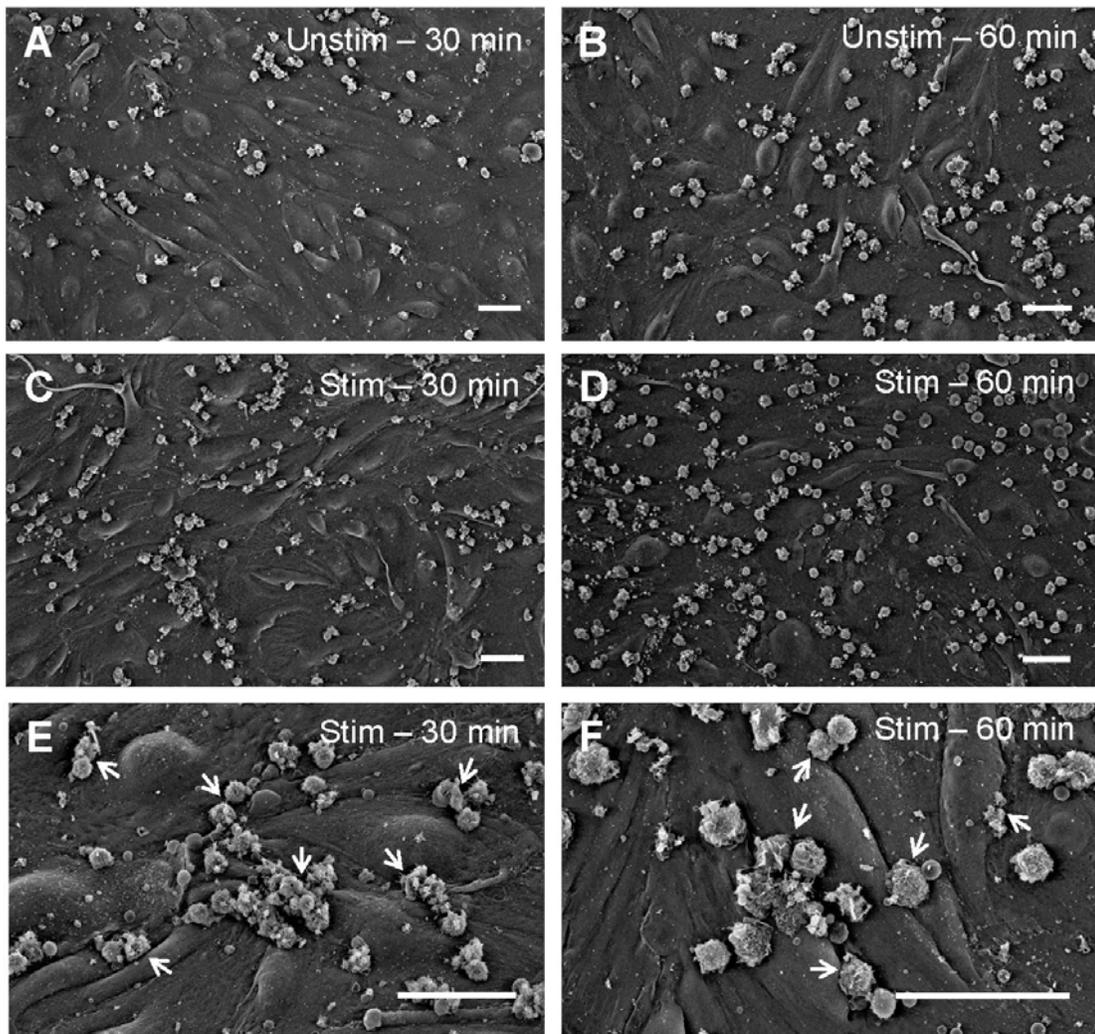


Figure 36 Scanning EM of monocyte adhesion to unstimulated or cytokine-stimulated HBMEC.

The small number of monocytes that adhere to unstimulated HBMEC by 30 min. (A), increased by 60 min. (B). Cytokine stimulated HBMEC become spindly and elongated (C to F). Significantly more monocytes adhere to stimulated HBMEC at 30 min (C) and 60 min (D) compared to unstimulated HBMEC. Adherent monocytes position themselves at or near interendothelial junctions (arrows) by 30 min (E) and monocytes migrate between endothelial junctions at 60 min (F). (bar = 50 μ m)

