BETA CHEMOKINE EXPRESSION AND REGULATION OF LYMPHOCYTE TRAFFIC AT THE BLOOD-BRAIN BARRIER

by

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ABSTRACT

The blood-brain barrier (BBB) formed by the endothelial lining of the cerebral vasculature contributes to the relative immunologic isolation of the brain. The BBB is compromised in ischemic, traumatic, infectious, inflammatory and autoimmune diseases resulting in increased permeability and leukocyte entry into the CNS. The chemokines RANTES (regulated upon activation normal T cell expressed and secreted) and MIP-1β (macrophage inflammatory protein 1β) have been implicated in the subset-specific chemoattraction and activation of memory and activated CD4+ T lymphocytes respectively. The presence of these chemokines in inflammatory brain lesions suggests a role in subset-specific lymphocyte trafficking which has not been previously explored.

The objective of this study was to characterize the expression of RANTES and MIP-1β by human brain microvessel endothelial cells (HBMEC) in vitro and to investigate their role in mediating T cell adhesion and migration across HBMEC monolayers. HBMEC were induced by pro-inflammatory cytokines and lipopolysaccharide (LPS) to secrete and bind to their surface RANTES and MIP-1β, suggesting chemokine availability for interactions with lymphocytes. The ability of RANTES and MIP-1β to attract specific lymphocyte subsets across HBMEC monolayers was studied in a double chamber chemotaxis system. After establishing chemokine gradients across the monolayers, resting, anti-CD3 activated, memory and naïve CD4+ T cells were added to the upper chamber and incubated with HBMEC for one to three hours. Concentration gradients of RANTES or MIP-1β stimulated significant in vitro adhesion and migration of resting, memory, antigen-specific, and activated T cells but not naïve T cells. Chemotaxis was observed only in the presence of cytokine-activated HBMEC. The chemokine

enhanced adhesion and migration were dependent upon integrins and EC adhesion molecules. Thus, neutralizing antibodies (mAb) to intercellular adhesion molecule-1 (ICAM-1) significantly reduced adhesion and migration of both resting and activated CD4+ T cells. mAb blocking of vascular cell adhesion molecule-1 (VCAM-1) and α4 integrin (the α subunit of VLA-4) also reduced the chemokine-enhanced adhesion and migration of activated T cells.

These studies indicate that RANTES and MIP-1β have chemoattractant as well as activating effects on T lymphocyte subsets, suggesting an important role of these chemokines in regulating lymphocyte subset-specific trafficking across the BBB.

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LIST OF ABBREVIATIONS

Ab.....antibody

Ag.....antigen

ANOVA .....analysis of variance

APC.....antigen presenting cell

BBB.....blood brain barrier

BM .....basement membrane

CNS.....central nervous system

CSF .....cerebrospinal fluid

DAB .....3,3'-diaminobenzidine

EAE.....experimental allergic encephalomyelitis

EC .....endothelial cell

ECGS .....endothelial cell growth supplement

ECM.....extracellular matrix

EM .....electron microscopy

FMLP .....formyl-methionyl-leucyl phenylalanine

FN .....fibronectin

GAG.....glycosaminoglycan

HBMEC .....human brain microvessel endothelial cell

HDMEC .....human dermal microvessel endothelial cell

HRP.....horseradish peroxidase

HUVEC.....human umbilical vein endothelial cell

ICAM .....intercellular adhesion molecule

IFN .....interferon

Ig .....immunoglobulin

IGSS .....immunogold silver staining

IL-1 .....interleukin-1

IL-8 .....interleukin-8

ISF .....interstitial fluid

LFA .....leukocyte function-associated antigen

LGL .....large granular lymphocytes

LPS .....lipopolysaccharide

LTB_4 .....leukotriene B_4

mAb .....monoclonal antibody

MCP .....monocyte chemoattractant protein

MHC .....major histocompatibility complex

MIP-1β .....macrophage inflammatory protein

MMP .....matrix metalloproteinase

MS .....multiple sclerosis

NAWM .....normal appearing white matter

NF-κB .....nuclear factor κB

NK .....natural killer

PBMC .....peripheral blood mononuclear cell

PBTC .....peripheral blood T cell

PECAM .....platelet/endothelial cell adhesion molecule

PF4 .....platelet factor 4

PMN.....polymorphonuclear leukocyte

PTX.....pertussis toxin

RANTES.....regulated upon activation, normal T cell expressed and secreted

RT-PCR .....reverse-transcriptase polymerase chain reaction

SIV .....simian immunodeficiency virus

TEM.....transmission electron microscopy

TNF .....tumor necrosis factor

VCAM.....vascular cell adhesion molecule

VLA .....very late activation antigen

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CHAPTER 1 INTRODUCTION

1.1 INFLAMMATION IN THE CENTRAL NERVOUS SYSTEM

1.1.1 A question of immune privilege

For years, scientists had regarded the brain as an immune privileged site, not normally accessible to leukocyte traffic. This conclusion was drawn based on a number of historical observations that many molecules thought to be required for inflammatory responses were not present within the central nervous system (CNS). Throughout the rest of the human body, inflammation is characterized by well established pathological processes responsible for the four classical local signs of inflammation: redness and heat from increased blood flow secondary to vascular dilatation at the site of challenge, swelling due to increased permeability to plasma and leukocytes, and pain due to mechanical compression of sensory nerves and the action of locally released inflammatory mediators by cells recruited to the battle. Numerous molecules and cellular processes are involved in this initial recruitment of cells which will respond to the injury or infection by destroying pathogens, removing debris, and aiding in wound repair. If the initial mobilization of leukocytes is not successful, then a second phase of the immune response will be initiated, calling for cells which respond in a more specific manner.

Within the CNS however, many crucial factors seemed to be absent. Most notably, very few immunocompetent cells such as lymphocytes were detected by histologists: indeed they were extremely rare in the brain (Hauser et al., 1983; Traugott et al., 1983). In addition, major histocompatibility (MHC) molecules, normally expressed on professional antigen presenting cells (APC) and required for antigen presentation and initiation of antigen-dependent immune responses, were undetectable. Accompanying the lack of immune cells was the absence of a

conventional lymphatic system within the brain. These in situ observations suggested that the CNS was anything but a hot spot for antigenic stimulation, and that immune responses were in some way restricted.

Historical *in vivo* studies supported this notion of immune privilege. Early in this century studies of transplantable tumors showed that intracerebral grafts were well tolerated compared to those transplanted into the skin (Medawar 1948; for a historical review, Bjorklund and Stenevi, 1985). Additional studies by Theiler supported immune privilege in the CNS with observations that the yellow fever virus survived quite well when injected into the brains of mice, such that this became a widely-used method for virus propagation (Theiler 1937).

Although there remains considerable truth to these early theories of a restricted immune response in the CNS, the idea that the brain is completely immunologically inert is no longer tenable. It has been shown that grafts into the brain are indeed rejected in a manner similar to those in extracerebral organs (Scheinberg et al., 1966) and that numerous viruses do indeed elicit immune responses within the CNS. There can be no doubt that the nervous system and immune system do in fact regularly communicate. At times appearing frankly unresponsive, the immune response in the CNS can change and come full circle in terms of complete immune readiness and response. When one considers autoimmune disorders such as multiple sclerosis (MS) or its animal model experimental allergic encephalomyelitis (EAE) which are characterized by inflammation and associated myelin destruction, it is difficult to imagine the notion of immune privilege (Lucchinetti et al., 1996). In addition, trauma (Olsson et al., 1992; Holmin et al., 1998), cerebral ischemia (Chopp et al., 1994; Clark et al., 1991), dementia related to acquired immune deficiency syndrome (AIDS) (Gendelman et al., 1994), other infections (Johnson et al., 1984) and even neurodegenerative disorders such as Alzheimer's or Parkinson's Disease (Kalaria et al., 1993; McGeer et al., 1988; Rogers 1995; Akiyama 1994) are all believed to have an immune

component. They share the presence of inflammatory infiltrates and resident immune cells whose products and stimulatory molecules serve to initiate, modify and enhance an immune response.

The previous observations merely stated the relative isolation of the CNS from the general immune system. While it was once believed that the efferent arm of the immune response was blocked by the barrier which restricted blood to brain passage, it was now clear that numerous cells could indeed enter the CNS. In addition, the concept of an afferent arm was confirmed when investigators found that protein injected into the brains of laboratory animals could be detected in the cervical lymphatics. Brain interstitial fluid (ISF) is linked to the cerebrospinal fluid (CSF) through special channels consisting of perivascular spaces or Virchow-Robin spaces which surround the vessels that penetrate the brain (reviewed in Bradbury 1985); it is believed that the secretion of interstitial fluid by the cerebral EC accompanied by the transport of water and solutes into the brain is the driving force for the ISF to join the CSF. From the subarachnoid space, ISF and CSF drain either into dural sinus blood or along certain cranial nerves to extracranial regions, thereafter entering capillaries or lymphatics respectively.

1.1.2 Acute versus chronic inflammation

Acute inflammation is typically characterized by polymorphonuclear cell (PMN) infiltrates in which the predominant type is the neutrophil. PMNs migrate into the CNS and form perivascular "cuffs" for 1-2 days following trauma, ischemia, or infection by bacteria or viruses. In these instances, PMNs invade where substantial destruction or necrosis has occurred or bacteria or fungi are present; this is associated with a breakdown of the barrier restricting blood to brain trafficking where products of microbial breakdown or complement components attract leukocytes into the brain. Here they phagocytose organisms and release numerous destructive enzymes and free radicals. This response to microorganisms within the CNS is not significantly

different from that seen extracerebrally. If unsuccessful in resolving the insult, the nature of the immune response changes. After four or five days, monocytes/macrophages, lymphocytes and plasma cells infiltrate the same perivascular space, replacing the PMNs to fight off infection; plasma cells produce antibodies that allow for opsonization of the organisms which macrophages then phagocytose. This initiates the chronic stages of inflammation as antigen presentation initiates the cascade of events and mononuclear cells are now primarily responsible for fighting off and isolating the invader.

Chronic inflammation is a more specific type of immune response occurring beyond 24 to 48 hours after the initial insult. It is governed by antigen presentation and interactions amongst various mononuclear cell types. During viral infection of the CNS, the T cells are responsible for recognizing and localizing the foreign antigen for which they are specific. In viral encephalitides, T lymphocytes are a major constituent of the inflammatory infiltrate.

The major difference in autoimmune diseases such as EAE or MS, is that the antigen is no longer foreign but self or presumed to be self, the immune response resulting in severe inflammation along with demyelination. In acute MS, the lesion is infiltrated by mononuclear phagocytes as well as CD4+ and CD8+ T lymphocytes (Hauser et al., 1986; Traugott et al., 1983). In chronic MS, numerous lymphocytes as well as some plasma cells and macrophages form such perivascular "cuffs". PMNs are virtually absent (Raine 1991). In addition to the roles played by leukocytes from the circulation, cells native to the brain also play an important role in mediating the initiation and progression of CNS inflammation.

1.2 IMMUNOLOGICALLY COMPETENT CELLS OF THE CNS

Qualities which distinguish the CNS as different from other tissues immunologically include the presence of specialized cells, native to the CNS, which can be called upon to initiate

an effective immune response. Dendritic cells, regularly present in tissues throughout the body as the classic antigen presenting cell, capturing antigen and presenting it to T cells to initiate an immune response, are not present in the CNS. However, several cells within the brain including microglia, astrocytes, perivascular cells and the cerebral endothelium are all believed to contribute immunologically in some way during CNS inflammation.

1.2.1 Microglia

Each tissue of the body has its own resident macrophage, cells which are the first line of defense against insult or injury; the resident macrophage in the CNS is the microglial cell. These cells are highly specialized macrophages with the ability to process and present antigen (Hickey and Kimura, 1988) and are considered to be facultative APC in the CNS, since class II MHC required for this process may be induced or upregulated. Microglia proliferate and express class II MHC molecules early in inflammation (Gehrmann et al., 1992). In addition, microglia produce numerous cytokines and chemoattractant cytokines and express important cell surface molecules involved in cell to cell adhesion (reviewed in Perry et al., 1995).

1.2.2 Astrocytes

Another important glial cell in the inflammatory response is the astrocyte. Astrocytes are considered the buffer cells of the CNS: these cells respond quickly to injury such as trauma or ischemia both spontaneously and also following stimulation with factors produced by the cells which are often affected most quickly in these conditions, the neurons. Astrocytes typically respond to injury by proliferation and hypertrophy, also termed gliosis. In addition to proliferation, astrocytes that have experienced insult are an important source of cytokines in the

CNS. Numerous cytokines and other soluble molecules are produced by astrocytes during inflammation, many of which are believed to be important in initiating and maintaining an immune response. The ability of astrocytes to present antigen, the hallmark characteristic of immune initiation, has been studied extensively. Inflammatory cytokines typically present in the milieu of an immune response have been shown to upregulate MHC class I molecules (Lavi et al., 1988) and to enhance MHC class II expression (Benveniste et al., 1989) by astrocytes. Others have failed to duplicate these results, or have shown that the presence of these molecules was not sufficient to efficiently present antigen.

1.2.3 Perivascular cells

Perivascular cells which surround the endothelium have recently been suggested to be key mediators of the immune response: their phagocytic capacity has been documented in trauma, ischemia and stroke (Jeynes 1985; Tagami et al., 1991). Further evidence for immune interactions by these cells includes the constitutive expression of low levels of the cell adhesion molecules (CAMs) VCAM-1 and ICAM-1 (Verbeek et al., 1995). In addition to cytokine production by these cells, the observation of interferon-γ (IFN-γ) induced upregulation of MHC class II molecules on perivascular cells in rat brain suggests a potential to present antigen and activate T cells, thus playing a potential role in the initiation of inflammation (Fabry et al., 1993a; Fabry et al., 1993b; Balabanov and Dore-Duffy, 1998). Additionally, EAE has been initiated in mice where the only MHC-compatible APC present in the CNS were perivascular macrophage-like cells (Hickey and Kimura, 1988; Hinrichs et al., 1987).

1.2.4 Endothelial cells

The cerebral endothelium is believed to play a key role in inflammation of the brain: as the primary cell in contact with immune cells in the circulation, cerebral endothelial cells (EC) are uniquely positioned to mediate immune cell trafficking into the brain. EC have been shown to de novo express class II MHC following stimulation with IFN-γ, in addition to expressing molecules important for leukocyte adhesion and migration (Huynh and Dorovini-Zis, 1993; Traugott et al., 1985; Stins et al., 1997; Wong and Dorovini-Zis, 1992). In vivo studies have indicated MHC class II localization to cerebral EC in MS autopsy specimens and mice during EAE, although reports suggest variability and apparent intermittence in positive staining (Traugott et al., 1986; Sakai et al., 1986).

1.3 THE BLOOD-BRAIN BARRIER (BBB)

Early experiments using trypan blue injection into the CSF or into the circulation first demonstrated that some form of barrier existed between the blood and the brain, as dye injected into one space was not later detected in the other (Ehrlich 1885). With the advent of the electron microscope, the anatomical basis of the BBB has been localized to the endothelium lining the vasculature of the brain (Reese and Karnovsky, 1967; Brightman and Reese, 1969) with two important morphological features that define the barrier properties unique to these endothelial cells (EC): 1) the presence of tight junctions with a high electrical resistance that prevent paracellular passage of ions, proteins [including antibodies/immunoglobulins (Ig)], and circulating blood cells into the brain, and 2) the paucity of cytoplasmic vesicles, most of which are involved only in pinocytosis. In addition, cerebral EC possess various specific transport proteins such as glucose and amino acid transporters which serve to ferry molecules essential for brain function across the BBB and into the brain (Pardridge and Oldendorg, 1975 & 1977).

Increased permeability of the BBB is considered a major event in CNS inflammation, and is noted in response to trauma, in ischemia, infection, and in autoimmune demyelinating diseases such as MS and its experimental model EAE (Holmin et al., 1995; Nag et al., 1997; Albayrak et al., 1997; Claudio et al., 1995). This barrier can be opened experimentally by hyperosmotic solutions such as urea or arabinose which shrink the endothelium and reversibly open the tight junctions making them permeable to ionic lanthanum as well as macromolecules such as horseradish peroxidase (HRP) (Dorovini-Zis et al., 1983; Brightman et al., 1973). *In vitro* studies confirmed that the interendothelial tight junctions do open in response to treatment with hyperosmotic media, and in a reversible manner (Dorovini-Zis et al., 1987). Tight junction permeability can be measured using either electron dense tracers such as HRP or by directly measuring the electrical resistance across a microvessel (*in vivo*) or a monolayer of cultured cells (*in vitro*). Electrical resistance measurements of cerebral microvessels in the rat are at least 1,500 to 2,000 $\Omega \cdot cm^2$ and are similar to the electrical resistance of the tightest epithelium (Bradbury, 1993). *In vitro* models using cerebral EC cultures exhibit resistances upwards of 200 $\Omega \cdot cm^2$ in comparison to large vessel EC *in vitro* which have a much lower electrical resistance at 35 $\Omega \cdot cm^2$ or non-BBB microvessel EC at 69 $\Omega \cdot cm^2$ (our observations; Furie et al., 1984).

Other factors known to increase the permeability of cerebral endothelium both *in vitro* and *in vivo* include pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and IFN-γ as well as lipopolysaccharide (LPS) (Burke-Gaffney and Keenan, 1993). TNF-α and IFN-γ increase monolayer permeability by increasing the permeability of tight junctions. In addition, LPS increases the size and number of endocytotic profiles of brain microvascular EC compared to untreated EC (Defazio et al., 1997).

Insight into the mechanisms regulating BBB permeability is not only important in understanding the process of CNS inflammation in an attempt to control it, but also in

investigating novel approaches to drug delivery to the brain for therapeutic purposes and in studying the basic exchange of peptides, proteins, nutrients and ions between the brain and the circulation.

1.4 LEUKOCYTE TRAFFICKING IN THE CNS

Inflammatory diseases of the CNS are characterized by increased leukocyte trafficking across the BBB and infiltration of the neuropil by inflammatory cells. Perivascular lymphocytic infiltrates are associated with cytokine release, edema formation and autoimmune demyelination in MS and EAE (Zamvil et al., 1990; Kennedy et al., 1987; Kalman et al., 1995). The factors that control the entry of leukocytes into the brain, across the BBB, during CNS inflammation have not been fully investigated. Several studies suggest that trafficking across the cerebral endothelium follows the accepted paradigm for trafficking elsewhere, which involves leukocytes rolling along the endothelium, regardless of their antigen specificity. Rolling may be followed by adhesion of these cells to the EC if the proper adhesion molecules are present, that is to say, the EC surface is conducive for interactions with leukocytes. Migration across the EC may then occur if a chemotactic gradient exists to draw cells into the tissue or the state of cell activation allows for T cell migration; if the trafficking signals on the endothelium or the leukocytes are insufficient, the cells may simply return to the circulation.

1.4.1 Phenotype of infiltrating cells

Throughout the human body different types and subsets of immune cells are able to traverse the endothelium with little restriction, acting as a sweeping surveillance. In contrast, it has been shown that not all T cells can readily access the CNS. Recent studies have shown that

resting T cells that have not recently encountered the antigen for which they are specific cannot cross an intact BBB. Conversely, T cells which have been activated are able to cross an intact BBB (Hickey et al., 1991; Male 1995), particularly those which are neuroantigen-specific (Cross et al., 1990). As such, it seems that an intact BBB keeps resting T cells at bay, and that the activation state and subset of the immune cell may determine its ability to access the CNS.

1.4.2 T cell subsets

T cells are divided empirically into two major subsets, CD4 (helper) or CD8 (cytotoxic), based on expression of cell surface proteins. CD4+ T cells are associated with MHC class II restricted helper-inducer activity and the secretion of cytokines which mediate their regulatory T cell functions. CD8+ T cells mediate direct cytotoxicity which is MHC class I restricted. Often included with the description of CD8 + T cells are the natural killer cells (NK). Historically, NK cells were cells grouped on the basis of natural killer properties: the ability to kill the target without prior exposure, restriction by MHC or involvement of other cells. Some cells of the CD8+ lineage and monocytes display this NK activity, although the major phenotype which has been most associated with this NK activity is a population of large granular lymphocytes (LGL). T cell subsets are often distinguished according to their activation state. Resting T cells are morphologically small, and don't express any typical T cell activation molecules. Once T cells encounter their antigen and become activated, they express the early activation marker CD69 (reviewed in Ziegler et al., 1994) within 24 hours; after 24 to 48 hours, T cells begin to express receptors for the autocrine cytokine IL-2, identified as the CD25 marker (review Allouche et al., 1990). Activated T cells also enter the blast phase and upregulate their expression of integrins, cytokines, and other cell adhesion molecules as well as matrix metalloproteinases (MMPs), enzymes which digest components of the extracellular matrix and are vital in leukocyte

trafficking (Romanic and Madri, 1994). A major event in converting T cells into a motile phenotype (characteristic of activated T cells) is a shift in cell polarity: cells show a leading edge with high sensitivity to antigen and chemoattractant cytokines, and a cytoplasmic projection termed a uropod which protrudes in the opposite direction to that of locomotion and serves to facilitate interactions with other T cells. Several cell adhesion molecules (CAMs) such as ICAM-3, ICAM-1 and CD44 have been localized to these uropods, and are believed to play an important role in the recruitment of other lymphocytes to inflammatory foci (reviewed in del Pozo et al., 1996)

T cells can also be divided in terms of naïve or memory cells. Naïve cells are those which have not encountered the antigen for which they are specific and are high in the expression of CD45RA at their cell surface. They are CD44 low and L selectin high, and readily undergo lymphocyte homing to lymph nodes (Mackay, 1993). Memory cells have lost the CD45 RA isoform and through activation have entered the blast phase; they exhibit an RA- or RO phenotype which is L selectin negative and denotes a cell which readily migrates to remote organs. Memory subsets show increased expression of CD44, VLA-4, -5, -6 and increased LFA-1, CD2 and LFA-3 expression (Shimizu et al., 1990; Sanders et al., 1988; Budd et al., 1987). Memory cells respond quickly to subsequent antigen exposure. While most distinctions made between naïve and memory are loosely based on phenotypic markers, it has been suggested that a better distinction is made on the basis of the activation requirements for each (Kedl and Mescher, 1998; Gause et al., 1997).

1.4.3 Th1 and Th2 responses

Following stimulation with cytokines or activation signals, naïve CD4+ helper T cells (Th0) differentiate into one of two subsets: Th1 or Th2. These subsets are quite distinct in

murine models, but less distinct in humans where they are distinguished on the basis of cytokine secretion profiles. Th1 cells and Th1 type responses are typically associated with delayed hypersensitivity type reactions or cytotoxic functions and involve the production of IFN-γ, IL-2 and IL-12. Th2 responses are involved more in humoral immunity and involve CD4+ T cells stimulated with IL-4 and possibly IL-10. Th2 cells secrete the cytokines IL-10, IL-4, and IL-5 which are generally downregulators of immune responses in addition to promoting B cell activation and class switching (Hsieh et al., 1992; Schmitt et al., 1994; reviewed by Seder and Paul, 1994).

It is apparent that the cells of each subset vary in their responsiveness to antigen and effector functions, thus one recognizes the importance of regulating the recruitment of these different immune cells to effect their varied functions, particularly within the CNS. Evidence accumulates for some sort of cross talk between cells of the brain, the vasculature, and of the immune system, cross talk that exists in both soluble and non-soluble forms.

1.5 CELL ADHESION MOLECULES

Cell adhesion molecules are the "sticky stuff" in the interactions between immune cells and the endothelium. They are the traffic signals which, when displayed by endothelium, are the key mediators of leukocyte extravasation. As a whole, they can be divided into three classes: selectins, integrins, and immunoglobulins.

1.5.1 Selectins

Selectins are a group of cell surface glycoproteins that support leukocyte adhesion through the recognition of carbohydrates. The group comprises three molecules: 1) L selectin is

expressed on circulating leukocytes. 2) P selectin is stored within EC and platelets. Thrombin or histamine, acute mediators of inflammation, cause the rapid transport of intracellular P selectin to the cell membrane for interaction with leukocytes. 3) E selectin is expressed on the surface of EC following stimulation with pro-inflammatory cytokines such as tumor necrosis factor, interleukin-1 or lipopolysaccharide. Selectins mediate the rolling and initial tethering of leukocytes flowing in circulation to the endothelial surface as they interact with their ligands: these are typically a sialylated carbohydrate or mucin such as isomers of sialyl Lewis x for P and E selectin, in addition to the binding of L selectin to at least two known mucins, CD34 and glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1).

Members of this family such as L selectin have been suggested to have a role in the immune response within the CNS (Huang et al., 1991). The detection of its receptor in myelinated areas of the CNS but not in the periphery may suggest an important role in the formation of inflammatory lesions.

1.5.2 Integrins and Immunoglobulins

Integrins are found on most cell types and facilitate cell-cell and cell to extracellular matrix (ECM) interactions. Acting in cooperation with selectins, integrins mediate the firm adhesion of leukocytes to EC, the next step in the attempt of a leukocyte to extravasate into the tissue. The cell to cell contacts which are formed also contribute to effective antigen presentation and generation of cytotoxicity. Integrins consist of an α and a β subunit which combine to form perhaps the most versatile of the cell adhesion molecules. Combinations of α subunits L, M, x, and 4 combine with β subunits 1, 2, and 7 to form five key integrins involved in leukocyte/EC interactions: LFA-1 ($\alpha L\beta 2$), Mac-1 ($\alpha M\beta 2$), p150,95 ($\alpha x\beta 2$) and the $\alpha 4$ integrins: VLA-4

($\alpha 4\beta 1$) and VLA-7 ($\alpha 4\beta 7$). LFA-1, VLA-4 and VLA-7 are all expressed on T lymphocyte subpopulations.

The function of integrins requires activation via cross-linking of a variety of cell surface molecules and serves to increase integrin adhesiveness within a few minutes; these changes do not result from increased surface expression of the integrins, but rather a state of higher affinity for ligands on EC or ECM.

Ligands for integrins consist of members of the immunoglobulin (Ig) superfamily which are expressed on endothelium (reviewed by Bevilacqua 1993). Intercellular adhesion molecule-1 (ICAM-1) was the first to be identified as the ligand for LFA-1 on endothelium and set a paradigm for other integrin-Ig interactions. ICAM-1, 2, and 3 are all distinct products of separate genes, each with the ability to bind LFA-1 (Rothlein et al., 1986; Staunton et al., 1989; Fawcett et al., 1992). ICAM-1 is induced on EC as well as several other cells by inflammatory cytokines including TNF-α, IFN-γ, IL-1β, and LPS. ICAM-2 is constitutively expressed, suggesting a role in leukocyte trafficking to non-inflamed tissues. Leukocytes are the only cell type that expresses ICAM-3, which is an important molecule in leukocyte-leukocyte interactions.

Vascular cell adhesion molecule-1 (VCAM-1) is another inducible CAM present on the surface of endothelial cells following treatment with pro-inflammatory cytokines. VCAM-1 interacts with VLA-4 and also with VLA-7: VCAM has two separate binding sites for $\alpha 4$, one which is activation dependent, and the other which is activation independent (Needham et al., 1994; Kilger et al., 1995).

1.5.3 Integrins and immunoglobulins in CNS inflammation

The key cell adhesion molecules involved in T cell/EC interactions within the CNS are believed to be the ligand pairs ICAM-1/LFA-1, and VCAM-1/VLA-4. VLA-4 is expressed primarily on infiltrating lymphocytes (T and B) in addition to macrophages and monocytes; VCAM-1 is expressed on activated endothelium. LFA-1 is expressed on leukocytes including lymphocytes or monocytes/macrophages as well as microglia, whereas ICAM-1 is expressed on endothelium, T and B cells, monocytes/macrophages, microglia as well as astrocytes (reviewed in Archelos and Hartung, 1997).

Trafficking into the CNS appears to rely heavily on the interactions of VLA-4 with VCAM, as antibodies to VLA-4 ameliorated disease in EAE (Keszthelyi et al., 1996). Lymphocytes infiltrating the CNS during inflammation also display VLA-4; this has been confirmed in studies showing infiltrating cells binding to VCAM-1, but not MAdCAM-1 (the ligand for $\alpha 4\beta 7$) (Engelhardt et al., 1995). An association has also been shown between VLA-4 or $\alpha 4$ integrin expression and the ability of myelin basic protein (MBP) specific T cells to enter the brain and have encephalitogenic properties (Kuchroo et al., 1993; Baron et al., 1993).

In addition, ICAM-1 has been shown to be upregulated on cerebral EC in EAE in comparison to its normally low expression on resting EC (Cannella et al., 1990). Also, antibodies to LFA-1, which is believed to play an important role in the adhesion of lymphocytes to the cerebral endothelium, reduced the severity of disease in EAE (Gordon et al., 1995). The importance of ICAM-1/LFA-1 in mediating adhesion between lymphocytes and cerebral endothelium has been documented (Male et al., 1994).

Accompanying their role in immune cell trafficking, LFA-1/ICAM-1 and VLA-4/VCAM-1 are also believed to play an active role in mediating antigen presentation, acting as accessory molecules during the interaction between APC and T cells (Damle and Arufo, 1991,

Damle et al., 1992, VanSeventer et al., 1990). This suggests an additional role for these molecules in governing the initiation of inflammation within the CNS.

1.6 LIPOPOLYSACCHARIDE AND CYTOKINES AS INFLAMMATORY MEDIATORS IN THE CNS

The first soluble mediators of cell to cell communication were termed the cytokines, small molecules which allowed for the temporal and spatial control of an immune response. Throughout the 70's it was generally understood that these factors were produced during interactions between monocytes and lymphocytes, however it is apparent today that a variety of hematopoietic and non-hematopoietic cells have the ability to produce these cytokines in addition to expressing their receptors, thus setting the stage for an inflammatory response. Tumor necrosis factors (TNF), the interleukins (IL) as well as the interferons (IFN) are among the most prevalent cytokines implicated as initiators of the immune response in the CNS. Their synthesis in the brain can be triggered by numerous stimuli including peripheral infection/endotoxin; CNS infection such as meningitis, malaria or HIV; trauma, ischemia, autoimmune disorders such as multiple sclerosis (MS) as well as in Alzheimer's disease (for review, Hopkins and Rothwell, 1995). Expression of each has been shown to correlate with induction of EAE and the progression of MS (Benveniste, 1995).

1.6.1 Lipopolysaccharide (LPS)

Lipopolysaccharide is the endotoxin component of the bacterial cell wall which is released by living or degraded gram negative bacteria. LPS is the key to an immediate response to bacterial infection as several cells including monocytes and macrophages, PMNs, lymphocytes, fibroblasts and EC respond quickly to LPS, resulting in induction of cytokine expression, cell activation, and release of colony stimulating factors. Monocytes and

macrophages are particularly responsive to LPS, secreting TNF, IL-1, prostaglandins and leukotrienes. LPS also activates the complement pathway; alternate and classical pathways are set in motion, along with the production of anaphylatoxins and chemoattractants which enhance the immune response. Endothelial responses to LPS include de novo expression or upregulation of cell adhesion molecule, chemokine and cytokine expression. The high cytotoxicity associated with LPS is largely due to the generalized and tumultuous nature of these responses in combination.

In vivo levels of LPS range widely from less then 25 μg to 260 μg/ml in plasma of meningitis patients and up to 210 to 260,000 μg/ml in patients with septicemia; concentrations are believed to be much higher within tissues (Brandtzaeg et al., 1989).

Several studies have traced the effects of LPS *in vivo* following injection directly into the brain or *in vitro* after the addition of LPS to cultures. Intracranial injection of LPS into mice rapidly upregulated the adhesion molecules ICAM-1 and VCAM-1 on cerebral endothelium (Bell and Perry, 1995), results which have been simulated *in vitro* (Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995).

1.6.2 Tumor Necrosis Factor α (TNF-α)

TNF was initially described as a soluble factor derived from monocytes which had cytotoxic effects on tumor cells (Imamura et al., 1987). Activated monocytes and macrophages are the major sources of TNF, however a wide variety of cell types including activated T cells, astrocytes, microglia and epithelium (Beutler et al., 1985) are also potential sources of this immediate early response gene which exists in two separate forms, TNF-α and TNF-β. The effectiveness of TNF-α as an inflammatory mediator is evident from its involvement in the

pathogenesis of endotoxic shock, cerebral malaria, rheumatoid arthritis, and multiple sclerosis. The local and immediate production of TNF-α is essential in fighting off many bacterial pathogens, but its uncontrolled expression can have deleterious consequences such as septic shock. It is believed that numerous responses to TNF-α are mediated through the nuclear factor transcription system NFκB (Lowenthal et al, 1989).

Endothelial cells from different vascular beds respond in a variety of ways to stimulation by TNF-α (Cotran 1987; Pober and Cotran, 1990), including changes in coagulant and fibrinolytic properties, upregulation of cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, the induction of cytokine expression, and forming an overall "pro-adhesive" phenotype for leukocytes. Endothelial activation occurs following the binding of TNFα to the TNFR1 receptor on EC (Slowik et al., 1993; Mackay 1993) and the subsequent translocation of NFκB from the cytoplasm to the nucleus (Heller and Kronke, 1994).

The principal sources of TNF-α in the CNS are astrocytes and macrophages in addition to infiltrating mononuclear cells. In patients with AIDS dementia complex, TNF-α was also localized *in vivo* to perivascular macrophages and some microglial and endothelial cells (Seilhean et al., 1997). TNF-α has been shown to upregulate MHC class I molecules on astrocytes (Lavi et al., 1988) and act in synergy with interferon-γ to enhance MHC class II expression (Benveniste et al., 1989), thereby increasing the ability of the astrocyte to serve as an APC. TNF-α has numerous effects on the cerebral endothelium, including the production of other cytokines such as interleukins 1 and 6 (Frigerio et al., 1998) and the induction of several CAMs including ICAM-1, VCAM-1 and E-selectin (Stins et al., 1997, Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995; Wong and Dorovini-Zis, 1996a-b), thus mediating lymphocyte-EC interactions at the level of the blood-brain barrier. TNF-α increases the

permeability of cerebral endothelial cells *in vitro*, and is associated with increased blood-brain barrier permeability *in vivo* (Brett et al., 1989). TNF-α has been implicated in immune-mediated demyelination, where it's upregulation has been recorded before or during exacerbations of MS (Beck et al., 1988; Sharief and Hentges, 1991). As such, TNF-α appears to play a key role in mediating inflammatory responses within the CNS.

Levels of TNF-α during responses to infection or during inflammation appear to range greatly, from 68 U/ml to 500 U/ml in serum from patients with Kawasaki disease and Guillain-Barre syndrome, respectively (Matsubara et al., 1990; Sharief et al., 1993). TNF-α has also been shown to increase up to 15 U per mg of tissue following fluid-percussion trauma injury in rats (Taupin et al., 1993). These levels have set general margins for the use of TNF-α in *in vitro* studies of TNF-α mediated immune responses.

1.6.3 Interleukin-1 (IL-1)

The IL-1 family consists of two members, IL-1α and IL-1β, which share structural homology, a receptor, and biological properties. IL-1 is in many ways similar to TNF-α in that both are acute-phase proteins, and monocyte or macrophage-like cells are the major sources in addition to a variety of immune and non-immune cells such as lymphocytes, neutrophils, fibroblasts and endothelial cells (Dinarello, 1997; Akira et al., 1990; Poubelle et al., 1990). Many effector functions of IL-1 are also shared with TNF-α including wound healing, elimination of antigens, inflammation and vasodilatation (review by Schobitz et al., 1994). Effects upon vascular endothelium are also shared with TNF-α including induction of cell adhesion molecule and chemokine expression in addition to enhancement of pro-coagulant activity; however, the cytotoxicity of TNF-α is not shared with IL-1 (Mantovani and Dejana, 1989).

In the CNS, IL-1β has been shown to be produced by endothelial cells, pericytes, perivascular macrophages/microglia and astrocytes (for review Fabry et al, 1994). IL-1β has many effects similar to TNF-α, including upregulation of ICAM-1 on astrocytes (Frohman et al., 1989), in addition to the *in vitro* stimulation of astrocyte proliferation (Giulian and Lachman, 1985) and the complementary induction of gliosis following IL-1β injection directly into the brain (Giulian et al., 1988). IL-1 has been shown to enhance adhesion of leukocytes to cerebral EC (Libby et al., 1986) and to upregulate ICAM-1, VCAM-1 and E-selectin (Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995; Wong and Dorovini-Zis, 1996a,b). Unlike TNF-α, IL-1 does not increase cerebral endothelial permeability. IL-1β can however, upregulate the expression of TNF-α receptors on the surface of cerebral endothelium (Bebo and Linthicum, 1995).

In vivo, up to 3 U/ml of IL-1β were released into rat brain following mechanical trauma (Woodroofe et al., 1991) and up to 18 U/ml were detected in cortex homogenate following fluid-percussion trauma (Taupin et al., 1993).

1.6.4 Interferon-γ (IFN-γ)

The interferons were first described and assessed for their ability to interfere with viral replication, hence their name and the distinction of a biological unit of activity. Other activities have since been defined, including the ability to prevent the activation and proliferation of cells of the immune system (reviewed in Baron et al., 1991). Interferons are split into 2 main families: the type I interferons interferon α, β, and ω which are produced by nearly all cells of the body (α predominantly by leukocytes and monocytes, β by fibroblasts), and type II or immune interferon γ which is produced predominantly by natural killer cells. Type I interferons are largely induced

by viral infection as well as by bacteria and some cytokines, whereas interferon-γ is typically produced following cell activation or encounter with antigen. Interferon-γ serves to activate a variety of functions in cells of lymphoid origin and otherwise, particularly the enhanced expression of major histocompatibility complex (MHC) class II on the surface of professional antigen presenting cells (APC) and their induction on facultative APCs. As such, interferon-γ is under intense investigation for its role in autoimmune diseases where its expression, or blocking thereof, can profoundly affect disease pathogenesis (review in Billiau, 1996).

IFN-γ has been shown to have several effects on vascular endothelium: in addition to inducing MHC class II expression (Pober et al., 1983), cytokine and chemoattractant cytokine expression are also induced, particularly acting in synergy with TNF-α (Marfaing-Koka et al., 1995); these observations are extended to cerebral endothelium (Huynh and Dorovini-Zis, 1993). The primary sources of IFN-γ in the CNS are infiltrating mononuclear cells during inflammation. Additional observations include IFN-γ induction of molecules which control lymphocyte adhesion to cerebral endothelium (Male et al., 1995) and the ability of IFN-γ to increase the permeability of cerebral endothelial monolayers *in vitro* (Huynh and Dorovini-Zis, 1993). IFN-γ can also augment expression of MHC class II on pericytes and perivascular macrophages (Male et al., 1987) suggesting potential effects for IFN-γ in regulating inflammation and antigen presentation in the central nervous system.

In vivo levels of interferon γ have ranged from 2.5 U/ml in patients with Kawasaki disease (Matsubara et al., 1990) or human T-lymphotropic virus-associated myelopathy (HTLV-1) to 3.5 U/ml in the cerebrospinal fluid of the latter (Kuroda and Matsui, 1993).

1.7 CHEMOKINES: CHEMOATTRACTANT CYTOKINES

Within the past decade, a rapidly growing family of molecules has emerged on the scene as secondary mediators of inflammation: proteins which are produced by immune as well as non immune cells following stimulation with LPS or pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β, and serve to enhance the interactions between leukocytes and their cell adhesion molecules during leukocyte trafficking. These molecules were first identified as secreted basic, heparin binding proteins with the unique ability to attract and activate distinct leukocyte subsets to sites of inflammation in addition to other later defined non-inflammatory roles. These initial discoveries opened the door for these chemoattractant cytokines – or *chemokines* – as key regulators of inflammatory responses.

1.7.1 Chemokine structure

The chemokines are a family of small, 8 to 12 kDa secreted proteins grouped into sub-families based on a two to four conserved cysteine residue motif at the NH_2 terminal end. The two largest subfamilies are the CXC or α chemokines which have one intervening amino acid between the 1st and 2nd cysteine, and the CC or β chemokines which have no such intervening amino acid. In contrast, only one member is known to date for each of the C or γ chemokines, which have a lone NH_2 terminal cysteine, and the CX_3C chemokines which have 3 intervening amino acids. The CX_3C chemokine, fractalkine, is also unique with a transmembrane region and mucin stalk which anchor the chemokine component to the cell surface; this chemokine component can also be cleaved to form a functional soluble molecule (Bazan et al., 1997). Genomically, chemokines typically have three exons and two introns. Hydrophobicity analysis of the α chemokine IL-8 and the β chemokine macrophage inflammatory protein (MIP)-1β

suggest that each subfamily appears to fold differently to form a dimer distinct from members of the other subfamily (Covell et al., 1994).

The genes for the CXC chemokines are clustered on chromosome 4, the genes for the CC family on chromosome 17. The lymphotactin gene is on chromosome 1 and the fractalkine gene located on chromosome 16q, suggesting that the chemokines within their subfamilies evolved together, whereas the different subfamilies evolved separately.

1.7.2 Chemokine function

Overall, the chemokines play significant roles in mediating angiogenesis, where IL-8 is a key angiogenic factor, interferon-inducible protein-10 (IP-10) and monocyte-chemoattractant induced by gamma (MIG) are angiostatic; in hematopoiesis, MIP-1α and PF4 have been implicated in precursor cell cycling and the inhibition of megakaryopoiesis, respectively (Baggiolini et al., 1994).

Of particular interest are the interactions between chemokines and leukocytes, where chemokines have been identified as molecules which can attract and/or activate distinct leukocyte subsets during an inflammatory response. The sub-families which are created according to structure also delineate the functional distinction of chemokine subfamilies (reviewed in Ward and Westwick, 1998). For example, the α chemokines IL-8, MIP-2 and others in this family have activating and chemoattractant effects on neutrophils, but little or no effects on other leukocyte subsets such as lymphocytes or monocytes. Conversely, the β chemokines serve to activate, induce granule release, as well as chemoattract lymphocytes, monocytes, and basophils, with little or no effect on neutrophils. In turn, lymphotactin, as its name suggests, recruits lymphocytes to inflammatory sites, and fractalkine attracts T lymphocytes as well as

natural killer cells (Imai et al., 1997). Boyden chamber assays have been used to study the chemotactic responses of different leukocyte subsets to chemokines, where chemokine is added to the lower chamber of the system and leukocytes specific for that chemokine migrate through a filter and down into the lower chamber; similar systems have been employed to study transendothelial migration in response to chemokines (Roth et al., 1995; Loetscher et al., 1994). In addition, chemokines can specifically recruit sub-types of lymphocytes; a given chemokine might have the highest affinity for T cells with a memory activated phenotype while another chemokine a higher affinity for naïve T cells.

1.7.3 Binding to glycosaminoglycans

The primary mechanism of action for chemokines is believed to be via haptotaxis, whereby chemokines are immobilized on a surface and "presented" to receptors on the surface of leukocytes or other cells. The ability of chemokines to bind glycosaminoglycans (GAGs), the polysaccharide components of cell-surface and extracellular-matrix (ECM) proteoglycans, makes them key suspects as "presentation" molecules. Investigators have found that different chemokines do in fact show differential binding to glycosaminoglycan subpopulations (Witt and Lander, 1994), and that GAGs are capable of mediating the cell surface oligomerization and polymerization of chemokines, thereby increasing their local concentration and enhancing interactions with chemokine receptors (Hoogewerf et al., 1997). It has been suggested that these proteoglycans on the surface of endothelial cells also present these adhesion-inducing cytokines to leukocytes (review by Tanaka et al., 1993b), and in accordance with studies showing IL-8 binding selectively to the luminal surface of microvascular EC (Rot 1992) and MIP-1β localization to high endothelial venules of reactive lymph nodes (Tanaka et al., 1993a), suggest that such strategic positioning may play a key role in transendothelial trafficking.