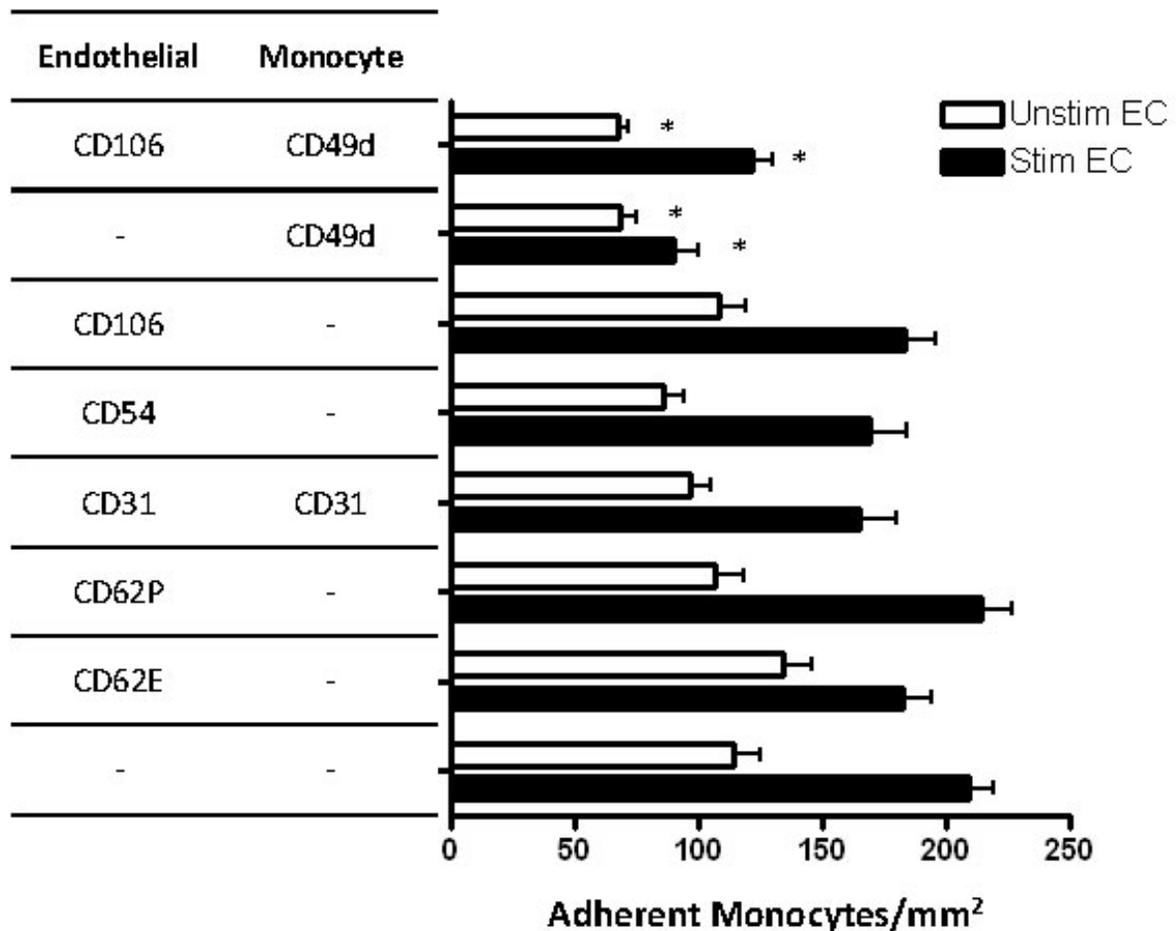


Figure 37 Role of adhesion molecules in monocyte adhesion to HBMEC.

Adhesion of monocytes is regulated by endothelial cell adhesion molecules and leukocyte integrins. The minimal adhesion of monocytes to unstimulated HBMEC is increased significantly after treatment of HBMEC with TNF- α and IFN- γ for 24 h. Preincubation of HBMEC with anti- ICAM-1 (CD54), VCAM-1 (CD106), PECAM-1 (CD31), P-selectin (CD62P) or E-selectin (CD62E) blocking antibodies 30 min prior to the adhesion assay did not significantly reduce monocytes adhesion to resting or cytokine-activated HBMEC. Adhesion was significantly reduced by blocking of α 4 integrin (CD49d) on monocytes 30 min before incubation with HBMEC monolayers. Combination of VCAM-1 and CD49d blocking antibodies did not have any additive effect in reducing monocyte adhesion. Values represent mean \pm SEM of triplicate wells from 2 experiments. * $p < 0.05$ as compared to controls.