1.7.4 Chemokine receptors

The specificity of a given chemokine for a leukocyte subset is largely governed by the presence of the receptor for that given chemokine. Chemokine receptors are G protein-coupled rhodopsin-like proteins which have seven transmembrane domains. As the chemokines were divided into subfamilies, so are the chemokine receptors, which can be loosely classified as specific, shared or promiscuous (for review, Premack and Schall, 1996). Whereas a given receptor for α chemokines may be specific for a single α chemokine, a given β chemokine receptor may have the ability to bind several β chemokines, albeit with varying affinities. Generally, structural similarities amongst α chemokine receptors, which differ greatly from those similarities seen amongst β chemokine receptors, are the keys to the strict α and β subfamily chemokine-receptor division. However, the Duffy blood group antigen receptor does exist as an exception, and is defined as promiscuous because it has the ability to bind members of both the α and β chemokine subfamilies (Szabo et al., 1995). Originally recognized as an erythrocyte chemokine receptor, no known signaling response occurs when chemokines bind to the receptor (Neote et al., 1994). Chemokines and their receptors expressed on T cells are listed in Table 1 (p. 114).

1.7.5 Chemokine receptor signaling

Upon binding of the chemokines to their receptors, a complex signaling pathway is initiated which is still under intense investigation. The ultimate result of chemokine receptor engagement is the movement of the cell. This involves the relay of the message to intracellular messengers, the rearrangement of the cytoskeleton, and the formation of focal adhesions which

allow for the detachment and reattachment of the cell to the substrate along the direction of movement, typically along a chemokine concentration gradient. The G proteins are linked to phospholipases which downstream form inositol triphosphates, leading to an increase in intracellular calcium and eventually protein kinase C (PKC) activation (reviewed in Bokoch 1995). Because this system is sensitive to *Bordetella pertussis* toxin (PTX) which block signaling in a number of experiments, the G_i family of proteins are believed to be involved. The Ras, Rac, and Rho families are also suggested to be involved in signaling, yet this is likely through tyrosine kinase pathways.

One of the best studied chemokines to date, RANTES, illicits a biphasic mobilization of intracellular calcium in T cell clones, one which is transient, sensitive to pertussis toxin and associated with chemotaxis, and a second calcium mobilization which is sustained but sensitive to tyrosine kinase inhibitors and blocks its cellular activation properties including increased secretion of IL-1and IL-5, increased IL-2 receptor expression and increased cellular proliferation (Bacon et al., 1995). These observations may serve to characterize the dual effects of chemokine signaling adhesion and migration through a single ligand-receptor interaction.

1.7.6 RANTES and MIP-1β

RANTES and MIP-1β are two β chemokines which have been shown to be extensively involved in the recruitment and functional regulation of T lymphocytes. Each is associated with a Th1 type immune response, as these molecules are typically induced by pro-inflammatory cytokines such as IFN-γ, and the upregulation of their receptors on T cells typically requires IL-2 stimulation (Sallusto et al., 1998). Additionally, Th1 cells preferentially respond to RANTES and MIP-1β versus Th2 cells which predominantly respond to other β chemokines (O-Garra et al.,

1998). RANTES is believed to be a major chemoattractant for CD4+ T cells, especially of the memory phenotype (Schall et al., 1990), in addition to monocytes/macrophages. RANTES attraction of T cells has been described extensively in extracerebral tissues both *in vivo* and *in vitro* (Murphy et al., 1994); MIP-1β on the other hand has been shown to chemoattract different CD4+ and CD8+ subsets as well as monocytes/macrophages, although reports are contradictory concerning the nature of preferred effector cells: these have included CD4+ T cells with some preference for a naïve phenotype (Schall et al., 1993) and a preference for activated CD4+ T cells (Taub et al., 1993a-b).

In addition, both chemokines have been shown to participate in the creation of a motile T cell phenotype in activated cells: the formation of the uropod into the circulation, away from the site of adhesion, is induced by RANTES and MIP-1β in T cell blasts. Accompanying cytoskeletal rearrangements are the redistribution of cell surface molecules such as ICAM-1 and ICAM-3, all part of RANTES and MIP-1β contributing to a pro-adhesive and pro-migratory phenotype on the T cell (del Pozo et al., 1997; Serrador et al., 1997). Another characteristic of this motile phenotype is the localization of several chemokine receptors to the leading edge of the T cell (Nieto et al., 1997). In addition, RANTES and MIP-1β both in soluble and immobilized forms have both been shown to enhance T cell adhesion to CAMs and ECM proteins (Lloyd et al., 1996).

Interestingly, each of these proteins has been documented to enhance not only the adhesion of T cells to EC, but also to promote transendothelial migration (Roth et al, 1998; Borthwick et al., 1997; Taub et al., 1993b). Combined with other observations these studies suggest a key role for RANTES and MIP-1β in mediating T cell adhesion and trafficking across EC in addition to their cell activating properties.

1.7.7 Chemokines and their receptors in the CNS

The expression of RANTES and MIP-1β has been reported within the CNS. In fact, numerous chemokines have been documented in a wide variety of infectious (Lahrtz et al., 1998; Schmidtmayerova et al., 1996; Sasseville et al., 1996; Spanaus et al., 1997), traumatic (Glabinski et al., 1996), ischemic (Gourmala et al., 1997) and autoimmune responses within the CNS (Berman et al., 1996) in addition to a suggested involvement in neuronal migration (Nishiyori et al., 1998; Bolin et al., 1998; Peterson et al., 1997). A summary of chemokine and receptor expression by cells of the CNS is provided in Table 2.

Consistently, the expression of β chemokines is associated with intense mononuclear cell infiltrates. Studies in AIDS encephalitis, MS and its animal model EAE show characteristic perivascular cuffs of mononuclear cells, evidence of a mass exodus of inflammatory cells from the circulation which cross the blood brain barrier and enter the CNS (Simpson et al., 1998). Several chemokines have been localized to the infiltrates of these perivascular cuffs, as well as to other cells in the neural environment such as astrocytes, neurons and microglia, both *in vitro* and *in vivo* (Barnes et al., 1996; McManus et al., 1998). Typically, *in vitro* studies have shown that the pro-inflammatory cytokines TNF-α, IFN-γ and IL-1β are key inducers of chemokine production from astrocytes, microglial cells, and also infiltrating mononuclear cells.

Both RANTES and MIP-1β have been documented in mononuclear cell infiltrates *in vivo*, in addition to their expression by numerous cells of the CNS *in vitro*. RANTES has been detected in astrocytes following induction with TNF-α, IFN-γ and IL-1β (Barnes et al., 1996) and in astrocytes and microglia infected with virus (Fisher et al., 1995) Message for RANTES has also been detected in activated encephalitogenic T cells present in EAE. Cytokines have been shown to induce MIP-1β and MIP-1α in human fetal microglia (McManus et al., 1998). RANTES and MIP-1β have both been found to be prevalent in MS and its animal model EAE;

RANTES positive cells were found to be infiltrating T cells, blood vessel EC, astrocytes and macrophages/microglia (Miyagishi et al., 1997; Simpson et al., 1998). Most MIP-1β reactive cells were found around blood vessels (Miyagishi et al., 1997). In MS, analysis of post-mortem specimens revealed that RANTES expression was seen on the perivascular cells and neighbouring astrocytes; MIP-1β was primarily localized to macrophages and microglia within the lesion (Simpson et al., 1998).

Chemokine receptors have also been identified in infiltrating leukocytes as well as cells intrinsic to the CNS such as astrocytes, microglia, EC and neurons during inflammation and in some neurodegenerative disorders (Lavi et al., 1998; Lavi et al., 1997; Xia et al., 1997; Horuk et al., 1997); receptors have also been documented in normal brain (Horuk et al., 1996; Nishiyori et al., 1998). This suggests the obvious potential for chemokine interaction with mononuclear cells, thus mediating the immune response. In particular, CCR3 a receptor for RANTES that is shared with monocyte chemoattractant proteins 1-3 (MCP 1-3) and CCR5, the receptor shared by RANTES, MIP-1β and MIP-1α have been localized in lymphocytic infiltrates of the CNS, particularly in models of EAE and AIDS encephalitis (Jiang et al., 1998; Westmoreland et al., 1998). In addition, the Duffy receptor which is able to bind RANTES, is also expressed within the brain (Horuk et al., 1996).

While it is apparent that both chemokines are present in the perivascular space during inflammatory processes in the CNS in accompaniment of mononuclear cell infiltrates, the functional significance of their presence has not been characterized. Although previous studies in other tissues would suggest a role in lymphocyte recruitment, the presence of these chemokines at the level of the BBB and the role which they play in directing lymphocyte trafficking into the CNS have not been previously investigated.

In multiple sclerosis, the prototype autoimmune demyelinating disease of the CNS, CD4+ T cells are believed to be the primary cells which initiate the inflammatory cascade within the CNS. MS and its animal model EAE are characterized by early breakdown of the BBB and leukocytic infiltration of the CNS. These infiltrates are seen as intense perivascular lymphocytic cuffs. Demyelination is the main pathological feature in these diseases. The believed mechanism for disease involves neuro-antigen specific T cells recognizing their antigen in the periphery and becoming activated. Activated T cells are the only cells believed capable of entering the CNS. Activated cells readily cross the intact BBB, and when they encounter their antigen again in the CNS, eg. in the form of myelin basic protein or proteolipid protein (the two most abundant antigenic determinants present within the CNS, known to play a role in CNS autoimmunity) (Zamvil and Steinman, 1990), these cells initiate an immune response. Studies have outlined the importance of activated lymphocytes (those bearing IL-2 receptors) in the development of MS (Bellamy et al., 1985) in addition to the involvement of Th1 types of responses (Del Prete, 1998; Nagelkerken, 1998). Numerous cytokines are secreted which serve to activate cells intrinsic to the CNS such as microglia and astrocytes, in addition to activating the cerebral endothelium thus increasing its permeability (reviewed in Benveniste 1997). TNF-α has been shown to be crucial in inducing cytokine production by glial cells, and also in mediating myelin and oligodendrocyte damage (Selmaj et al., 1991). Additional observations of increased TNF-α levels in the CSF of MS patients that correlate with the disruption of the BBB in active MS patients suggest a crucial role in MS pathogenesis (Sharief et al., 1991). IFN-γ levels are higher in peripheral blood mononuclear cells (PBMC) from MS patients than normal controls in addition to increased numbers of IFN-γ secreting cells within the CSF (Lu et al., 1993). Clinical trials involving IFN-γ administration to MS patients showed exacerbation of disease, indicating a role in disease progression (Panitch et al., 1987). IL-1 is constitutively secreted by PB macrophages and is

localized to microglia in MS plaques, and combined with its numerous pro-inflammatory effects upon glial and non-glial cells likely plays a key role in MS (Hofman et al., 1991; Merrill et al., 1989). These cytokines serve to prime an otherwise unwielding cerebral endothelium, priming the EC to facilitate the traffic of T lymphocyte subsets and monocytes into the brain without discrimination. The cell adhesion molecules ICAM-1 and VCAM-1 which are key in lymphocyte trafficking are upregulated and induced respectively (Cannella and Raine, 1995). Chemokine expression is also induced: RANTES, MIP-1β and others have all been shown to be expressed at increased levels in MS, in addition to their localization in cells of perivascular cuffs, although the role which they play in the pathogenesis of MS is unclear (Simpson et al., 1998). Studies showing a predominance of memory T cells in MS plaques and in the lesions of EAE (Pitzalis et al., 1988) in addition to the presence of other T cell subsets shadowing the absence of PMNs may suggest a role for chemokines such as RANTES and MIP-1β in attracting such distinct lymphocyte subsets while excluding others during the course of MS.

While investigators have addressed many questions concerning the involvement of these molecules in autoimmune disorders within the CNS, extensive investigation is still required to gain a full understanding of the mechanisms which regulate the trafficking of lymphocytes across the BBB. This BBB must be primed in order to allow trafficking which is the stepping stone in the inflammatory response that results in severe neurological deficits in the course of T cell mediated autoimmune demyelination.

1.8 ENDOTHELIAL HETEROGENEITY

Recent developments in endothelial cell research have established beyond doubt that EC from different species and vascular beds are heterogeneous in their phenotype, cell surface molecules, antigenic determinants, metabolic, immunologic and permeability properties.

Therefore, it is no longer acceptable to use the more readily available large vessel EC cultures or lines such as human umbilical vein EC and to extrapolate these results to EC of different vascular beds (Gordon et al., 1991; Miyazoto et al., 1991; Page et al., 1992; Male et al., 1990). In no case is this more likely to be apparent than when making comparisons to the cerebral endothelium, which is distinctly different from EC in extracerebral tissues. It has also been well established that EC in culture lose many of their primary morphological characteristics through passaging in addition to changing characteristics such as cell adhesion molecule induction in response to cytokine stimulation, making them less and less comparable to their *in vivo* counterparts (Klein et al., 1995). Again, this rings particularly true for cerebral EC, which lose BBB enzyme properties (Meyer et al., 1990) in addition to exhibiting decreased electrical resistance through passaging, characteristics which may be partially restored using astrocyte conditioned media or cAMP analogs (Sun et al., 1995; Wolburg et al., 1994).

1.8.1 Human brain microvessel endothelial cells (HBMEC) *in vitro*

In order to carry out such studies, many have used *in vivo* models to block the activities of chemokines and determine the effects on disease pathogenesis and leukocyte trafficking across the BBB. Whereas such models may be useful for studying such properties as molecular transport across the BBB (Bonate 1995), they become less useful when trying to focus on direct cell to cell interactions occurring at the level of the BBB. Due to the complicated nature and multitude of cells present in the natural system, *in vitro* models have become a powerful if somewhat more simplistic means of analyzing events *in vivo* since they allow for better control of the cellular environment. Numerous *in vitro* systems have been characterized to mimic the BBB *in vivo*, typically consisting of brain endothelial cells with or without contributions from

astrocytes or astrocyte conditioned medium which have been recorded to influence the BBB characteristics of these cells.

An *in vitro* model of the BBB was developed and characterized in this laboratory and consists of primary cultures of human brain microvessel endothelial cells (HBMEC) (Dorovini-Zis et al., 1991). These cultures retain important endothelial and BBB properties and respond to experimental manipulation. In addition to characteristics common to all EC such as Factor VIII related antigen immunoreactivity, alkaline phosphatase reactivity and the binding of *Ulex europaeus* I lectin, key BBB characteristics are also observed. These include the presence of tight junctions which are impermeable to HRP, and electrical resistance much higher than other EC cultures at up to 200 $\Omega \cdot cm^2$ or greater (Dorovini-Zis et al., 1991).

Immunologically, HBMEC in this system have been shown to express MHC class II antigens in response to stimulation with IFN-γ (Huynh and Dorovini-Zis, 1993) and also to express PECAM-1 and upregulate the expression of CAMs ICAM-1, VCAM-1 and E-selectin in response to treatment with pro-inflammatory cytokines (Wong and Dorovini-Zis, 1996; Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995).

1.9 GENERAL AIM OF THIS STUDY

The overall objective of this project is to characterize the expression and upregulation of the β-chemokines RANTES and MIP-1β along with their receptors CCR3 and CCR5 by human brain microvessel endothelial cells (HBMEC), and to study the role these molecules play in the adhesion and migration of T-lymphocyte subsets across primary cultures of HBMEC.

1.9.1 Specific objectives of this study

Phase I. Investigation of the expression of β chemokines by human brain microvessel endothelial cells *in vitro*

1. To study the expression/upregulation of the β chemokines RANTES and MIP-1β in unstimulated HBMEC as well as cells stimulated with TNF-α, IL-1β, IFN-γ, LPS, and combinations of these cytokines.
2. To study the expression/upregulation of the chemokine receptors CCR3 and CCR5 by HBMEC prior to and following stimulation with TNF-α, IL-1β, IFN-γ, LPS, and combinations of these cytokines.

Phase II. Investigation of the role of β chemokines in mediating T cell subset adhesion and transmigration across human brain microvessel endothelial cell monolayers

1. To determine the role of RANTES and MIP-1β in adhesion and migration of T lymphocyte subsets across confluent monolayers of HBMEC.
2. To investigate the involvement of endothelial cell adhesion molecules in the chemokine-mediated adhesion and migration of T lymphocyte subsets across confluent HBMEC monolayers.

CHAPTER 2 MATERIALS & METHODS

2.1 EXPRESSION OF CHEMOKINES

2.1.1 Human Brain Microvessel and Umbilical Vein EC Culture

Human brain microvessel endothelial cells (HBMEC) were isolated from brains removed at autopsy and primary HBMEC cultures were established as previously described (Dorovini-Zis et al., 1991). The clinical history was reviewed at the time of post mortem examination to ensure that brains were free of pathological processes. Cerebral cortex was obtained less than 12 hours post mortem. Following removal of the meninges and leptomeningeal blood vessels, the tissue was minced into 1-2 mm cubes and incubated for 3 hours in 0.5% dispase (Boehringer Mannheim, IN) in a 37°C shaking water bath. Following centrifugation at 1000g for 10 minutes, the resultant pellets were resuspended in 15% dextran (Sigma, MO) and centrifuged at 5800g for 10 minutes. The pellets containing the microvessels were washed in Medium M199 (Gibco, ON) and then incubated overnight in 0.1 % collagenase/dispase (Boehringer Mannheim, IN) in order to remove the basement membrane and pericytes. At the end of the incubation period the microvessels were subjected to differential centrifugation on Percoll gradients (Sigma, MO) for 10 min at 1000g in order to separate EC from pericytes, red blood cells and basement membranes. The resultant EC band was aspirated, washed, and plated on fibronectin-coated plastic wells (Corning Plastics, NY).

EC cultures were maintained in M199 supplemented with 10% heat inactivated horse serum (Hyclone Labs, UT), 100 μg/ml heparin, 20 μg/ml endothelial cell growth supplement (Sigma, MO), 100 μg/ml antibiotics/antifungal containing streptomycin, penicillin and amphotericin (Gibco, ON) and 300 μg/ml glutamine. Culture media were changed every other

day. EC reached confluence 7 to 10 days after plating. The endothelial nature and purity of the cultures were confirmed by their strongly positive staining for Factor VIII-related antigen, binding of *Ulex europeaus* I lectin, acetylated low-density lipoprotein uptake, and alkaline phosphatase immunoreactivity. In order to make direct comparisons of chemokine expression between brain microvascular and large vessel extracerebral endothelium, primary cultures of human umbilical vein EC were isolated according to methods described previously (Jaffe et al., 1973). Briefly, umbilical cords were rinsed with PBS, and then incubated with 1 mg/ml collagenase for 15 minutes at 37°C. Cords were then flushed with warm M199 and collected in 20% horse serum in M199. The endothelial origin and purity of the cultures were determined by staining for Factor VIII related antigen and acetylated low density lipoprotein uptake. Endothelial cells were plated on plastic culture wells coated with fibronectin and cultured under identical conditions with HBMEC until confluent. Several different primary cultures of HBMEC and HUVEC were used for the experiments.

2.1.2 Induction of chemokine expression by cytokines and LPS

Confluent monolayers of HBMEC and HUVEC grown in replicate wells were treated for 24, 48, and 72 hrs with 100 U/ml tumor necrosis factor-α (TNF-α,Sigma), 200-500 U/ml interferon-γ (IFN-γ, Collaborative Biomedical), 10 U/ml interleukin-1β (IL-1β, Genzyme), 5 μg/ml bacterial lipopolysaccharide (LPS from *E. coli* O55:B5, Sigma) and combinations of these cytokines. ECGS and heparin were removed from the culture media for the last 24hrs. Cytokine-conditioned media were replenished every 24 hrs. In experiments designed to detect MIP-1β release into the media by cytokine-treated HBMEC or HUVEC using sandwich ELISA of the supernatants, cytokines were replenished only once after the first 24 hours to allow for detectable

levels to accumulate in the supernatants.

2.1.3 Semi-quantitative Reverse-Transcriptase PCR

HBMEC and HUVEC were grown to confluence on fibronectin coated plastic 60 mm diameter culture dishes and used untreated or following treatment with TNFα (100 U/ml) in combination with IFN-γ (500 U/ml) for 24 hrs. Total cellular RNA was prepared using reagents from the Invisorb II kit (ID Labs). Briefly, frozen pellets of HBMEC and HUVEC were lysed and phenol/chloroform extracted. 2 μg of cellular RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase for 90 minutes at 42°C. PCR was performed on 2μl of the cDNA preparation using AmpliTaq Gold (Perkin Elmer) with primers from Gibco BRL (Table 3) under the following conditions: dissociating at 94°C for 9 min., annealing at 57° for 30 sec, extension at 72°C for 3 min., then cycling for total stated number of cycles (Table 1) with 30 sec at 90°C, 30 sec. at 57°C, and 1 min. at 72°C. The housekeeping gene GAPDH was used as the standard to control for variations in RNA isolation, cDNA synthesis and PCR performance. RNA isolated from PBMC of healthy donors collected using Ficoll-Hypaque was also used as a positive control in a resting state and following treatment for 24 hours with TNF-α (100 U/ml) and IFN-γ (500 U/ml). PCR products were run on a 6% TBE polyacrylamide gel and stained with EtBr. Confirmation that the desired RANTES or MIP-1β fragments had indeed been amplified was performed using restriction enzyme (RE) analysis of the PCR products for each chemokine. Following two hours of incubation at 37°C, the RE products were run on a 8% agarose gel and stained with EtBr for viewing under UV light.

2.1.4 Antibodies and Reagents

The following primary antibodies (Abs) were used for immunohistochemistry: monoclonal mouse anti-human RANTES IgG_1, polyclonal goat anti-human RANTES IgG (10 μg/ml), monoclonal mouse anti-human MIP-1β IgG_{2b} (10 μg/ml); polyclonal goat anti-human MIP-1β IgG (10 μg/ml; all from R&D Systems, MN). Secondary Abs included goat anti-mouse IgG LM and rabbit anti-goat IgG LM linked to 5 nm gold particles (diluted 1:40; Auroprobe™ LM RAG IgG and Auroprobe™ LM GAM Ig, Janssen/Cedarlane Labs Ltd., Hornby, ON). Isotype-matched control Abs (10 μg/ml) included mouse anti-human leukocyte common antigen IgG_1 (Incstar, MN), mouse anti-human Leu 14/CD22 IgG_{2b} (Becton-Dickinson), and polyclonal goat anti-hepatitis B surface antigen (Dako, CA). For the detection of the chemokine receptors CCR3 and CCR5, Abs 5C7 (CCR3) and 12G5 (CCR5) (both at 10 μg/ml) were obtained from the NIH AIDS Reference and Reagent program (McKesson Bioservices, MD). Secondary Ab was biotinylated goat anti-mouse IgG (1:400) from Caltag, CA.

2.1.5 Enzyme-linked immunosorbent assay (ELISA)

In order to detect and quantitate the release of RANTES and MIP-1β by HBMEC before and after cytokine stimulation, supernatants of untreated and cytokine-treated HBMEC and HUVEC were collected and analyzed by sandwich ELISA (RANTES and MIP-1β Quantikine ELISA Kits, R&D Systems, Minneapolis). Microtitre plates coated with an α-RANTES or α-MIP-1β mAb were incubated with supernatants, washed, and a second enzyme-linked polyclonal Ab was added. Following incubation, the plates were washed to remove unbound Ab. Color substrate was added and allowed to react with horseradish peroxidase (HRP) for 20 minutes. The reaction was stopped with HCL. Absorbances read at 450 nm revealed protein concentrations

with sensitivities of 12.5 and 25 pg/ml for RANTES and MIP-1β kits respectively. Controls included unstimulated HBMEC and HUVEC, supernatants from human platelets treated with TNF-α (100 U/ml) as a positive control for RANTES (Klinger et al., 1995), and human peripheral blood monocytes treated with LPS (5 μg/ml) as a positive control for MIP-1β (Ziegler et al., 1991). All treatments were performed in triplicate and each experiment performed at least three times.

2.1.6 Immunogold silver staining (IGSS) for light microscopy

2.1.6.1 Surface localization of RANTES and MIP-1β

Following cytokine stimulation, HBMEC monolayers were washed with PBS containing 1% BSA, 1% normal goat or rabbit serum and 0.03% sodium azide (wash buffer) and incubated for 40 minutes at room temperature with the primary Ab diluted at 10 μg/ml in carrier buffer containing 5% BSA, 4% normal goat or rabbit serum and 0.05% sodium azide. At the end of the incubation period the cells were washed with wash buffer and then incubated for 60 min. at room temperature with the secondary Ab (1:40 dilution). Sodium azide was added to prevent internalization of Ag-Ab complexes. After incubation, cultures were washed, fixed for 30 seconds in acetone/buffered formaldehyde, washed in distilled water, and incubated with silver enhancement solution (IntenSE M^{TM}, Janssen/Cedarlane) for 25 to 28 min. Cells were counterstained with Giemsa.

Controls included unstimulated HBMEC and incubation with isotype matched irrelevant Abs, normal mouse or goat IgG or carrier buffer instead of the primary Ab, or with supernatants derived from RANTES or MIP-1β peptide affinity-adsorbed polyclonal Ab preparations.

Immunostained cultures were examined under a Nikon Labophot microscope.

Quantitation of chemokine expression was performed by counting cells as labeled or unlabeled in one central and four peripheral fields in each well with an ocular grid at 20X magnification. All counts were performed blindly. Each experiment was repeated at least three times, using HBMEC from different isolations.

2.1.6.2 Intracellular localization

Cytokine or LPS treated HBMEC and HUVEC monolayers were washed with wash buffer as described above without sodium azide and fixed in acetone/buffered formaldehyde containing 0.03% Triton X-100 for 10 minutes. After washing, monolayers were incubated with primary Ab for 60 min. at 10 μg/ml and then incubated with secondary Ab at 1:10 dilution for 90 minutes. Cultures were washed, incubated with silver enhancement solution and counterstained with Giemsa.

Controls included unstimulated HBMEC and incubation with isotype matched irrelevant antibodies, normal mouse or goat IgG or carrier buffer instead of the primary Ab, or with supernatants derived from RANTES or MIP-1β peptide affinity-adsorbed polyclonal Ab preparations.

Immunostained cultures were examined under a Nikon Labophot microscope. Quantitation of chemokine expression was performed by counting cells as labeled or unlabeled in one central and four peripheral fields in each well with an ocular grid at 20X magnification. All counts were performed blindly. Each experiment was repeated at least three times, using HBMEC from different isolations.

2.1.7 Data collection & statistics

Statistics were performed using the computer program Sigma Stat (Jandel Scientific, San Rafael, CA). ANOVA was first performed to determine significant differences. The Tukey multiple comparisons was next performed to determine differences between control unstimulated and cultures stimulated with cytokines. Where populations were not normally distributed the Mann Whitney Rank Sums test was performed. $P<0.05$ was taken as statistically significant for all analyses.

2.2 ROLE OF CHEMOKINES IN THE ADHESION AND MIGRATION OF T CELL SUBSETS ACROSS HBMEC MONOLAYERS.

2.2.1 HBMEC culture

HBMEC were cultivated in a double chamber chemotaxis system the floor of which consists of solubilized, pyrogen-free collagen (Fig. 3; Cellagen discs, ICN Biomedical, OH). Cells were grown in culture media as described previously in Materials and Methods for 7 to 10 days until confluent. Upon reaching confluence, HBMEC were used in a resting state or following co-incubation with TNF-α (100 U/ml) plus IFN-γ (200 U/ml) for 24 hours to maximally upregulate cell adhesion molecule (Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995) and chemokine expression. Heparin and ECGS were again depleted from the culture media for the last 24 hours of culture.

2.2.2 Measurement of electrical resistance

The electrical resistance of the HBMEC monolayers was measured and recorded daily throughout the culture period to the time of adhesion or migration studies. Measurements were

performed using an Endohm electrical resistance monitor (Fig. 4; World Precision Instruments, FLA) immediately following removal of the cultures from the incubator and at least 12 hours following the last media change to account for any changes due to environmental changes.

2.2.3 CD4+ T cell subset isolation and activation

Peripheral blood mononuclear cells (PBMC) were isolated from anti-coagulated venous blood of healthy donors by Histopaque (Sigma) density centrifugation. The isolated PBMC were passed through human CD4+ T cell Recovery Columns (Cedarlane) or human Naïve ($CD45RA^+$) or Memory ($CD45RO^+$) CD4+ T cell columns (R&D Systems) to negatively select for CD4+ T cell subsets. Monocytes and B lymphocytes were trapped in the column by size and by binding to anti-immunoglobulin Abs on the column beads, respectively. All preparations were greater than 99% viable by trypan blue exclusion.

FACS analysis of T cell subset fractions was performed by incubating lymphocyte preparations with mouse mAbs to CD45, CD2, CD20, CD56 and CD14 (all from Dako) to detect leukocyte, T cell, B cell, NK cell or monocyte populations respectively. Following a 30min incubation at 4°C, cells were washed with 2% fetal calf serum in TC 199 (Gibco) and fixed in 1% paraformaldehyde. Fluorescence was read on an Epic Profile I (Coulter Corp) where dead cells were gated out and quadrants were set using isotypic controls. Isolated subsets were consistently greater than 95% pure for CD4, CD45RO and CD45RA expression, with less than 1% contaminating B, NK, CD8+ T or monocytes.

CD4+ T cells were activated with immobilized mouse anti-human CD3 (Becton Dickinson) at 3.125 μg/ml with 30 U/ml IL-2 in 10% AB serum in RPMI for 24 hrs at 37°C in a 10% CO_2/90% air incubator. AB serum was heat inactivated and therefore contained no

functional serum proteins including complement components. FACS analysis was performed using mAbs to CD69 and CD25 (IL-2 receptor) (both from Dako) to assess T cell activation.

2.2.4 Antigen-specific CD4+ T cell clones

CD4+ T cell clones were a generous gift from Dr. Rick Shimonkevitz (Columbia Swedish Medical Center, CO). These clones designated HA 307-319 are specific for the peptide sequence of amino acids #307 to 319 of the Influenza hemagglutinin. The cytokine secretion profile for these cells when stimulated consists of significant amounts of IL-4, IL-10, IFN-γ and IL-2. FACS analysis of cells on day 11 or 12 (the day of experiment) revealed the following FACS profile: 60% CD45 RO high, 40 % IL-2R high, and 55% α4 integrin high.

To clonally expand the population, every 18 to 21 days the clones were restimulated with feeder layers consisting of gamma-irradiated PBMC (5000 rad) in 10% fetal calf serum containing Lymphocult LT and Lymphocult HP containing IL-2 and IL-4, glutamine and antibiotics for 3 days. Following stimulation on 12 well plates (Corning Plastics, NY) cells were split to 75cm^2 flasks and allowed to expand until day 10 or 11 when they were used for adhesion experiments. T cell clones were centrifuged at 250 g for 10 minutes, washed twice with 1X HBSS without Ca^{2+} or Mg^{2+} to remove cytokines present in the growth media and resuspended in 10% AB serum in RPMI at 1 x 10^6 cells/ml.

2.2.5 Chemokine receptor expression on CD4+ T cell subsets

FACS analysis for CCR3 and CCR5 was performed using the 5C7 and 12G5 antibodies respectively. These antibodies were obtained from the NIH AIDS Reference and Reagent program and were also used for immunocytochemistry. Resting, memory and naïve CD4+ T cells

were investigated for expression of CCR3 and CCR5. CD4+ T cells activated for 24 hours with either immobilized anti-CD3 plus IL-2 as described above or with immobilized anti-CD3 alone were also examined for expression of these chemokine receptors.

2.2.6 Chemokine receptor expression on HBMEC

Immunohistochemistry was used to determine whether or not the β-chemokine receptors CCR3 and CCR5 were expressed by HBMEC *in vitro*. Confluent cultures of HBMEC were used untreated or following treatment with TNF-α (100 U/ml) and IFN-γ (200 U/ml). Primary Abs provided by the NIH AIDS and Reference Reagent Program were used at 10 μg/ml following the same protocol used for silver enhanced immunogold cytochemistry.

2.2.7 Chemokines and chemokine diffusion assay

In order to characterize the diffusion of RANTES and MIP-1β from the lower chamber through the cellagen discs into the upper chamber during the adhesion and migration assays, a chemokine diffusion assay was performed. Human recombinant RANTES and MIP-1β were purchased from Peprotech (NJ) and used at 10 or 100 ng/ml in RPMI for chemokine adhesion and migration studies. For diffusion studies, ^{125}I-labeled RANTES and MIP-1β were kind gifts from Dr. Ian Clarke-Lewis (University of British Columbia) labeled at $1.65x10^5$ cpm/pmole and $3.3x10^5$ cpm/pmole respectively. For diffusion studies, untreated and cytokine-treated confluent HBMEC and HUVEC monolayers maintained in 100μl of 10% horse serum in M199 in the upper chamber were placed above 350 μl of M199 in the lower chamber containing 100 ng/ml of radiolabeled RANTES or MIP-1β. EC were incubated at 37°C for 3 ½ hours and radioactivity was determined at .5, 1.5 and 3.5 hours, in the upper and lower chamber supernatants using a

Beckmann Gamma 5500 and expressed as % equilibration, i.e. the concentration of radioactive counts (cpm/μl) in inserts (upper well) divided by the concentration of radioactive counts in bottom wells. Each experiment was performed twice using duplicate wells to test for chemokine diffusion. Controls consisted of chemokine diffusion across collagen discs without EC monolayers.

2.2.8 Antibodies to cell adhesion molecules and integrins

Blocking monoclonal antibodies to EC adhesion molecules on HBMEC as well as to corresponding ligands on T cells were used to determine the role of these molecules in mediating T cell subset adhesion and migration. Anti-human ICAM-1 (mouse IgG1, clone RR1/1, Immunocorp) and humanized mouse anti-human alpha 4 integrin (gift from Dr. Ted Yednock, Athena Neurosciences, CA) were both used at 10 μg/ml; anti-human VCAM-1 (mouse IgG1, 2G7 gift from Dr. Walter Newman, Leukosite MA) was used at 20 μg/ml. All of the above have been shown to bind their ligands on EC and to block interactions between these molecules and their ligands (Dustin et al., 1988; Graber et al., 1990; Keszthelyi et al., 1996).

2.2.9 T cell adhesion assay

Confluent HBMEC monolayers grown on cellagen discs in duplicate wells were treated with a combination of TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 24 hours to maximally upregulate the expression of RANTES, MIP-1β, and the EC adhesion molecules ICAM-1 and VCAM-1. In some experiments culture media from the upper chamber of the double chamber chemotaxis system were replaced with 10% horse serum in M199 containing anti-ICAM-1 or anti-VCAM-1 blocking antibodies 30 min before the assay. At this time, gradients of RANTES

or MIP-1β were established by adding 10 or 100 ng/ml of each chemokine to the lower chamber to allow for the diffusion of the chemokines into the collagen membrane. These concentrations have been shown to be effective in several transendothelial migration studies investigators (Roth et al., 1995; Roth et al., 1998). Controls included untreated EC, treated EC in the absence of chemokine gradients or presence of chemokines in both chambers (no gradients). In some experiments blocking antibodies to alpha 4 integrin were also added to the T cell preparations. All blocking Abs were present during the adhesion assay.

After the 30 minutes of blocking and chemokine diffusion into the membrane, the contents of the upper chamber were aspirated and 100 µl of CD4+ T cell subset suspensions at 1 to 2 X 10^6 cells/ml were added to the upper chamber and swirled gently 4 times to allow for even dispersion of the cells. Following 1 hour of incubation at 37°C, the non-adherent cells were washed away with RPMI by gently pipetting up and down at each lateral wall of the wells. HBMEC monolayers with adherent lymphocytes were fixed with 1:1 acetone/100% EtOH for 7 minutes at 4°C and stained for leukocyte common antigen (LCA) with the immunoperoxidase technique. Following wash with Tris buffer containing Tween-20 (pH 7.6) for 10 minutes, endogenous peroxidase activity was blocked using 1:4 3%H_2O_2/MeOH for 30 minutes. Lymphocytes were stained for leukocyte common antigen using a monoclonal anti-LCA antibody linked to HRP (Dako, ON) and DAB (Sigma, MO) as the chromogen. HBMEC were counterstained with hematoxylin.

The number of lymphocytes bound per mm^2 of the monolayers was determined by counting the number of adherent lymphocytes in one central and eight peripheral fields using a 20X objective lens and a 1 mm^2 grid. Treatment labels were masked during enumeration to prevent bias.

2.2.10 Migration assay

Primary HBMEC cultures were grown to confluence on cellagen discs and treated with TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 24 hours. Gradients (100 ng/ml) of RANTES or MIP-1β in RPMI were established by adding these chemokines to the lower chamber of the system and allowing for diffusion into the membrane for 30 minutes. The supernatants were then removed from the upper chamber of the double chamber chemotaxis system and replaced with 100 µl of 10% horse serum in M199 containing CD4+ T cells at $1x10^5$ cells per well. In control experiments 100 ng/ml of RANTES or MIP-1β were added to both chambers or 10 ng/ml of either chemokine was added to the upper chamber only (no gradients). Additional controls included unstimulated HBMEC and cytokine-treated cultures in the absence of chemokine gradients. In some experiments following 30 minutes of incubation during which the cells have initiated adhesion, 20 µl of 10% AB serum in RPMI was added to control wells, whereas experimental wells received 20 µl of the same media containing anti-ICAM-1, anti-VCAM-1 or anti-alpha-4 integrin antibodies to make up to 10, 20, and 20 µg/ml respectively and rotated gently four times to allow for antibody dispersal. Following incubation for 3 hours at 37°C, the upper chambers were washed four times to remove non-migrated lymphocytes and fixed for 1 hour at 4°C with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.2M Na cacodylate buffer, pH 7.15, Osm 1049. After washing in 0.2M Na cacodylate buffer, cells were fixed in 1% Osmium tetroxide for 1 hour at 4°C, washed in Na cacodylate buffer for 30 mins followed by Na acetate buffer (pH 5.15) for 30 mins, and block stained overnight at 4°C with uranyl magnesium acetate. Cellagen discs with HBMEC monolayers and migrated lymphocyte subsets were dehydrated in graded series of methanol and embedded in Epon-Araldite. One hundred 1 µm thick sections taken 40 µm apart were cut and stained with toluidine blue. The number of

adherent and migrated lymphocytes per mm of monolayer length was enumerated in each section under the light microscope at 200X magnification. Thin sections stained with uranyl acetate and lead citrate were examined on a Zeiss 10 Electron Microscope.

2.2.11 Statistics

For adhesion studies, the number of adherent cells per mm^2 in one central and eight peripheral fields of duplicate wells were compared using student's t test and ANOVA. For migration studies, the number of adherent and migrated cells in each of 100 sections were compared between treatments using ANOVA and student's t test. Non-normally distributed populations were analyzed using an ANOVA on RANKS and Mann Whitney Rank Sums test to compare treatments found to be significant following the ANOVA. P values for tests are reported, and statistical significance was regarded as $p<0.05$.

CHAPTER 3 RESULTS

3.1 HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELLS (HBMEC)

HBMEC in primary culture form confluent, contact-inhibited monolayers that show strong positive staining for Factor VIIIR:Ag (Fig. 1a), bind *Ulex europeaus* I lectin (Fig. 1b) and have high alkaline phosphatase activity (Fig. 1c). Furthermore, cultured HBMEC resemble their *in vivo* counterparts in that they contain few pinocytotic vesicles and are joined together by tight junctional complexes that restrict the transendothelial passage of horseradish peroxidase (Dorovini-Zis et al., 1991). Growth of HBMEC on fibronectin-coated plastic wells (Fig. 2a) is comparable to growth on cellagen discs (Fig. 2b).

3.2 CHEMOKINE EXPRESSION BY HBMEC IN PRIMARY CULTURE

3.2.1 RANTES

3.2.1.1 Detection of RANTES RNA by semi-quantitative RT-PCR

The primer pairs designed for PCR allow for the amplification of 290 and 287 bp fragments for RANTES and MIP-1β respectively (see Table 3). RT-PCR on untreated and cytokine-treated HBMEC and HUVEC revealed differences in chemokine expression between the two types of endothelial cells (Fig. 5). Small amount of RANTES RNA was detected in untreated HBMEC but not in untreated HUVEC. Following co-stimulation with TNF-α and IFN-γ (100 and 500 U/ml respectively), RNA levels for RANTES increased significantly in both HBMEC and HUVEC cultures. Restriction enzyme analysis of the amplified fragments confirmed that the desired fragments had indeed been amplified (Fig. 6).

3.2.1.2 RANTES release by endothelial cells in culture

HBMEC and HUVEC were cultured to confluence and incubated for 72 hours with TNF-α, IFN-γ, IL-1β or LPS alone or in combination. Supernatants were collected every 24 hours for measurement of chemokine concentrations and media were replenished. Minimal amounts of RANTES in the range of 80 to 160 pg/ml were detected in supernatants of untreated HBMEC (Fig. 7a). Treatment with TNF-α increased protein release up to 1000 pg/ml. These levels were maintained over the 72 hours of treatment. RANTES release was not greatly increased following treatment with IL-1β. IFN-γ did not significantly augment RANTES release into the media during the first 24 hours of treatment, however, protein levels doubled in supernatants collected between 24 and 48 and were maintained from 48 to 72 hours following daily re-stimulation with IFN-γ at 200 U/ml. Incubation with LPS induced RANTES secretion up to 3200 pg/ml over the first 24 hours, although levels dropped off significantly in supernatants collected between 24 to 72 hrs.

Co-incubation with TNF-α and IFN-γ was most effective at enhancing RANTES production, since at least 5000 pg/ml of RANTES were consistently released into the media during each 24 hour incubation period (Fig.7b). Combination of TNF-α with IL-1β resulted in RANTES release at levels that were greater than for IL-1β alone, but not as high as TNF-α treatment alone. IFN-γ in combination with IL-1β also showed negligible protein in the supernatants.

Untreated HUVEC cultures released negligible amounts of RANTES into the culture media. Only TNF-α (100 U/ml) alone was able to increase the levels of RANTES above those of unstimulated cultures (Fig. 8a). IFN-γ and IL-1β had no such effect over the 72 hour incubation

period. Interestingly, HUVEC monolayers were induced to secrete RANTES following co-stimulation with TNF-α and IFN-γ (Fig. 8b) at levels similar to those released by HBMEC cultures. However in contrast to HBMEC, RANTES secretion by HUVEC consistently dropped off dramatically after the initial 24 hours of TNF-α/IFN-γ treatment, indicating an apparent decrease in responsiveness to cytokine treatment.

3.2.1.3 Intracellular localization of RANTES

Silver enhanced intracellular immunogold staining was performed on cultures of HUVEC and HBMEC to examine the presence of RANTES or MIP-1β in the cell cytoplasm. Unstimulated HBMEC showed light staining in the form of fine, black granular deposits indicating the presence of small amounts of cytoplasmic RANTES (Fig. 9a). Following 24 to 72 hours of stimulation with TNF-α and IFN-γ or other cytokines, the intracellular staining intensity was greatly increased (Fig. 9b), indicating increased synthesis of RANTES. Following cytokine treatment, 100% of cells consistently stained positive for RANTES, although slight differences were seen in staining intensity from cell to cell. Controls including normal mouse or goat IgG or buffer in place of the primary antibody showed no staining.

In contrast, unstimulated primary cultures of HUVEC showed negligible intracellular staining for RANTES (Fig. 10a). However, cytokine treatment of HUVEC did result in strong cytoplasmic staining in the perinuclear region of cells indicating increased production of RANTES. Similar to HBMEC, 100 % of HUVEC stimulated with TNF-α and IFN-γ showed increased staining for RANTES (Fig. 10b).