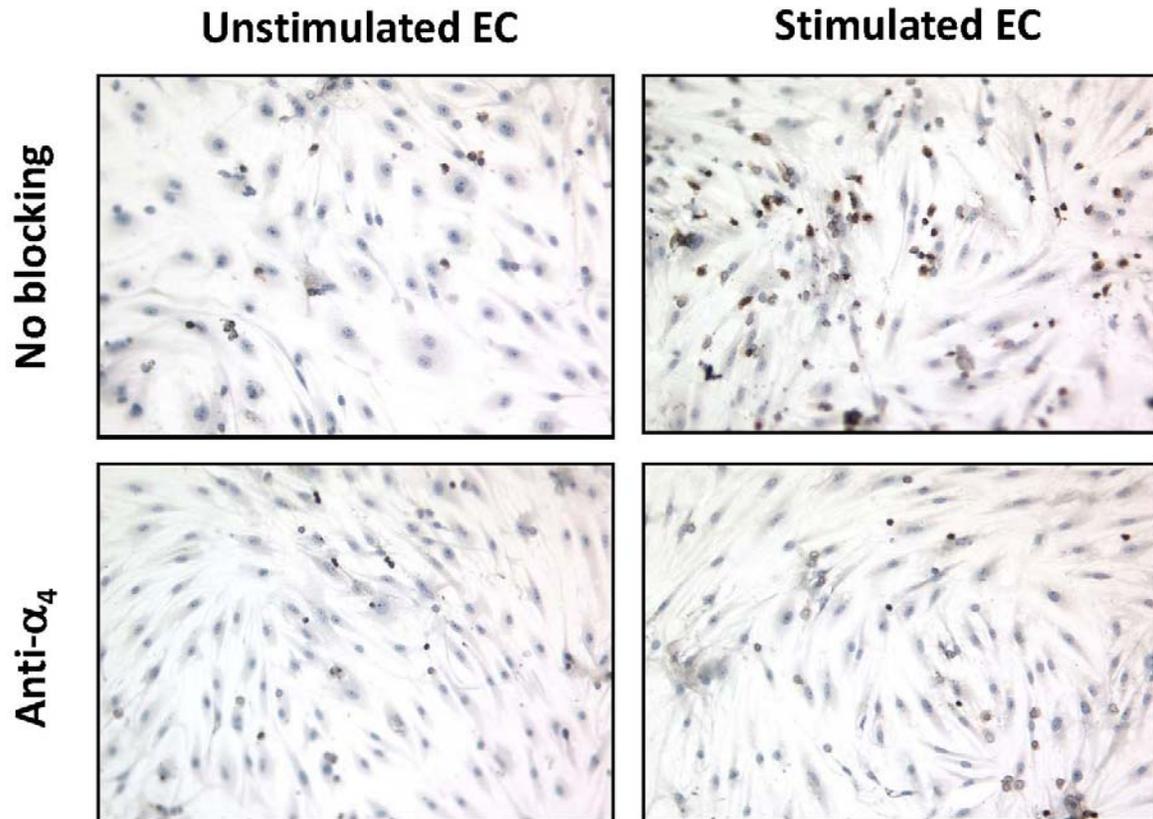


Figure 38 Blocking of α_4 -integrin (CD49d) on monocytes decreases monocyte adhesion to HBMEC.

In the absence of blocking antibodies, a small number of monocytes adhere to unstimulated HBMEC and a greater number monocytes adhere to cytokine stimulated HBMEC. Pre-incubation of monocytes with an anti- α_4 blocking antibody significantly inhibited monocytes adhesion to both unstimulated and cytokine stimulated HBMEC. The effectiveness of anti- α_4 blocking is most apparent in cytokine stimulated HBMEC where reduction in the number of adherent monocytes is most significant.

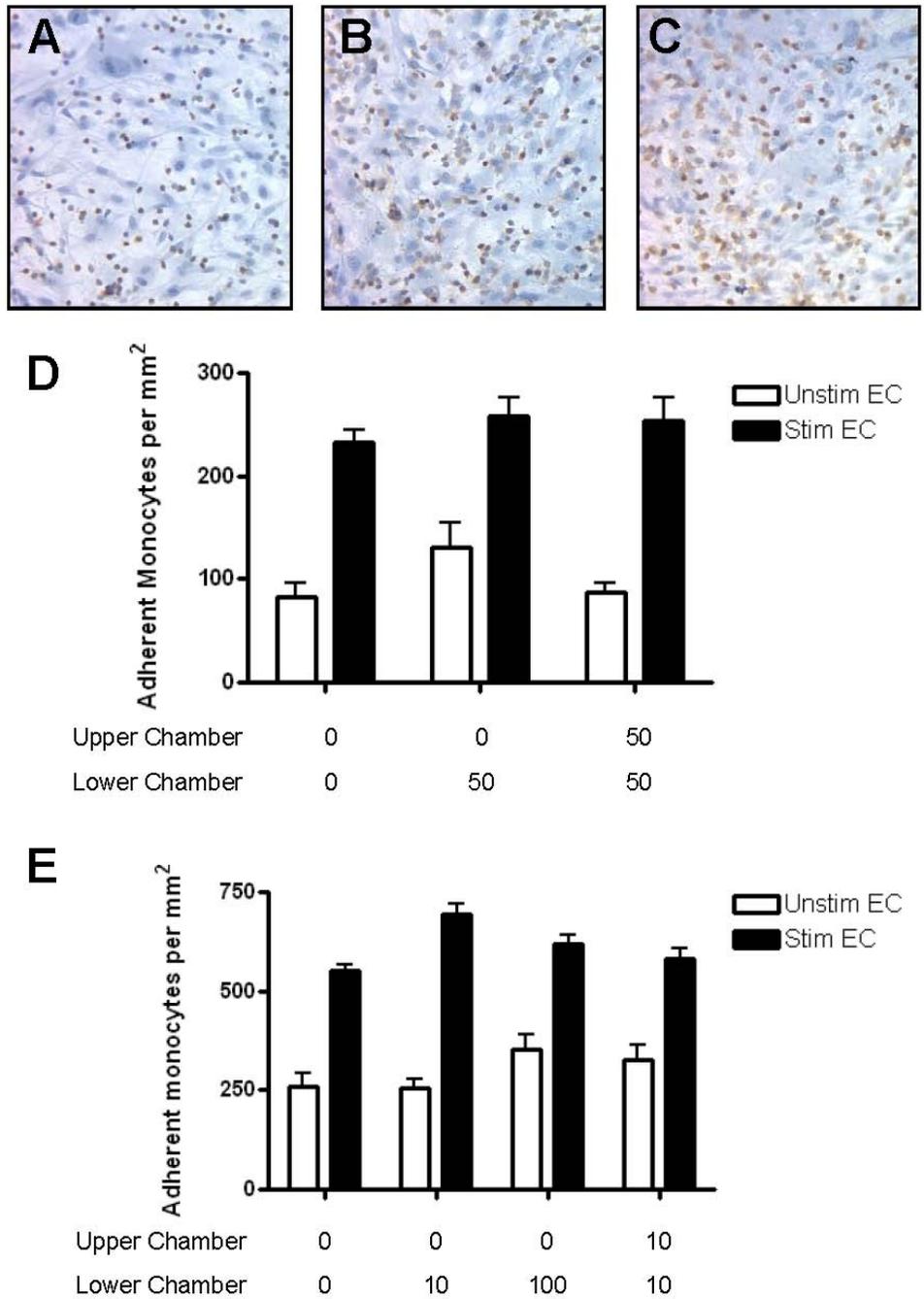


Figure 39 Adhesion of monocytes to resting or cytokine-treated HBMEC in the presence of CCL2 or CCL3 gradients.

Monocyte adhesion to cytokine activated HBMEC in the absence of chemokine gradients (A) and in the presence of CCL2 (B) and CCL3 (C) gradients. Monocytes appear bigger and monocytes with pseudopod are observed more often in the presence of CCL2 (B) and CCL3 (C) compared to control (A). Quantification of monocyte adhesion in the presence of CCL2 (D) or CCL3 (E) gradients. Mean \pm SEM in 1 of 2 experiments performed in duplicate wells is shown.

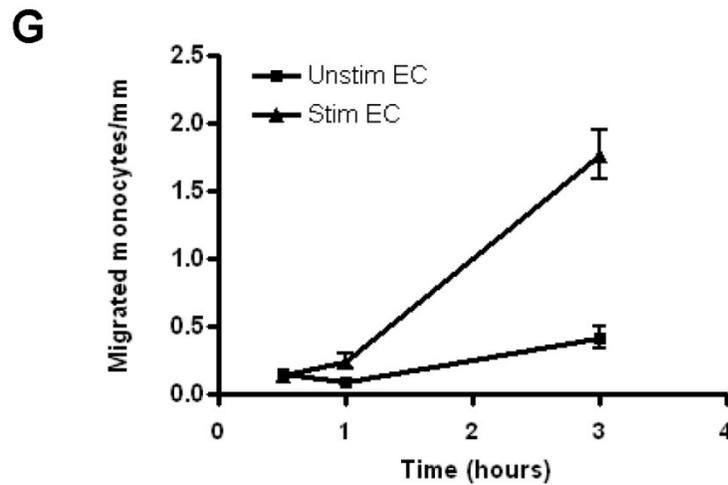
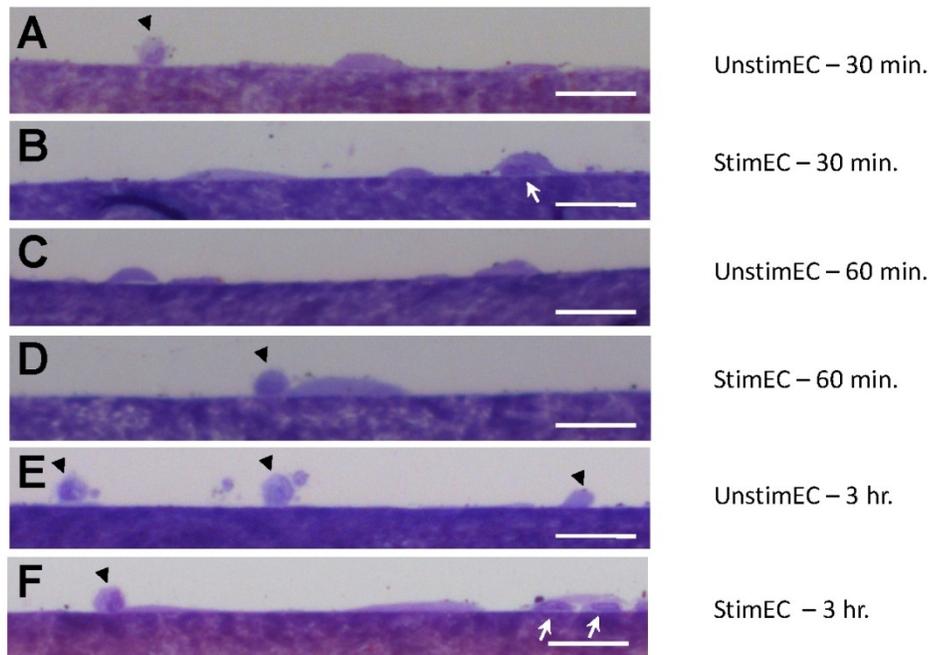


Figure 40 Kinetics of monocyte migration across resting or cytokine-treated HBMEC.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated monocytes. Transmigrated monocytes are indicated by arrows and adherent monocytes are indicated by arrow heads. Bars = 20 μ m.