3.2.1.4 Surface localization of RANTES

The chemokines are typically secreted molecules, yet their action has been implicated to be via haptotaxis, whereby these molecules are presented while bound to glycosaminoglycans in the extracellular matrix or at the endothelial cell surface. Surface immunogold silver staining of untreated or cytokine-treated HBMEC monolayers demonstrated that cytokine activation of HBMEC not only upregulates synthesis and secretion of RANTES, but in addition, results in detectable amounts of the chemokine bound to the endothelial cell surface (Fig. 11). Unstimulated cultures showed negligible surface staining for RANTES (Fig. 12a). Cytokine treatments induced expression and surface binding of these chemokines as indicated by the fine black granular deposits distributed randomly over the cell surface. Compared to untreated controls, 60 to 90 % of HBMEC were induced to express RANTES at the cell surface following 24, 48, or 72 hours of cytokine treatment (Fig. 12b,c). Variability in staining intensity was noted amongst endothelial cells of the same stimulated culture, although little difference was noted in staining patterns amongst cultures from different donors.

3.2.2 MIP-1β

3.2.2.1 Detection of MIP-1β RNA by semi-quantitative RT-PCR

MIP-1β RNA was virtually undetectable in unstimulated HBMEC cultures but was induced following activation of HBMEC with TNF-α and IFN-γ at 100 and 500 U/ml respectively. HUVEC, however, did not express MIP-1β RNA in controls and could not be induced to express MIP-1β RNA following similar cytokine treatment (Fig. 13).

3.2.2.2 MIP-1β release by endothelial cells in culture

Untreated HBMEC released low levels of MIP-1β into the culture media in the range of 15 to 20 pg/ml (Fig. 14).The amount of MIP-1β in the supernatants increased following cytokine and LPS stimulation of EC. LPS as well as TNF-α in combination with IL-1β (at 100 and 10 U/ml respectively) doubled MIP-1β protein release to 50 pg/ml. Conversely, MIP-1β release by HUVEC following similar cytokine treatments could not be induced to levels above the controls (0 to 15 pg/ml).

3.2.2.3 Intracellular localization of MIP-1β

Immunogold silver staining after cell membrane permeabilization allowed for intracellular localization of MIP-1β. The staining intensity was weak in unstimulated HBMEC cultures (Fig. 15a). Following co-stimulation with TNF-α and IFN-γ, finely granular perinuclear staining was observed in 80 to 90% of HBMEC which had exhibited MIP-1β secretion into the culture media (Fig. 15b). Overall, the staining intensity for MIP-1β was considerably more weak than that for RANTES among cells of the same culture suggesting that comparatively less MIP-1β protein is produced by HBMEC.

Staining of HUVEC for MIP-1β showed that neither resting nor cytokine-stimulated cells contained detectable protein intracellularly (data not shown), correlating well with the lack of detectable RNA and presence of negligible amounts of protein in culture supernatants.

3.2.2.4 Surface localization of MIP-1β

In accordance with the RT-PCR, ELISA, and intracellular immunocytochemistry results, resting HBMEC showed negligible MIP-1β protein at the cell surface (Figs. 16, 17a). However,

following stimulation with pro-inflammatory cytokines, MIP-1β was readily detected on the surface of HBMEC (Figs. 16, 17b). Following treatment with TNF-α in combination with IFN-γ or IL-1β, granular deposits of silver-enhanced immunogold particles were detected at the surface. As was true with LPS stimulation, some cells were stained more intensely than others. Again, although the intensity of the immunoreactivity varied amongst cells within a culture, consistency was seen in the staining patterns in response to similar cytokine treatments from culture to culture.

3.2.3 Chemokine Receptor Expression

3.2.3.1 CCR3

In order to determine whether or not chemokines may indeed be having effects on the cerebral endothelium itself, HBMEC *in vitro* were investigated for expression of the relevant chemokine receptors. CCR3 is a receptor which is shared by RANTES and MCP-1, 2 & 3. Intracellular immunogold cytochemistry revealed that resting HBMEC cultures have negligible expression of CCR3. Staining of HBMEC for CCR3 was negative following treatment with pro-inflammatory cytokines including TNF-α and IFN-γ for 24 hours.

3.2.3.2 CCR5

CCR5 is a chemokine receptor shared amongst the β chemokines RANTES, MIP-1α and MIP-1β. Intracellular immunocytochemistry showed lack of staining for CCR5 in primary cultures of HBMEC. Treatment with the pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β or LPS, either alone or in combination, did not induce detectable expression of CCR5 in these cells over 24 or 48 hours.

3.3 REGULATION OF T LYMPHOCYTE SUBSET ADHESION AND TRANSENDOTHELIAL MIGRATION BY THE β-CHEMOKINES RANTES AND MIP-1β

3.3.1 Electrical resistance of untreated and cytokine or chemokine treated HBMEC monolayers

The measurement of electrical resistance across a monolayer is considered a valid method for assessing permeability of the monolayer. Primary cultures of HBMEC were plated onto cellagen membranes and grown to confluence for 9 to 11 days. The cellagen membrane discs were transferred from the double chamber chemotaxis system to a chamber with an electrode positioned in the center of the chamber. Another electrode was placed on top of the monolayers. The collagen disc itself had a mean electrical resistance of 27.5 $\Omega \cdot cm^2$, which was subtracted from all measurements. A drop in resistance indicates an increase in the permeability of the monolayers.

Resistance measurements were taken once the cultures became semi-confluent and daily thereafter. Typically, as the monolayers reached confluence, the electrical resistance rose to about 180 $\Omega \cdot cm^2$ (usually by 8-10 days), and thereafter dropped slightly, usually after day 11 or 12. Resistance measurements for two different cultures of HBMEC are shown (cultures A and B). Generally, cultures plated at a higher density reached a higher peak resistance than those plated at a lower density. Solid lines indicate the resistance across a resting culture. Following 24 hour treatment of confluent HBMEC monolayers with TNF-α and IFN-γ at 100 and 200 U/ml respectively, the electrical resistance significantly decreased to 28 to 40 $\Omega \cdot cm^2$ (broken lines) (Fig. 18a, b). In comparison, studies of culture B (Fig. 18b) which had been plated at identical plating densities, suggest that similar plating densities resulted in cultures from numerous

experiments reaching similar maximal levels of resistance over a similar time course. Again, cytokine treatment of HBMEC caused a dramatic reduction in the electrical resistance of the monolayer compared to the resistance of resting cultures.

In order to determine whether RANTES or MIP-1β alter the permeability of HBMEC monolayers, the electrical resistance of HBMEC monolayers was measured following the addition of these chemokines to the lower chamber. Resistance was monitored daily from day 7 and 8 and once cells reached confluence and peak resistance, chemokines were added to either the upper or lower chambers at 100 ng/ml. Resistance was then measured over the next 24 hours in both chemokine-treated and control cultures. The addition of chemokines to either the upper or the lower chambers did not significantly change the electrical resistance as compared to control monolayers over the 24 hour period (data not shown).

3.3.2 Radiolabeled chemokine diffusion across EC monolayers

Since chemokine gradients were going to be established across HBMEC monolayers, tests were first carried out to determine whether or not RANTES and MIP-1β indeed diffused both through the collagen disc and also through resting or cytokine-treated HBMEC monolayers [cytokines: TNF-α (100 U/ml) and IFN-γ (200 U/ml)]. Parallel studies were carried out using HUVEC monolayers for comparison.

RANTES and MIP-1β readily diffused across cellagen discs, although the increase in % equilibration over time did increase beyond 0.5 hours. HBMEC monolayers had significantly less % equilibration compared to cellagen discs at 26%; this was much lower than the % equilibration observed at 0.5 hours for HUVEC ($p<0.05$). In addition, cytokine treatment of HBMEC increased the % equilibration of RANTES to 42% at 0.5 hours ($p<0.05$), therefore

cytokine treatment did enhance chemokine passage through HBMEC monolayers (Fig. 19a). Similar results were obtained for diffusion of MIP-1β (Fig. 19b). Chemokine diffusion was not significantly greater across cytokine-treated versus resting HUVEC monolayers, suggesting that HUVEC are quite permeable to chemokine diffusion, irrespective of activation state. While monolayers of both cell types significantly reduced chemokine diffusion compared to collagen discs alone, resting HBMEC were significantly more impermeable than HUVEC particularly at points prior to 1.5 hours ($p<0.05$), whereas cytokine-treated HBMEC allowed for levels of chemokine diffusion similar to cytokine-treated HUVEC.

3.3.3 CD4+ T cell subsets

FACS analysis was performed on isolated T cell subsets to determine the purity of T cell preparations. All isolates contained less than 1% contaminating NK, B, $CD8^+$ T cells or monocytes. Resting CD4+ T cell cultures were greater than 95% pure; analysis of donor lymphocytes revealed that an average of 26.4 ± 6.1 and 24.3 ± 1.4% were of the CD45RO high and CD45RA high phenotype respectively. Isolated memory and naïve cultures were greater than 95% CD45RO and CD45RA high respectively. FACS analysis of anti-CD3 activated CD4+ T cells revealed that greater than 90% of cells were high in the expression of CD69 and greater than 75% were high in the expression of CD25 (IL-2 receptor) after 24 hours of activation: this was significantly increased compared to resting peripheral CD4+ T cell cultures of which 4% were high in expression of CD69 and 7% high in expression for CD25.

Chemokine receptor expression on T cells

FACS analysis was performed to determine the proportion of cells within each isolated T cell subset which expressed the chemokine receptors CCR3 and CCR5; these results are

summarized (Table 4). Activated CD4+ T cells showed highest expression of both chemokine receptors followed by memory, resting and naïve cells in that order. Interestingly, it was largely only the blast-appearing cells in each population which expressed the receptors.

3.3.4 T cell subset adhesion to human brain microvessel endothelial cells

3.3.4.1 Resting CD4+ T cell adhesion

Very few CD4+ T cells from a non-stimulated peripheral population adhered to unstimulated HBMEC monolayers (Figs. 20a,b; 21a,b). Pretreatment of HBEMC with TNF-α plus IFN-γ increased adhesion by two fold compared to untreated monolayers. This increase in adhesion was seen in all experiments, although slight variation in basal levels of adhesion was seen between T cell and HBMEC preparations from different donors.

The presence of RANTES or MIP-1β in the lower chamber of the double chamber chemotaxis system did not enhance adhesion of resting CD4+ T cells to unstimulated EC monolayers. Similarly, no changes in adhesion were noted when RANTES or MIP-1β were added to both chambers.

However, adhesion of resting CD4+ T cells to HBMEC monolayers was increased in the presence of RANTES in the subendothelial region of cytokine-treated monolayers. Addition of 10 ng/ml RANTES to the lower chamber was not consistently effective at enhancing adhesion ($p=.70$, $p=.042$) amongst different donors of T cells and HBMEC, however 100 ng/ml of RANTES in the lower chamber consistently enhanced adhesion to cytokine-treated monolayers (Fig. 21c). Interestingly, the presence of RANTES in both chambers during the adhesion assay further augmented adhesion to cytokine-treated monolayers, resulting in numerous cells adherent to the monolayers (Fig. 21d).

Gradients of MIP-1β were also effective at enhancing resting CD4+ T cell adhesion to cytokine-treated HBMEC monolayers (Figs. 20b; 21e). Consistently, 100 ng/ml of MIP-1β were more effective than 10 ng/ml in raising the level of adhesion. In addition, the presence of MIP-1β in both upper and lower chambers of the system, augmented the adhesion of resting CD4+ T cells to cytokine-treated HBMEC, suggesting a pro-adhesive effect above and beyond that induced by the presence of a gradient (Fig. 21f).

3.3.4.2 Activated CD4+ T cell adhesion

Peripheral CD4+ T cell populations from different donors were activated for 24 hours using immobilized anti-CD3 mAb in the presence of 30 U/ml human IL-2. Activated CD4+ T cells readily adhered to untreated HBMEC monolayers (Figs. 22, 23a) in the area of 600 to 1000 cells/mm^2. This level of adhesion was increased significantly when HBMEC were pre-treated with TNF-α and IFN-γ (Figs. 23b, 24a).

The presence of RANTES in the lower chamber had no significant effect on the adhesion of activated CD4+ T cells to untreated HBMEC monolayers. Similarly, the presence of RANTES concurrently in both chambers of the system did not augment T cell adhesion. Conversely, 100 ng/ml of RANTES in the lower chamber upregulated adhesion to cytokine-treated HBMEC by as much as thirty percent (Fig. 24c). Lower concentrations of RANTES (10 ng/ml) had less consistent effects on adhesion. Addition of RANTES to both chambers consistently enhanced adhesion to cytokine-treated HBMEC at 100 ng/ml, although less consistently at 10 ng/ml, again suggesting a direct effect on T cells in addition to any chemoattractant function (Fig. 24d).

MIP-1β did not augment adhesion of activated CD4+ T cells to resting EC monolayers, however, adhesion of activated CD4+ T cells to cytokine-activated HBMEC was significantly

enhanced by the presence of 10 or 100ng/ml MIP-1β in the subendothelial region (Figs. 23b; 24e). When MIP-1β was present in both chambers of the system, adhesion was also augmented (Fig. 24f). Interestingly, adhesion was slightly but significantly increased when MIP-1β was added only to the upper chamber, in the absence of chemotactic gradients (Fig. 22b).

3.3.4.3 Effects of cell adhesion molecule blocking on lymphocyte adhesion

Resting CD4+ T cells

In order to investigate the involvement of cell adhesion molecules in T cell adhesion to HBMEC, mAbs to ICAM-1 or VCAM-1 were added to the upper chamber of the chemotaxis system for 30 minutes prior to the addition of lymphocytes, and during the 1 hour co-incubation of the lymphocytes with the HBMEC monolayers. mAb to α4 integrin was added during the co-incubation of lymphocytes with HBMEC monolayers.

Since chemokines exerted their effects on adhesion only in the presence of a cytokine activated monolayer, the involvement of cell adhesion molecules was examined during resting T cell subset adhesion to activated HBMEC monolayers. In the absence of chemokine gradients, anti-ICAM-1 mAb decreased adhesion by 48 to 76% (Fig. 25). In the presence of RANTES under the EC monolayers (10 or 100 ng/ml), the anti-ICAM-1 mAb blocked adhesion by 65%. When RANTES was added to both chambers, ICAM-1 blocking decreased adhesion by 61 to 85% (Fig. 25a). Similarly, adhesion in response to MIP-1β was significantly reduced ($p<0.05$) by anti-ICAM-1 mAb (Fig. 25b): the increased adhesion observed in the presence of MIP-1β in the lower chamber or when MIP-1β was added to both chambers was blocked by 58 to 71% and 74 to 87% respectively. In each case, adhesion was reduced to the same level as in controls in the absence of gradients following anti-ICAM-1 blocking.

The anti-VCAM-1 mAb had variable and less pronounced effects compared to the anti-ICAM-1 mAb on the adhesion of resting CD4+ T cell subsets to activated HBMEC, both in the presence and absence of chemokine gradients. Blocking of VCAM-1 decreased adhesion in some experiments by 21 to 23%. In the presence of RANTES in both or only the lower chamber, mAb to VCAM-1 decreased adhesion by 17 to 25% and 14 to 46% respectively. VCAM-1 blocking similarly reduced adhesion by up to 40% and 12 to 15% in the presence of MIP-1β in both chambers or MIP-1β only in the subendothelial region.

A humanized antibody to the α4 integrin, the α portion of VLA-4 currently being tested in clinical trials, was also tested for its ability to reduce the chemokine-enhanced T cell subset adhesion to HBMEC. This antibody has been previously shown to reduce the intensity of lymphocytic infiltrates and neurological symptoms in EAE. Anti-α4 blocking during the adhesion assay in the presence of RANTES or RANTES gradients decreased adhesion of resting CD4+ T cells to activated HBMEC by up to 10%; enhanced adhesion in the presence of MIP-1β in both chambers or in the subendothelial region was similarly reduced by up to 45 and 40% respectively.

Activated CD4+ T cells

Similar mAb blocking studies were performed during adhesion of activated CD4+ T cells on resting and activated HBMEC monolayers (Fig. 26). Anti-ICAM-1 significantly reduced activated CD4+ T cell adhesion to resting HBMEC by 39 to 46%; a similar effect was seen when RANTES or MIP-1β were present, even though these chemokines did not enhance the adhesion of these cells to resting HBMEC. VCAM-1 blocking did not significantly reduce the adhesion of activated CD4+ T cells to resting HBMEC.

Adhesion of activated CD4+ T cells to activated HBMEC was consistently reduced by 55 to 81% with mAb to ICAM-1, in the presence of chemokines in both chambers or in the subendothelial region and in control experiments in the absence of chemokines.

Anti-VCAM-1 Ab was at times ineffective in reducing adhesion of activated CD4+ T cells to activated HBMEC, but in other instances it reduced adhesion by up to 35%. When RANTES was present in the upper and lower chambers, or in the subendothelial compartment, adhesion was reduced by 12 to 36% and up to 37% respectively. Similarly, the MIP-1β enhanced adhesion was affected to different degrees by anti-VCAM-1: in the presence of MIP-1β in both chambers or in the lower chamber alone was reduced by up to 35% and 36 to 38% respectively. Anti-α4 mAb reduced adhesion of activated CD4+ T cells to activated HBMEC by 21%. The upregulated adhesion induced by RANTES present as a gradient or in both chambers was reduced by 34 to 40% by blocking α4. The increased adhesion in response to MIP-1β in the subendothelial compartment or in both chambers was decreased by up to 35 and 25% respectively.

3.3.4.4 Antigen specific CD4+ T cell clone adhesion

Significant numbers of HA 307-319 specific T cell clones adhered to untreated HBMEC monolayers *in vitro*. Cytokine stimulation of HBMEC monolayers with TNF-α and IFN-γ resulted in further increases in adhesion by 160 to 200 % (Figs. 27; 28a,b).

The presence of RANTES in the lower chamber did not enhance adhesion of T cell clones to resting HBMEC monolayers (Fig. 27a). RANTES present in the lower chamber at 10 or 100 ng/ml did, however, significantly increase adhesion to cytokine-treated HBMEC ($p=0.025$ and 0.013 respectively) (Fig. 28c). When RANTES was present in the upper and lower chambers, 10 ng/ml was not sufficient to enhance adhesion, however 100 ng/ml markedly

enhanced adhesion of Ag-specific T cells (p=0.047) (Fig. 28d). When 10 ng/ml of RANTES was present only in the upper chamber, adhesion was not notably increased.

MIP-1β did not markedly affect the adhesion of HA 307-319 specific T cell clones to resting HBMEC monolayers. Presence of 10 ng/ml in the subendothelial region were sufficient to enhance T cell clone adhesion to one endothelial culture by 190% (p=0.013 data not shown) but did not significantly increase adhesion to a different HBMEC culture (Figs. 27b; 28e) (p=0.33). Increasing the concentration of MIP-1β in the lower chamber to 100 ng/ml augmented adhesion to both cultures by 162% (p=0.023; data not shown) and 150% (p=0.048). The presence of 10 or 100 ng/ml of MIP-1β in both chambers of the system did not further increase the adhesion to activated HBMEC monolayers, nor did the presence of 10 ng/ml in the upper chamber alone (Fig. 28f).

3.3.4.5 Memory CD4+ T cell adhesion

Memory CD4+ T cells were isolated from immunocolumns and were found to be greater than 95% CD45RO high, and less than 2 % CD45RA high. Generally, 100 to 200 memory cells adhered per mm^2 of untreated monolayer (Figs. 29; 30a). This was increased by two to three fold following 24 hours pre-incubation of the monolayers with TNF-α and IFN-γ (Figs. 29; 30b).

The presence of RANTES in the subendothelial compartment or in the upper chamber had no effect on the adhesion of memory CD4+ T cells to resting EC monolayers. In contrast, 100 ng/ml of RANTES added to the lower chamber beneath activated HBMEC increased adhesion by 40 to 50 %. In addition, presence of RANTES at 100 ng/ml in both chambers enhanced adhesion to activated HBMEC (Figs. 29a; 30c,d).

The presence of MIP-1β in either the upper or lower chambers had no effect on the levels of memory T cell adhesion to resting HBMEC monolayers. MIP-1β beneath the monolayers slightly augmented the adhesion of memory CD4+ T cells to cytokine-treated HBMEC. Concentrations at 10 ng/ml were variably effective at enhancing adhesion. However 100 ng/ml consistently enhanced the adhesion of memory T cells to cytokine-treated HBMEC. In addition, 100 ng/ml in both chambers consistently enhanced adhesion to cytokine-treated HBMEC monolayers by 20 to 30 %. 10 ng/ml of MIP-1β was less effective (Figs. 29b; 30e,f).

3.3.4.6 Naïve CD4+ T cell adhesion

Naïve CD4+ T cell populations were isolated using a process of negative selection through immunocolumns. Very few naïve CD4+ T cells adhered to resting HBMEC monolayers. Following stimulation of the EC monolayers with cytokines for 24 hours, adhesion was significantly increased (Figs. 31, 32a,b).

Neither RANTES nor MIP-1β present beneath the endothelium or in both chambers had any effect on the adhesion of naïve T cells to resting EC monolayers compared to basal levels. Similarly, neither chemokine enhanced adhesion of naïve T cells to cytokine-treated HBEC monolayers (Figs. 31a,b).

3.3.5 Effects of RANTES and MIP-1β on migration of T cell subsets across human brain microvessel endothelial cells

3.3.5.1 Resting CD4+ T cell migration

Only occasional resting CD4+ T cells migrated across untreated HBMEC monolayers (Fig. 33; Fig. 34a). Chemokines present beneath untreated monolayers did not enhance T cell migration. Similarly, the presence of RANTES or MIP-1β in both chambers of the system had no effect on migration, suggesting that these β chemokines are not capable of enhancing lymphocyte adhesion of resting lymphocytes to a non-activated blood-brain barrier endothelium.

Approximately 35% more resting CD4+ T cells migrated across cytokine-treated HBMEC monolayers (Fig. 34b). The presence of RANTES in the subendothelial compartment of activated monolayers showed a trend towards enhanced migration by 35%, although these values were not statistically significant (Fig. 34c). MIP-1β gradients across activated HBMEC also showed increased means for migration although these values were also statistically insignificant (Fig. 34d). The presence of either chemokines in the upper and lower chambers of the assay as a control for chemotaxis neither augmented or decreased migration across HBMEC.

3.3.5.2 Activated CD4+ T cell migration

Anti-CD3 activated CD4+ T cell subsets migrated more readily across resting HBMEC than the resting CD4+ T cell population (Figs. 35; 36a). Again, neither the presence of chemokine gradients of RANTES or MIP-1β, nor their presence in the upper and lower chambers affected the migration of activated CD4+ T cells across resting HBMEC monolayers (Fig. 36b).

Cytokine activation of HBMEC with TNF-α and IFN-γ for 24 hours augmented the migration of activated CD4+ T cells (Fig. 36c). This migration was enhanced by 175% and

193% respectively in the presence of RANTES and MIP-1β beneath activated HBMEC monolayers (Figs. 36d,e). Interestingly, the presence of RANTES in both upper and lower chambers also increased subset migration across activated monolayers by 167% (Fig. 36f). Addition of MIP-1β to both upper and lower chambers also enhanced migration, although to a lesser degree.

3.3.5.3 Effects of cell adhesion molecule blocking on migration of resting and anti-CD3 activated CD4+ T cell subsets across HBMEC monolayers.

Migration of CD4+ T cells across resting HBMEC is blocked by anti-ICAM-1

In order to examine the involvement of the cell adhesion molecules ICAM-1, VCAM-1 and its ligand VLA-4 in T cell subset migration across cerebral EC *in vitro*, mAb to these molecules were added to the upper chamber during the migration assay. In each instance T cell subsets were allowed to adhere for 30 minutes before the addition of blocking mAb to cell adhesion molecules to allow for the process of adhesion to occur unhindered during this time.

The effects of mAb blocking of cell adhesion molecules on CD4+ T cell migration across resting HBMEC monolayers are summarized in Figs. 37a and b. Migration of resting and activated CD4+ T cells to resting HBMEC was all but completely eliminated by mAb to ICAM-1. mAbs to VCAM-1 and α4 integrin had no effect on the migration of resting CD4+ or activated CD4+ T cells across resting HBMEC ($p<0.05$).

Resting CD4+ T cell migration in response to chemokines is integrin-dependent

The migration of resting CD4+ T cells across cytokine-treated HBMEC was greatly reduced to 5% of that seen in controls using mAb to ICAM-1 (Fig. 38). As seen in Fig. 38a, the increased migration induced by RANTES in the subendothelial compartment was greatly

reduced by anti-ICAM-1 mAb to levels comparable to those observed in controls without RANTES treated with anti ICAM-1. The blocking effect of ICAM-1 was also observed when migration proceeded in the presence of RANTES in both chambers of the system, suggesting that resting CD4+ T cell migration is largely ICAM-1 dependent, as is increased migration resulting from the presence of RANTES. Enhanced migration of resting CD4+ T cells in response to RANTES gradients across cytokine-treated HBMEC was also reduced slightly but significantly with mAb to both VCAM-1 and α4, whereas neither had any effect upon migration occurring in the presence of RANTES in both upper and lower chambers of the assay.

Fig. 38b summarizes the results of cell adhesion molecule blocking on the responses of resting CD4+ T cells to MIP-1β and MIP-1β gradients across cytokine-treated HBMEC. Again, mAb to ICAM-1 greatly reduced migration to control levels in spite of the presence of MIP-1β in the subendothelial compartment; migration was also reduced, although not significantly when MIP-1β was present in both upper and lower chambers. VCAM-1 and α4 integrin appear to play a role in the response of resting CD4+ T cells to concentration gradients of MIP-1β, as mAb to these two molecules significantly reduced migration across cytokine-treated HBMEC ($p<0.05$). Again, these molecules had no effect upon resting CD4+ T cell migration when concentration gradients were absent and MIP-1β was present both above and below the HBMEC monolayer.

Migration of activated CD4+ T cells relies upon integrin involvement

Antibodies to cell adhesion molecules were also effective at reducing activated CD4+ T cell migration across cytokine-treated HBMEC (Fig. 39). Anti-ICAM-1 practically eliminated activated CD4+ T cell migration across activated HBMEC. While a trend toward decreased migration following treatment with mAb to VCAM-1 was observed, it was not as significant as that seen with anti-α4 treatment or mAb to α4 combined with mAb to VCAM-1.

Increased migration in response to RANTES beneath activated HBMEC appeared to be largely ICAM-1 and partially VCAM-1 and α4 dependent; mAb to ICAM-1 reduced transendothelial migration to levels seen in α-ICAM-1 treated controls (Fig. 39a). Interestingly, antibodies to ICAM-1, VCAM-1 and α4 all decreased migration when RANTES was present in both the upper and lower chambers. However, mAb to ICAM-1 could not bring the level of transendothelial migration down to that seen in controls without RANTES treated with ICAM-1, suggesting that increased migration in response to RANTES in the upper and lower chambers is not completely dependent upon ICAM-1.

The effects of cell adhesion molecule blocking during activated T cell responses to MIP-1β were very similar to those observed with RANTES (Fig. 39b). Again, ICAM-1 drastically reduced the level of transendothelial migration in response MIP-1β in the subendothelial region, whereas blocking of VCAM-1 and α4 integrin also reduced migration across activated HBMEC although to a lesser degree. In the presence of MIP-1β in the upper and lower chambers of the assay, mAb to ICAM-1 significantly reduced but did not completely inhibit transendothelial migration; while migration was reduced to levels seen in controls without MIP-1β present, migration was not reduced to levels seen in controls treated with α-ICAM-1. While VCAM-1 blocking significantly reduced transendothelial migration in response to MIP-1β present in the subendothelial compartment or in both chambers, mAb to α4 significantly reduced transendothelial migration only in response to MIP-1β present beneath activated monolayers ($p<0.05$).

3.3.5.4 Memory CD4+ T cell migration

Small numbers of memory CD4+ T cells migrated across resting HBMEC monolayers (Fig. 40; Fig. 41a). Migration was not enhanced by the presence of RANTES or MIP-1β under the monolayers or in both chambers. Transendothelial migration of memory CD4+ T cells was six times greater across cytokine-treated HBMEC (Fig. 41b). The presence of RANTES or MIP-1β in the subendothelial compartment of activated monolayers enhanced migration by 150 and 200% respectively ($p=0.072$ and 0.001) (Fig. 41c,d). Presence of MIP-1β in both upper and lower chambers doubled the number of memory CD4+ T cells which migrated across activated HBMEC monolayers ($p=0.073$); no significant increase was noted in the presence of RANTES in the upper and lower chambers of the system.

3.3.5.5 Naïve CD4+ T cell migration

Transendothelial migration of naïve CD4+ T cells across resting HBMEC monolayers was negligible (Fig. 42). Migration was not enhanced in the presence of RANTES or MIP-1β in the upper or lower chambers or both. While cytokine treatment of the EC monolayer resulted in two fold increase in migration, RANTES present in the subendothelial compartment had no effect on the transendothelial migration of these cells. MIP-1β beneath activated monolayers had no effect on the migration of naïve CD4+ T cells. However the presence of MIP-1β in the upper and lower chambers of the system was sufficient to enhance the migration of a small but significantly larger number of cells ($p<0.05$).

3.3.5.6 T cell adhesion and migration by transmission electron microscopy (TEM)

Treatment of HBMEC with TNF-α and IFN-γ induced elongation and focal overlapping of EC and formation of prominent finger-like cytoplasmic projections on the apical surface of the endothelium (Fig. 43). The process of adhesion and transendothelial migration was similar for the different CD4+ T cell subsets regardless of their state of activation. Lymphocytes made the initial contact with the EC by extending thin foot-like cytoplasmic processes (pseudopodia) to the apical surface of the endothelium (Fig. 44a). The pseudopodia of the lymphocytes often appeared to come in contact first with the finger-like apical projections of the endothelium (fig. 44b). This step was followed by firm adhesion to the endothelium (Figs. 44b, 45b) during which the cytoplasmic membranes of the two cells remained in close contact. The adherent lymphocytes then became elongated, so that the contact area between EC and lymphocytes increased considerably (Figs. 45a, 46a). In the presence of chemokine gradients, the adhering lymphocytes often displayed a long cytoplasmic process (uropod) protruding from the side of the cell opposite to the adhering front (Fig. 47a). In the initial stage of migration, lymphocytes inserted a long cytoplasmic process between two adjacent HBMEC (Figs. 44c, 47b). Throughout the migration process, the EC remained in close contact with the migrating lymphocytes (Figs. 44c, 45c, 46a, 47c). Eventually, the rest of the cytoplasm and nucleus moved across the monolayer. Upon completion of their migration, lymphocytes assumed an elongated shape and positioned themselves between the overlying EC and underlying collagen often in close association with both (Figs. 45c, 46b, 47c,d). At the end of the migration, the monolayers resumed their continuity and interendothelial junctional complexes appeared intact (Figs. 45c, 46b, 47c,d).

CHAPTER 4 DISCUSSION

4.1 HBMEC *IN VITRO* AS A MODEL OF THE HUMAN BBB

Since it is difficult to study the factors that regulate the function, permeability, immunological and angiogenic properties of neural microvessels in intact animals, culture techniques have been developed over the last 15-20 years in order to establish functional *in vitro* models of the BBB from different species. The use of primary cultures of human cerebral endothelial cells in the present studies allows for direct investigation of the human BBB function. Several important characteristics of the BBB endothelium *in vivo* that indicate preservation of the BBB phenotype are maintained in culture, including high electrical resistance, presence of tight junctions and paucity of pinocytotic vesicles in addition to EC markers such as alkaline phosphatase activity, Factor VIIIR:Ag expression, binding of *Ulex europeaus* I lectin and uptake of acetylated low density lipoproteins. This *in vitro* model is independent of extraneous factors such as astrocytic co-culture, glioma cell conditioned media or addition of second messengers such as cyclic AMP (cAMP) which are required to maintain BBB EC characteristics in other models which utilize passaged HBMEC or EC derived from other vascular beds (Beuckmann et al., 1995; reviewed in Deli and Joo, 1996). This model has allowed for extensive study into the regulation of cell adhesion molecule expression by HBMEC (Wong and Dorovini-Zis, 1996a; Wong and Dorovini-Zis, 1996b; Wong and Dorovini-Zis, 1995; Wong and Dorovini-Zis, 1992), interactions between neurotropic viruses and cerebral EC (Cabirac et al., 1995), the antigen-presenting capabilities by HBMEC and their MHC class II expression (Huynh et al., 1995), characterization of interactions between leukocytes and cerebral EC, and the investigation of factors which regulate the permeability of the BBB (Wong et al.,

1999). These observations lend important relevance to the function of cerebral endothelial cells as mediators of CNS inflammation *in vivo*.

4.1.1 Endothelial cell permeability

The permeability of endothelial cell monolayers can be studied either by measuring the electrical resistance across confluent cultures or with electron dense tracer techniques. For electrical resistance measurements, cells are cultivated in a double chamber chemotaxis system. Electrical resistance measurements allow the monitoring of monolayer permeability before, during and following experimental treatments in a highly reproducible manner. Decreased resistance indicates increased permeability and increased resistance denotes decreased permeability.

The electrical resistance of HBMEC cultures increased as the monolayers became increasingly confluent and continued to rise once confluent. Typically, 24 to 48 hours post-confluence the resistance reached a plateau, and then began to drop slightly. The peak electrical resistance of HBMEC cultures was 146.4 ± 4.9 $\Omega \cdot cm^2$. The average electrical resistance on the date of experiment was 117.6 ± 11.2 $\Omega \cdot cm^2$ which is comparable to results obtained in all cultures. Monolayers of bovine brain EC clones had resistances ranging from 157.4 ± 4.5 $\Omega \cdot cm^2$ to a high of 783.2 ± 7.0 $\Omega \cdot cm^2$ (Rutten et al., 1987). The average resistance recorded across the capillary wall of microvessels at the surface of the frog brain was 1870 $\Omega \cdot cm^2$, which was attributed to EC as glial investment was absent (Crone and Olesen, 1982). The electrical resistance of pial microvesels in anaesthetized rats was found to be 1800 $\Omega \cdot cm^2$ (Butt, 1995). To date, the *in vivo* electrical resistance of human brain microvessels has not been reported.

In contrast to HBMEC, primary cultures of HUVEC had a peak resistance of 18 ± 1.7 $\Omega \cdot cm^2$. These values are identical to those published by Burke-Gaffney and Keenan (1993) for low passage HUVEC cultures, yet higher than those previously reported by Yamada et al. (1990) (9.6 ± 0.6 (S.E.M.) $\Omega \cdot cm^2$) for higher passage cultures plated at lower densities.

Microvascular EC monolayers tend to have higher electrical resistance than larger vessel EC. Bovine microvascular EC in primary culture exhibited a resistance of 69 ± 28 $\Omega \cdot cm^2$, (Furie et al., 1984) while EC from bovine aorta and rat epididymal fat pads show resistance values of 13.5 ± 0.2 and 0.45 ± .03 $\Omega \cdot cm^2$ respectively (Rutten et al., 1987).

Trauma, infection, chemical and immunological insults increase the permeability of the cerebral microvessels. In particular, cytokines such as TNF-α and IFN-γ have been shown to increase permeability (decrease the electrical resistance) of HBMEC *in vitro* (Huynh and Dorovini-Zis, 1993; Dorovini-Zis et al., 1995). In the present study, treatment of HBMEC with TNF-α and IFN-γ significantly decreased the electrical resistance of HBMEC monolayers to 27.7 ± 6.3 $\Omega \cdot cm^2$. Treatment of HUVEC with TNF-α plus IFN-γ decreased transendothelial resistance to 4.6 ± 0.4 $\Omega \cdot cm^2$. These results are similar to treatment of HUVEC monolayers with TNF-α, IFN-γ, or IL-1α/β which increased permeability; combinations of IFN-γ with either TNF-α or IL-1β increased permeability to a degree greater than seen with either cytokine alone (Burke-Gaffney and Keenan, 1993).

4.2 EXPRESSION OF RANTES AND MIP-1β BY HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELLS

4.2.1 RANTES

In view of the fact that chemokines are considered secondary mediators of inflammation, typically produced following some initial pro-inflammatory stimulus, studies were undertaken to

investigate the expression and/or upregulation of RANTES by HBMEC *in vitro*. At the molecular level, minimal RNA for RANTES was detected in resting HBMEC cultures, suggesting that HBMEC *in vitro* produce RANTES at very low levels. Synthesis of RANTES RNA was greatly increased following stimulation with pro-inflammatory cytokines. Protein studies using ELISA of culture supernatants in addition to surface and intracellular immunohistochemistry, was in accordance with these results. HBMEC *in vitro* showed constitutive production of RANTES at very low levels in the area of 100 pg/ml over a 24 hour period. LPS treatment alone and TNFα in combination with IFN-γ were the most effective treatments at increasing the expression of RANTES to 5000 to 6500 pg/ml. TNF-α alone also raised levels significantly, as did combinations of TNF-α with IL-1β. Interestingly, IFN-γ resulted in significant production of RANTES only after the first 24 hours of treatment.

Several studies have focused on RANTES expression by extracerebral EC *in vitro*. HUVEC have shown negligible RANTES expression in the absence of pro-inflammatory stimulus which was confirmed by both RNA and protein secretion analysis (Marfaing-Koka et al., 1995). While little or no RANTES was produced following stimulation with IFN-γ (100 U/ml), IL-β (10 ng/ml) or TNF-α alone (100 U/ml), the combination of TNF-α plus IFN-γ resulted in significant RANTES RNA and protein expression. These results correlate well with our studies of HUVEC, where indeed TNF-α alone only slightly increased protein release, whereas in combination with IFN-γ, large amounts of RANTES were released. Although our studies did not investigate the mechanisms of this synergistic induction, previous studies in HUVEC (Marfaing-Koka et al., 1995) suggested that IFN-γ in fact sensitizes HUVEC to the stimulation by TNF-α, which may also be the case for HBMEC. Additional studies on HUVEC revealed less than 200 pg/ml produced by unstimulated cultures within a 24 hour period, and an increase up to 2300 pg/ml following stimulation with TNF-α plus IFN-γ (both at 200 U/ml)

(Devergne et al., 1994). In the present studies, HUVEC responded in a very similar fashion to these stimuli, and were equally unresponsive to the application of an even higher concentration of IFN-γ (500 U/ml).

Apparent differences in the expression of RANTES between microvascular and large vessel are suggested by our results as well as others. Human dermal microvascular endothelial cells were examined for RANTES production following treatment with inflammatory cytokines, and both primary cultures and a cell line were only weakly induced to produce RANTES in response to IL-1β (Goebeler et al., 1997). TNF-α treatment at 10 ng/ml increased RANTES production to approximately 400 pg/ml while co-incubation of TNF-α with IFN-γ resulted in significant upregulation of RANTES production.

Since both HUVEC and HBMEC were cultured under identical conditions, differences in RANTES production between the two endothelial systems cannot be attributed to variations in culture conditions. Whereas only TNF-α in combination with IFN-γ and to a lesser extent TNF-α could induce RANTES production by HUVEC, both cytokines as well as LPS, significantly upregulated RANTES expression by HBMEC. Moreover, RANTES production by HBMEC was sustained for 72 hours in the presence of cytokines, while RANTES release by HUVEC dropped significantly after 24 hours even though cytokines were present for 72 hours. The striking similarities between dermal microvascular EC and HBMEC would suggest similarities in chemokine expression between the two microvascular EC systems, that in turn distinguish them from large vessel EC. While reports of RANTES expression by HDMEC do not show effects of IFN-γ alone, those studies were only carried out over the 24 hours following stimulation. Therefore the increase which we detected over 24 to 48 hours in HBMEC, if present in HDMEC, would not have been detected.

One common element in cytokine-induced chemokine expression is the synergism between TNF-α and IFN-γ in upregulating RANTES production. Synergy between these two cytokines has been described in a variety of instances, including macrophage cytotoxicity (Feinman et al., 1987), nitric oxide and oxygen radical production (Liew et al., 1990) and induction or upregulation of adhesion molecules by endothelium (Doukas and Pober, 1990; Johnson and Pober, 1990). This synergy is at least partially dependent upon cooperation between Signal Transducer and Activator of Transcription (STAT-1) and nuclear factor κB (NF-κB) (Ohmori et al., 1997), and, in the case of IL-8 production, activation of activator protein-1 and NF-κB (Yasumoto et al., 1992). RANTES production is consistently upregulated following synergistic treatment of several cell types including epidermal and oral keratinocytes (Li et al., 1996), bronchial epithelium (Stellato et al., 1995) and smooth muscle cells (John et al., 1997). RANTES production has been shown to be dependent upon NF-κB activation (Zoja et al., 1998; Moriuchi et al., 1997), and with both NF-κB and AP-1 sites in the upstream regulatory region of RANTES (Nelson et al., 1993), these molecules may also be important in transcriptional responses in HBMEC.

Several reports have described RANTES-positive astrocytes and perivascular T cells during the course of CNS inflammation in AIDS encephalitis, MS or EAE (Miyagishi et al., 1997; Hvas et al., 1997), indicating the presence of RANTES in perivascular regions during inflammation. *In vivo* studies have shown diffuse staining for RANTES in the perivascular space in MS lesions and also specifically within the blood vessel EC (Simpson et al., 1998) by in situ hybridization and immunohistochemistry. In addition, RANTES was localized to EC in the normal appearing white matter (NAWM) of these patients, suggesting a potential involvement in the earlier stages of lesion formation. Endothelial expression of RANTES was elevated in macaques with SIV encephalitis, where it was upregulated in cerebral vessels with and without

perivascular inflammatory cuffs (Sasseville et al., 1996). Our study provides insight into the regulation of RANTES production by cerebral EC, where cytokines produced by the invading mononuclear cells as well as resident cells such as astrocytes and microglia are capable of inducing and upregulating RANTES expression by cerebral EC.

4.2.2 MIP-1β

RT-PCR analysis of HBMEC *in vitro* revealed that unstimulated cells show negligible RNA for MIP-1β. However, following co-incubation with TNF-α and IFN-γ, faint bands were indeed present indicating the presence of MIP-1β RNA. The presence of MIP-1β in the supernatants of untreated cultures as well as those treated with proinflammatory cytokines was tested using sandwich ELISA. Unstimulated HBMEC produced very small amounts of MIP-1β (10 to 20 pg/ml) in the culture media, whereas treatment with TNF- α in combination with IFN-γ or IL-1β doubled the amount of detectable protein to 40 to 64 pg/ml. LPS was also effective at upregulating MIP-1β release from HBMEC. The detection of minimal RNA in unstimulated cells is comparable to their lack of expression using immunohistochemistry; the increase in RNA expression following cytokine treatment also parallels the increased localization of MIP-1β to these activated HBMEC.

Parallel studies revealed that HUVEC neither expressed MIP-1β RNA in the presence of pro-inflammatory cytokines, nor released MIP-1β in the supernatants even after cytokine stimulation. The levels detected in supernatants were consistently less than 20 pg/ml, which is at the lower detection limit of the assay, making these values insignificant. Since identical culture conditions were used for the growth of HUVEC and HBMEC, it seems apparent that HBMEC but not HUVEC can be induced to produce MIP-1β in response to pro-inflammatory cytokines.

Very few studies have been performed to investigate the regulation or induction of MIP-1β expression by EC isolated from different vascular beds, allowing for little discussion as to variations in the literature. While cytokine treatment of HDMEC *in vitro* could not induce MIP-1β expression by these cells, hepatic endothelium *in vivo* was shown to express MIP-1β following liver transplantation (Adams et al., 1996; Afford et al., 1998), which required the use of in situ hybridization to ascertain that the MIP-1β was in fact a product of the endothelium, rather than being bound to GAGS or receptors at the endothelial surface following production from another cell type. As such, it appears that MIP-1β production by EC may be selective within certain vascular beds during inflammatory processes.