(G) Migration of monocytes across resting and cytokine activated HBMEC was measured at 0.5, 1.0 and 3.0 hours. Stimulation of HBMEC with TNF- α (100U/ml) and IFN- γ (200U/ml) significantly augments monocyte migration at 1.0 and 3.0 hours. There is minimal difference in monocyte migration between resting and cytokine-activated HBMEC at 0.5 hour.

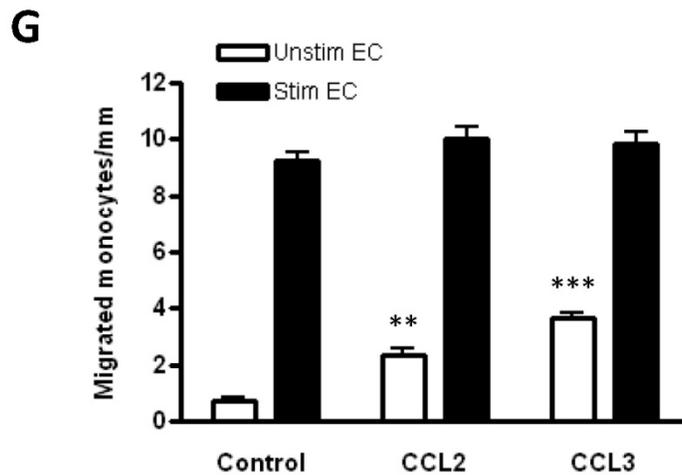
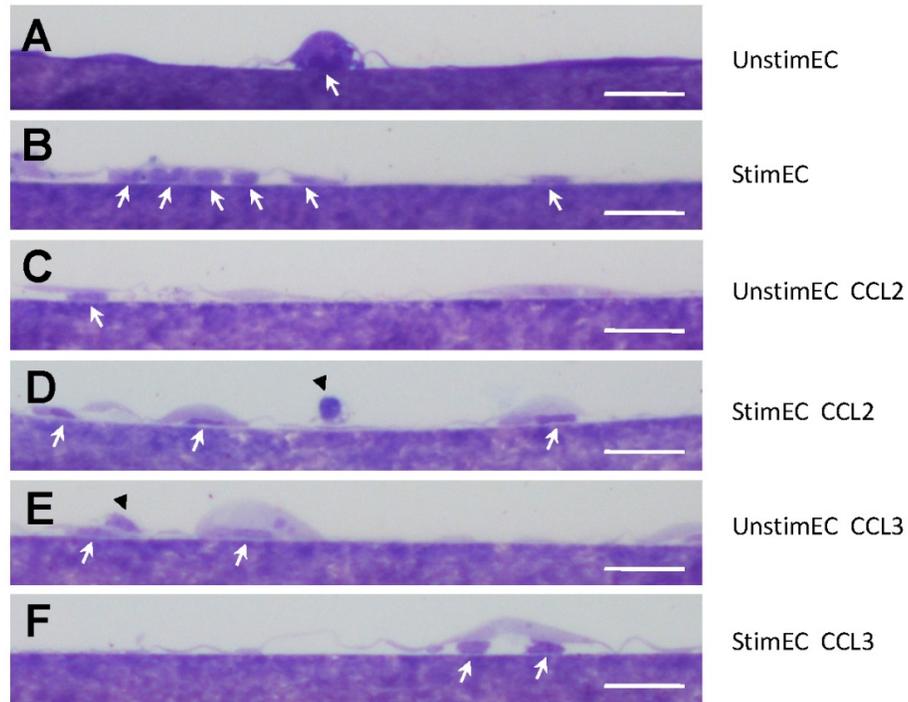


Figure 41 Migration of monocytes across resting or cytokine-treated HBMEC in the presence of chemokine gradients.

(A-F) Toluidine blue stained, 1 μ m thick plastic cross-sections of HBMEC monolayers with migrated monocytes. Transmigrated monocytes are indicated by arrows and adherent monocytes are indicated by arrow heads. Bars = 20 μ m.

(G) Cytokine-treatment of HBMEC significantly increases monocyte migration. CCL2 or CCL3 increased monocyte migration across resting but not cytokine-treated HBMEC.