MIP-1β expression is upregulated and has been localized to the perivascular space during CNS inflammation in AIDS encephalitis, MS, EAE as well as trauma (Sasseville et al., 1996; Simpson et al., 1998; Miyagishi et al., 1997; Hausmann et al., 1998). The strongest expression was found in glial cell populations and in infiltrating mononuclear cells. Specifically, Simpson et al. (1998) localized MIP-1β to cerebral endothelium; while mRNA was not detected, this may be explained by the lower sensitivity resulting from the combination of immunohistochemistry with in situ hybridization. Similar to RANTES, endothelial expression of MIP-1β was present in vessels with and without inflammatory infiltrates during SIV encephalitis (Sasseville et al., 1996), suggesting the potential involvement of MIP-1β as a mediator of lymphocyte trafficking at the BBB.

4.2.3 Binding of RANTES and MIP-1β to the surface of HBMEC

The presence of RANTES and MIP-1β bound to the surface of HBMEC was detected using immunogold silver staining. While neither RANTES nor MIP-1β was detected on

unstimulated HBMEC, treatment with pro-inflammatory cytokines resulted in the presence of both chemokines at the cell surface. The weak peppery staining observed on stimulated cells is in accordance with the secreted nature of these molecules; the majority of the protein is likely detected in the supernatants while only a portion remains bound to ligands at the EC surface during the release process.

It is difficult to imagine how soluble cytokines might serve to enhance leukocyte adhesion and migration unless in close proximity to or in association with the vascular wall; it has been argued that without such an association, chemokines would be immediately diluted into the bloodstream. Notably, several chemokines have been localized *in situ* to the vascular endothelium using immunohistochemical techniques (Tanaka et al., 1993a), although it has remained unclear as to whether these chemokines were bound to specific saturable receptors at the endothelial cell surface, or maintained in the cytoplasm of the EC which produced them.

Potential presentation molecules at the endothelial surface are the receptors for these chemokines. The failure of TNF-α in combination with IFN-γ to induce the expression of either CCR3 or CCR5 on HBMEC *in vitro* is in keeping with results obtained *in vitro* using HUVEC (Gupta et al., 1998). Yet studies by Hohki et al., (1997) demonstrated the expression of CCR3 in human mucosal microvascular EC. CCR5 has been localized to the EC of lymph node, spleen and brain (Rottman et al., 1997). These conflicting observations might be attributed to the use of different antibodies, as we have noted in this study, that several different Abs to CCR5 applied to frozen sections of tonsil and brain identify different cells expressing CCR5. Another molecule which may be a ligand for chemokines at the EC surface is the promiscuous Duffy antigen receptor which binds RANTES and several other β and α chemokines, but not MIP-1β. The Duffy antigen has been localized to the microvessels of normal brain (Desbaillets et al., 1997), although its expression on HBMEC *in vitro* was not investigated in this study. Based on our

immunocytochemical observations on HBMEC *in vitro*, it is unlikely that the receptors CCR3 or CCR5 are present at the EC surface binding RANTES and MIP-1β.

It has previously been established that chemokines bind heparin and heparin-like molecules, and studies have shown that glycosaminoglycans (GAGs) are the binding sites for IL-8, RANTES and MCP-1 on endothelial cells: competition assays demonstrated competition with immobilized heparin while EC showed no mRNA for known chemokine receptors (Hoogewerf et al., 1997). The scenario thus depicts chemokine "presentation" by glycosaminoglycans to leukocytes at the endothelial cell surface. Elegant studies carried out by Hub et al. (1998) describe the binding of RANTES, MCP-1 and MIP-1α to ECs in human skin. RANTES bound specifically to EC of venules and small veins but not arteries or capillaries. While it is known that heparin sulfate proteoglycans are abundant on the luminal surface of EC, there is considerable structural diversity due to post-translational processing (Kjellen and Lindahl, 1991); a well described PG, syndecan is only expressed on capillaries in granulation tissue (Elenius et al., 1991), whereas for other GAGs, structural changes become evident as tissues respond to injury or in wound repair (Wight 1989). As such, the GAG composition of a particular EC may influence its ability to bind and present chemokines. In particular, cytokines have been shown to induce quantitative changes in GAGs on the surface of HUVEC and aortic EC resulting in altered distribution of anionic sites. Although the anionic sites, which are the potential chemokine presenters on rat brain EC, are stable and remain unchanged after activation with cytokines (dos Santos et al., 1996), it remains to be determined whether the same stability exists within human brain EC. It is not presently known which endothelial proteoglycans are involved and which are physiologically relevant to the presentation of chemokines. Candidates include molecules such as CD44 because 1) it can carry heparan sulfate side chains which would allow them to bind chemokines, 2) it is expressed on EC and becomes upregulated with activation, 3) it

has been implicated in T cell adhesion, 4) the alternative splicing of different forms of CD44 in different tissues might affect the binding of chemokines, and thus the tissue specificity of chemokine presentation and 5) its presentation of MIP-1β to T cells has already been documented *in vitro* (Tanaka et al., 1993a; Tanaka et al., 1993b). These proteoglycans and GAGs are the most likely candidates for chemokine presentation at the EC surface.

While several studies have addressed the binding of exogenously added chemokines to EC or localized chemokines to EC *in vivo*, this is the first study to show RANTES or MIP-1β at the surface of cells which synthesize them. Proteoglycan-immobilized MIP-1β was found to induce the binding of T cells to VCAM-1 *in vitro* (Tanaka et al., 1993b) and GAG-bound chemokines including RANTES, MCP-1, IL-8 and MIP-1α induced the polymerization of chemokines to increase their local concentration and enhance effects upon high-affinity receptors (Hoogewerf et al., 1997). This availability of RANTES and MIP-1β at the EC surface for interaction with receptors on leukocytes in the circulation is what potentiates their functional roles in mediating T cell adhesion and migration at the blood and tissue interface.

4.3 FUNCTIONAL ROLE OF RANTES AND MIP-1β IN MEDIATING T CELL SUBSET ADHESION TO AND MIGRATION ACROSS HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYERS

4.3.1 Effects of cytokines and chemokines on human brain microvessel EC permeability

Since the next goal of these studies was to investigate the effects of chemokine gradients on the adhesion and transendothelial migration of T lymphocytes across HBMEC monolayers, it was imperative to determine the pattern of diffusion of RANTES and MIP-1β through the solubilized collagen substrate and the HBMEC monolayers during the adhesion and migration

assays. Studies performed by Roth et al., (1995) have shown that untreated HUVEC grown in a transwell filter chemotaxis chamber reduced diffusion of the chemokines IL-8 and MCP-1 from the lower to the upper chamber compared to diffusion across the filter alone. Our studies compared the % equilibration as a function of radiolabeled chemokine in the upper chamber compared to the lower chamber across the collagen disc alone, and across HUVEC or HBMEC cultures grown to confluence on collagen discs. The diffusion of RANTES and MIP-1β across collagen membranes was similar. Diffusion of both chemokines was greatly retarded by the presence of HBMEC, whether untreated or following treatment with TNF-α and IFN-γ for 24 hours. Cytokine treatment of the HBMEC monolayer, however, increased the diffusion of chemokines into the upper chamber. In comparison, chemokines diffused much more readily across resting HUVEC reflecting the comparatively increased permeability of HUVEC monolayers in keeping with their low electrical resistance. The % equilibration of activated HBMEC was in fact similar to that of resting HUVEC cultures. It is notable that these differences in chemokine diffusion were most marked during the first 0.5 to 2 hours after the addition of chemokines to the lower chamber. Beyond 2.5 hours the % equilibration was not significantly different between the HBMEC and HUVEC cultures, either untreated or cytokine-treated. Separate studies showed that the addition of RANTES or MIP-1β did not by themselves significantly affect the electrical resistance/permeability of resting HBMEC cultures over an 8 hour period (data not shown). These data show that RANTES and MIP-1β pass through the endothelial monolayer. However passage is much more restricted by intact EC than by a cytokine-treated monolayer. Treatment of confluent HBMEC cultures with 200 U/ml IFN-γ induces morphological changes of the monolayers which are associated with increased permeability to macromolecules (Huynh and Dorovini-Zis, 1993). Similarly, TNF-α treatment of HBMEC decreases the electrical resistance of confluent monolayers so that they become

permeable to large molecules (Wong 1995). The increased permeability resulting from these treatments is due to increased permeability of the interendothelial tight junctions in the absence of increased vesicular transport. It is, therefore, possible that the increased passage of chemokines across the HBMEC monolayers after treatment with TNF-α and IFN-γ in the present studies is due to increased paracellular diffusion through permeable tight junctions.

Resting HBMEC restrict passage to a much greater extent than resting HUVEC. This difference is most likely related to the greater permeability of tight junctions between HUVEC and may account for the reported differences in the effects of chemokine gradients on leukocyte migration across EC monolayers derived from different vascular beds. One potential mechanism for this increased passage would be receptor-mediated endocytosis, however neither CCR3 or CCR5 were localized to these cells. While increased permeability due to cytokine treatment would explain the increased passage of chemokines across the cytokine-treated monolayers, additional mechanisms may be involved to account for the transport of chemokines across resting HBMEC and for the much greater diffusion across intact HUVEC.

In addition to increased permeability, a number of morphological changes occur in EC following exposure to TNF-α and IFN-γ. These include changes in morphological characteristics consistent with alterations in monolayer permeability such as reorganization of monolayers, the upregulation of cell adhesion molecules and chemokines and the induction of a pro-adhesive and pro-migratory state for leukocytes (Oppenheimer-Marks et al., 1988; Stolpen et al., 1986; Frigerio et al., 1998).

4.3.2 Chemokines mediate T cell adhesion to HBMEC *in vitro*

The mechanisms involved in T cell recruitment to sites of inflammation have been characterized on a general level to consist of the following: rolling along the EC surface,

attachment and firm adhesion, followed by migration, typically along a chemotactic gradient, to the site of antigenic challenge. Within this dogma of lymphocyte trafficking or "homing" it has become accepted that naïve lymphocytes tend to traffic through lymph nodes, whereas memory lymphocytes tend to traffic through non-lymphoid tissues and remote organs (Mackay 1993; Mackay et al., 1992). However, primed memory or recently activated T cells are the predominant subset found at sites of inflammation, such as graft rejection, delayed type hypersensitivity, or otherwise (reviewed in Shimizu et al., 1992). T cell infiltration of the CNS is quite similar: memory CD45 RO cells are the major constituent of infiltrates seen in EAE lesions, MS plaques, viral encephalitis and chronic inflammation models of *Corynebacterium parvum* (Jensen et al., 1992; Pitzalis et al., 1988; Engelhardt et al., 1995). However, during the recruitment phase of the disease, several other subsets of mononuclear cells have been identified within lesions, yet the mechanism for their recruitment has not been fully characterized. The aforementioned localization of various chemokines to perivascular cuffs in CNS inflammation, as well as their localization to other inflammatory sites undergoing extensive leukocyte-subset specific infiltration, suggests a role for these molecules in promoting the adhesion as well as migration of distinct leukocyte subsets during CNS inflammation.

4.3.2.1 Resting and activated CD4+ T cell subsets

Several studies have demonstrated the ability of RANTES and MIP-1β to enhance the adhesion of T lymphocytes to endothelial cells or endothelial cell ligands. A large body of evidence indicates that each chemokine specifically attracts certain T cell subsets to sites of inflammation. In our studies, very few resting CD4+ T cells adhered to resting EC monolayers, and adhesion was not increased in the presence of RANTES or MIP-1β in the subendothelial region or when chemokines were present in both upper and lower chambers. Adhesion was

increased by 2 to 5 fold when HBMEC were treated with TNF-α and IFN-γ. These results are consistent with previous studies showing increased adhesion of T lymphocytes to both cerebral and extracerebral EC following EC treatment with pro-inflammatory cytokines (Wysocki and Wykretowic, 1993; Oppenheimer-Marks et al., 1990), particularly studies by Hughes et al. (1988), which showed a 2 to 3 fold increase in lymphocyte adhesion to rat cerebral capillary EC following stimulation with a combination of TNF-α plus IFN-γ. Adhesion of T cells to HBMEC *in vitro* has been previously shown to increase following stimulation of cultures with 100 U/ml of TNF-α (Wong et al., 1998).

The adhesion of activated CD4+ T cells to resting HBMEC was consistently two to five fold greater than resting CD4+ T cells adhesion, supporting the accepted theory that activated T cells are more likely to adhere to BBB EC than resting CD4+ T cells. In general, activated lymphocytes adhere more readily than resting lymphocytes to unstimulated EC, (Oppenheimer-Marks et al., 1990) and antigen-challenged rat lymphoblasts adhere much better to resting EC than small lymphocytes (Wysocki et al., 1992). However, it has been suggested that the mode of T cell activation plays an important role in the adhesion pathways used by the T cell. *In vitro* stimulation of T cells with phorbol myristate acetate (PMA) and concanavalin A for 1-4 hours caused a 7 to 12 fold increase in adhesion to unstimulated and IFN-γ-stimulated EC which was partially inhibited by anti-VLA-4, whereas enhanced T cell adhesion following anti-CD3 or IL-2 stimulation required 2 to 3 days of activation and was not inhibited by anti-VLA-4 (suggesting greater involvement of the LFA-1 pathway). Since it has been observed that different cytokines regulate CAM expression on EC differently, it is likely that modes of activation of EC would also affect adhesion pathways followed during lymphocyte/EC interactions.

Although the role of chemokines in T cell adhesion to cerebral EC has not been previously addressed, several studies have investigated the effects of chemokines on T cell

adhesion to extracerebral EC. RANTES increased the adhesion of resting T cells to IL-1 treated but not resting HUVEC at 10 ng/ml (Taub et al., 1993a). Resting or anti-CD3 activated T cell subsets treated with RANTES and MIP-1β for 6 hours adhered more readily to IL-1 activated HUVEC but no effect was seen with unstimulated EC (Taub et al., 1993b). Specifically, RANTES upregulated the adhesion of resting T cells, whereas MIP-1β had an effect only on the adhesion of anti-CD3 activated T cells. Further separation of these cells into CD4+ subsets showed that RANTES induced adhesion of resting and activated CD4+ T cells, whereas MIP-1β induced the adhesion of activated CD4+ T cells. In another study, soluble MIP-1β (0.1 to 10 ng/ml) induced adhesion of T cells to untreated EC isolated from the synovium of patients with rheumatoid arthritis; this increase was further enhanced once MIP-1β was immobilized upon these cells (Tanaka et al., 1998). The authors suggest that these EC were likely activated in some way, as the majority of observations describe increased adhesion only to cytokine-treated EC. Blocking of heparan sulfate proteoglycan synthesis reduced the amount of MIP-1β bound to the surface of adult T cell leukemia cells and prevented these cells from integrin-mediated adhesion to IL-1 treated HUVEC and ICAM-1 (Tanaka et al., 1996), suggesting not only a role for MIP-1β in enhancing T cell adhesion, but stressing the importance of GAGs in presenting these molecules to their receptors.

Studies to date have focussed on the effects of T cell subset exposure to chemokines on T cell adhesion, yet the role of chemokine gradients in these events has not been characterized. The above results are similar to those obtained in the present study, where RANTES and MIP-1β at concentrations of 10 to 100 ng/ml significantly enhanced adhesion of resting CD4+ T cells to activated EC. It is important to note that 5 to 10 % of the resting CD4+ T cell population consisted of activated CD4+ T cells high in IL-2 R expression; this is in accordance with data suggesting that 10 % of peripheral blood T lymphocytes are activated (Hannet et al., 1992). The

presence of RANTES in the subendothelial region increased adhesion compared to controls by up to 27%. MIP-1β was slightly more effective and enhanced adhesion up to 50 %, although variations were detected amongst T cells and EC from different donors. Similar responses were noted in controls, where chemokines were present in both the upper and lower chambers, suggesting that the effect of soluble chemokines in the upper chamber as well as their presence in the lower chamber in the form of a gradient can enhance T cell subset adhesion.

Activated CD4+ T cells were also responsive to chemokine gradients; RANTES was able to enhance adhesion to activated HBMEC by 20 to 30 %, whereas MIP-1β was more effective and enhanced the adhesion of activated CD4+ T cells to activated HBMEC by 75 to 90%. The presence of RANTES and MIP-1β in the upper and lower chambers also enhanced adhesion of activated CD4+ T cells to activated HBMEC, again suggesting that soluble chemokines in the upper chamber also enhance the adhesion of activated CD4+ T cells. A possible scenario is that RANTES or MIP-1β diffuse from the lower to the upper chamber through or perhaps between cytokine-activated and to a lesser degree resting HBMEC. Once bound to GAGs at the endothelial surface, RANTES and MIP-1β would enhance the potential for interactions with receptors on T cell subsets leading to enhanced adhesion. Increased permeability of the HBMEC monolayers and passage of chemokines through the endothelium is not sufficient to enhance adhesion however, since neither chemokine gradients nor the presence of chemokines in the upper chamber without gradients was capable of enhancing adhesion to resting HBMEC. This suggests that some element consistent with or present on the surface of activated HBMEC is required.

A possible mechanism responsible for enhanced adhesion in the presence of chemokines is increased binding to adhesion molecules or ECM at the EC surface. Six hour incubations with RANTES or MIP-1β at 1 to 10 ng/ml induce the adhesion of both resting and anti-CD3 activated

T cells to purified ICAM-1 and VCAM-1 (Lloyd et al., 1996). Adhesion was significantly enhanced following as little as 15 minutes of peripheral blood T cell (PBTC) treatment with RANTES or MIP-1β. 60 minutes of pre-incubation with RANTES or MIP-1β also enhanced adhesion of PBTC to fibronectin (FN), collagen, and laminin; a preference for CD4+ over CD8+ T cell induced adhesion to FN by MIP-1β was observed; however, no such preference was observed for RANTES. Interestingly, similar studies by Gilat et al., 1994 suggest that RANTES and MIP-1β enhanced adhesion to ECM requires chemokine binding to heparan sulfate (HS), because HS removal by heparinase eliminates the effect as does pre-treatment of chemokines with heparin or HS. Again, responses were detectable within 15 minutes of chemokine exposure. The increased adhesion to ICAM-1 and VCAM-1 by the chemokines is a likely event in our observations of enhanced adhesion of resting and activated CD4+ T cell subsets to activated but not resting HBMEC, as VCAM-1 is first induced on HBMEC by cytokine treatment, and the low basal expression of ICAM-1 is greatly upregulated following cytokine treatment (Wong and Dorovini-Zis, 1992; Wong and Dorovini-Zis, 1995).

Antibody blocking studies have suggested that enhanced adhesion in response to chemokines is in fact integrin dependent. Chemokine-induced T cell adhesion to ICAM-1 and VCAM-1 was markedly reduced by treatment with neutralizing β2 (LFA-1) and β1 (the β component of VLA-4) integrin antibodies respectively (Lloyd et al., 1996), as was the background adhesion in the absence of a stimulus. Furthermore, antibodies to the α4 region of VLA-4 but not to CD18 significantly inhibited the augmented T cell adhesion to VCAM-1 caused by chemokines. Notably, antibodies to VLA-4 also inhibited the chemokine-induced adhesion to fibronectin. In addition, CD44 immobilized MIP-1β-induced T cell adhesion to VCAM-1 was substantially reduced in the presence of neutralizing antibodies to VCAM-1, the

α4 subunit of VLA-4, and the β1 subunit; no effect was seen with antibodies to β2, suggesting that MIP-1β triggers the adhesion of T cell subsets to VCAM-1 via β1 integrins.

In our studies, antibodies to ICAM-1 significantly reduced the control and chemokine-enhanced adhesion of both resting and activated CD4+ T cells to activated HBMEC suggesting a dependence upon ICAM-1. In particular, anti-ICAM-1 reduced the adhesion of resting and activated T cells to resting HBMEC; these results correspond with observations that a mAb to CD18 markedly decreased the binding of control T cells to resting HUVEC (Oppenheimer-Marks et al., 1990). ICAM-1 present on cerebral EC participates in the adhesion of activated lymphocytes to cerebral EC (McCarron et al., 1993) and the interaction between LFA-1 and ICAM-1 is important in MS patient lymphocyte adhesion to brain EC (Tsukada et al., 1993; Tsukada et al., 1994). The adhesion of peripheral T lymphocytes to rat cerebral EC treated with TNF-α plus IFN-γ was inhibited by 60 to 70% with antibodies to β1 integrins (Hughes et al., 1988). This reduction was observed in the presence of chemokine gradients and when chemokines were added to both chambers. Anti-VCAM-1 antibodies had more variable effects: mAb to VCAM-1 decreased resting CD4+ T cell adhesion to activated HBMEC by up to 23% in the absence of gradients compared to 17 to 25% and 17 to 46% when RANTES was present in both chambers or in the subendothelial region. The increased adhesion of resting CD4+ T cells to activated HBMEC by MIP-1β or MIP-1β gradients was reduced by up to 40% and 15 to 12% respectively. Activated CD4+ T cell adhesion to activated HBMEC was reduced by up to 35% with anti-VCAM-1. In addition, VCAM-1 blocking reduced the increased adhesion in the presence of RANTES or MIP-1β. Consistently, the anti-α4 Ab reduced activated CD4+ T cell adhesion to activated HBMEC by 21 to 25 % and the RANTES induced adhesion by 34 to 40%. α4 mAb had no effect on the MIP-1β gradient–induced adhesion, but reduced the increased

adhesion due to MIP-1β in both chambers by 34%. These data indicate that cytokine activation of HBMEC is required for CAM-dependent enhanced T cell adhesion mediated by chemokines.

Studies by Lloyd et al. (1996), showed that chemokine treatment of resting or anti-CD3 activated PBTC for 60 minutes did not result in increased expression of the molecules CD18, CD29, LFA-1, VLA-4, ICAM-1 or VCAM-1. This suggests that while the increased adhesion in response to chemokines appears to be β1 and β2 integrin dependent due to interactions between LFA-1/ICAM-1 and VLA-4/VCAM, the chemokine effect on these cells is not a result of increased T cell integrin expression. The effects of chemokine exposure on T cell CAM expression were not addressed in the present study. However, treatment of HBMEC with 100 ng/ml of RANTES or MIP-1β does not upregulate the expression of ICAM-1 or VCAM-1 on the surface of EC (data not shown), suggesting that the effects of chemokines are not mediated by the increased expression of CAMS on EC. Instead, the effect is likely mediated via chemokine-induced upregulation in the affinity of these integrins for their ligands.

While the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways mainly mediate interactions between lymphocytes and endothelium, the interactions between LFA-1 and ICAM-1,-2, and –3 play an important role not only in lymphocyte/EC interactions but also in lymphocyte/lymphocyte interactions. In fact, the high expression of ICAM-3 on both resting and activated T cells suggests an important role for this CAM in the early phase of the immune response (Fawcett et al., 1992). Another effect of chemokines on lymphocytes is the induction of cellular polarization and the redistribution of adhesion receptors during interactions with EC and ECM. As T cells become activated they become polarized and display a cellular uropod (Wilkinson and Higgins, 1987). ICAM-3, the most prominent ligand for LFA-1 on resting T lymphocytes, has been localized in high concentrations to this uropod, as have several other CAMS including ICAM-1 and CD43 (del Pozo et al., 1995); from this structure these CAMS

may be involved in the "trapping" of lymphocytes at a given site. Specifically, ICAM-3 has been implicated in the aggregation of lymphocytes, where ICAM-3 localized in high density to the uropod interacts with ICAM-3 on other lymphocytes, resulting in low levels of aggregation. As the aggregates of T cells become larger, the uropods are exposed to the external milieu, where they can further encourage T cell aggregation through LFA-1/ICAM-1 interactions (Campanero et al., 1993). Uropod formation and ICAM-3 redistribution has been observed during interactions between HUVEC and T cells: uropods were noted in 5 and 10% of freshly isolated T lymphocytes and T lymphoblasts respectively within 30 minutes. These numbers were significantly increased by a mAb to ICAM-3; uropod formation was further increased at 4 hours (Campanero et al., 1993).

Both RANTES and MIP-1β have been found to induce uropod formation and redistribute ICAM-3 to uropods during T lymphoblast adherence to ICAM-1 or VCAM-1 (del Pozo et al., 1995). In our studies, uropods were often observed towards the end of the adhesion assay, most commonly during resting, activated or memory T cell subset adhesion to cytokine-activated HBMEC. Uropod formation was prominent when RANTES or MIP-1β were present. This increase in uropod formation and also cell aggregation in response to chemokines may provide an additional explanation for the increased adhesion/aggregation of recently activated lymphocyte subsets to activated EC. Interestingly, kinetics of chemokine-mediated uropod formation show uropods forming within 5 minutes following exposure to chemokines in the range of 1 to 100 ng/ml and declining beyond 4 to 6 hours (del Pozo et al., 1995). Other studies have shown that RANTES treatment of pertussis-toxin specific CD4+ T cell clones resulted in homotypic cell aggregation within 15 minutes, and increased up to 20 hours: similar results were obtained using CD45RO+ T cells, although aggregation occurred to a lesser degree (Szabo et al., 1997). This RANTES-induced homotypic cell aggregation was virtually abolished by pre-

treatment of T cells with mAb to LFA-1 or β2 integrins, whereas mAb to ICAM-1 had a significant but lesser effect. These results were not observed with other chemokines including MIP-1α and MIP-1β. Interestingly, this aggregation was found to be pertussis toxin insensitive, but was inhibited by herbamycin A, suggesting tyrosine-kinase dependence. While this study was able to report increased expression of cell surface molecules on memory and naïve T cells exposed to RANTES suggesting an activating potential for RANTES on these cells, increases in VLA-4, CD18, and VLA-6 were only observed after 20 hours of incubation. It is highly unlikely that such increased CAM expression on T cells would occur in our one hour long adhesion assay. The timing of aggregation and uropod formation events suggest that the exposure of resting, activated or memory T cell subsets to RANTES or MIP-1β, either present in both the upper and lower chamber, or having diffused through a cytokine treated HBMEC monolayer, enhances uropod formation and cell aggregation during the one and three hour time periods of the adhesion and migration assays (del Pozo et al., 1995).

4.3.2.2 Memory, antigen-specific clone and naïve CD4+ T cell subsets

Memory CD4+ T cells and antigen-specific T cell clones used in this study share several characteristics, including recent activation and high expression of CD45RO. *In vitro* studies have shown that memory T lymphocytes adhere readily to IFN-γ-activated HDMEC compared to unstimulated EC; adhesion was much greater than that of naïve T cells and was partially inhibited by anti-E-selectin antibody (Lee et al., 1995). Anti-ICAM-1 did not reduce this adhesion significantly, although anti-ICAM-1 greatly reduced memory CD4+ T cell adhesion to IL-1 activated HDMEC, as did VCAM-1 although to a lesser degree. This observation may be only partly relevant to HBMEC in our study; HBMEC upregulate E-selectin only in response to TNF-α, but not to IFN-γ, and anti-E-selectin Abs had no effect on the adhesion of a T cell

population to TNF-α treated HBMEC (Wong et al., 1999). These observations may differ due to the differential expression of E-selectin in these two cell types in response to different cytokines, or the effects observed with memory cells may have been significant because a pure population of the memory T cell subset was analyzed. In our studies, memory and antigen specific CD4+ T cells responded highly to RANTES and MIP-1β with enhanced adhesion up to 200% in the presence of RANTES or MIP-1β concentration gradients. Controls where chemokines were present in both the upper and lower chambers also enhanced adhesion, indicating that receptors for RANTES and MIP-1β were indeed responding to the chemokines and were present on a significant proportion of the memory cells assayed. Other *in vitro* studies suggest that RANTES enhances the ability of unstimulated memory T cells to adhere and migrate (Taub et al., 1993b) whereas similar studies with MIP-1β have not been carried out. While CAM blocking studies were not performed on the chemokine-induced adhesion of these two CD4+ T cell subsets, it is generally accepted that the same involvement of LFA-1/ICAM-1 and VLA-4/VCAM-1 is expected, as recently activated subsets typically show increased levels of LFA-1 and VLA-4 in addition to CD44, similar to the activated CD4+ T cell subsets described above.

4.3.2.3 Chemokine receptor expression by CD4+ T cell subsets

It is likely that regulation of the receptors for RANTES and MIP-1β on these T cell subsets dictates the response to chemokines within this system. The resting CD4+ T cell population, which responded to a small degree to RANTES and MIP-1β, showed high levels of CCR3 and CCR5 expression in only 2.5% of cells. CCR3 and CCR5 expression on memory cells were slightly higher at 3.8 and 7.9% respectively. Similarly, antigen specific clones exhibited 12 and 15% expression of CCR3 and CCR5 respectively, suggesting that this population should

respond to RANTES and MIP-1β in a similar manner to memory cells if responses were indeed consistent with receptor expression. The anti-CD3 + IL-2 activated population exhibited much higher levels of CCR3 and CCR5 expression at 25 and 26% respectively, as did the population activated with anti-CD3 alone. As such, it appears that the subsets exhibiting greater expression of the receptors showed greater responses to chemokines and chemokine gradients.

Accordingly, increases in cell adhesion of up to 90% were observed in response to chemokine gradients as well as chemotactic controls with chemokine in both the upper and lower chambers. In contrast, only 0.4% of the naïve CD4+ T cell population showed CCR3 and CCR5 expression on their surface; the minimal of receptor expression for RANTES and MIP-1β most likely accounts for the lack of effect of RANTES and MIP-1β on naïve CD4+ T cell adhesion to resting or activated HBMEC.

Upregulation of CCR3 and CCR5 mRNA expression in activated and memory T cells has been described by several investigators, and appears to be dependent on exogenous cytokines such as IL-2. Expression of these two receptors may also distinguish CD4+ T cells into the Th1 and Th2 subtypes as Th2 lymphocytes preferentially express CCR3, and CCR5 was expressed at high levels on Th1 cells and is virtually absent from Th2 cells (Loetscher et al., 1998). In these instances, CCR5 expression is rapidly lost in the absence of IL-2, and activation by anti-CD3 has actually resulted in CCR5 downregulation. Although we did not assess the levels of CCR5 expression after longer periods of time to test for further increases, we did not observe significant differences between CCR3 and CCR5 expression in CD4+ T cells activated with anti-CD3 alone or in combination with IL-2 for 24 hours. This discrepancy is likely due to the short activation period of 24 hours used in this study, whereas downregulation of receptors is most significant two days after T cell activation. Our results are comparable to studies on Th1 and Th2 cells which revealed lack of CCR3 and CCR5 expression on naïve lymphocytes, whereas a small

proportion of memory/activated T cells, 1-10% and 18-32% expressed CCR3 and CCR5 receptors respectively (Sallusto et al., 1998). These studies also revealed the loss of CCR5 expression after 2 days of IL-2 withdrawal, whereas no such decrease in CCR3 expression was observed following IL-2 withdrawal. Another study gated specifically for CD4+ memory T cells in adult peripheral blood showed 26.1% of cells expressing CCR5 (Mo et al., 1998), supporting our observations that CCR3 and CCR5 expression are expressed in increasing order of magnitude on naïve (at zero) < resting < memory < activated CD4+ T cell subsets.

4.3.3 Chemokines mediate the migration of CD4+ T cell subsets across human brain microvessel endothelial cells

4.3.3.1 Resting and activated CD4+ T cell migration across resting and cytokine-treated HBMEC

Migration of resting CD4+ T cells across resting HBMEC monolayers was extremely low as only .240 resting CD4+ T cells migrated per mm of monolayer. This migration was increased by 200% following activation of HBMEC monolayers with TNF-α and IFN-γ for 24 hours. Studies in mice have shown a 2.5 fold increase in migration of splenocytes across murine brain microvessel endothelial cells following cytokine treatment of EC; the ability of optimal concentrations of cytokines to enhance migration was greatest for TNFα>IFN-γ=IL-1α (Fabry et al., 1995). Activation of rat retinal EC with IFN-γ did not enhance the level of lymphocyte or retinal-antigen specific T lymphocyte migration however, suggesting that IFN-γ was most likely incapable of inducing CAM expression on these EC (Greenwood and Calder, 1993). IL-1 or IFN-γ pretreatment of HUVEC resulted in a 17 or 31% increase in the number of migrating T lymphocytes compared to controls (Pietschmann et al., 1992). Anti-CD3 activation of CD4+ T cells augmented migration across resting HBMEC monolayers by 42% (.342 cells per mm of

monolayer). Several studies have shown that T cell activation is a requirement for T cells to migrate across unactivated EC (Masuyama et al., 1992).

4.3.3.2 Cell adhesion molecule blocking of CD4+ T cell migration across HBMEC

The anti-ICAM-1 mAb significantly blocked resting CD4+ T cell migration across resting HBMEC to 3.5% of controls; similarly, anti-ICAM-1 mAb blocked migration of activated CD4+ T cells across resting HBMEC (11% of controls). Neither anti-VCAM-1 nor anti-α4 mAb had any effect on the migration of resting or activated CD4+ T cells across resting HBMEC suggesting a VLA-4/VCAM-1 independent pathway. Antigen-specific T cell migration across unstimulated rat cerebral EC was inhibited to 8.0% and 6.8% of control values by blocking of LFA-1 or ICAM-1; again, blocking VLA-4 or VCAM-1 had no effect (Pryce et al., 1997). Studies on HUVEC also showed no decrease in transendothelial migration of resting T cells across resting EC by anti-VCAM-1 treatment (Oppenheimer-Marks et al., 1993a; Oppenheimer-Marks and Lipsky, 1993b). An interesting consideration is that activated T cells have been shown to activate and induce the expression of CAMS and cytokines by resting HBMEC following direct cell/cell contact (Lou et al., 1996). E-selectin, ICAM-1 and VCAM-1 were all substantially upregulated to levels observed with TNF-α treatment at 1000 U/ml (with the exception of VCAM-1 which was slightly decreased compared to TNF-α treatment). These increases were found to be at least partly modulated by the production of TNF-α. Therefore, it is possible that activated CD4+ T cells serve to activate the HBMEC, and may in fact have contributed to the ICAM-1 dependent pathway, thus potentiating the anti-ICAM-1 mAb effects on a previously resting HBMEC monolayer over a period of 3 hours. Interestingly, HBMEC have been shown to be more responsive to stimulation with TNF-α than HUVEC in subsequent expression of CAMs such as ICAM-1 (Hess et al., 1994). This possibility raises an interesting

potential for the induction of CAMS on BBB EC by activated T cells prior to the production of soluble inflammatory mediators, resulting in the adhesion of T cells to HBMEC and therefore contributing to the pathogenesis of inflammatory diseases.

Other studies of T cell migration across resting HUVEC monolayers had varied results: mAb to CD18 inhibited migration of activated T cells through resting HUVEC, but had no effect on migration of resting T cells across resting or activated HUVEC. These differences can most likely be attributed to differences within the assay. In our studies, T cells were added, allowed to incubate and adhere for 30 minutes, after which time blocking mAb to ICAM-1, VCAM-1, and α4 integrin were added to the upper chambers. As a result, mAb would affect both migration as well as adhesion which would still be taking place after the first 30 minutes. Previous studies have shown that the adhesion of both resting and activated T cells to HUVEC cultures is LFA-1 dependent, as mAb to LFA-1 significantly reduce adhesion. Furthermore, incubation of resting T cells with HUVEC for as little as 2 hours in the absence of mitogenic stimulation resulted in the induction of LFA-1 dependent T cell binding to HUVEC (Oppenheimer-Marks et al., 1990). On the basis of these observations, it is quite likely that LFA-1 is induced in resting CD4+ T cells during their 3 hour incubation with HBMEC. Thus, the significant decrease in migration observed with anti-ICAM-1 antibodies might in fact be the result of blocking of the LFA-1/ICAM-1 adhesion pathway. Although the blocking of migration with anti-ICAM-1 may be partly due to blocking of the adhesion process, the present study indicates that migration of activated T lymphocytes across HBMEC monolayers is largely dependent upon the LFA-1/ICAM-1 pathway. The 75% decrease in adhesion of activated T cells to resting HUVEC after LFA-1 blocking observed by Oppenheimer-Marks et al. is in accordance with our own observations on HBMEC.

A number of studies have investigated the effects of RANTES and MIP-1β as chemoattractants using Boyden chambers rather than transendothelial assays. Such studies have been useful in establishing the responses of lymphocyte subsets to various chemokines: RANTES has been shown to preferentially attract CD4+ T cells, particularly those expressing a memory phenotype, with lesser yet significant effects upon pan T cell populations (Schall et al., 1990). MIP-1β at 100 pg/ml was reported to attract CD4+ T lymphocytes, with some preference for T cells of the naïve phenotype; however, in another study, MIP-1β attracted only anti-CD3 activated T cells but not unstimulated lymphocytes and had an affinity for CD4+ T cells (Schall et al., 1993; Taub et al., 1993a). Since vascular endothelium may serve to present chemoattractants to lymphocytes and establish the permeability barrier which maintains the chemotactic gradients, transendothelial migration assays are much more relevant for studying the effects of chemokines on lymphocyte transendothelial extravasation from the circulation.

The presence of RANTES or MIP-1β in the subendothelial region of HBMEC *in vitro* had no effect on the transendothelial migration across resting HBMEC. Similarly, no changes in resting CD4+ T cell migration across resting HBMEC were noted in the presence of chemokines in both chambers. In contrast, RANTES gradients established across cytokine-treated HBMEC resulted in a 25% increase in the number of migrated cells while MIP-1β had a lesser effect which was not statistically significant. The presence of chemokines in both chambers did not have any effect on the migration of resting CD4+ T cells across activated HBMEC. This suggests that RANTES gradients enhance migration of a non-stimulated peripheral CD4+ T cell population.

Similarly, the migration of activated CD4+ T cells across resting HBMEC was not enhanced by chemokine gradients or chemokines present as a chemotaxis control. However, gradients of RANTES and MIP-1β enhanced the migration of activated CD4+ T cell subsets

across cytokine-treated HBMEC by 175 and 193% respectively. Interestingly, chemotaxis controls which contained chemokines in both the upper and lower chambers also augmented migration across activated HBMEC. Since adhesion was upregulated under the same conditions, it is likely that more cells adhered during the three hour incubation period compared to controls. In the absence of a chemotactic gradient to direct them, these cells which have already been shown to display a pro-migratory phenotype remain firmly adherent to the endothelium and are exposed to chemokines which promote their aggregation and the potential to migrate.

The effects of blocking mAbs to ICAM-1, VCAM-1 and VLA-4 upon chemokine-induced migration across activated HBMEC monolayers were investigated. Just as anti-ICAM-1 mAb had reduced the level of resting CD4+ T cell migration across activated HBMEC, it also significantly reduced migration enhanced by RANTES gradients or presence of RANTES in the upper and lower chambers. Levels were reduced to those seen in anti-ICAM-1 treated controls. Similar results were obtained by blocking ICAM-1 in the presence of MIP-1β in the subendothelial region as well as in the upper and lower chambers. Antibodies to VCAM-1 and VLA-4 reduced migration in response to RANTES gradients by 38% and 35 % respectively, but had no effect on the migration of resting CD4+ T cells in controls without gradients or with RANTES in both the upper and lower chambers. Similarly, VCAM-1 and VLA-4 had no effects on controls with MIP-1β present, whereas they reduced migration of resting CD4+ T cells by 56 and 82% respectively ($p<0.05$). These results suggest that resting CD4+ T cell responses to RANTES and MIP-1β are largely LFA-1/ICAM-1 dependent, and that the VCAM-1/VLA-4 pathway is only partially responsible for chemokine-enhanced migration.

The involvement of the LFA-1/ICAM-1 and VCAM-1/VLA-4 pathways in the activated CD4+ T cell migration in response to RANTES and MIP-1β was also investigated. Anti-ICAM-1 mAb almost completely blocked migration in response to RANTES or MIP-1β gradients. mAb

blocking of migration augmented by the presence of RANTES or MIP-1β in both the upper and lower chambers of the assay gave interesting results: ICAM-1 blocking was only capable of reducing the migratory response to RANTES and MIP-1β by one-half to two-thirds, which suggests an additional ICAM-1-independent effect of RANTES or MIP-1β on activated CD4+ T cells. mAb blocking of VCAM-1 and α4 each decreased the enhanced migration in response to RANTES or MIP-1β similarly: anti-VCAM by 72 and 88% and anti-α4 by 64 and 70% respectively. Identical blocking conditions gave similar results in chemotaxis controls with chemokine present in the upper and lower chambers, with the exception of anti-α4 which did not reduce migration of activated CD4+ T cells when MIP-1β was present in the upper and lower chambers.

4.3.3.3 Memory and antigen-specific T cell migration is enhanced by chemokine gradients

Chemokines in the subendothelial region had no effect on memory CD4+ T cell migration across resting HBMEC monolayers. RANTES or MIP-1β gradients, however, had the greatest effect on the migration of these cells in comparison with other subsets, and augmented migration across cytokine-treated HBMEC by 150 and 200% respectively. The presence of MIP-1β in the upper and lower chambers nearly doubled migration across activated HBMEC, whereas no such effect was observed for RANTES. These observations are consistent with the pro-migratory phenotype of memory cells.

Naïve CD4+ T cells did not migrate across resting HBMEC monolayers. Migration was not enhanced by chemokine gradients. While cytokine treatment of HBMEC resulted in few cells migrating across the monolayer, these observations are consistent with naïve cells lacking a migratory phenotype responsive to RANTES or MIP-1β.

As was observed in the adhesion experiments, the type of chemokine receptor expressed on the CD4+ T cell subsets appears to dictate the nature of their response to different chemokines. Adhesion and migration in response to chemokines is indeed lymphocyte subset specific; and the greater the expression of the receptor on a given cell type, the greater its response to chemokines. One possible exception to this is the tremendously enhanced migration of memory CD4+ T cells which, in our studies, displayed lower levels of chemokine receptors for RANTES or MIP-1β. However, this enhanced migration may be a synergistic effect, resulting not only from the treatment with chemokines, but also from some other characteristic of these cells, perhaps also coinciding with the high migratory tendencies of this population.

Several studies have focussed on RANTES and MIP-1β induced T cell subset transendothelial migration. RANTES, MIP-1α and MIP-1β have been shown to augment transendothelial chemotaxis of CD3+ T lymphocytes across HUVEC-covered membranes (Roth et al., 1995). Although chemokine diffusion across these monolayers was inhibited compared to non-HUVEC covered membranes, significant chemokine-induced effects were described across untreated EC. The chemokines tested selectively recruited a memory subset of T lymphocytes across untreated HUVEC at optimal concentrations of 10 to 100 ng/ml; RANTES and MIP-1β enhanced CD45 RO T cell migration by 3.0 and 6.0 times respectively. These greater responses may be due not only to the greater diffusion of chemokines across HUVEC monolayers compared to HBMEC monolayers, but also to the relative density of the collagen disc in our assays which would likely restrict diffusion to a greater degree than the membrane coated with HUVEC alone. Naïve T cells reportedly did not respond to any of the β chemokines tested, including RANTES and MIP-1β. In these studies, however, migration only occurred when a gradient of chemokines was present, suggesting that migration was not chemokinetic, but rather purely chemotactic in nature (Roth et al., 1998).