** $p < 0.01$ and *** $p < 0.001$ as compared to controls.

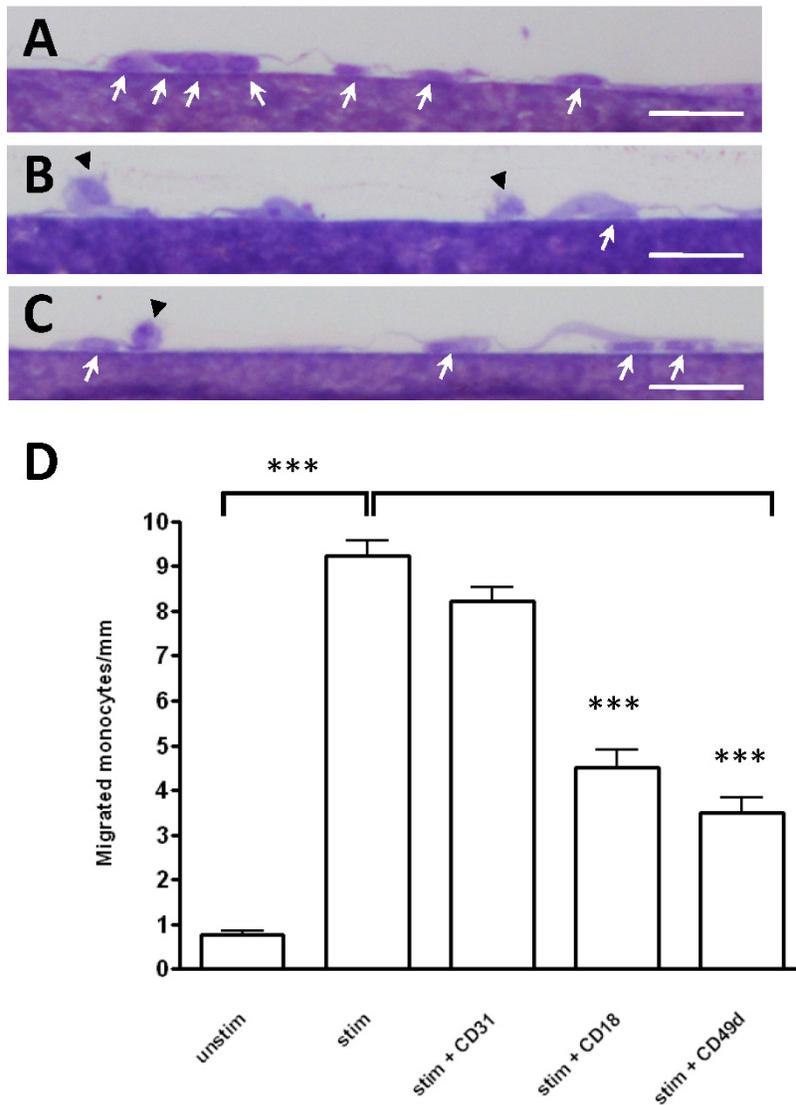


Figure 42 Migration of monocytes across resting or cytokine-treated HBMEC in the presence of blocking antibodies.

(A-C) Toluidine blue stained, 1 μ m thick plastic cross-sections of cytokine stimulated HBMEC monolayers with migrated monocytes in the presence of blocking antibodies to (A) CD31/PECAM-1, (B) CD18/ β 2-integrin and (C) CD49d/VLA-4. Transmigrated monocytes are indicated by arrows and adherent monocytes are indicated by arrow heads. Bars = 20 μ m.

(D) Blocking of CD18 or VLA-4 (CD49d) significantly decreased migration of monocytes across cytokine-treated HBMEC. *** $p < 0.001$.

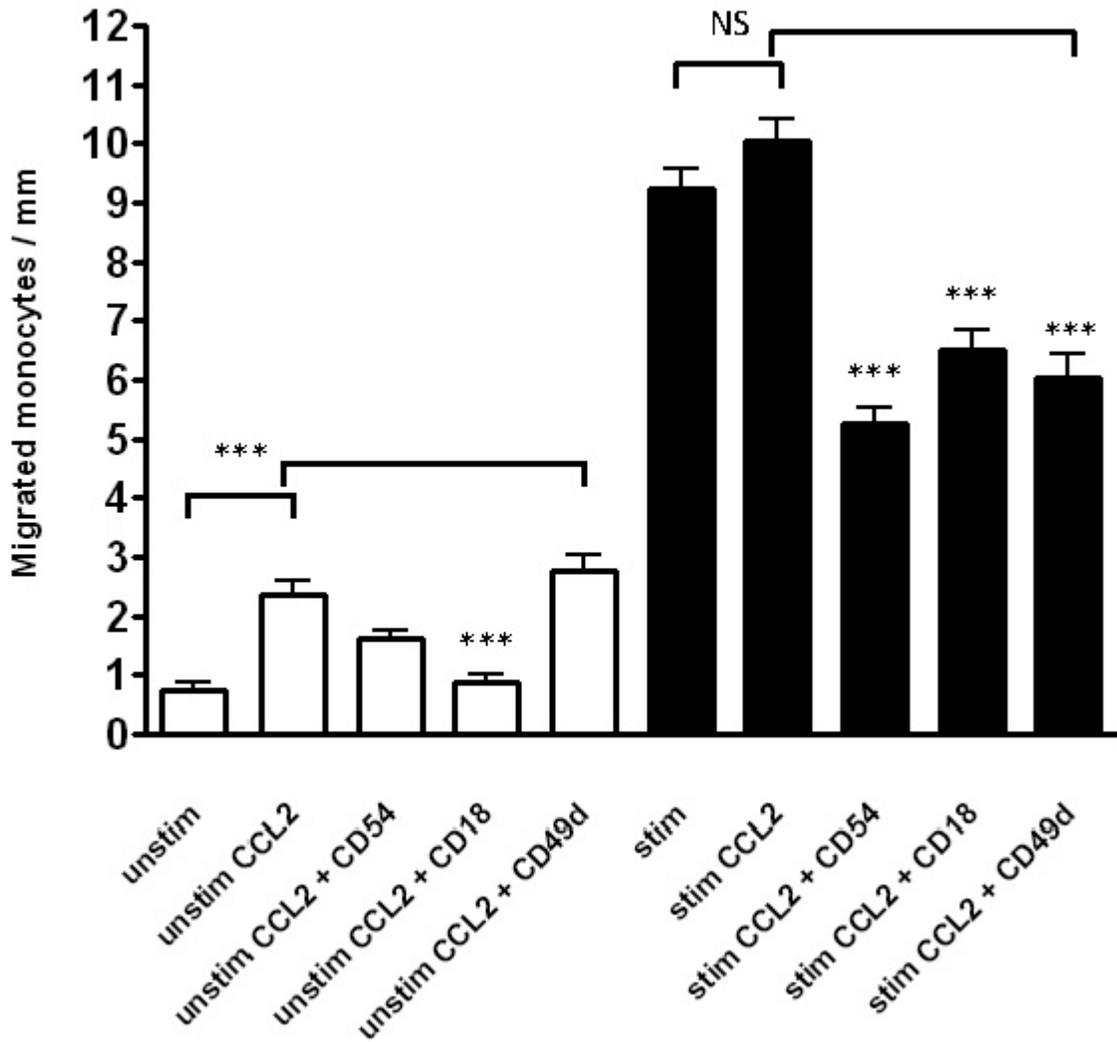


Figure 43 Monocyte migration across resting or cytokine-treated HBMEC in the presence of CCL2 and blocking antibodies.

Cytokine-treatment of HBMEC significantly increases monocyte migration. CCL2 increases migration of monocytes across resting HBMEC and this is significantly reversed by anti-CD18 blocking antibody. Blocking of CD49d did not reduce monocyte migration across resting HBMEC monolayers in the presence of CCL2 gradient. All 3 blocking antibodies effectively reduced migration of monocytes across cytokine-treated HBMEC in the presence of CCL2 gradient.