One obvious difference between the above studies and our observations concerns the role of chemokine gradients in augmenting migration across an EC monolayer and its dependence upon integrins. To date, there are no known studies on the involvement of CAMs in T lymphocyte chemokine-enhanced migration across EC. Monocyte migration across non-activated HUVEC monolayers in response to RANTES or MIP-1α gradients was blocked up to 90% by mAb to CD18. Migration across IL-1 treated HUVEC was no longer inhibited by mAb to CD18, but was blocked by combination of mAb to α4 chain of VLA-4 with the mAb to CD18, suggesting that the VLA-4 /VCAM-1 pathways play a greater role in monocyte transendothelial migration across activated monolayers (Chuluyan et al., 1995). Our studies also suggest that molecules of the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways are important for chemokine-enhanced migration to occur across cytokine-activated EC, but varies according to the activation state of the T cell. Similar studies performed on unstimulated HUVEC however, (Roth et al., 1995) would suggest that these molecules are less important as ICAM-1 is only very minimally expressed and VCAM-1 not at all by resting HUVEC cultures. This discrepancy may be explained by the heterogeneity between HUVEC and HBMEC, however it strongly suggests that transendothelial migration is far more likely to occur across resting HUVEC than resting HBMEC, lending support to the hypothesis that resting BBB EC are not permissive to leukocyte trafficking, even in the presence of chemokines.

An important event in the process of T cell migration is the production of matrix metalloproteinases (MMPs) which are capable of cleaving components of the basement membrane (Xia et al., 1996). Specifically, MMP-2 and MMP-9, the type IV gelatinases, are important in cleaving type IV collagen, thus attacking the backbone of the basement membrane. Interestingly, both RANTES and MIP-1β have been reported to augment the CD4+ T cell secretion of proMMP-9 and MMP-9, which then can be activated by other MMPs or proteases

such as plasminogen activators (Johnatty et al., 1997; Xia et al., 1996). While an increase in MMP secretion typically doesn't occur until close to 24 hours following exposure to RANTES or MIP-1β, and is likely of little relevance in our 3 hour migration assay, one cannot deny the significance of this induction. The production of MMP-2 and MMP-9 is also induced in T cells following VCAM-1 dependent adhesion to EC (Romanic and Madri, 1994), further suggesting its role as regulator of transendothelial migration. The involvement of these MMPs in immunity has been well described (reviewed by Goetzel et al., 1996). MMP-2 and MMP–9 have been shown to cleave and release the pro-inflammatory cytokine TNF-α from its membrane-bound precursor form, and have been implicated in the pathology of MS, particularly in the breakdown of the BBB, recruitment of leukocytes, myelin destruction and the release of immunogenic peptides such as TNF-α (reviewed in Chandler et al., 1997). Notably, demyelinating MS lesions display enhanced expression of MMP-9 (Cossins et al., 1997) suggesting that chemokine/T cell/MMP interactions may be important in autoimmune-mediated demyelinating events.

Our present in vitro studies support current theories on T cell subset-specific adhesion and migration patterns. Cells exhibiting pro-migratory phenotype would be expected to be pro-adhesive, lending to the importance of adhesive interactions during migration. Unstimulated CD4+ T cells minimally adhere to resting HBMEC monolayers. Recently activated T cells are the only subset able to cross an intact HBMEC monolayer in keeping with in vivo observations in EAE that activated, antigen-specific T cells cross an apparently intact BBB. In our studies, anti-CD3 activated CD4+ T cells were two to five times more likely than untreated CD4+ T cells to adhere to and migrate across untreated HBMEC.

A comparison of memory versus naïve CD4+ T cells revealed that naive CD4+ T cell migration across resting HBMEC was negligible. Memory CD4+ T cells, unlike naïve T cells, readily migrate across untreated HBMEC. Similar trends were observed when the HBMEC

monolayer was activated with cytokines, however adhesion and migration of all subsets was usually doubled across a cytokine-"primed" endothelium. Most importantly, chemokines further enhanced the migration of recently activated subsets across cytokine-actived EC. These results directly correlate with observations of recently activated T cells trafficking primarily at remote sites while naïve cells are excluded from these sites under normal conditions.

Our studies suggest that RANTES and MIP-1β play a significant role in mediating T cell subset trafficking, particularly of recently activated cells, across the BBB EC. Several reports have investigated the effects of limiting lymphocyte recruitment in response to these chemokines. It has been reported that neutralizing antibodies to MIP-1α, a particularly potent monocyte chemoattractant as well as chemoattractant for CD8+ and some CD4+ T cells which shares 70% homology with MIP-1β, prevented the development of EAE following the transfer of neuroantigen peptide-activated T cells (Karpus et al., 1995). Mononuclear cell infiltration was limited following this treatment, suggesting that the role of MIP-1α in recruiting mononuclear cells, when blocked, affects disease progression. Similar studies have not been performed using RANTES or MIP-1β, but it is likely that similar effects may be observed, as both RANTES and MIP-1β have been described as potent monocyte as well as lymphocyte chemoattractants (Chuluyan et al., 1995). In EAE, chemokine expression is first detected on and attributed to infiltrating mononuclear cells; early signs of inflammation exist before chemokine expression (reviewed by Ransohoff et al., 1997). Additional studies investigating the injection of several recombinant β chemokines into the parenchyma of mice failed to illicit significant leukocyte recruitment (Bell et al., 1996). These studies suggest, as have our studies, the importance of a primed endothelium in mediating chemokine enhanced adhesion and migration at the level of the BBB. Although not the initial instigators of the immune reaction, following the initial pro-inflammatory stimulus these molecules play a significant role as the amplifiers of the immune-

mediated response in addition to shaping the profile of recruited leukocytes. The importance of these chemokines in mediating inflammation is underscored by the therapies currently under development to restrict chemokine-mediated leukocytic infiltration.

CHAPTER 5 CONCLUSIONS

5.1 SUMMARY AND SIGNIFICANCE

The aim of this study was to 1) investigate the expression, regulation and binding of the chemokines RANTES (regulated upon activation, normal T cell expressed and secreted) and MIP-1β (macrophage inflammatory protein-1β) by human brain microvessel endothelial cells (HBMEC) in primary culture; 2) characterize the role of these chemokines in mediating CD4+ T cell subset adhesion and migration across HBMEC monolayers. Our initial hypothesis proposed that pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β and bacterial lipopolysaccharide (LPS) have the ability to induce the expression of these chemokines by cerebral EC. These chemokines, when present as haptotactic concentration gradients at the level of the BBB, would then mediate T cell subset specific adhesion and migration across the BBB and accumulation at sites of CNS inflammation.

Our findings show that HBMEC in primary culture constitutively express and secrete very low levels of RANTES, whereas MIP-1β expression by resting HBMEC is negligible. Activation of HBMEC with TNF-α, IFN-γ, IL-1β, and LPS alone or in combination significantly increased both RNA and protein expression of RANTES. Induction of RANTES was observed within 24 hours with most cytokine treatments with the exception of IFN-γ that required more than 24 hours of treatment for significant expression and release. MIP-1β expression and secretion was induced following treatment with combinations of TNF-α and IFN-γ or IL-1β as well as LPS. The most significant upregulation of chemokine expression by HBMEC was obtained by combined cytokine treatments, particularly TNF-α with IFN-γ.

Direct comparisons between human umbilical vein EC (HUVEC) and HBMEC cultures revealed significant differences in chemokine induction and expression between cerebral and extra-cerebral EC. Unstimulated HUVEC cultures showed negligible expression of RANTES and MIP-1β; while treatment with TNF-α slightly increased RANTES release, only TNF-α in combination with IFN-γ resulted in media concentrations of RANTES comparable to those observed with HBMEC. Interestingly, while RANTES production by HBMEC was maintained over 3 days under continuous cytokine stimulation, RANTES production by HUVEC dropped considerably beyond 24 hours in the presence of cytokines. Unlike HBMEC, none of the cytokine treatments applied induced MIP-1β expression in HUVEC. Together, these results suggest that HBMEC can serve a greater role as mediators of inflammation through production of pro-inflammatory molecules than large vessel EC such as HUVEC.

These studies also confirm that RANTES and MIP-1β produced by HBMEC bind to the surface of these cells. Surface immunocytochemistry demonstrated that unstimulated cultures fail to show RANTES or MIP-1β bound to their surface, whereas following stimulation with pro-inflammatory cytokines which upregulate their expression, both chemokines are detected at the surface of HBMEC. This provides a functional basis for chemokine expression by HBMEC, as chemokines secreted apically or basally will bind to the cell surface or components of the extracellular matrix, thus generating haptotactic gradients towards which inflammatory cells can migrate.

Several chemokines have been localized to perivascular cuffs of inflammatory cells in CNS inflammation; these cuffs are particularly prominent in autoimmune demyelinating diseases and viral infections. Traditionally considered to be immunoprivileged, the brain is rampaged by mononuclear cells under these conditions. Several mononuclear cells present within these cuffs are sources of chemokines, and thus help to establish chemoattractant gradients across the BBB.

Our studies show that resting HBMEC monolayers significantly reduce the diffusion of chemokines, which correlates well with their low permeability to macromolecules *in vivo* and *in vitro*. Cytokine stimulation of HBMEC increases not only the permeability of these monolayers as measured by electrical resistance, but also allows for significant diffusion of chemokines across HBMEC monolayers.

Concentration gradients of RANTES and MIP-1β were shown to significantly augment the adhesion and migration of memory, activated, and antigen-specific CD4+ T cell subsets to and across cytokine-treated HBMEC monolayers. Resting CD4+ T cell populations were less responsive, whereas naïve CD4+ T cell populations were completely unresponsive to chemokines. The presence of RANTES or MIP-1β in the absence of gradients enhanced the adhesion of resting, memory, antigen-specific and activated CD4+ T cell subsets to cytokine-treated HBMEC with varying affinities and enhanced the migration of memory and activated CD4+ T cell subsets across cytokine-activated HBMEC. Most importantly both chemokines exerted their effects of T cell subsets only in the presence of cytokine stimulated HBMEC cultures. The increase in adhesion and migration was found to be integrin and EC adhesion molecule dependent, as anti-ICAM-1 antibodies significantly reduced adhesion of both resting and activated CD4+ T cell subsets in response to gradients, and anti-VCAM-1 and anti-α4 integrins also reduced the chemokine-enhanced migration of activated CD4+ T cells. These results suggest that RANTES and MIP-1β can play an important role in mediating the selective adhesion and transendothelial migration of previously activated lymphocyte subsets at the level of the BBB.

We propose that following some initial insult within the CNS, or in the event of an activated T cell crossing a resting BBB endothelium and encountering its cognate antigen, an inflammatory cascade is initiated (Fig. 48). Several pro-inflammatory cytokines are secreted by

these activated T cells including IFN-γ and TNF-α. These serve to stimulated the cerebral EC, as well as other cells intrinsic to the CNS such as astrocytes and microglia, which will in turn respond by the synthesis and release of cytokines and chemoattractant cytokines (chemokines). Once the BBB EC have been activated or "primed" with cytokines, they are induced to express or upregulate their expression of cell adhesion molecules such as ICAM-1 and VCAM-1, in addition to chemokines such as RANTES and MIP-1β. RANTES and MIP-1β bind to the surface of HBMEC, available for interactions with leukocytes in the circulation. The increased permeability of an activated BBB allows for the establishment of chemotactic gradients as RANTES and MIP-1β, in addition to other chemokines produced by astrocytes, microglia, or perivascular cells, diffuse into the endothelial lining and establish haptotactic gradients toward which cells can migrate.

The present data do not support the involvement of RANTES or MIP-1β in the initial recruitment of activated antigen-specific T cells as the initiators of immune responses. Activated T cells are capable of crossing an intact BBB in our *in vitro* model in the absence of RANTES or MIP-1β. This is supported by *in vivo* studies which indicate that neuroantigen-specific T cells readily cross an intact BBB (Cross et al., 1990; Hickey et al., 1991). Furthermore *in vivo* animal studies suggest that chemokine production follows the entry of activated neuroantigen-specific T cells into the brain (Glabinski et al., 1995). Such a pattern of expression suggests a greater role for chemokines in the promotion and amplification of the immune response by further recruiting as well as activating not only non-neuroantigen-specific T cells, but also other mononuclear cells at the inflammatory site.

Lymphocyte subsets in the circulation roll along the endothelial lining of blood vessels independent of antigen specificity via several selectins. Chemokines such as RANTES and MIP-1β at the surface of the cerebral endothelium serve to activate integrins including LFA-1 and

VLA-4 on the surface of lymphocytes, particularly memory or recently activated CD4+ T cells, and to upregulate the affinity for their respective ligands ICAM-1 and VCAM-1. This results in firm adhesion, enabling the cells to respond to the chemotactic gradient which has been established across the cerebral EC and migrate towards higher chemokine concentrations in the CNS.

The successful completion of these interactions depends upon two important events, both of which are related to the activation state of the cerebral EC: 1) Activated EC express the cell adhesion molecules ICAM-1 and VCAM-1 upon which chemokine-enhanced migration is dependent for interactions with LFA-1 and VLA-4; and 2) Activated BBB EC show enhanced permeability in response to cytokines, thus allowing for proper diffusion of chemokines through the BBB and ECM to establish the chemokine gradient toward which cells will later migrate. Antibodies against the adhesion ligand pairs involved, ICAM-1/LFA-1 and VCAM-1 VLA-4 can significantly reduce this response. This suggests that therapeutic interventions aimed at blocking the action of chemoattractant cytokines such as antibody blocking experiments or the use of newly developed "altered-chemokine" chemokine receptor antagonists would provide possible means of reducing lymphocytic infiltration and thus lymphocyte-mediated cytotoxicity and tissue damage during CNS inflammation.

5.2 FUTURE EXPERIMENTAL CONSIDERATIONS

The present studies have addressed several questions related to the expression and function of the β chemokines RANTES and MIP-1β during cerebral EC-lymphocyte interactions and, in addition, have raised interesting questions that could form the basis for future investigations.

One project would involve characterization of the way chemokines are presented by the human cerebral endothelium. As mentioned in the discussion, proteoglycans or glycosaminoglycans (GAGs) are the most obvious molecules with chemokine-presentation capabilities at the EC surface. In addition, several different reports describe the localization of chemokine receptors to EC, particularly during inflammation. Experiments using enzymes such as heparinases which cleave the heparins at the cell surface would effectively reduce chemokine levels if GAGs were in fact the molecules responsible. Sensitive binding and competition assays using radiolabeled chemokines in addition to immunohistochemistry would rule out high-affinity receptor involvement in chemokine presentation at the BBB EC surface.

The second study which addresses the mode of chemokine diffusion or passage across the BBB indirectly relates to the first study. It is not currently known whether RANTES or MIP-1β might in fact be transported across cerebral EC, a mechanism which has been shown for other chemokines such as IL-8 in extracerebral EC (Rot 1992). While our diffusion studies suggest that differences in permeability amongst cerebral and extracerebral EC may account for differences in chemokine diffusion across EC monolayers, it would be relevant to CNS inflammation to determine whether or not chemokines produced on the "tissue" side of the BBB might in fact be transported across the BBB EC for presentation to lymphocytes at the brain-blood interface during the initiation of inflammation. Such transport has been suggested for other cytokines, and may also be relevant to chemoattractant cytokines.

Our data suggest that the enhanced adhesion and migration in response to RANTES and MIP-1β gradients is mediated by one or both of the following: 1) the requirement for increased permeability of the BBB to allow for diffusion of chemokines from the subendothelial compartment through a paracellular route to the luminal surface of the endothelium or for active transport across or presentation upon cerebral EC; and 2) the involvement of cell adhesion

molecules, including ICAM-1, VCAM-1 and possibly others at the EC surface which may be involved in this apparent integrin-dependent enhancement. Each of these fundamental changes during the process of EC activation seem to be required. An interesting set of experiments that could differentiate between chemokine diffusion across concentration gradients with presentation on the luminal surface and involvement of EC adhesion molecules would involve placing chemokines beneath an untreated HBMEC monolayer, and treating EC with a hyperosmotic agent such as arabinose. Following exposure to 1.6 M arabinose for up to 30 minutes, the interendothelial junctions become reversibly open, and the permeability to tracers such as HRP is increased (Dorovini-Zis et al., 1984). This would permit the diffusion of chemokines via a paracellular route through opened tight junctions thus establishing a concentration gradient toward which cells can migrate, in the absence of cell adhesion molecules such as ICAM-1 and VCAM-1. Such studies would combine with the above to characterize the differences in chemokine-enhanced adhesion and migration to and across an activated BBB.

The third study involves analysis of the *in vivo* blocking of chemokine-induced effects. Animal models of EAE using the adoptive transfer of encephalitogenic T cells with the administration of various agents that would block interactions between chemokines and their receptors on lymphocytes would be useful in determining the physiological relevance of chemokine-mediated recruitment. As already mentioned, neutralizing antibodies have been applied to MIP-1α and have reduced the clinical deficits and mononuclear cell infiltration observed during EAE. The application of monoclonal antibodies however, even though species-specific, still results in the formation of antibodies to these antibodies. As such, therapeutic intervention involving the application of modified chemokines with modified or deleted N-terminal domains that maintain their ability to bind and thus block receptors without signaling (Struyf et al., 1998; Bostick et al., 1998) is useful: no activation of the leukocyte occurs, thus

blocking the cascade of inflammatory events. Such models hold significant promise both in experimental models, as well as in CNS inflammation and related diseases. Of particular interest would be the analysis of the specific effects of these blocking studies. For example, would matrix metalloproteinase production, previously induced by chemokines, be curbed, and what effect would this have on the progression of disease? Would any observed decreases in lymphocyte adhesion and migration due to blocking of these interactions indeed be significant, and of the "activating" or "chemoattractant" properties of these molecules, blocking of which would be more pathophysiologically significant?

Clearly, much of the story regarding chemokine involvement in CNS inflammation remains to be told. Our studies combined with previous investigations by others provide insight into the basic functions of chemokines *in vitro*, and when combined with future studies including those described above, could contribute significantly to the understanding of chemokine-mediated recruitment of specific inflammatory cell subsets in the course of CNS inflammation.

Table 1 Chemokines and chemokine receptor expression on T cells.

Receptor	T cell Expressed?	Chemokine
CXCR1	?	IL-8
CXCR2	?	IL-8, GRO-α/β, NAP-2
CXCR3	Th1, Th2(±)	IP-10, MIG
CXCR4	Naïve, activated	SDF-1α,β
CXCR5	No	BCA-1, BLC
CCR1	Yes	RANTES, MIP-1α, MCP-2, MCP3
CCR2	Yes	MCP-1, -2, -3, -4
CCR3	Th2	EOTAXIN 1,-2 RANTES, MCP-2,-3,-4
CCR4	Th2	TARC, RANTES, MIP-1α, MCP-1
CCR5	Th1,(Th2±)	RANTES, MIP-1α, MIP-1β
CCR6	Yes	LARC,/MIP-3α, EXODUS
CCR7	Yes	ELC/MIP-3α
CCR8	Th2	I-309, (MIP-1β)
Unknown	Yes	Lymphotactin
CX_3CR1	Yes	FRACTALKINE

Table 2 Chemokine and chemokine receptor expression by cells of the CNS.

Chemokines	Cell expression in CNS	References
IP-10/Mig	Astrocytes	Vanguri et al. 1996
	Microglia	Glabinski et al. 1995
	Ependyma, choroid plexus	Asensio and Campbell 1997
MCP-1	Brain macrophages	Calvo et al, 1996
	Astrocytes	Glabsinski et al, 1995
	Endothelium	Frigerio et al, 1998
MIP-1α	Glial cells, neurons	Ishizuka et al, 1997
	Endothelium	Sasseville et al, 1996
MIP-1β	Macrophages, reactive astrocytes	Ghirnakar et al, 1996
	Microglia	McManus et al, 1998
	Endothelium	Sasseville et al, 1996
Fractalkine	Microglia	Pan et al, 1997
	Astrocytes	Nishiyori et al, 1998
RANTES	Brain macrophages	Ghirnakar et al, 1996
	Astrocytes	Barnes et al, 1996
	Endothelium	Sasseville et al, 1996
Chemokine receptors		
CCR1	Astrocytes	Hesselgesser et al, 1997
	Neuronal cells (culture)	Tanabe et al, 1997
CCR3	Neurons	He et al, 1997; Lavi unpublished
	Microglia	
CCR5	Microglia	He et al, 1997 Horuk et al, 1997
	Neuronal cells (culture)	Hesselgesser et al, 1997
	Astrocytes	Rottman et al, 1997
CCR8	Perivascular cells (?)	Jinno et al, 1998
CXCR4	Neurons, endothelium,	Lavi et al, 1997
	Microglia, neurons	Tanabe et al, 1997
	Microglia, astrocytes	Hesselgesser et al, 1997
Duffy	Purkinje cells	Horuk et al, 1997
	Endothelium	

Table 3 Primers and cycling for reverse-transcriptase PCR.

	Primer sequence	Amplified fragment (bp)	# cycles
RANTES	AS 5' CCACACCCTGCTGCTTTGCCTACAT S 5' GAGCGGGTGGGGTAGGATAGTGAGG	290	27,32
MIP-1β	AS 5' CCATGAAGCTCTGCGTGACTGTCCT S 5' AGCAGCTCAGTTCAGTTCCAGGTCA	287	28,33
GAPDH	AS 5' CCATGTTCGTCATGGGTGTGAACCA S 5' GCCAGTAGAGGCAGGGATGATGTTC	272	20,25

RT-PCR primer sequences and cycles. Primer pair sequences which span introns were chosen for RANTES and MIP-1β from GenBank accession numbers M21121 and J04130 respectively.

Table 4 CCR3 and CCR5 receptor expression on CD4+ T cell subsets.

	Resting	Activated α-CD3 + IL-2	Activated α-CD3 alone	Antigen specific clone	Memory	Naive
Blast population						
CCR3	2.7 ± .5	25.0 ± 6.2	28.2 ± 5.4	17.0 ± 3.3	3.8 ± .6	0.4 ± .2
CCR5	2.3 ± .6	26.3 ± 4.6	21.4 ± 4.8	23.2 ± 3.8	7.9 ± .9	0.4 ± .3
Whole population						
CCR3	3.7 ± 1.5	8.7 ± 2.2	11.7 ± 2.4	12.5 ± 2.3	2.4 ± .7	1.1 ± .6
CCR5	5.3 ± 1.6	8.2 ± 1.6	14.7 ± 2.8	12.1 ± 3.8	4.4 ± .9	0.8 ± .3

Table represents the percentage of T cells in each subset exhibiting high expression of the chemokine receptors. Gates were set on the blast population as well as gating on the entire population.

Fig. 1 Characterization of human brain microvessel endothelial cells in primary culture. a) Intense granular, perinuclear staining for Factor VIIIR:Ag with the immunoperoxidase technique. X 280. b) Binding of Ulex europaeus lectin I is indicated by positive immunoperoxidase staining for UEA I. X 460. c) Alkaline phosphatase reactivity is high, and is maintained for up to two weeks in culture. X 470.

Fig. 1 Characterization of human brain microvessel endothelial cells in primary culture.

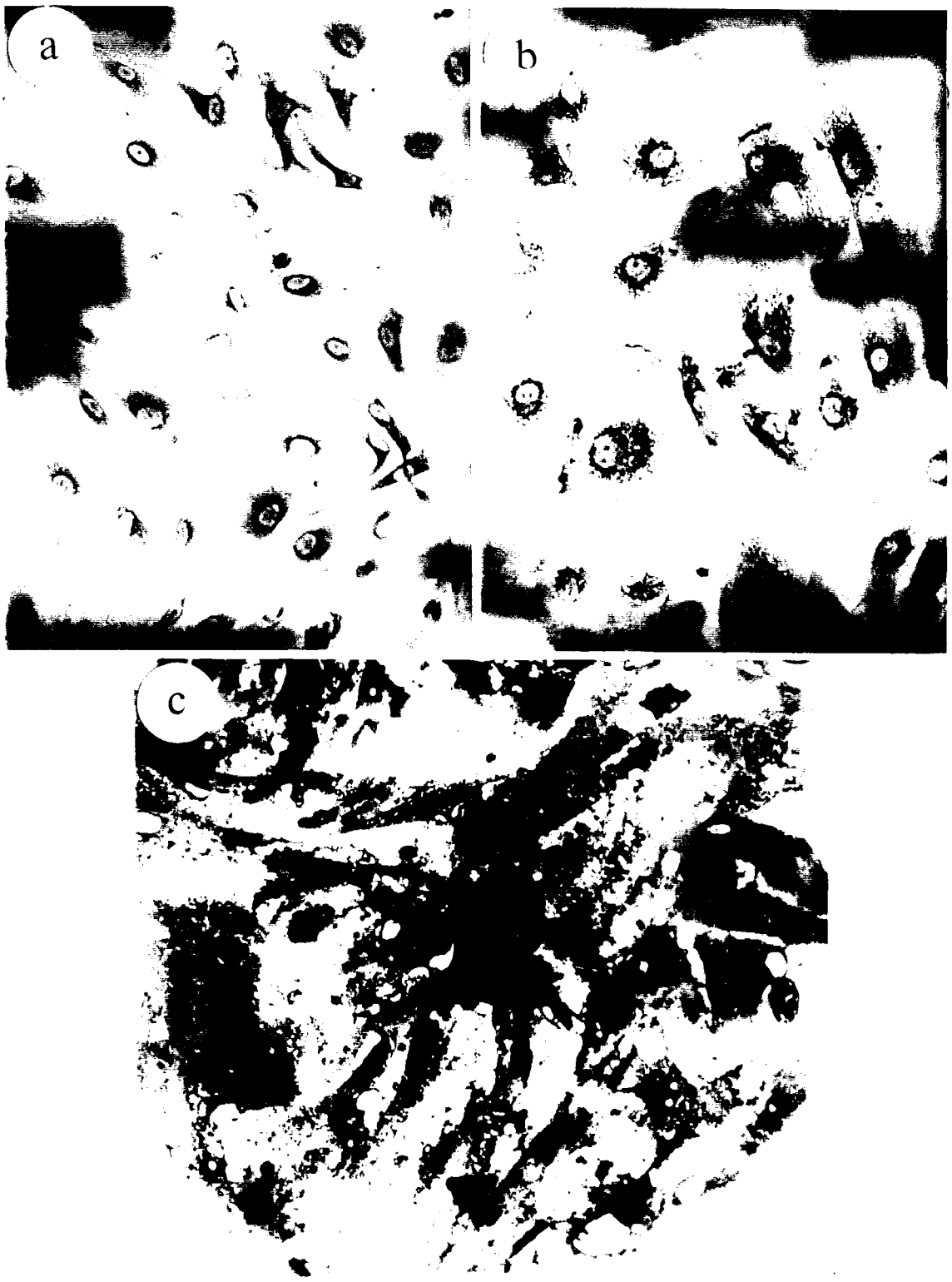


Fig. 2 Phase contrast micrograph of primary HBMEC cultures grown to confluence on a) fibronectin coated plastic wells and b) cellagen discs forming the bottom of the upper well in the double chamber chemotaxis system. The morphology and growth of HBMEC is similar on these two substrates. X 230.

Fig. 2 Phase contrast micrograph of primary HBMEC cultures.

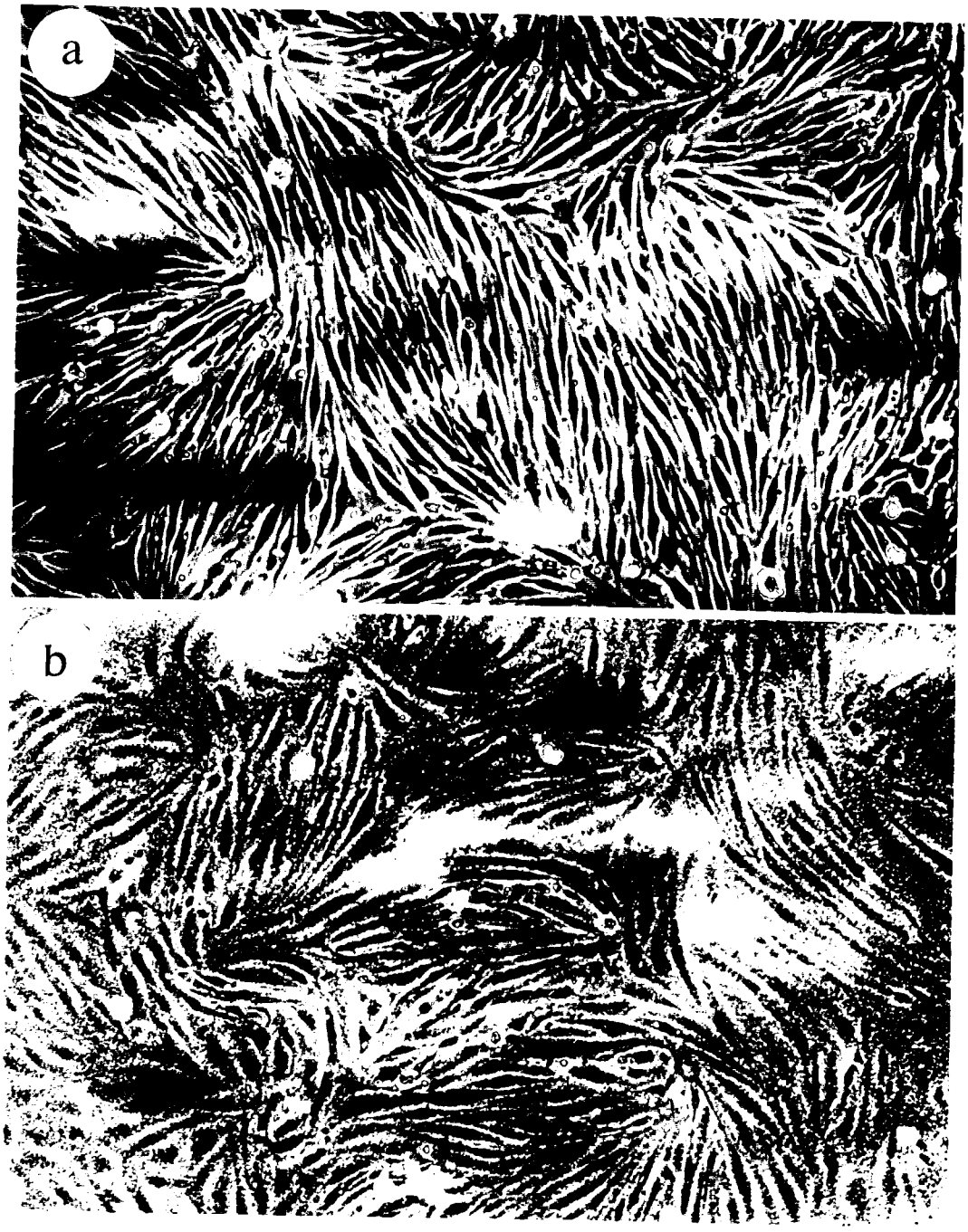


Fig. 3 Double chamber chemotaxis system used in adhesion and transendothelial migration assays. HBMEC are grown to confluence on collagen membranes attached to plastic supports, which are placed inside wells of 24 well plates to form a double chamber system. Chemokines are added to the lower chamber to obtain concentration gradients.

Fig. 3. Double chamber chemotaxis system used in adhesion and transendothelial migration assays.

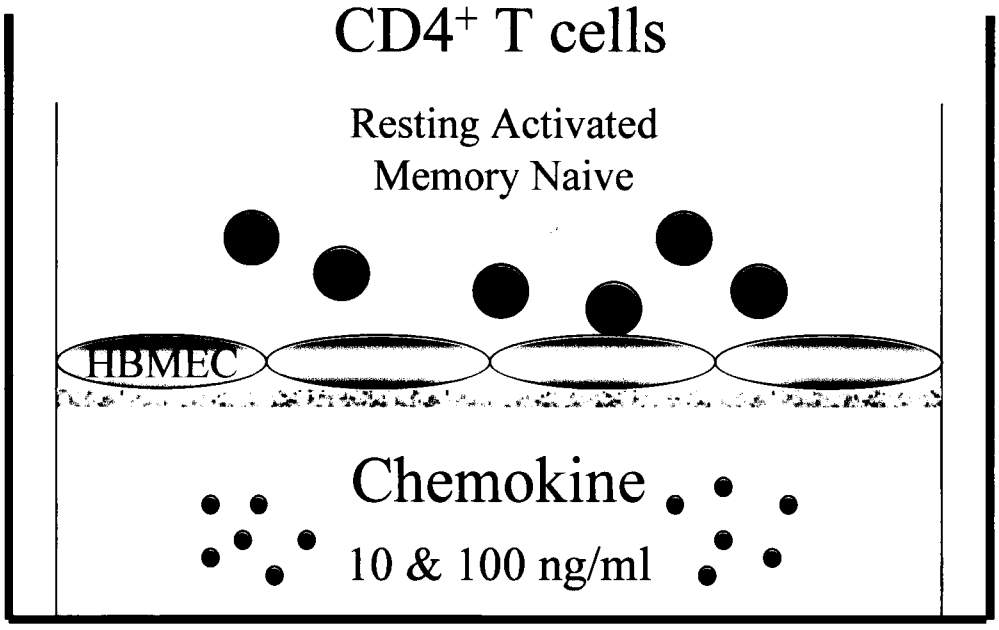


Fig. 4 Ohmeter used to measure the electrical resistance across endothelial monolayers. The EC cultures grown on cellagen discs with plastic supports are placed into the chamber which contains 2 ml of sterile M199. The upper electrode is secured to the lower portion of the unit for a tight fit, and the electrical resistance across the HBMEC monolayer is read. The background resistance of the disc (18 Ω) is subtracted from the reading of the Ohmeter and the new value is multiplied by the surface area of the collagen disc to give the resistance across the HBMEC monolayer ($\Omega \cdot cm^2$).

Fig. 4 Ohmeter used to measure the electrical resistance across endothelial monolayers.

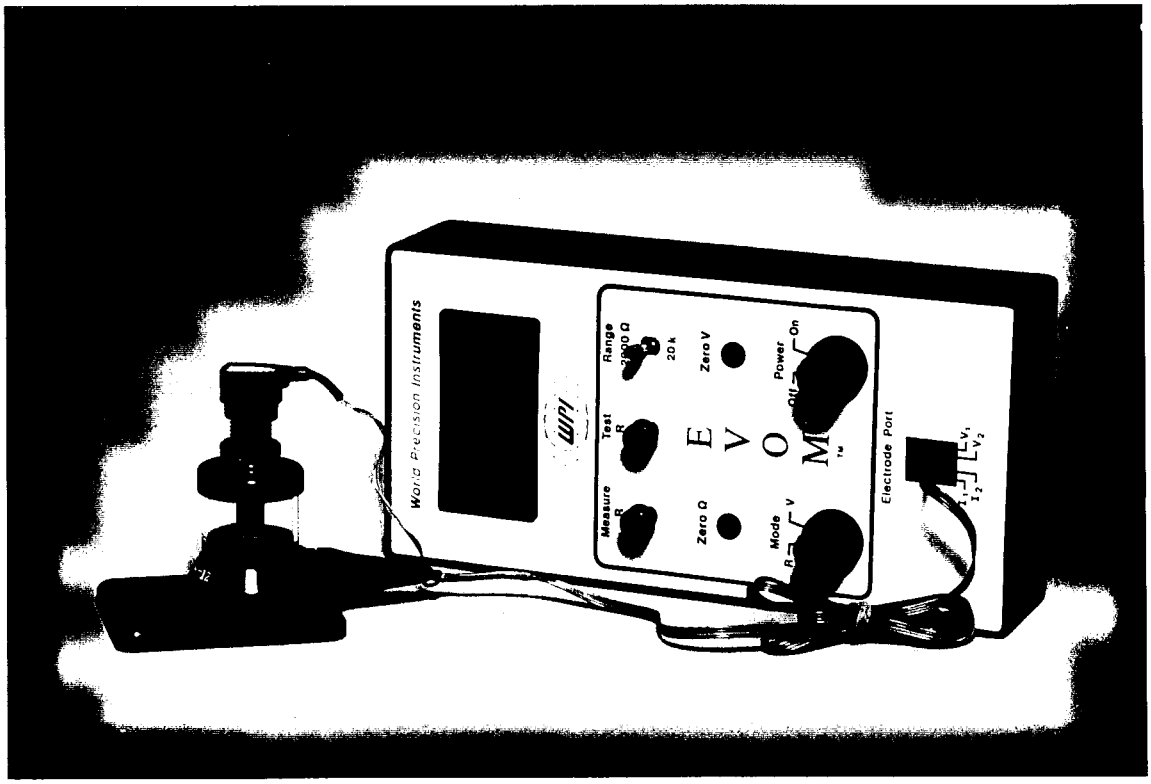


Fig. 5 Reverse-transcriptase PCR for RANTES expression in untreated and cytokine-treated [TNF-α(100 U/ml) and IFN-γ (500 U/ml)] primary cultures of HBMEC and HUVEC. Data from 2 experiments on each culture system are shown. Results obtained using 27 and 32 cycles for RANTES. Control levels of RNA are shown for GAPDH.

Fig. 5 Reverse-transcriptase PCR for RANTES RNA expression.

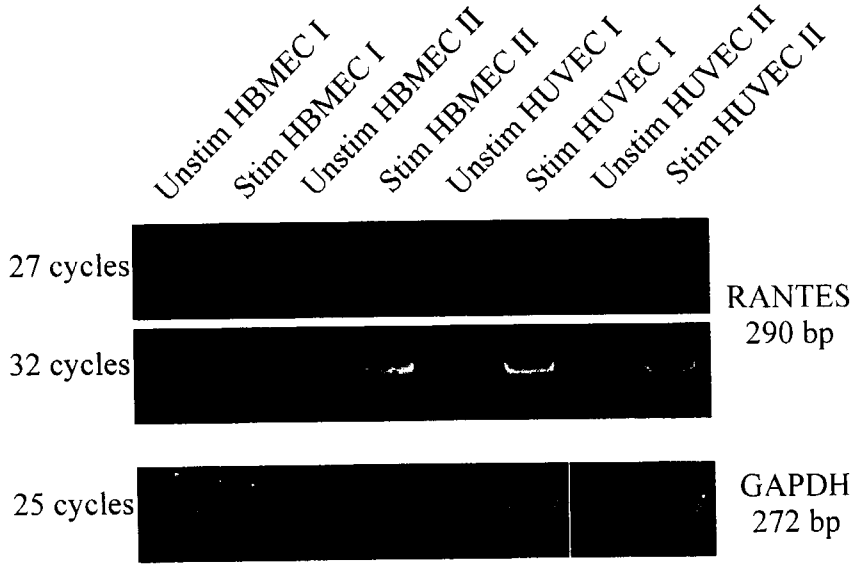


Fig. 6 Restriction enzyme analysis of RT-PCR amplified fragments. The 287 bp fragment of MIP-1β was cleaved with Hha I (62, 225 bp), Hind III (125, 162 bp), and Ava II (53, 224 bp) to yield the predicted fragments (listed in parenthesis). Similarly, the 290 BP amplified fragment of RANTES was cleaved with Alu I (40, 67, 183 bp), Tsp 509I (70, 220 bp) and Alu I with Tsp 509I (30, 37, 40, 183 bp) to yield the predicted fragments.

Fig. 6 Restriction enzyme analysis of RT-PCR amplified fragments

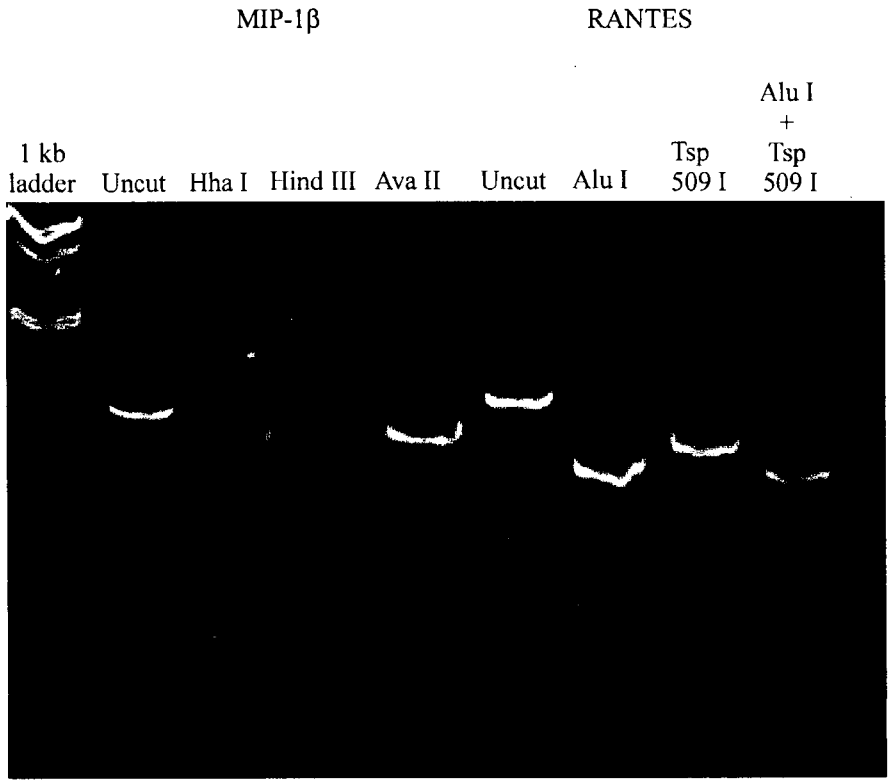


Fig. 7 Sandwich ELISA for detection of RANTES protein in supernatants of primary HBMEC cultures treated with (a) TNF-α (100U/ml), IFN-γ (200 U/ml), IL-1β (10 U/ml) or LPS (5 μg/ml) and (b) combinations of TNF-α (100 U/ml), IFN- (200 U/ml), and IL-1β (10 U/ml). Media and cytokines were changed every 24 hours. Data represent one of three experiments with similar results. Error bars represent the SEM. n=3. * = $p<0.05$.

Fig. 7 Sandwich ELISA for detection of RANTES protein in the supernatants of primary HBMEC cultures.

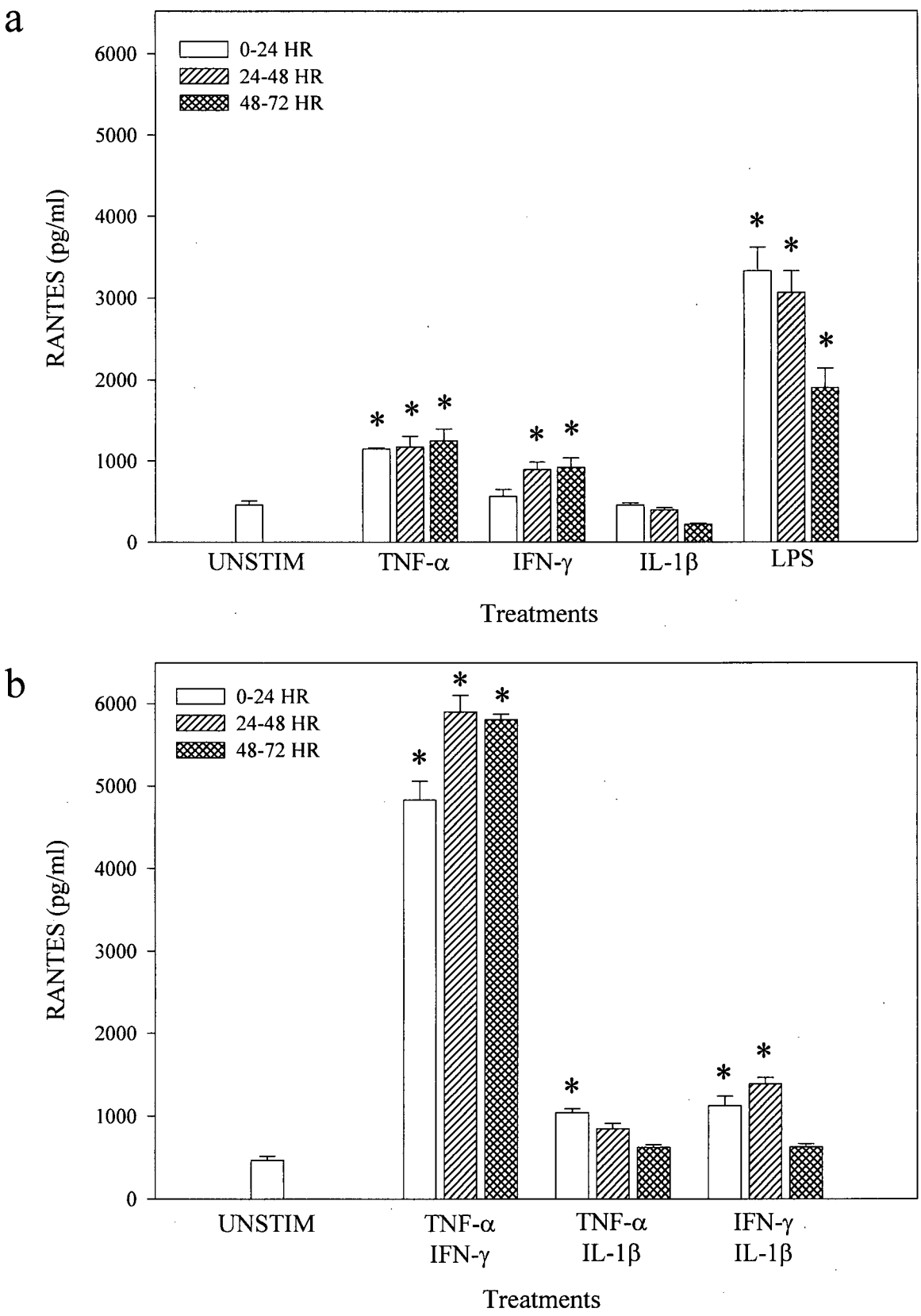


Fig. 8 Sandwich ELISA for detection of RANTES protein in supernatants of primary HUVEC cultures treated with (a) TNF-α (100U/ml), IFN-γ (200 U/ml), IL-1β (10 U/ml) or LPS 5 μg/ml and (b) combinations of TNF-α (100 U/ml), IFN-γ (200 U/ml), and IL-1β (10 U/ml). Media and cytokines were changed every 24 hours. Error bars represent the SEM. Data represent one of three experiments with similar results. n=3.
* = $p<0.05$.

Fig. 8 Sandwich ELISA for detection of RANTES protein in the supernatants of primary HUVEC cultures.

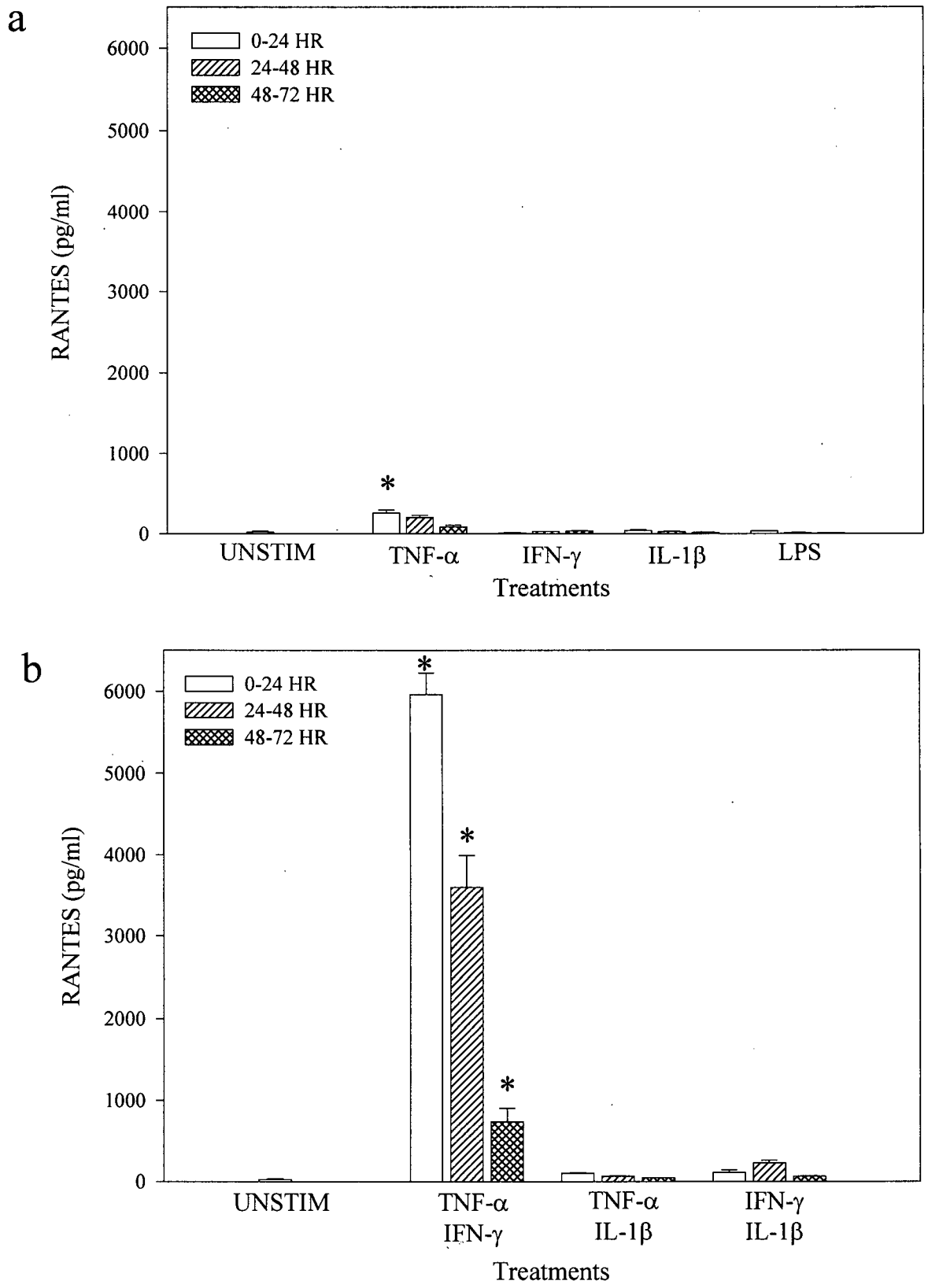


Fig. 9 Immunogold silver staining for intracellular localization of RANTES in HBMEC cultures. a) In unstimulated cultures most cells show faint, peppery staining for RANTES protein. The intensity of staining was significantly increased following stimulation with (b) IFN-γ (500 U/ml) for 72 hours and c) treatment with TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 72 hours. 430X.

Fig. 9 Immunogold silver staining for the intracellular localization of RANTES in HBMEC cultures.

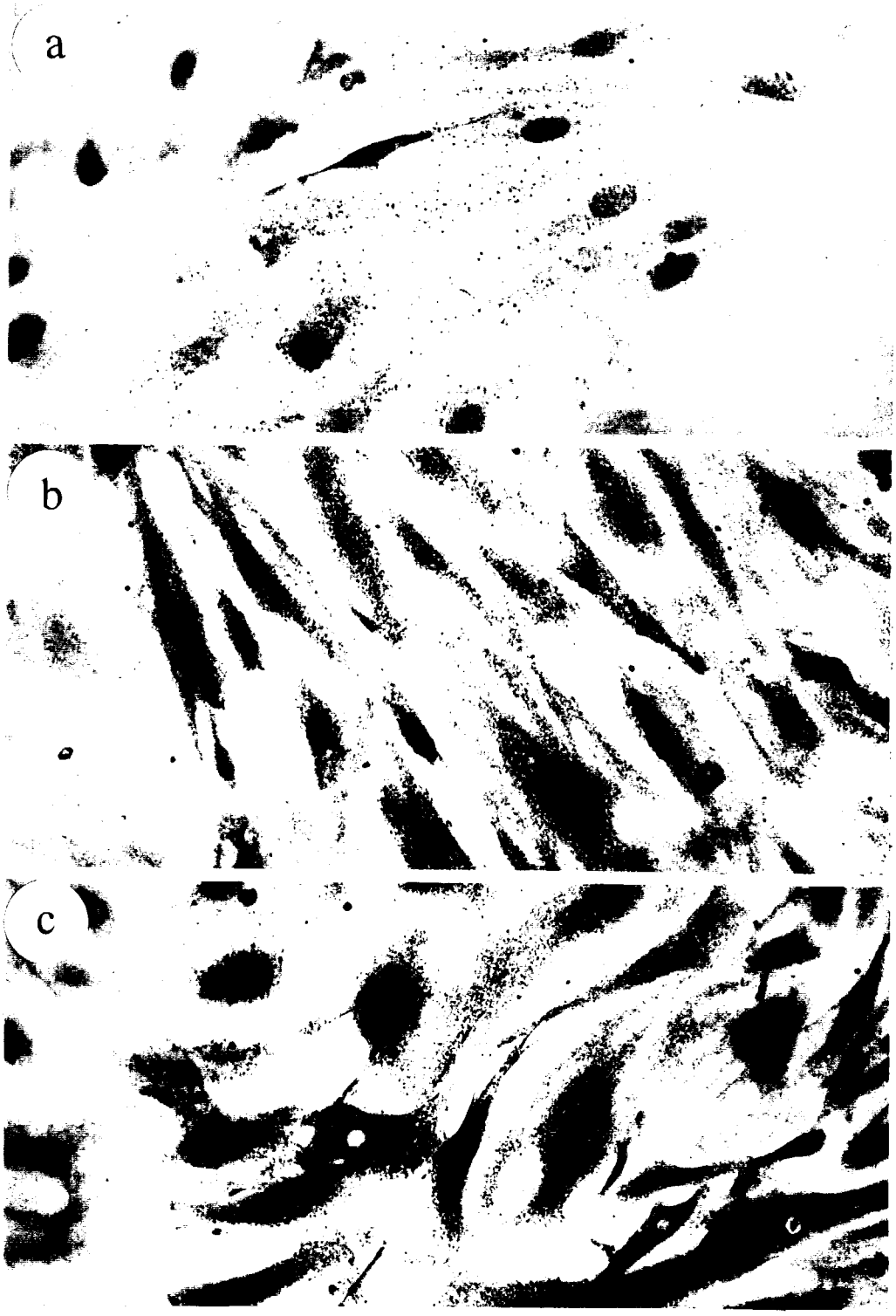


Fig. 10 Immunogold silver staining for intracellular localization of RANTES in HUVEC cultures. a) Unstimulated cultures show negligible staining for RANTES protein in most cells. b) Treatment with TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 24 hours resulted in a significant increase in staining intensity as indicated by fine black granules distributed throughout the cell. 490X.

Fig. 10 Immunogold silver staining for the intracellular localization of RANTES in HUVEC cultures.

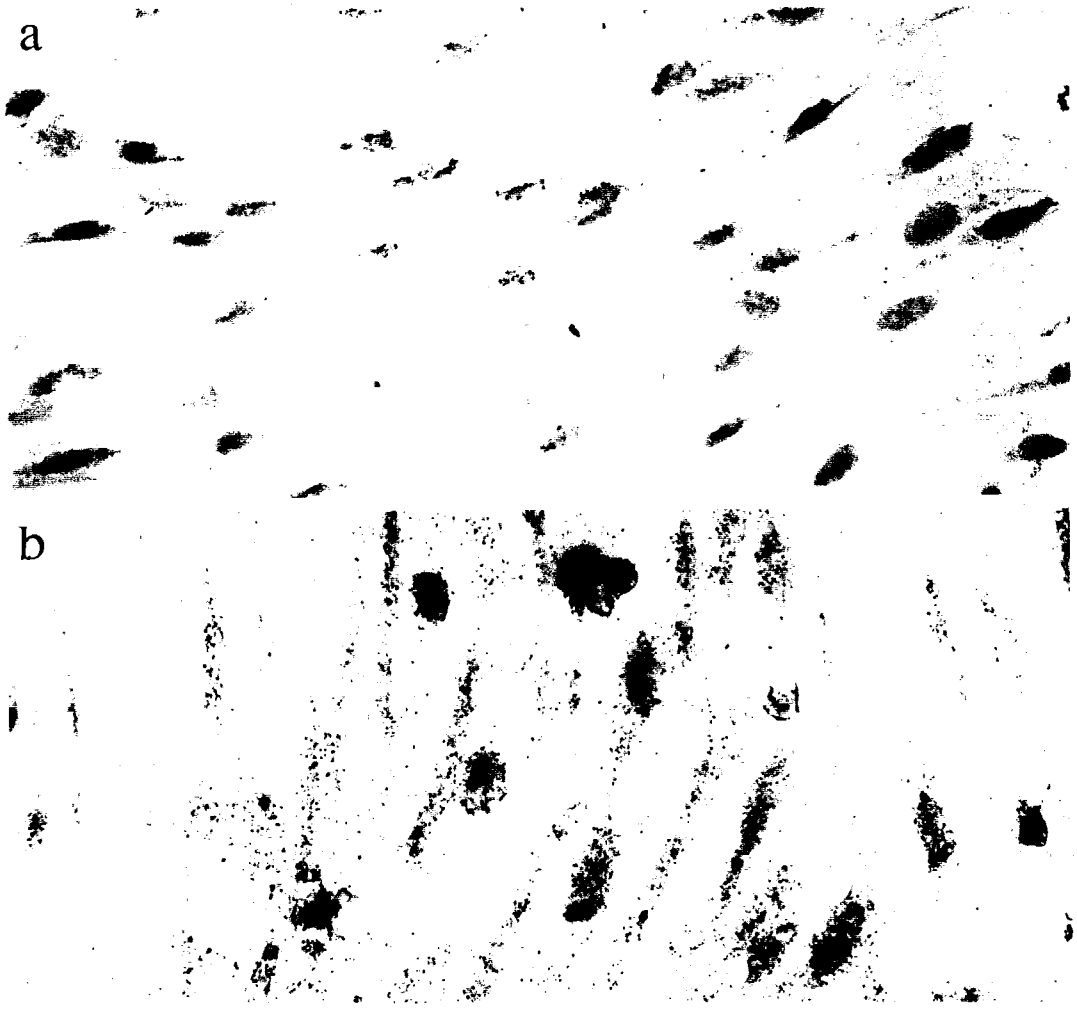


Fig. 11 Quantitation of surface expression of RANTES on HBMEC using immunogold silver staining. Graph displays the % of cells exhibiting surface staining in untreated HBMEC monolayers and cultures treated with TNF-α (100U/ml), IFN-γ (200 U/ml), IL-1β (10 U/ml) and LPS (5 μg/ml) alone or in combination. Error bars represent the SEM. All treatments were significant ($p<0.05$) except IL-1β. n=3.

Fig. 11 Quantitation of surface expression of RANTES on HBMEC using immunogold silver staining.

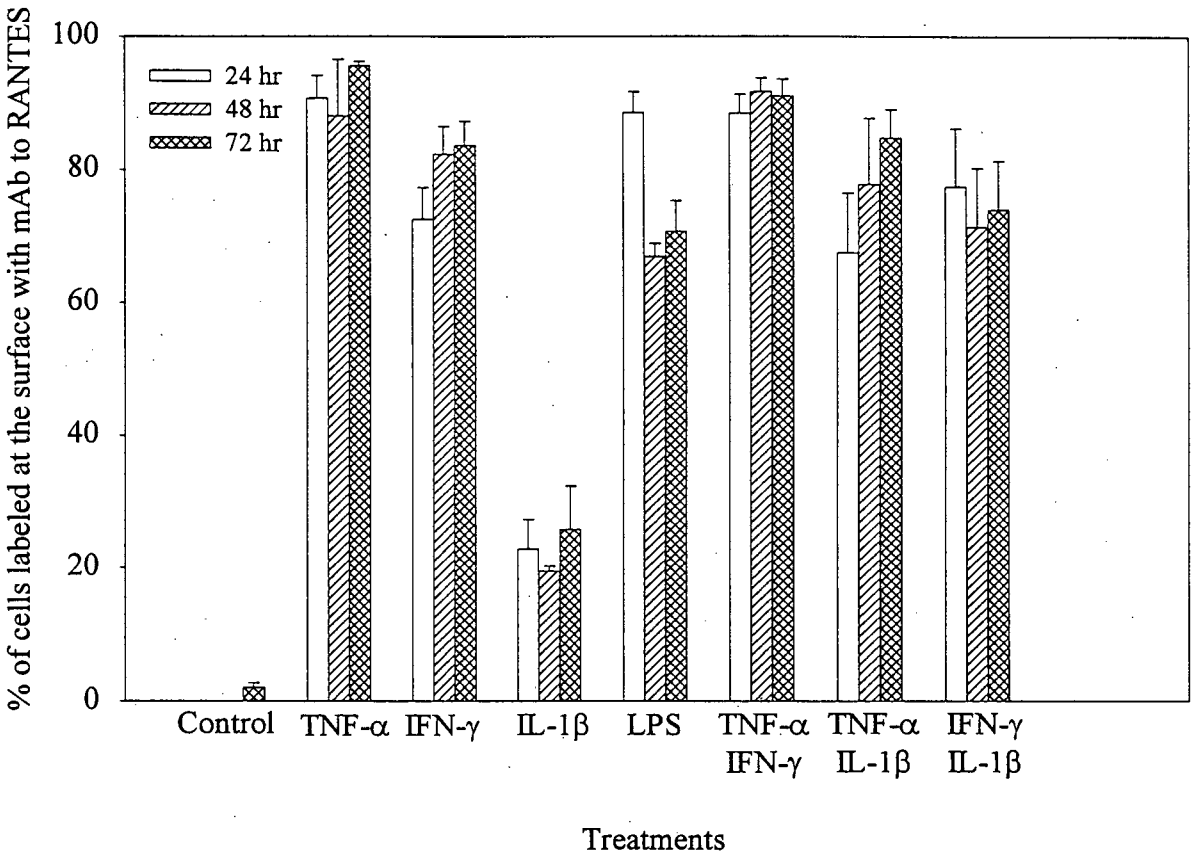


Fig. 12 Surface localization of RANTES to primary cultures of HBMEC. a) Unstimulated HBMEC show negligible staining for RANTES at the cell surface, whereas treatment with (b) LPS (5 μg/ml) for 24 hours or c) TNF-α (100 U/ml) and IL-1β (10 U/ml) for 24 hours resulted in increased surface expression in the form of fine, peppery surface staining. X 450.

Fig. 12 Surface localization of RANTES to primary cultures of HBMEC.

Fig. 13 Reverse-transcriptase PCR for MIP-1β expression in untreated and cytokine-treated [TNF-α(100 U/ml) and IFN-γ (500 U/ml)] primary cultures of HBMEC and HUVEC. Data from 2 experiments on each culture system are shown. Results were obtained using 28 and 33 cycles for MIP-1β. Control levels of RNA are shown for GAPDH.

Fig. 13 Reverse-transcriptase PCR for MIP-1β RNA expression.

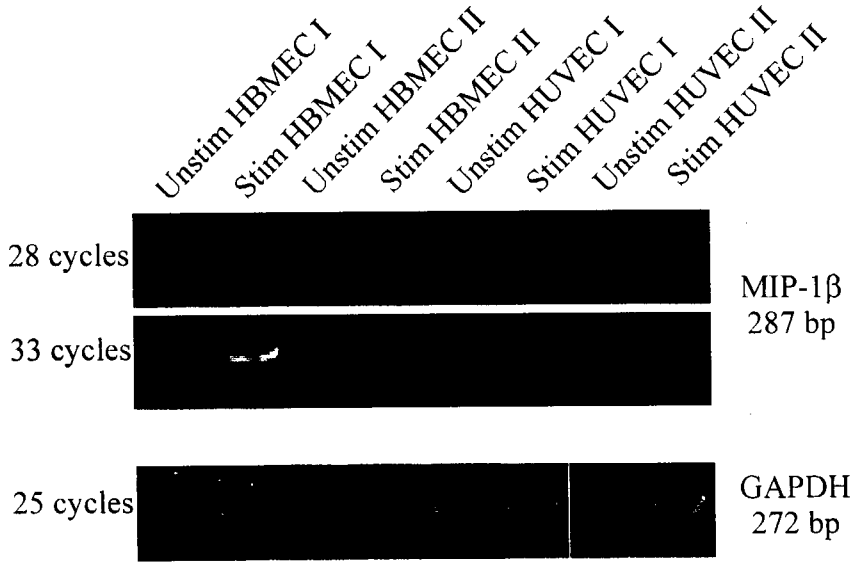


Fig. 14 Sandwich ELISA for detection of MIP-1β protein in supernatants of primary HBMEC cultures treated with TNF-α (100U/ml), IFN-γ (200 and 500 U/ml), IL-1β (10 U/ml) or LPS (5 μg/ml) alone or in combination. Protein content in pg/ml indicates the total protein present in supernatants collected after 24, 48, or 72 hours of treatment to allow for the pooling of detectable levels. Data represent one of three experiments. Error bars represent the SEM. * = $p<0.05$. n=3.

Fig. 14 Sandwich ELISA for detection of MIP-1β protein in the supernatants of primary HBMEC cultures.

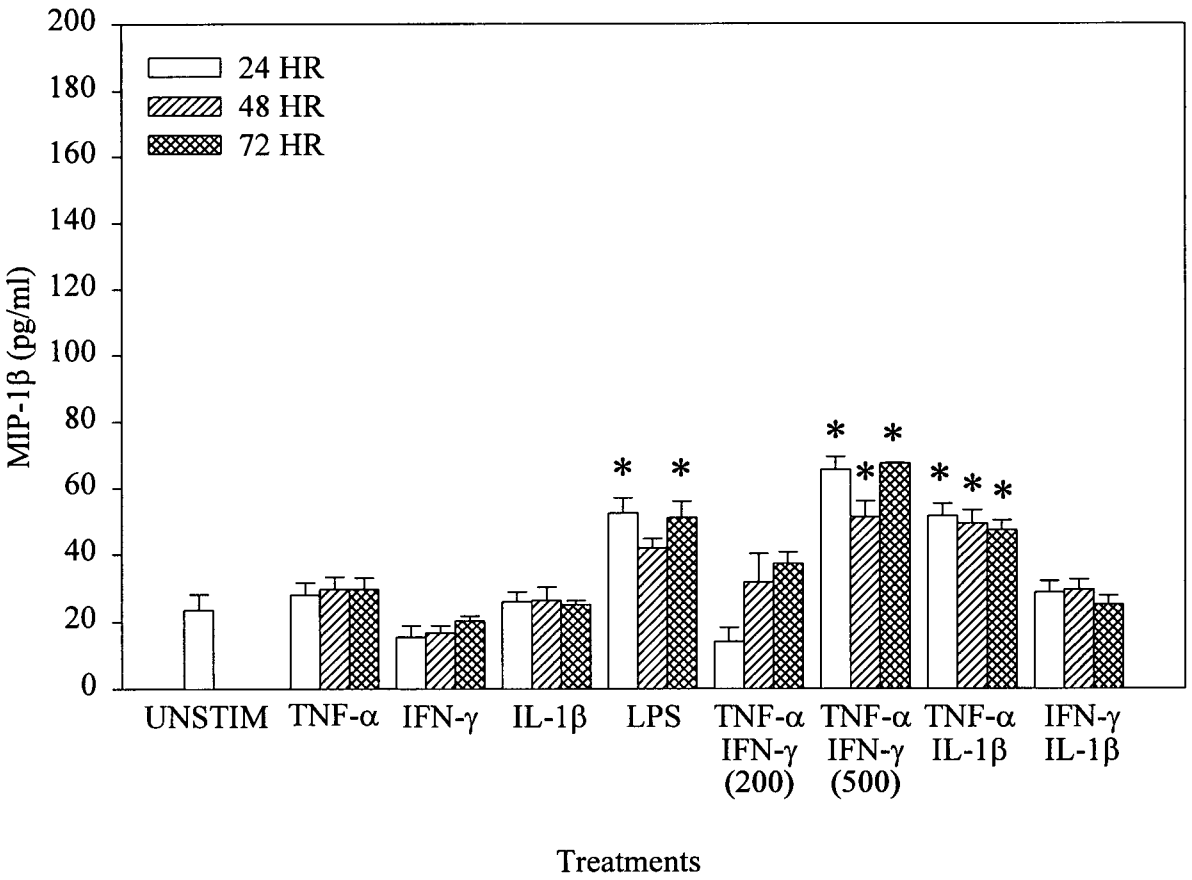


Fig. 15 Immunogold silver staining for intracellular localization of MIP-1β in HBMEC cultures. a) Most cells in untreated cultures show negligible staining for MIP-1β; b) treatment with TNF-α (100 U/ml) and IL-1β (10 U/ml) for 24 and c) TNF-α (100 U/ml) and IFN-γ (500 U/ml) for 24 hours resulted in a significant increase in staining intensity as indicated by fine black granules distributed throughout the cell. X 490.

Fig. 15 Immunogold silver staining for the intracellular localization of MIP-1β in HBMEC cultures.

Fig. 16 Quantitation of surface expression of MIP-1β on HBMEC using immunogold silver staining. Graph displays the % of cells showing surface staining in untreated HBMEC monolayers and cultures treated with TNF-α (100U/ml), IFN-γ (200 U/ml), IL-1β (10 U/ml) and LPS (5 μg/ml) alone or in combination. Error bars represent the SEM. n=3. * = $p < 0.05$.

Fig. 16 Quantitation of surface expression of MIP-1β on HBMEC using immunogold silver staining.

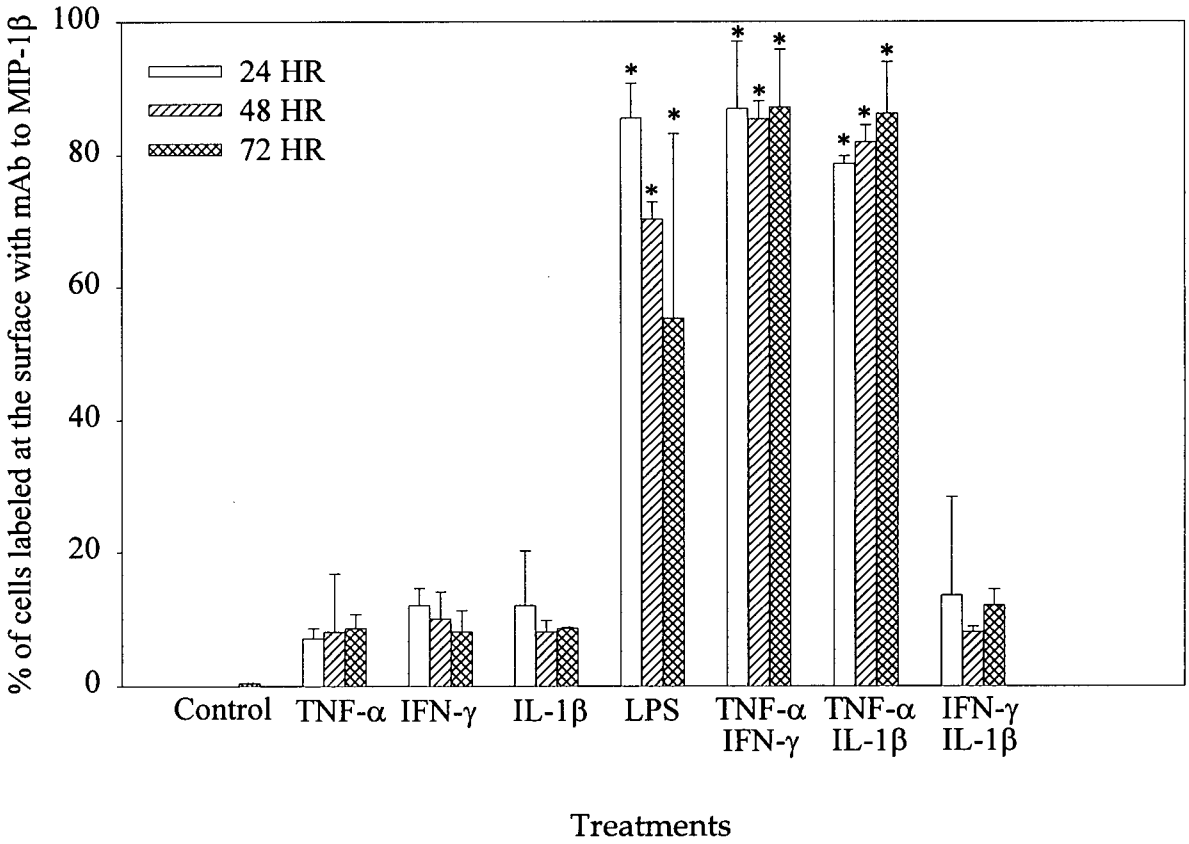


Fig. 17 Surface localization of MIP-1β to primary cultures of HBMEC. (a) Unstimulated HBMEC show negligible surface staining for MIP-1β. (b) Treatment with TNF-α (100 U/ml) and IL-1β (10 U/ml) for 24 hours resulted in significant amounts of MIP-1β as detected by fine, peppery staining over the surface of the cells. X 440.

Fig. 17 Surface localization of MIP-1β to primary cultures of HBMEC.

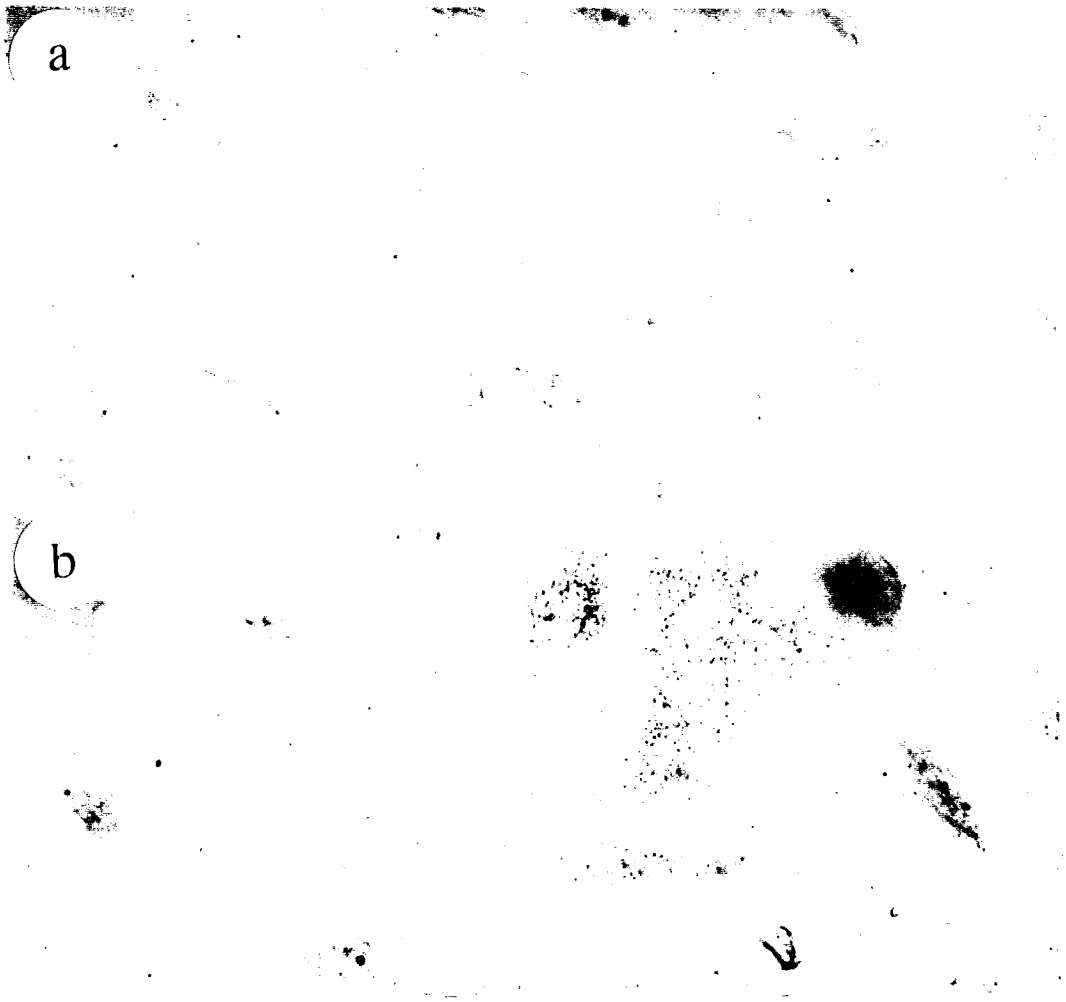


Fig. 18 Assessment of HBMEC monolayer permeability by electrical resistance measurements across the monolayers. The electrical resistance ($\Omega \cdot cm^2$) across HBMEC Isolation A (a) and Isolation B (b) was measured from day 8 to 13. Cultures became confluent on day 10 or 11. The solid line indicates the resistance of unstimulated cultures, and the dotted line the resistance during the 24 hours following treatment of HBMEC monolayers with TNF-α (100 U/ml) and IFN-γ (200 U/ml). Different symbols represent separate experiments. Error bars represent the SEM. n=12.

Fig. 18 Assessment of HBMEC monolayer permeability by electrical resistance measurements across the monolayers.

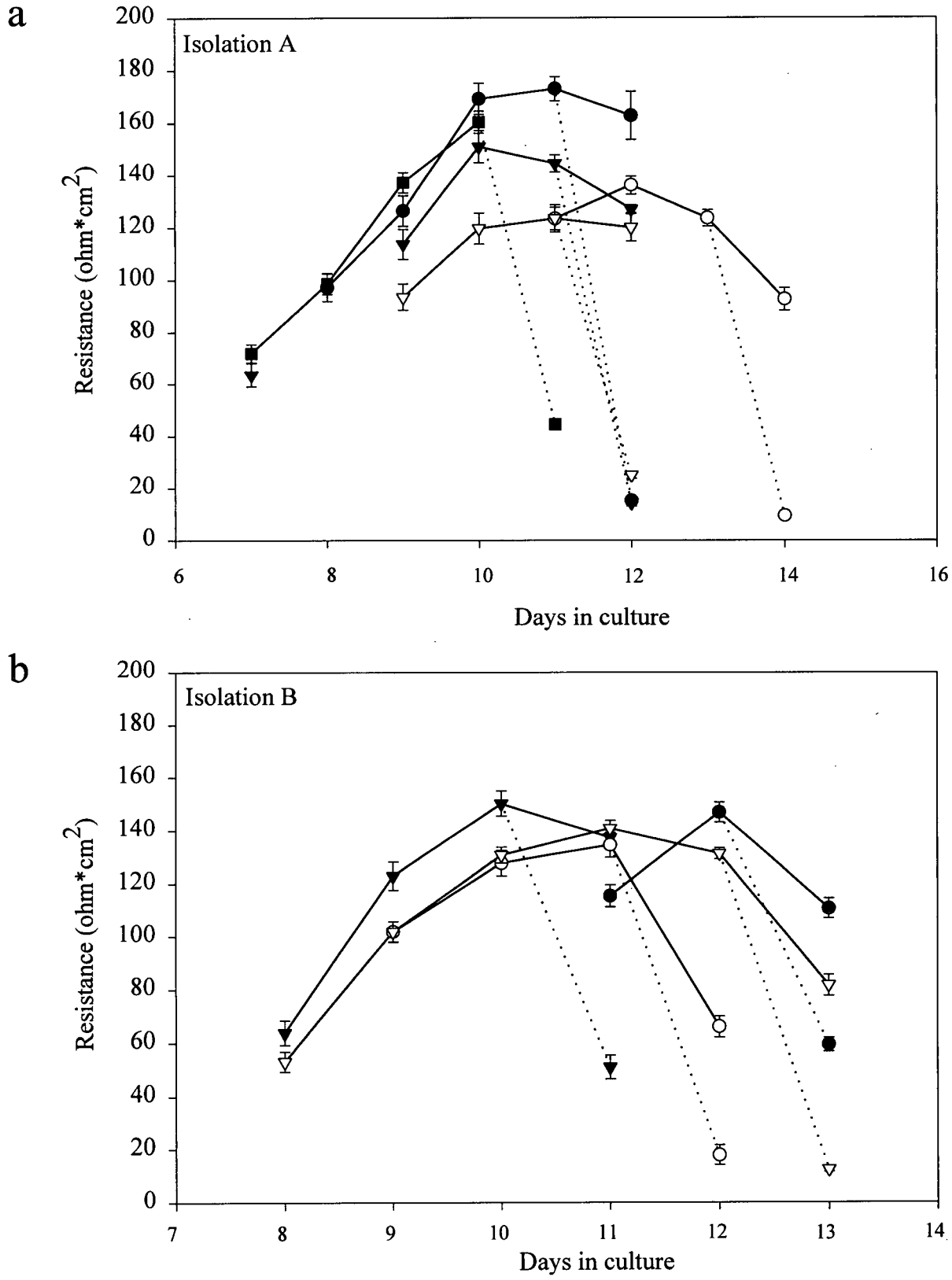
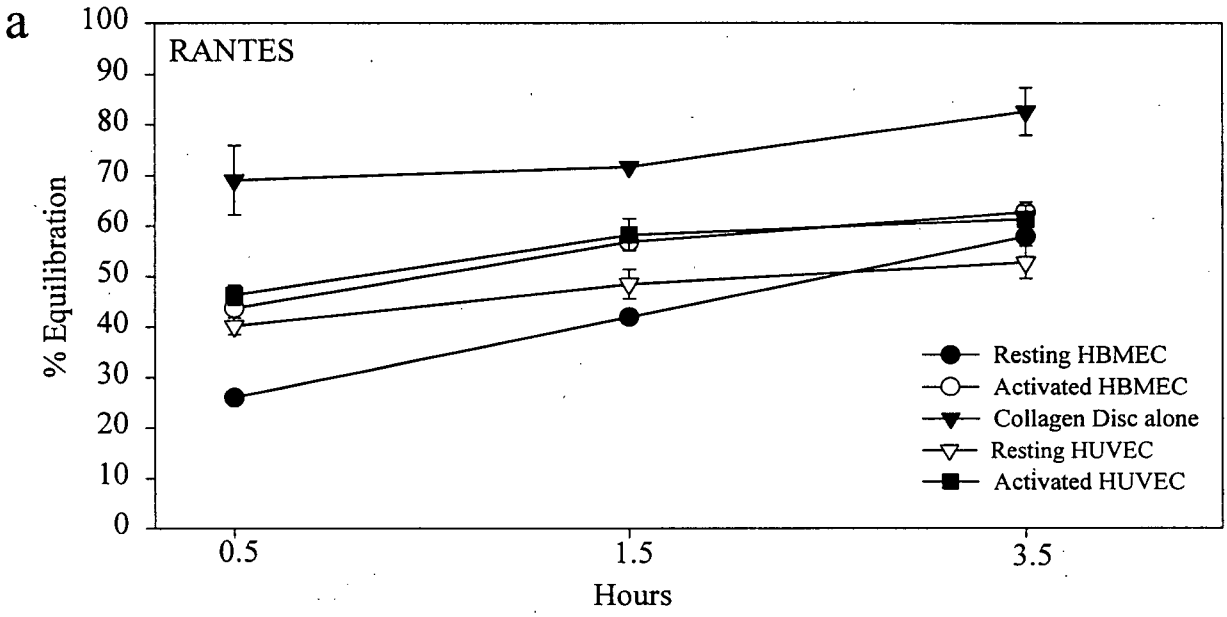


Fig. 19 Diffusion of ^{125}I-labeled chemokines across HBMEC and HUVEC monolayers cultivated on cellagen discs. TNF-α and IFN-γ (100 and 200 U/ml respectively) were used to activated EC cultures for 24 hours. Controls consisted of diffusion across the collagen disc alone. 350 μl of M199 containing 100 ng/ml of radiolabeled (a) RANTES or (b) MIP-1β were placed in the lower chamber of the chemotaxis system and allowed to diffuse to the upper chamber for up to 3.5 hours. Samples were aspirated from the upper chamber at .5, 1.5 and 3.5 hours to determine the % equilibration at each time point.

$$\% \text{ equilibration} = \frac{\text{cpm/100μl (upper chamber)}}{\text{cpm/350μl (lower chamber)}}$$

Error bars represent the SEM. n=3.

Fig. 19 Diffusion of ^{125}I labeled chemokines across HBMEC and HUVEC monolayers.



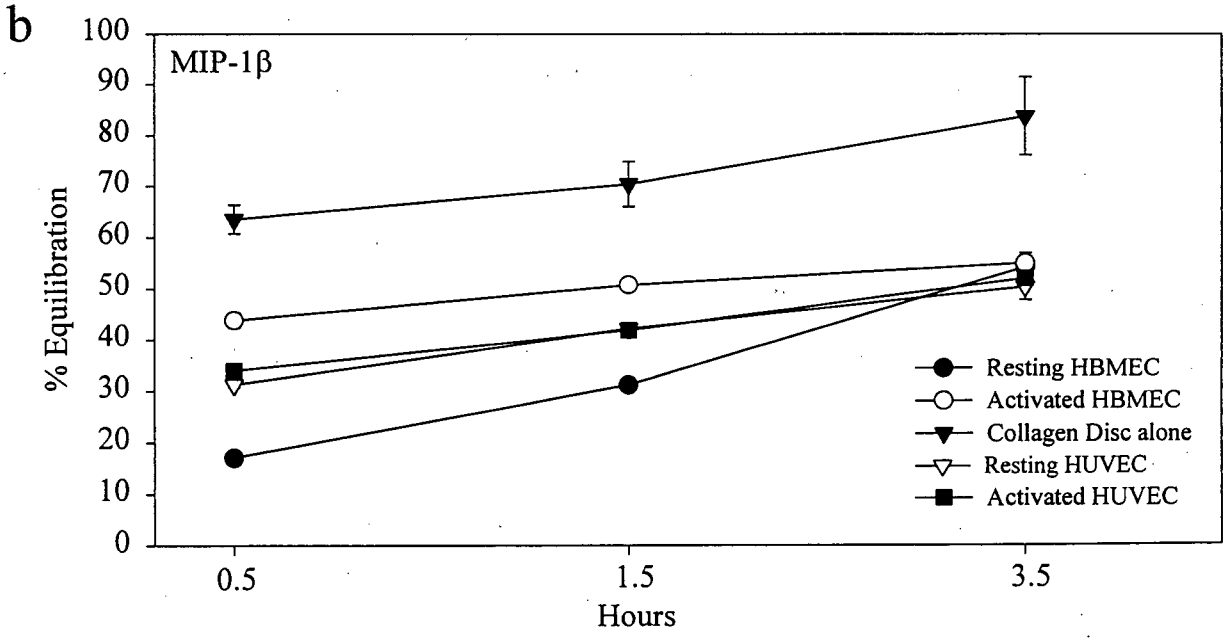
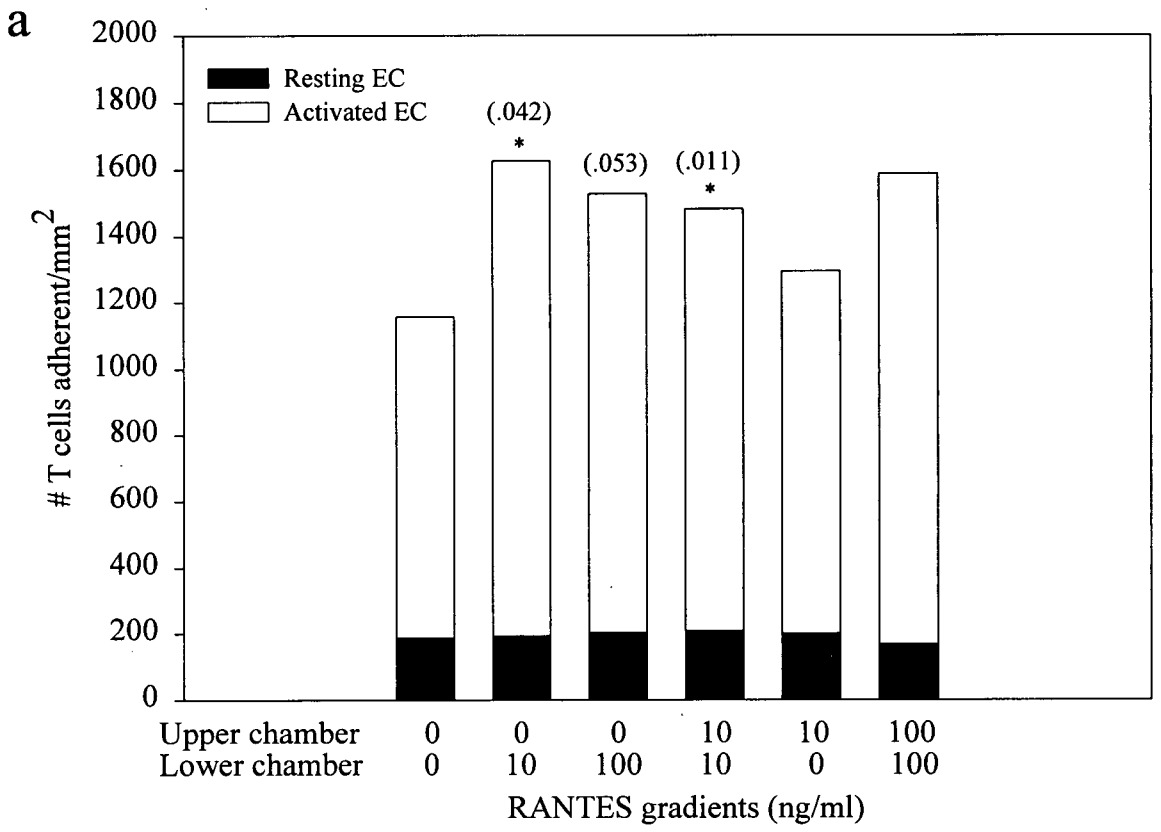


Fig. 20 Effects of RANTES and MIP-1β on resting CD4+ T cell adhesion to HBMEC monolayers. (a) RANTES augments the adhesion of resting CD4+ T cells to activated monolayers but has no effect on the adhesion to unstimulated HBMEC. (b) MIP-1β enhances the adhesion of resting CD4+ T cells to activated HBMEC monolayers but has no effect on the adhesion to unstimulated HBMEC. 1 x 10^5 T cells were added per well. Data represent one of three representative experiments each performed in duplicate.
* = $p<0.05$. n=2.