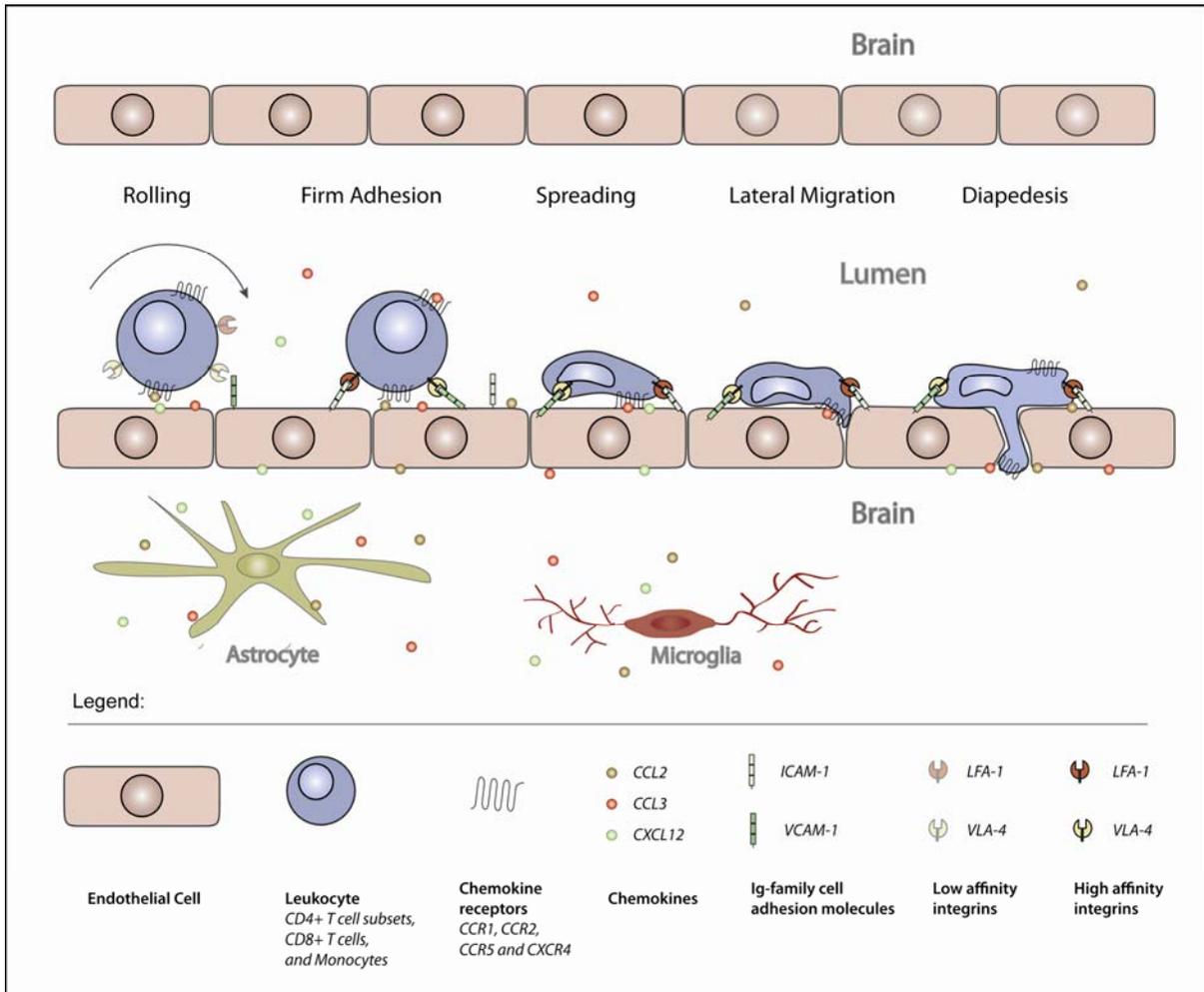


Figure 44 Proposed hypothesis for leukocyte transmigration across the human BBB in response to chemokines.

Rolling of leukocytes is primarily mediated by the selectins which slow down the circulating leukocytes and allow contact with the endothelium. The rolling leukocytes can then be activated by chemokines bound to the apical surface of the endothelium via G protein coupled receptors. Upon activation, integrins on leukocytes change into a high affinity state and by binding to their corresponding ligands on endothelial cells, they lead to firm adhesion to the endothelium. Firm adhesion is followed by spreading of the leukocytes. They then crawl along the luminal surface of the endothelium, seeking preferred sites of transmigration. Finally, monocytes or T cell subsets diapedese across the cerebral endothelium via a paracellular or a transcellular route (not shown). Thus, chemokines appear to play an important role in mediating the adhesion and migration of monocytes and T cell subsets across the BBB.

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APPENDIX – CERTIFICATES OF ETHICS APPROVAL



The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver,
BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

| | | |
|---|---|--------------------------------------|
| PRINCIPAL INVESTIGATOR: Katerina Dorovini-Zis | DEPARTMENT: UBC/Medicine, Faculty of/Pathology & Laboratory Medicine/Neuropathology | UBC CREB NUMBER: H97-70439 |
| INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT: | | |
| <small>Institution</small> | <small>Site</small> | |
| Vancouver Coastal Health (VCHRI/VCHA) Children's and Women's Health Centre of BC (incl. Sunny Hill) Providence Health Care Other locations where the research will be conducted: N/A | Vancouver General Hospital Children's and Women's Health Centre of BC (incl. Sunny Hill) St. Paul's Hospital | |
| CO-INVESTIGATOR(S): N/A | | |
| SPONSORING AGENCIES: - Multiple Sclerosis Society of Canada - "Human Cerebral Endothelium-Lymphocyte Interactions in Immune-Mediated CNS Disease" - Multiple Sclerosis Society of Canada - "Human cerebral endothelium-lymphocyte in immune-mediated CNS diseases" | | |
| PROJECT TITLE: Human Cerebral Endothelium-Lymphocyte Interactions in Immune-Mediated CNS Disease | | |

EXPIRY DATE OF THIS APPROVAL: January 5, 2010

APPROVAL DATE: January 5, 2009

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by :

**Dr. Stephen Hopton
Cann , Associate Chair**



The University of British Columbia
 Office of Research Services
 Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver,
 BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

| | | |
|---|---|--------------------------------------|
| PRINCIPAL INVESTIGATOR: Katerina Dorovini-Zis | DEPARTMENT: | UBC CREB NUMBER: H04-70561 |
| INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT: | | |
| <small>Institution</small> | <small>Site</small> | |
| Vancouver Coastal Health (VCHRI/VCHA) | Vancouver General Hospital | |
| Children's and Women's Health Centre of BC (incl. Sunny Hill) | Children's and Women's Health Centre of BC (incl. Sunny Hill) | |
| Other locations where the research will be conducted: N/A | | |
| CO-INVESTIGATOR(S): N/A | | |
| SPONSORING AGENCIES: - Multiple Sclerosis Society of Canada - "Entry of Monocytes and Dendritic Cells into the Brain: Regulation by Endothelial Cell Adhesion Molecules and Chemokines" | | |
| PROJECT TITLE: Entry of Monocytes and Dendritic Cells into the Brain: Regulation by Endothelial Cell Adhesion Molecules and Chemokines | | |

EXPIRY DATE OF THIS APPROVAL: November 10, 2009

APPROVAL DATE: November 10, 2008

CERTIFICATION:
In respect of clinical trials:
 1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
 2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
 3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by

**Dr. Stephen Hopton
 Cann, Associate Chair**