Fig. 20 Effects of (a) RANTES and (b) MIP-1β on resting CD4+ T cell adhesion to HBMEC



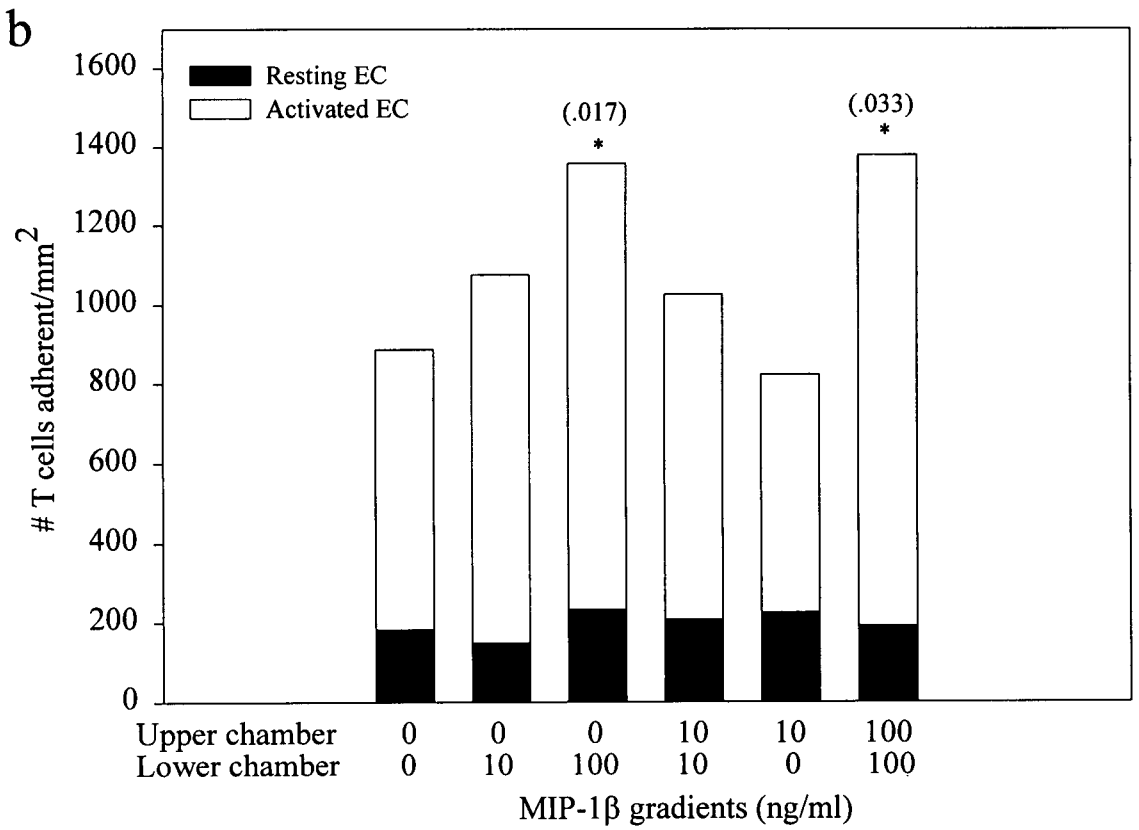


Fig. 21 Resting CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β concentration gradients. (a) Few resting CD4+ T cells adhere to resting HBMEC monolayers; (b) cytokine activation of HBMEC with TNF-α (100 U/ml) and IFN-γ (200 U/ml) doubles the number of adherent cells. The presence of RANTES (100 ng/ml) (c) and MIP-1β (100 ng/ml) (e) in the lower chamber enhanced adhesion of resting CD4+ T cells to cytokine-treated HBMEC. The presence of RANTES (d) or MIP-1β (f) in both upper and lower chambers also increases adhesion to cytokine-treated HBMEC. X 205.

Fig. 21 Resting CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β.

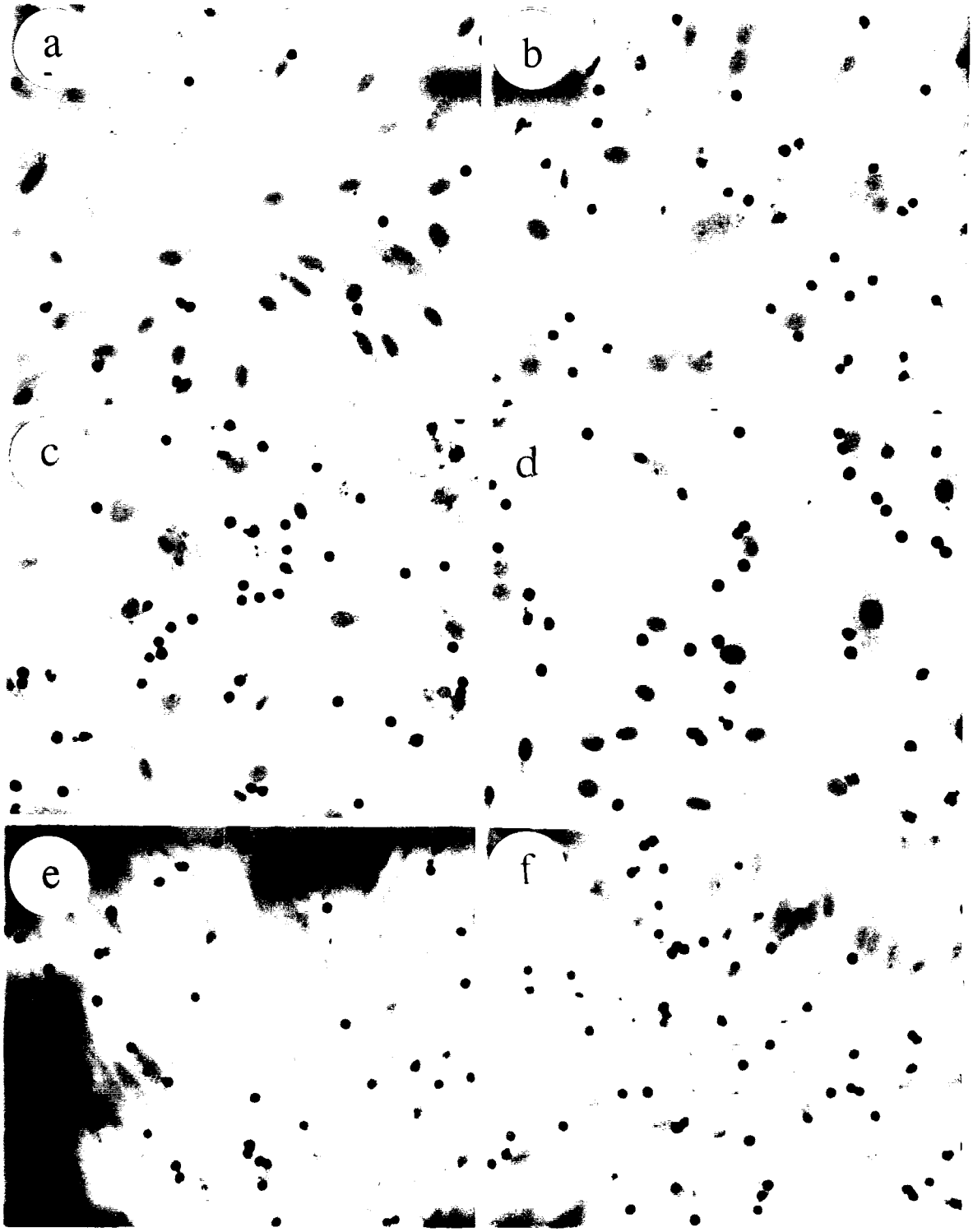
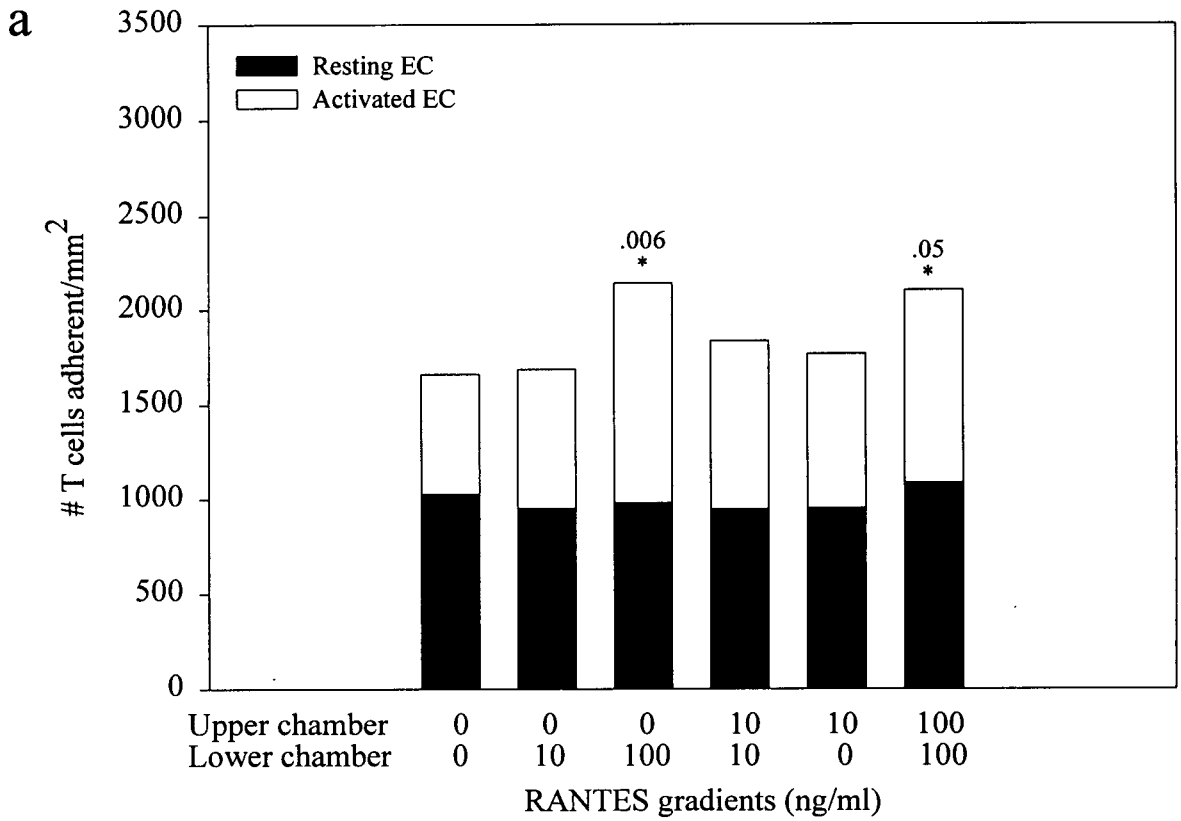


Fig. 22 Effects of RANTES and MIP-1β on anti-CD3 activated CD4+ T cell adhesion to HBMEC monolayers. (a) RANTES upregulates the adhesion of activated CD4+ T cells to activated monolayers but has no effect on the adhesion to resting HBMEC. (b) MIP-1β enhances the adhesion of activated CD4+ T cells to activated HBMEC monolayers but has no effect on the adhesion to resting HBMEC. 1 x 10^5 T cells were added per well. Data represent one of three representative experiments each performed in duplicate.
* = $p<0.05$. n=2.

Fig. 22 Effects of (a) RANTES and (b) MIP-1β on activated CD4+ T cell adhesion to HBMEC



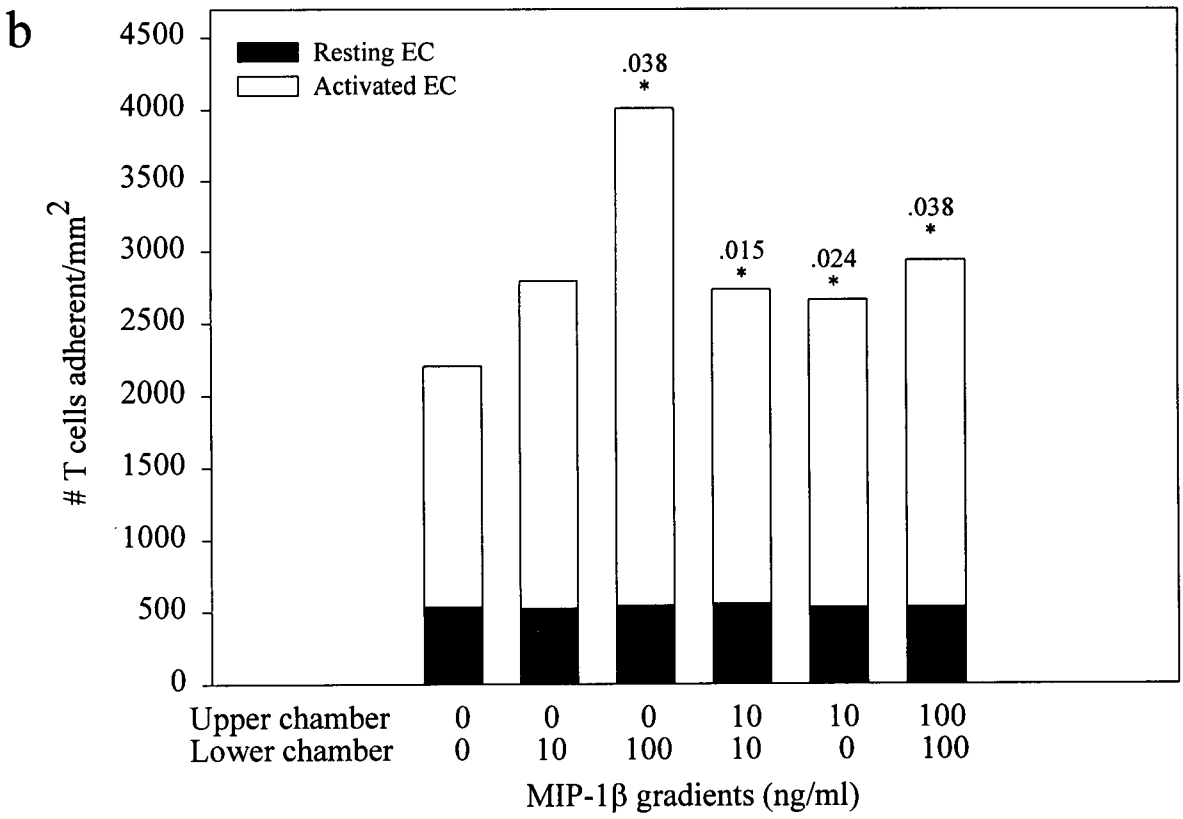


Fig. 23 Phase contrast micrographs of anti-CD3 activated CD4+ T cells adhering to HBMEC monolayers. (a) Activated CD4+ T cells adhere to cytokine-treated HBMEC monolayers [TNF-α (100 U/ml) and IFN-γ (200 U/ml)]. Adherent lymphocytes appear as refractile, round particles; lymphocytes which have migrated through the monolayer are non-refractile and ovoid or irregular in shape. (b) Anti-ICAM-1 mAb greatly reduces the number of adherent activated CD4+ T cells to activated HBMEC. (c) Increased numbers of activated CD4+ T cells adhere to activated HBMEC in the presence of an MIP-1β concentration gradient of 100 ng/ml; (d) this enhanced adhesion is drastically reduced by the presence of anti-ICAM-1 mAb. (e) 100 ng/ml of MIP-1β present in both upper and lower chambers resulted in increased adhesion of anti-CD3 activated CD4+ T cells to cytokine-treated HBMEC; (f) this increased adhesion was slightly reduced by mAb to VCAM-1. (c, e, f) Arrows indicate the presence of elongated CD4+ T cells which are extending cell processes (likely uropods) in the presence of MIP-1β. X 270.

Fig. 23 Phase contrast micrographs of anti-CD3 activated CD4+ T cells adhering to HBMEC monolayers.

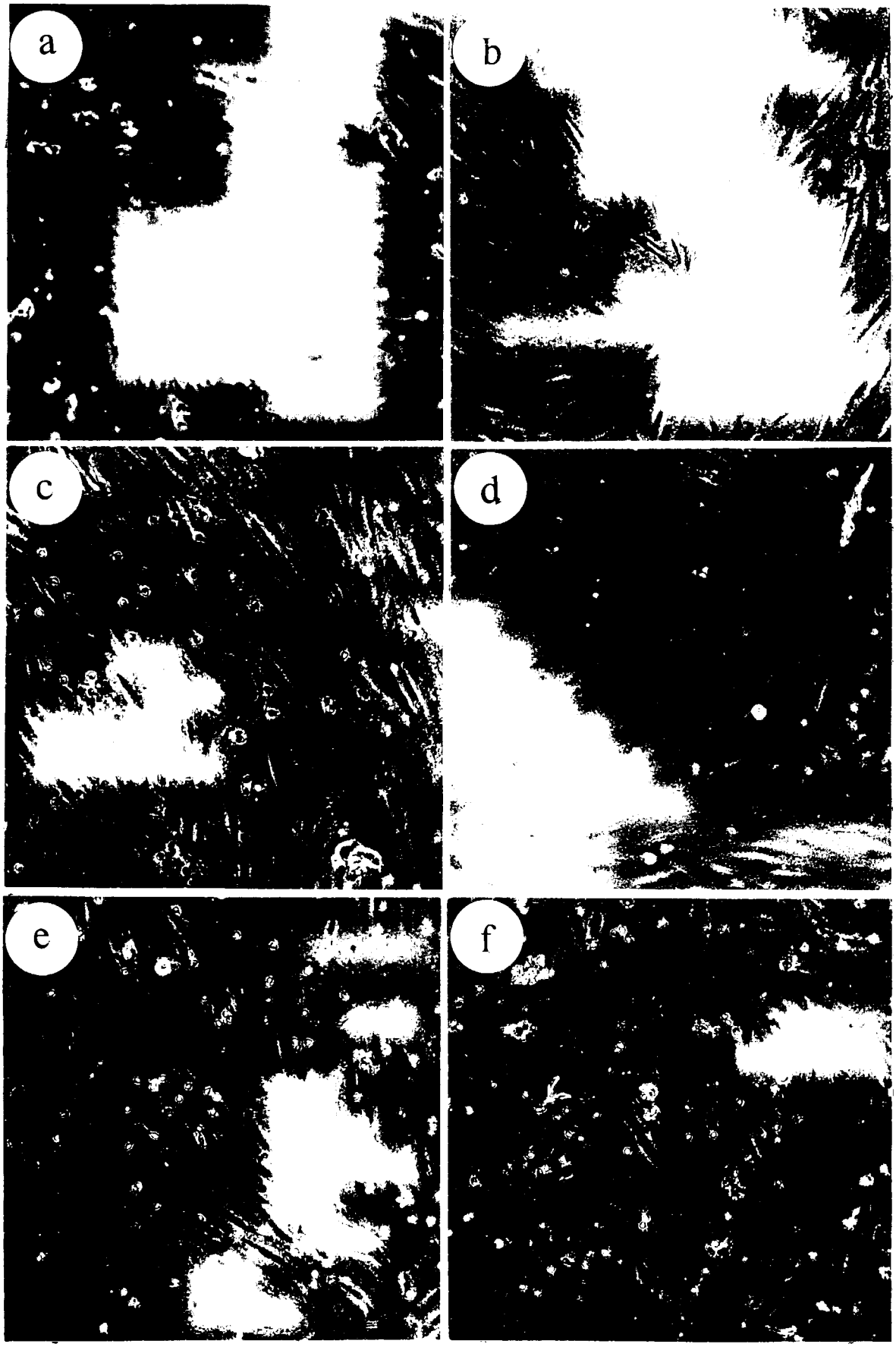
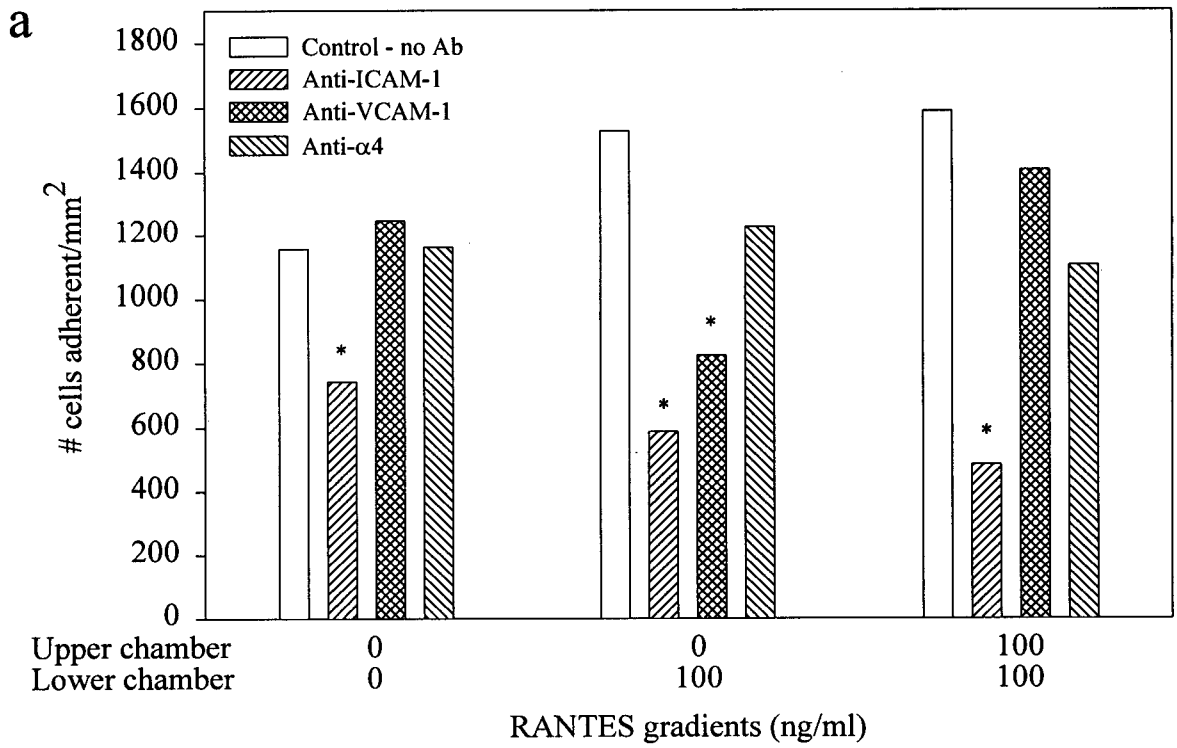


Fig. 24 Anti-CD3-activated CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β. (a) Several activated CD4+ T cells adhere to unstimulated HBMEC monolayers; (b) cytokine activation of HBMEC with TNF-α (100 U/ml) and IFN-γ (200 U/ml) doubles the number of adherent cells. Gradients of RANTES (100 ng/ml) (c) and MIP-1β-100 ng/ml (e) enhance the level of activated CD4+ T cell adhesion to cytokine-treated HBMEC. The presence of RANTES (d) or MIP-1β (f) in both the upper and lower chamber also enhanced adhesion to cytokine-treated HBMEC. Note the extensive cell aggregation in (e) as cells respond to MIP-1β in the lower chamber. (d, f) Numerous T cells processes (possibly uropods) are evident in the presence of RANTES and MIP-1β. X 205.

Fig. 24 Anti-CD3-activated CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β.

Fig. 25 Effects of cell adhesion molecule blocking on chemokine-enhanced adhesion of resting CD4+ T cells to cytokine-treated HBMEC with mAb to cell adhesion molecules. 1 hour of cytokine-treated HBMEC pre-incubation with mAbs to cell adhesion molecules reduced the adhesion of (a) RANTES enhanced and (b) MIP-1β enhanced adhesion of resting CD4+ T cells to activated HBMEC in the absence or presence of concentration gradients. 1 x 10^5 T cells were added per well. * = $p < 0.05$. n=2.

Fig. 25 Effects of cell adhesion molecule blocking on chemokine-enhanced adhesion of resting CD4+ T cells to cytokine-treated HBMEC.



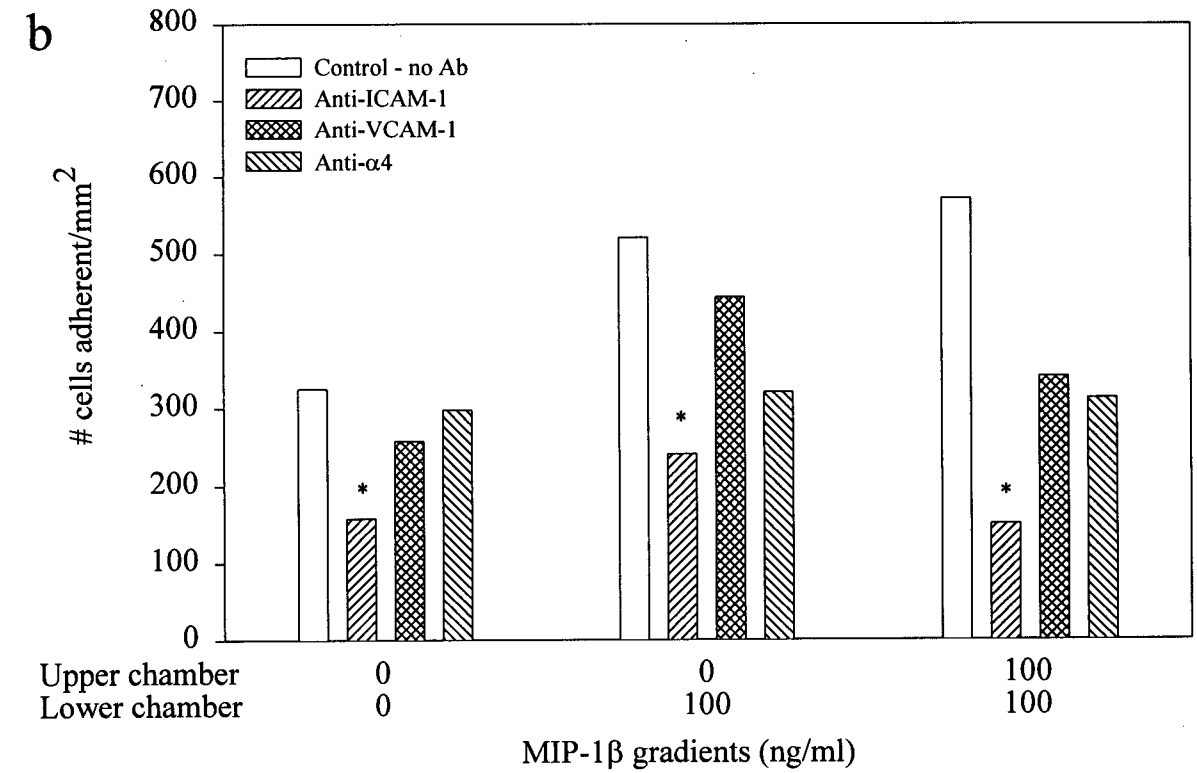
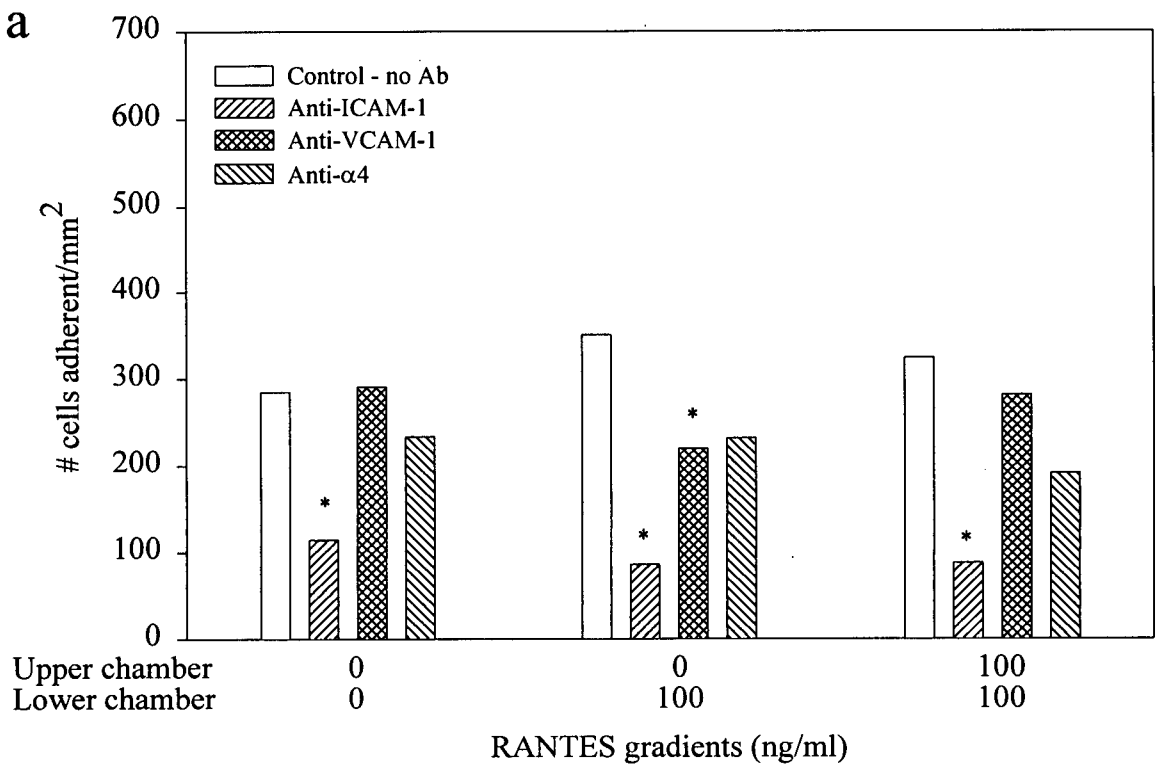


Fig. 26 Effects of cell adhesion molecule blocking on chemokine-enhanced adhesion of activated CD4+ T cells to cytokine-treated HBMEC. 1 hour pre-incubation of cytokine-treated HBMEC with mAbs to cell adhesion molecules reduced the adhesion of (a) RANTES enhanced and (b) MIP-1β enhanced adhesion of activated CD4+ T cells to activated HBMEC in the absence or presence of RANTES.
1×10^5 T cells were added per well. * = $p < 0.05$. n=2.

Fig. 26 Effects of cell adhesion molecule blocking on chemokine-enhanced adhesion of activated CD4+ T cells to cytokine-treated HBMEC.



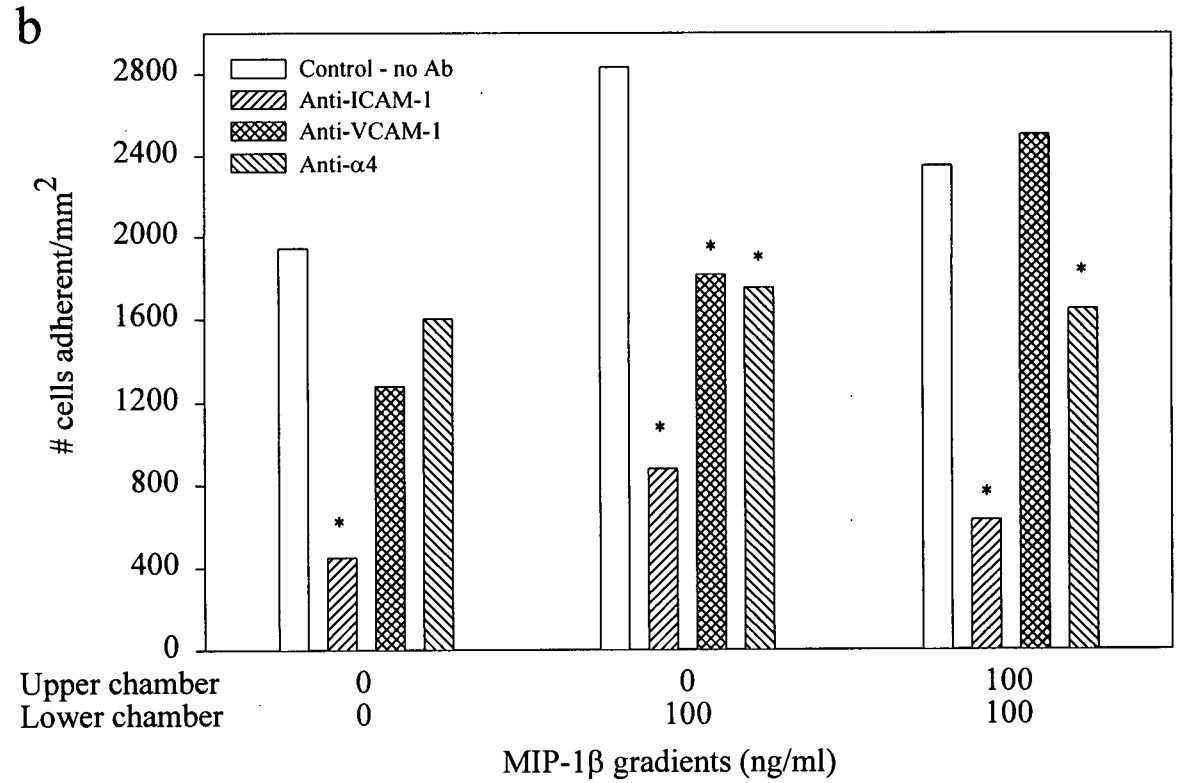
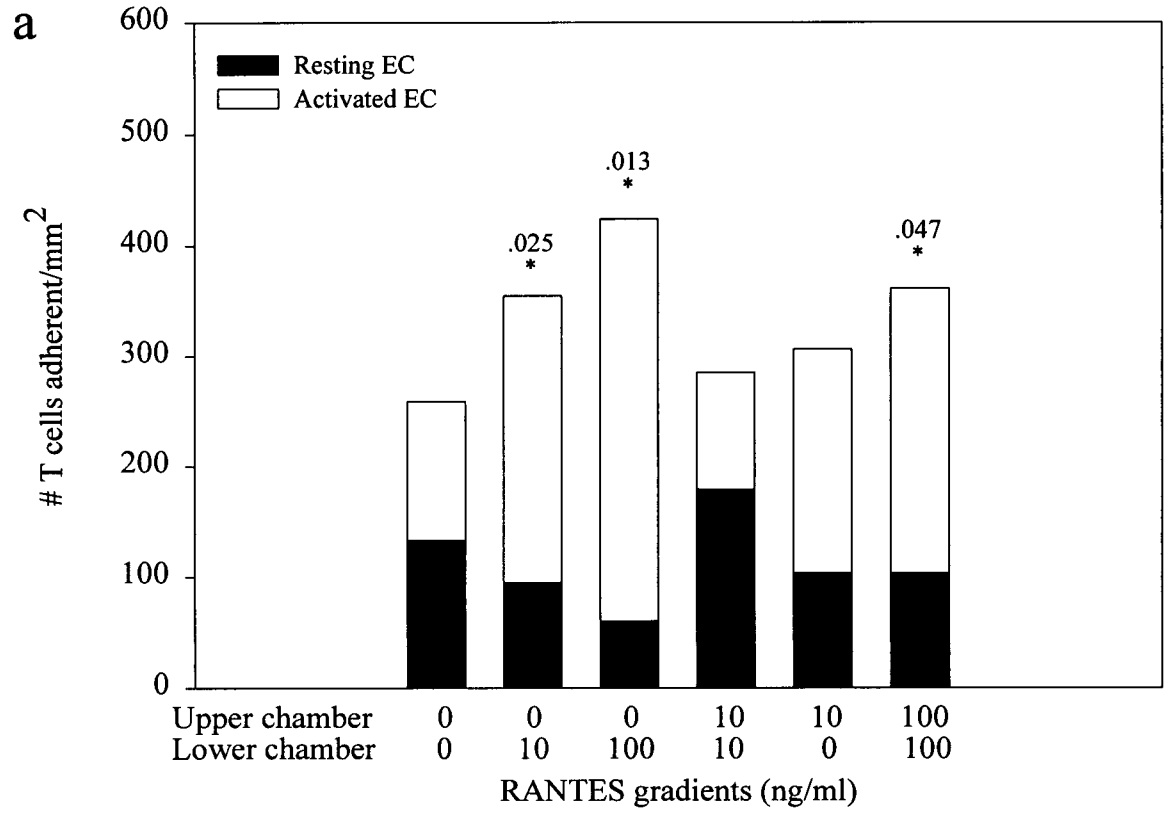


Fig. 27 Effects of RANTES and MIP-1β on HA 307-319 Ag specific CD4+ T cell clone adhesion to HBMEC monolayers. (a) RANTES augments the adhesion of memory CD4+ T cells to activated monolayers but has no effect on the adhesion to resting HBMEC. (b) MIP-1β enhances the adhesion of memory CD4+ T cells to activated HBMEC monolayers but has no effect on the adhesion to resting HBMEC.
1×10^5 T cells were added per well. Data represent one of three representative experiments each performed in duplicate. * = $p<0.05$. n=2.

Fig. 27 Effects of (a) RANTES and (b) MIP-1β on HA 307-319 Ag-specific T cell adhesion to HBMEC monolayers.



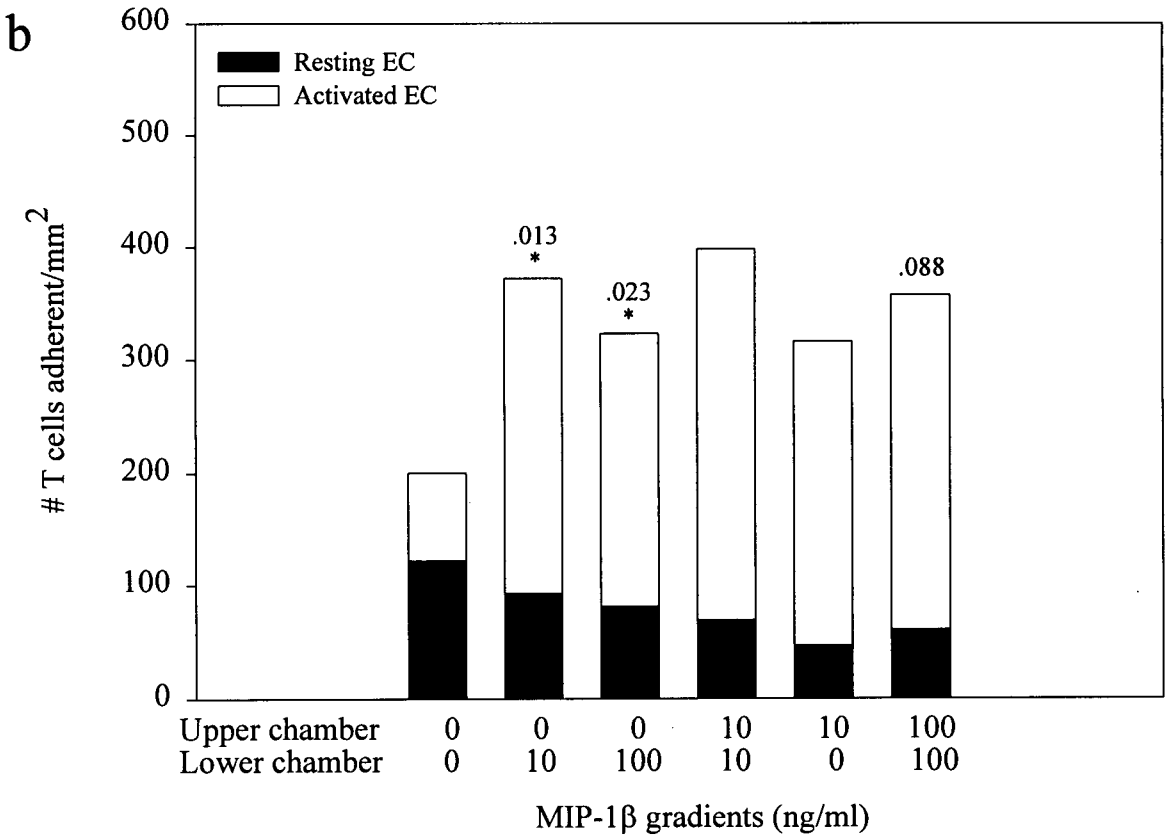
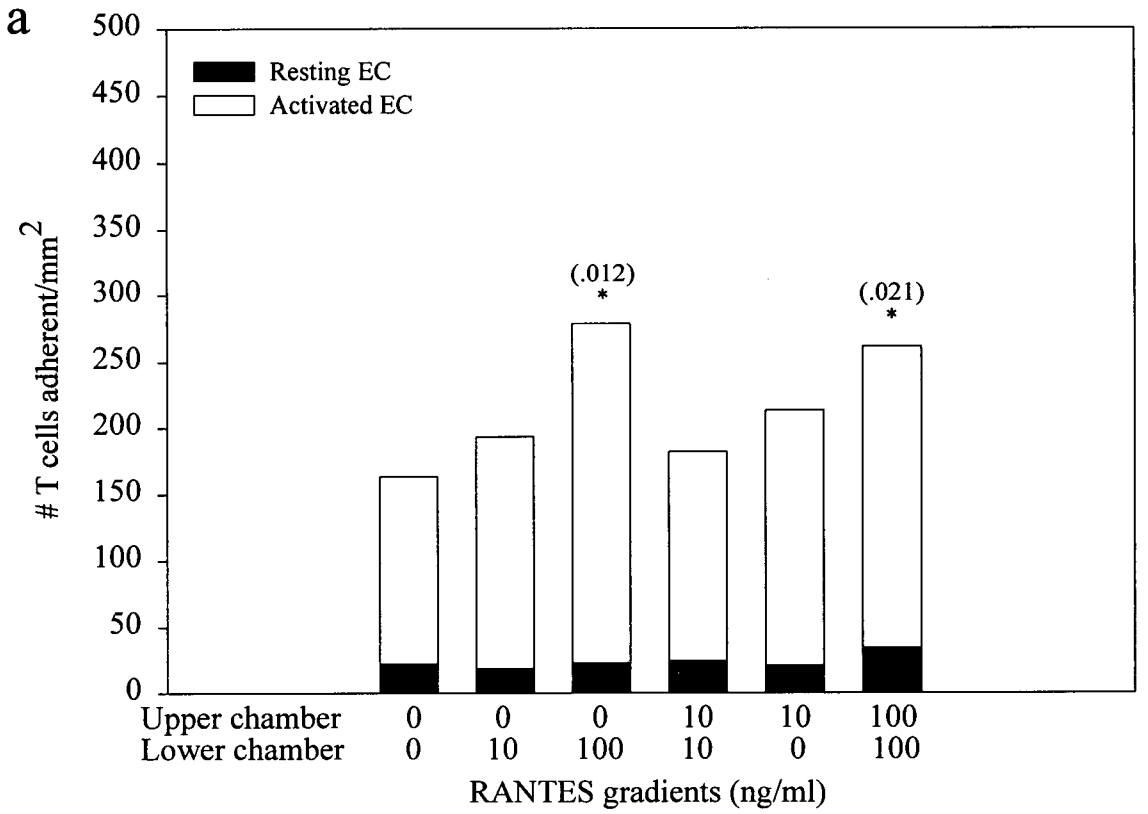


Fig. 28 HA 307-319 Ag specific CD4+ T cell clone adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β. (a) Few T cell clones adhere to untreated HBMEC monolayers; (b) cytokine activation of HBMEC with TNF-α (100 U/ml) and IFN-γ (200 U/ml) doubles the number of cells which adhere. Gradients of RANTES-100 ng/ml (c) and MIP-1β-100 ng/ml (e) enhance the level of memory CD4+ T cell adhesion to cytokine-treated HBMEC. The presence of RANTES (d) or MIP-1β (f) in both the upper and lower chamber also enhanced adhesion to cytokine-treated HBMEC. X 205.

Fig. 28 HA 307-319 Ag specific CD4+ T cell clone adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β.

Fig. 29 Effects of RANTES and MIP-1β on memory CD4+ T cell adhesion to HBMEC monolayers. (a) RANTES augments the adhesion of memory CD4+ T cells to activated monolayers but has no effect on the adhesion to resting HBMEC. (b) MIP-1β enhances the adhesion of memory CD4+ T cells to activated HBMEC monolayers but has no effect on the adhesion to resting HBMEC. 1 x 10^5 T cells were added per well. Data represent one of three representative experiments each performed in duplicate. * = $p<0.05$. n=2.

Fig. 29 Effects of (a) RANTES and (b) MIP-1β on memory CD4+ T cell adhesion to HBMEC monolayers.



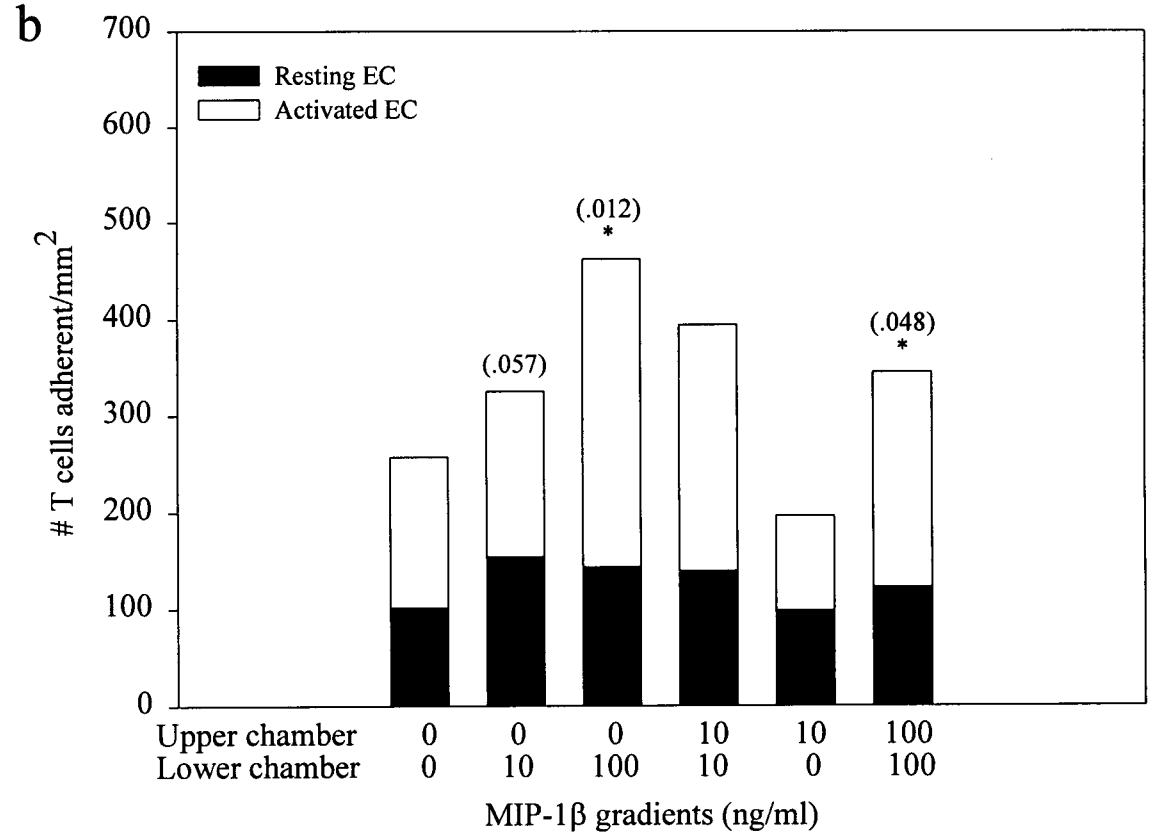


Fig. 30 Memory CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β. (a) Few memory CD4+ T cells adhere to resting HBMEC monolayers; (b) cytokine activation of HBMEC with TNF-α (100 U/ml) and IFN-γ (200 U/ml) doubles the number of adherent cells. Gradients of RANTES (100 ng/ml) (c) and MIP-1β (100 ng/ml) (e) upregulate the adhesion of memory CD4+ T cells to cytokine-treated HBMEC. The presence of RANTES (d) or MIP-1β (f) in both upper and lower chambers also enhance adhesion to cytokine-treated HBMEC. X 205.

Fig. 30 Memory CD4+ T cell adhesion to HBMEC monolayers is enhanced by RANTES and MIP-1β.

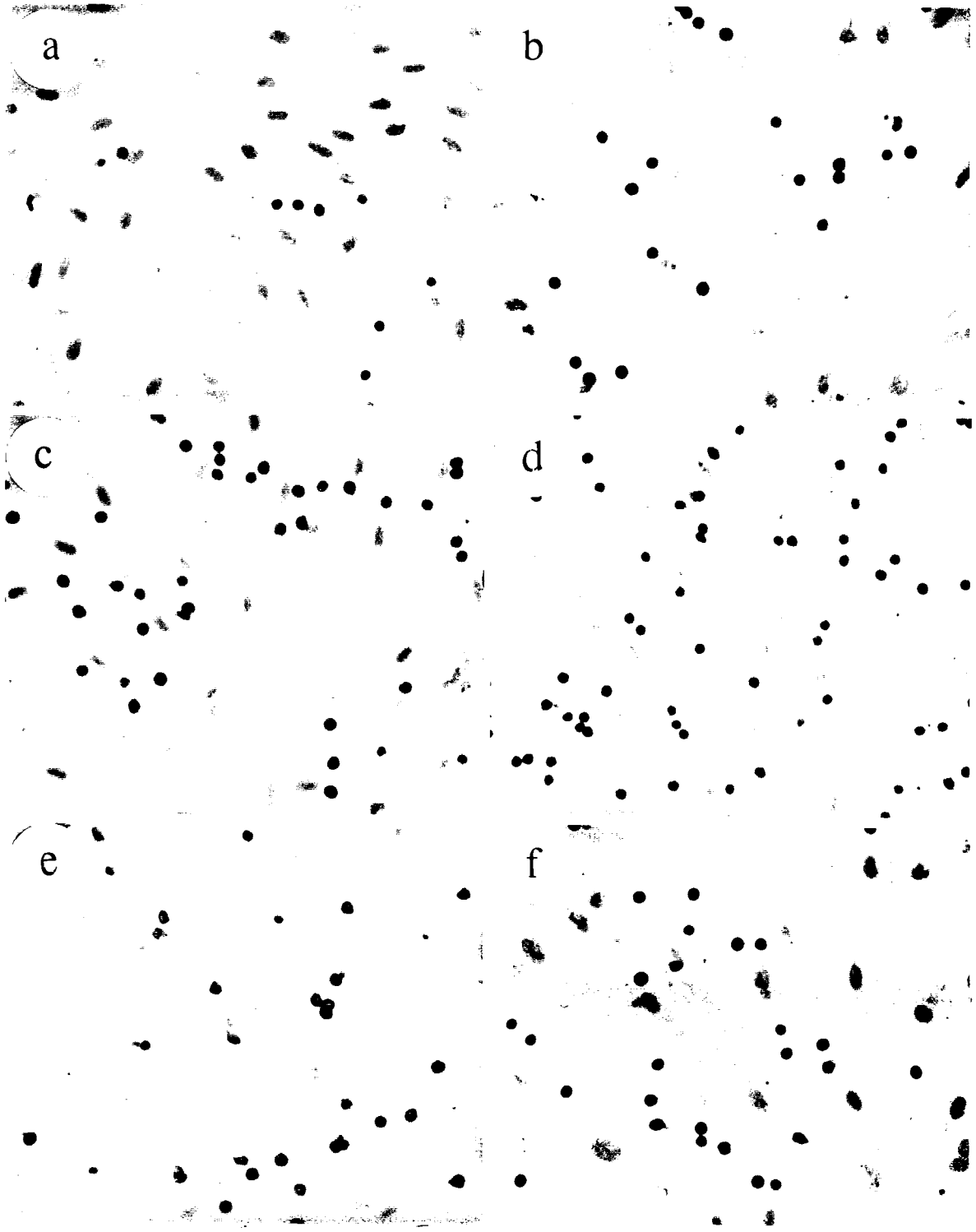
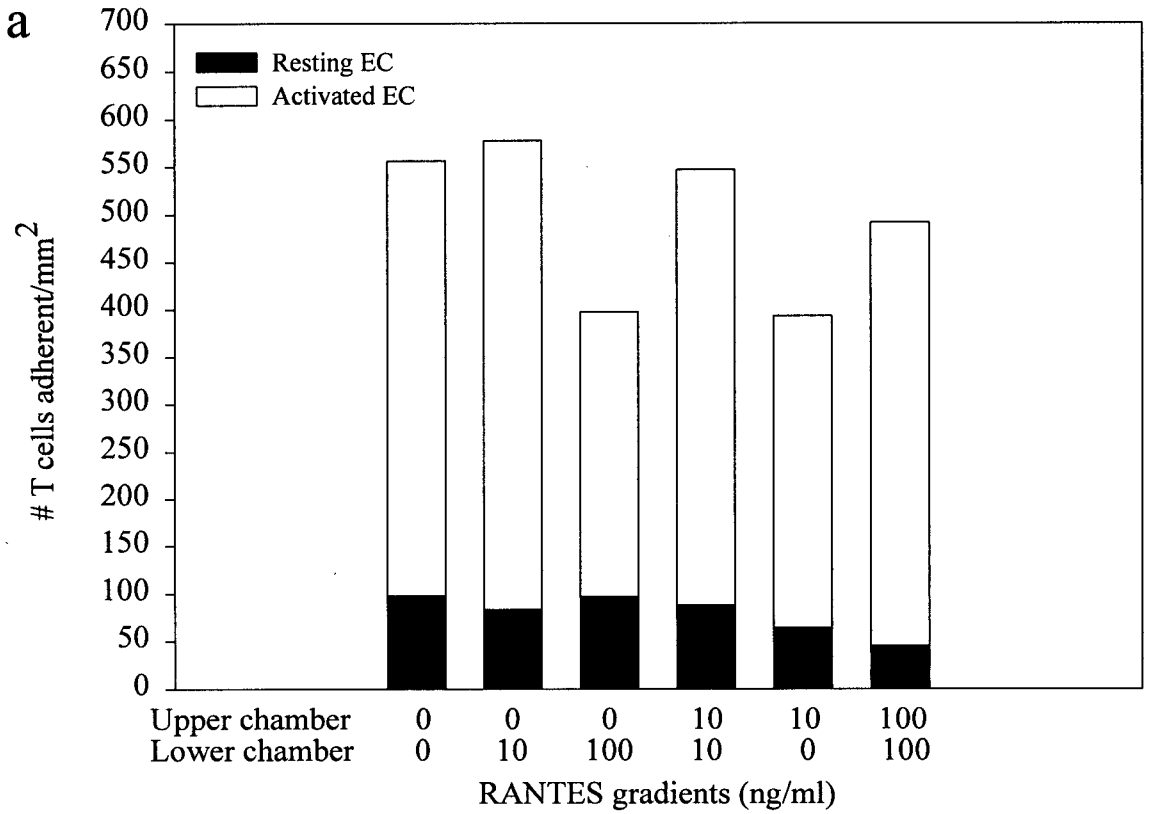


Fig. 31 Naïve CD4+ T cell adhesion to HBMEC monolayers. Neither RANTES (a) nor MIP-1β (b) have any effects on the adhesion of naïve CD 4+ T cell subsets to unstimulated or cytokine-treated HBMEC. 1 x 10^5 T cells were added per well. Data represent one of three representative experiments each performed in duplicate. * = $p<0.05$. n=2.

Fig. 31 Effects of (a) RANTES and (b) MIP-1β on naive CD4+ T cell adhesion to HBMEC monolayers.



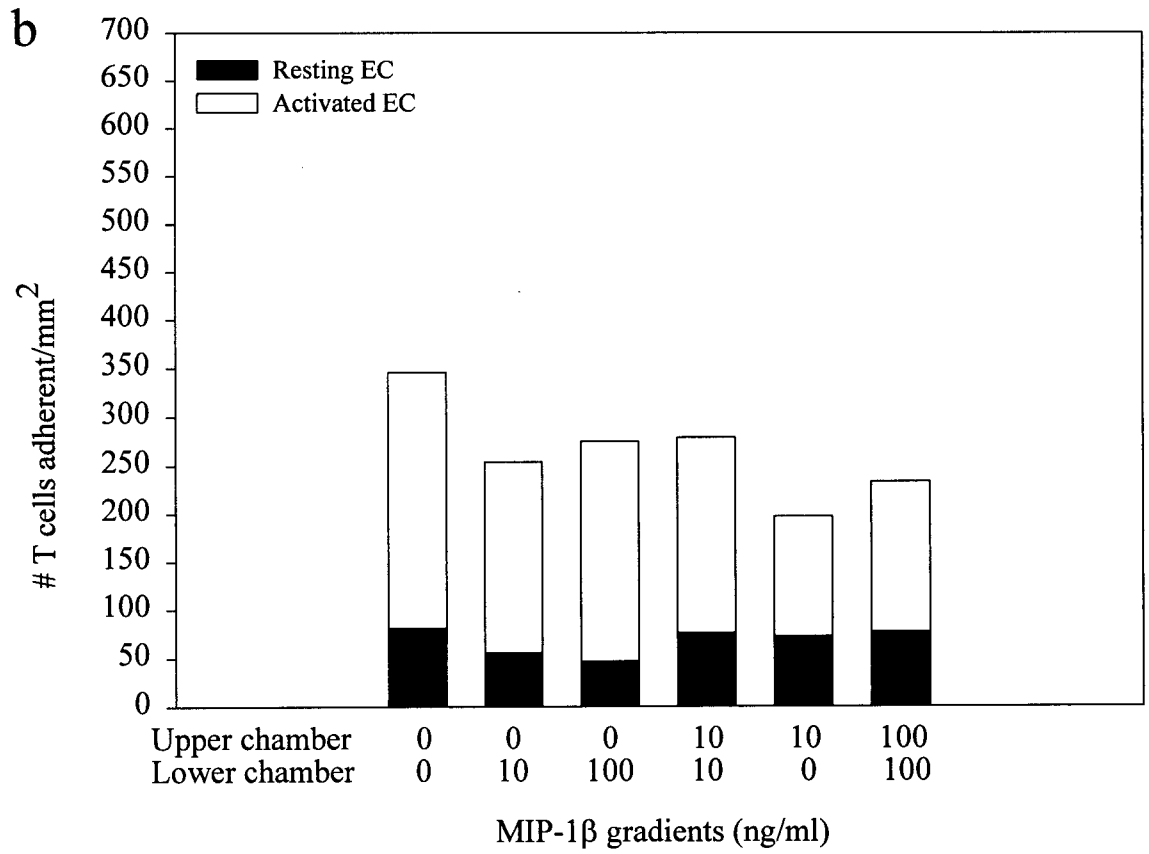


Fig. 32 Naïve CD4+ T cell adhesion to HBMEC monolayers. While neither RANTES nor MIP-1β have any effects on the adhesion of naïve CD4+ T cell subset adhesion to HBMEC, the basal level of adhesion of naïve CD4+ T cells to unstimulated HBMEC (a) is increased up to two fold by cytokine-treatment of HBMEC (b). X 205.

Fig. 32 Naïve CD4+ T cell adhesion to HBMEC monolayers.

Fig. 33 Effects of (a) RANTES and (b) MIP-1β upon resting CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers. Black bars and white bars represent migration across untreated and cytokine-treated HBMEC monolayers respectively. Neither chemokine affected migration across resting HBMEC monolayers, whereas a trend was seen towards increased migration in response to chemokine gradients. 1×10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment. * = $p < 0.05$.

Fig. 33 Effects of RANTES and MIP-1β upon resting CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers.

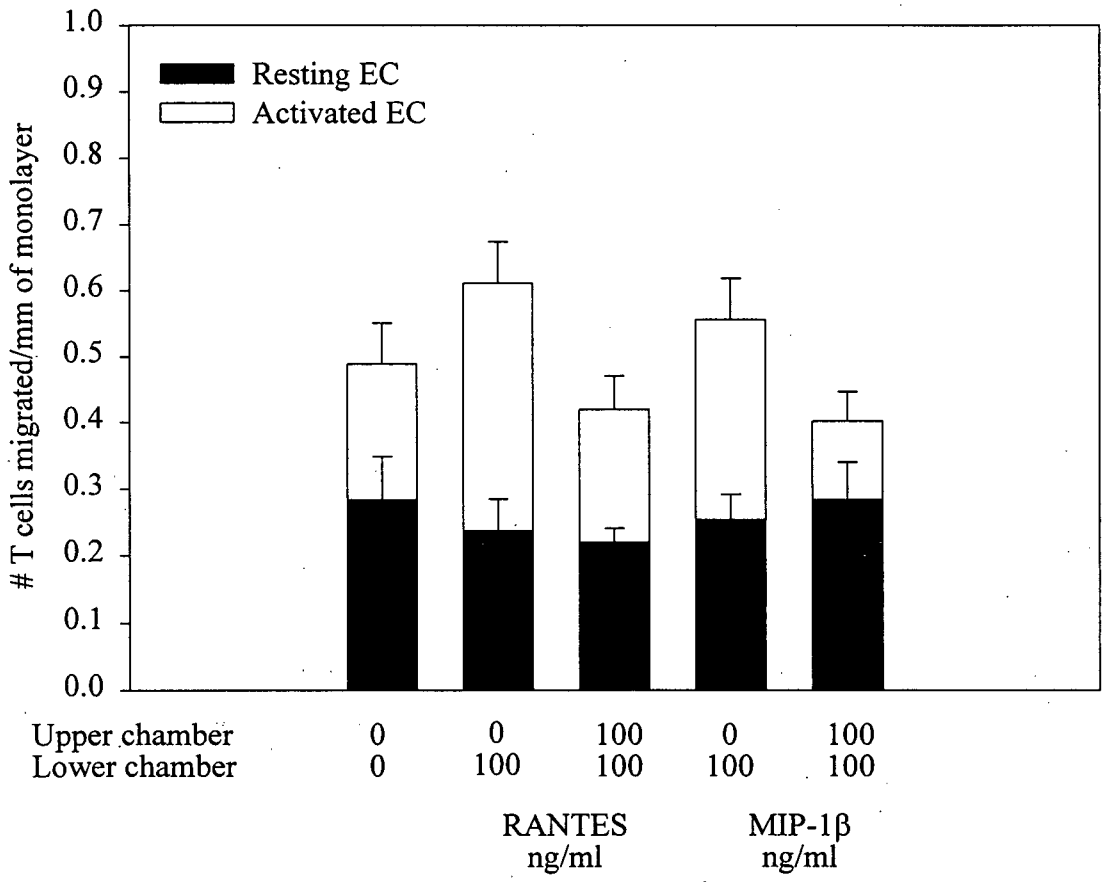


Fig. 34 Light micrographs of resting CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue. (a) Three untreated endothelial cells resting upon the cellagen membrane are pictured; no migrated resting CD4+ T cells are present. Lymphocytes are distinguishable by their intensely stained nuclei. Cytokine treatment with TNF-α and IFN-γ allowed for greater migration of resting T cells; a lymphocyte has just migrated across and is sandwiched between two EC and the cellagen membrane (b). (c) RANTES present in the lower chamber enhanced migration; one migrated lymphocyte is shown with another migrating through the monolayer with a protruding foot. (d) MIP-1β in the lower chamber showed a trend towards enhanced migration across activated HBMEC. Two lymphocytes have migrated and are sandwiched between the EC monolayer and the cellagen membrane. X 1010.

Fig. 34 Light micrographs of resting CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue.

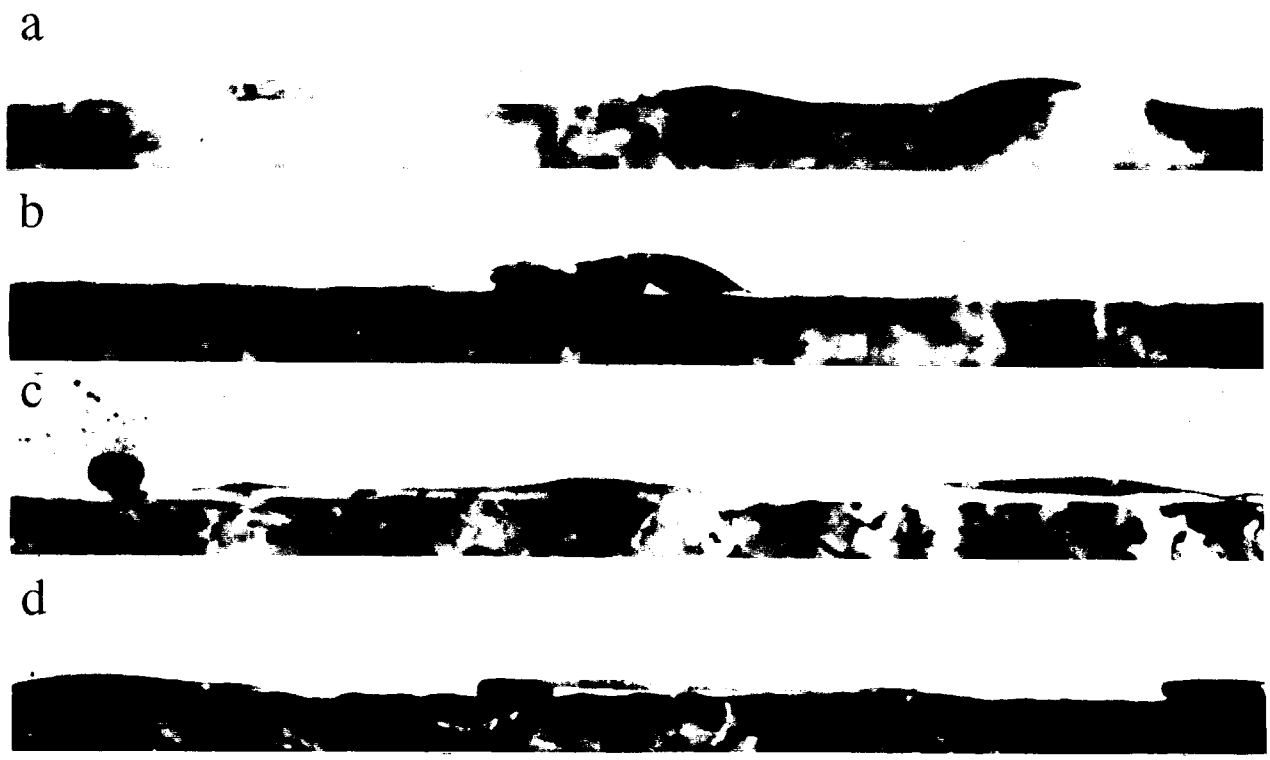


Fig. 35 Effects of (a) RANTES and (b) MIP-1β upon activated CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers. Black bars and white bars represent migration across resting and cytokine-treated HBMEC monolayers respectively. Neither chemokine affected migration across untreated HBMEC monolayers, whereas migration increased significantly in response to chemokine gradients. 0.75×10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment.* = $p < 0.05$.

Fig. 35 Effects of RANTES and MIP-1β upon activated CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers.

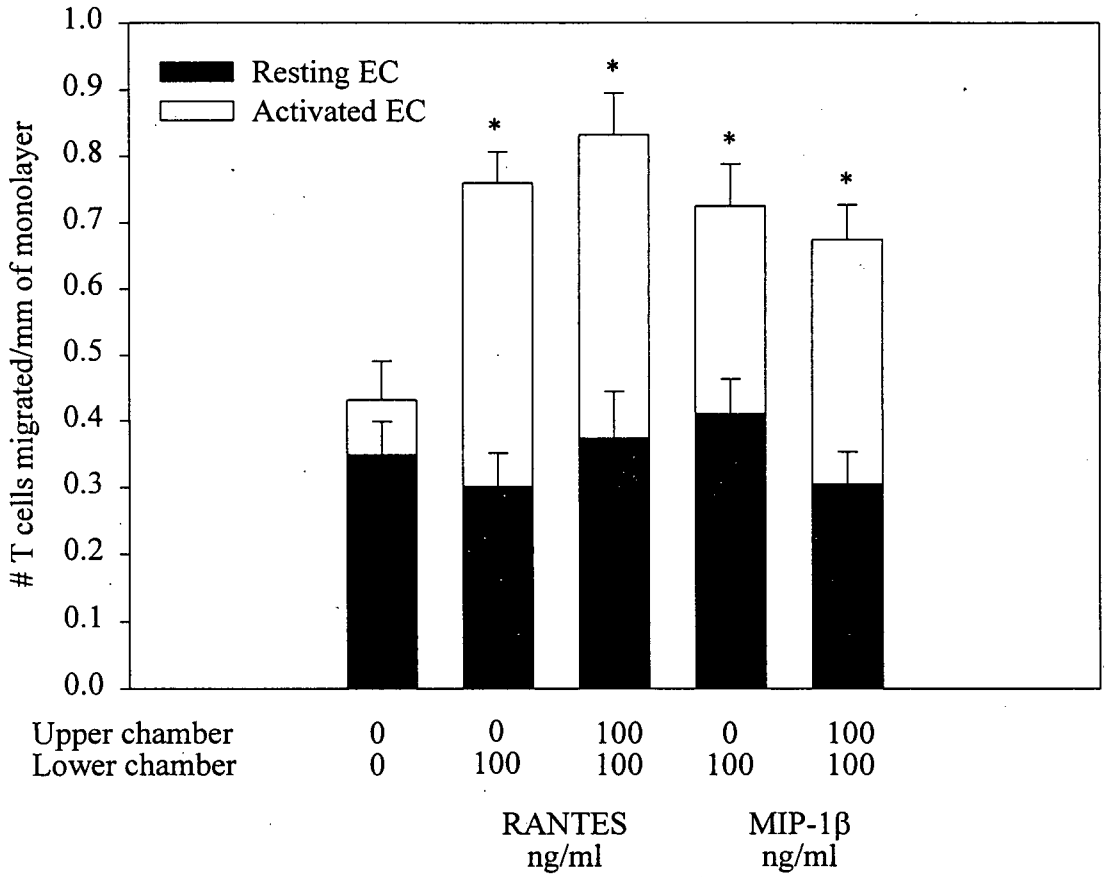


Fig. 36 Light micrograph of activated CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue. Lymphocytes are distinguishable with their densely-stained nuclei. (a) Activated CD4+ T cell migration was notable across untreated HBMEC monolayers and was not affected by (b) chemokine gradients (100 ng/ml RANTES). (a) One cell has migrated and a portion of another has also migrated. The EC monolayer is distended away from the cellagen membrane by the presence of the lymphocyte. (b) Two cells have migrated and 2 are adherent, showing that RANTES does not enhance migration across resting HBMEC monolayers. Migration was enhanced by cytokine-treatment of HBMEC (c). Gradients of (d) RANTES and (e) MIP-1β (100 ng/ml) enhanced migration across activated HBMEC monolayers, with several migrated lymphocytes per field. (f) RANTES in both upper and lower chambers also enhanced the migration of activated CD4+ T cells. X 1070.

Fig. 36 Light micrographs of activated CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue.

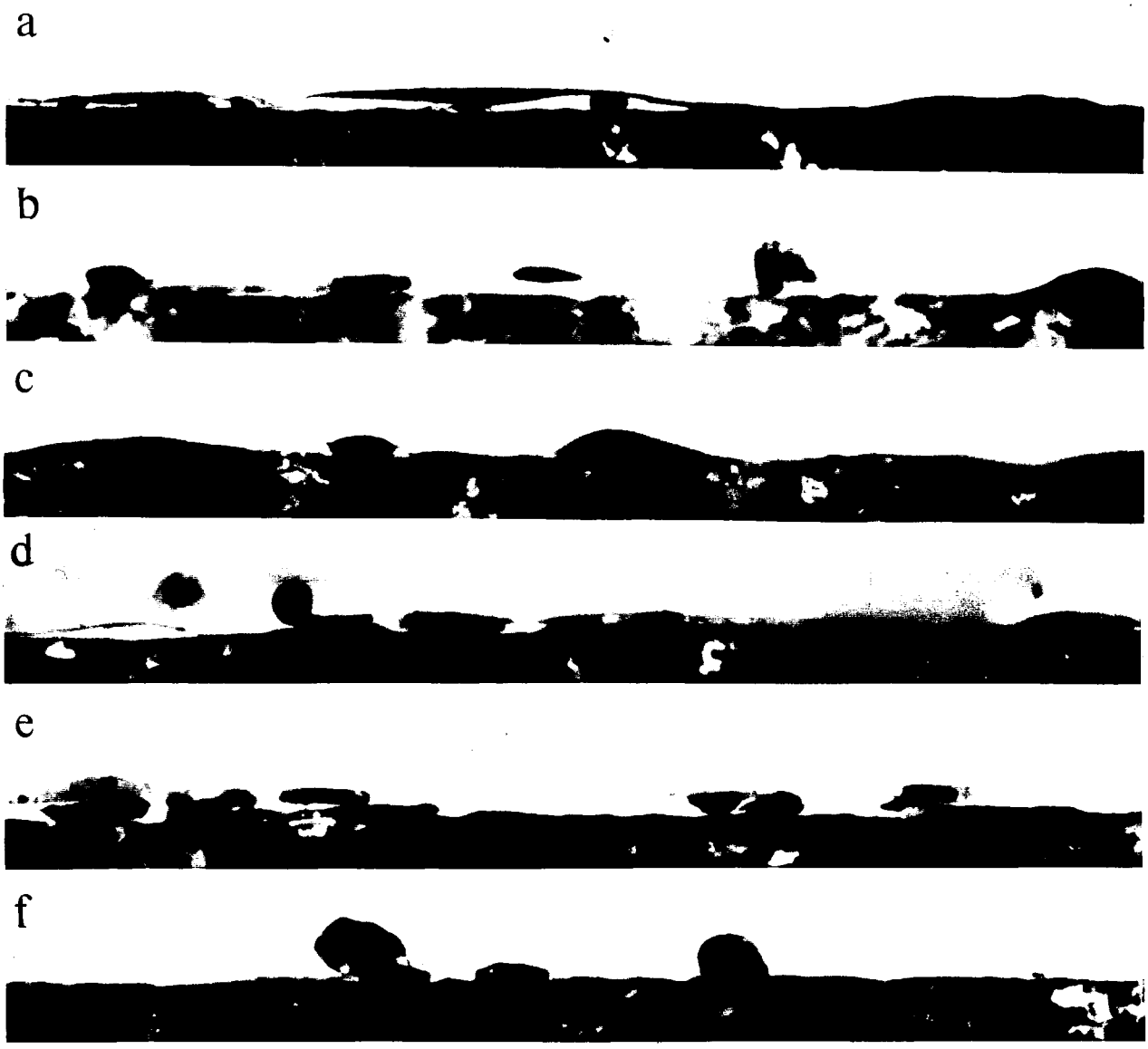


Fig. 37 Effects of cell adhesion molecule blocking on migration of resting (a) and activated CD4+ T cells (b) across untreated HBMEC. mAbs to ICAM-1, VCAM-1, and α4 were added after 30 mins. of T cell adhesion to unstimulated HBMEC. After a total of 3 hours co-incubation, the assay was stopped. 1 x 10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment.* = $p < 0.05$.

Fig. 37 Effects of cell adhesion molecule blocking on migration of resting CD4+ T cells and activated CD4+ T cells across untreated HBMEC.

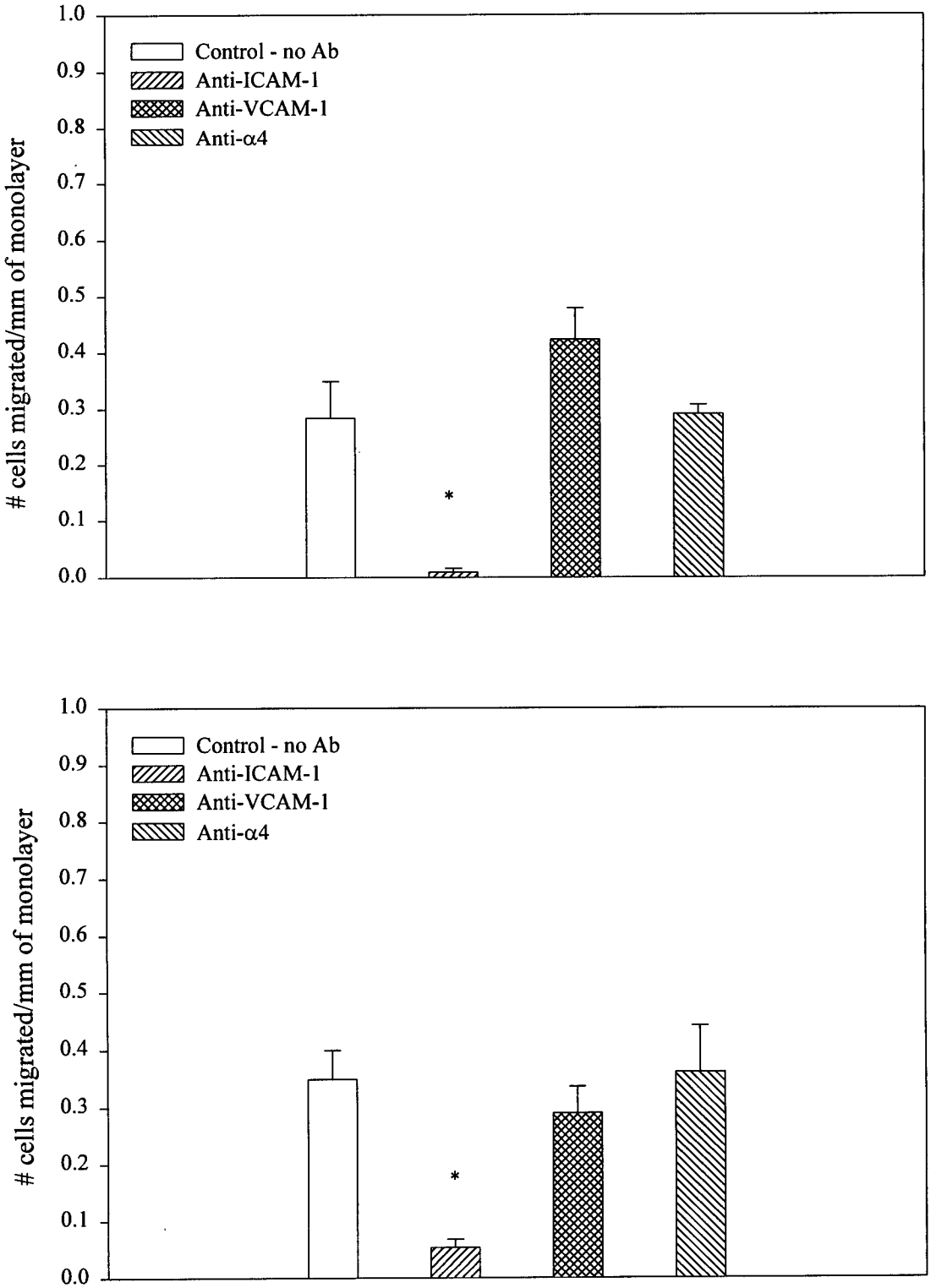
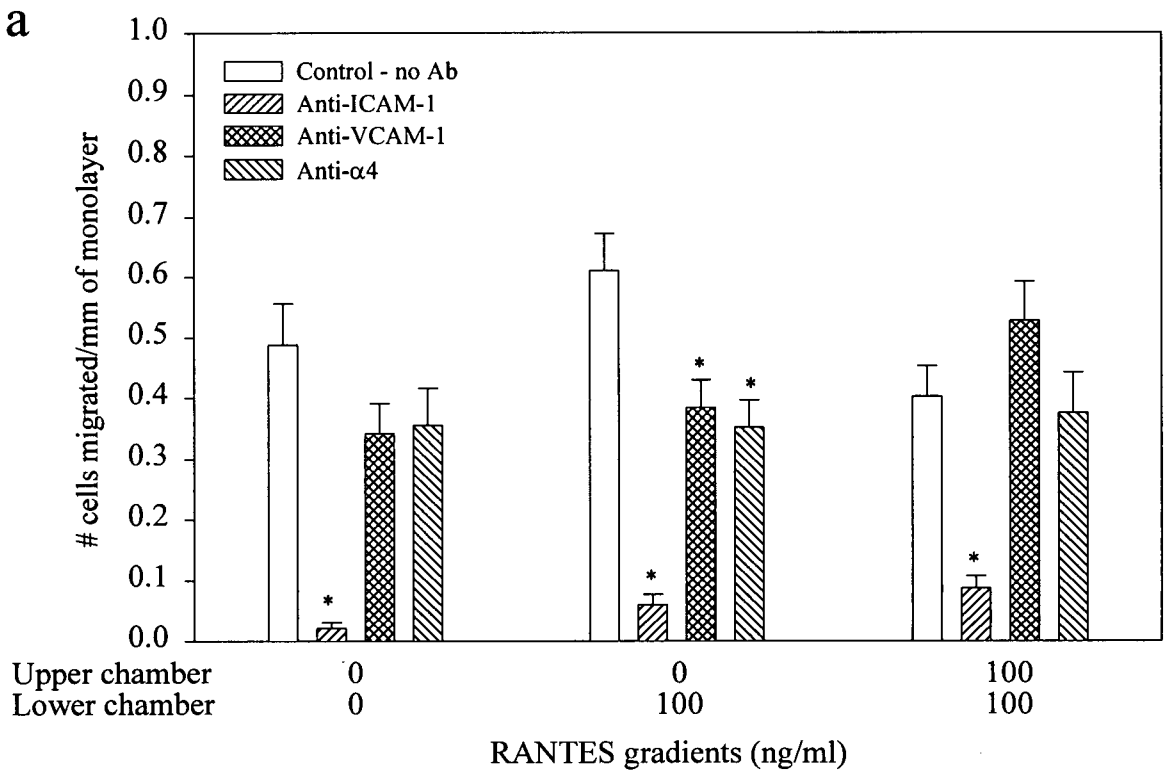


Fig. 38 Effects of cell adhesion molecule blocking on chemokine-enhanced migration of resting CD4+ T cells across cytokine-treated HBMEC. Adhesion was allowed to proceed uninhibited for 30 minutes before the addition of blocking mAbs. Blocking adhesion molecules reduced the migration of (a) RANTES enhanced and (b) MIP-1β enhanced migration of resting CD4+ T cells to activated HBMEC in the absence or presence of concentration gradients. 1 x 10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment.* = $p < 0.05$.

Fig. 38 Effects of cell adhesion molecule blocking on chemokine-enhanced migration of resting CD4+ T cells across cytokine-treated HBMEC.



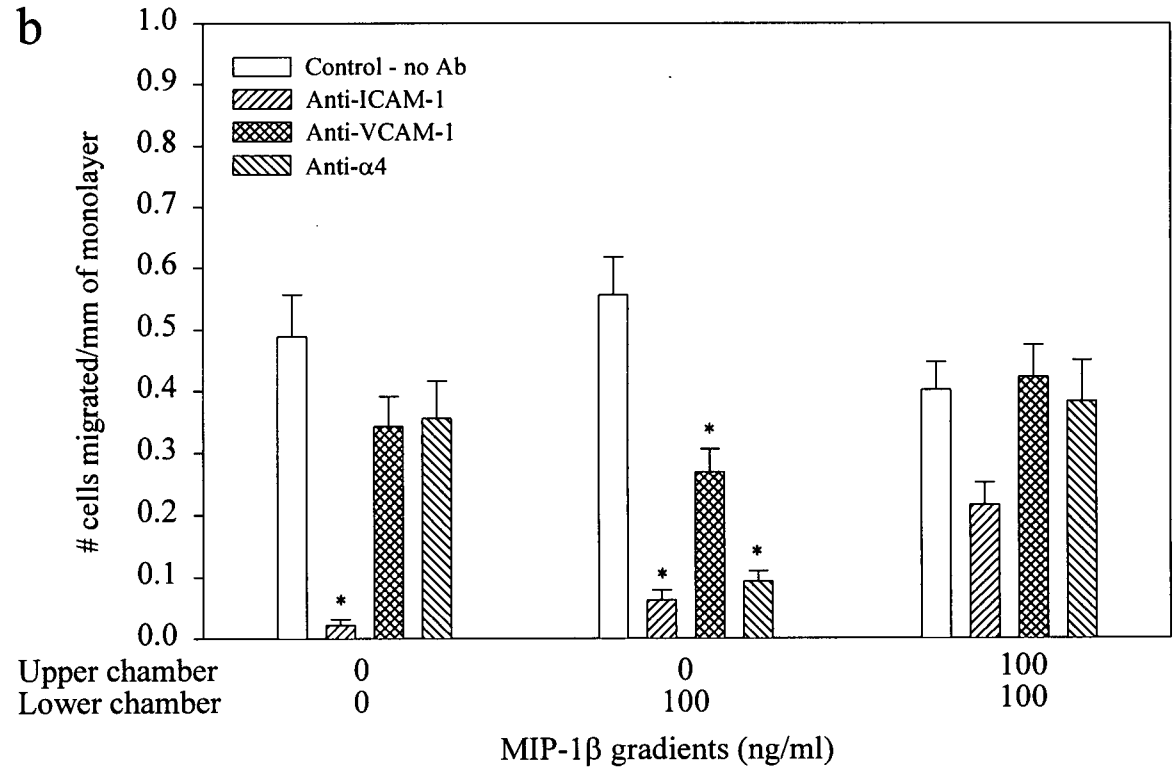
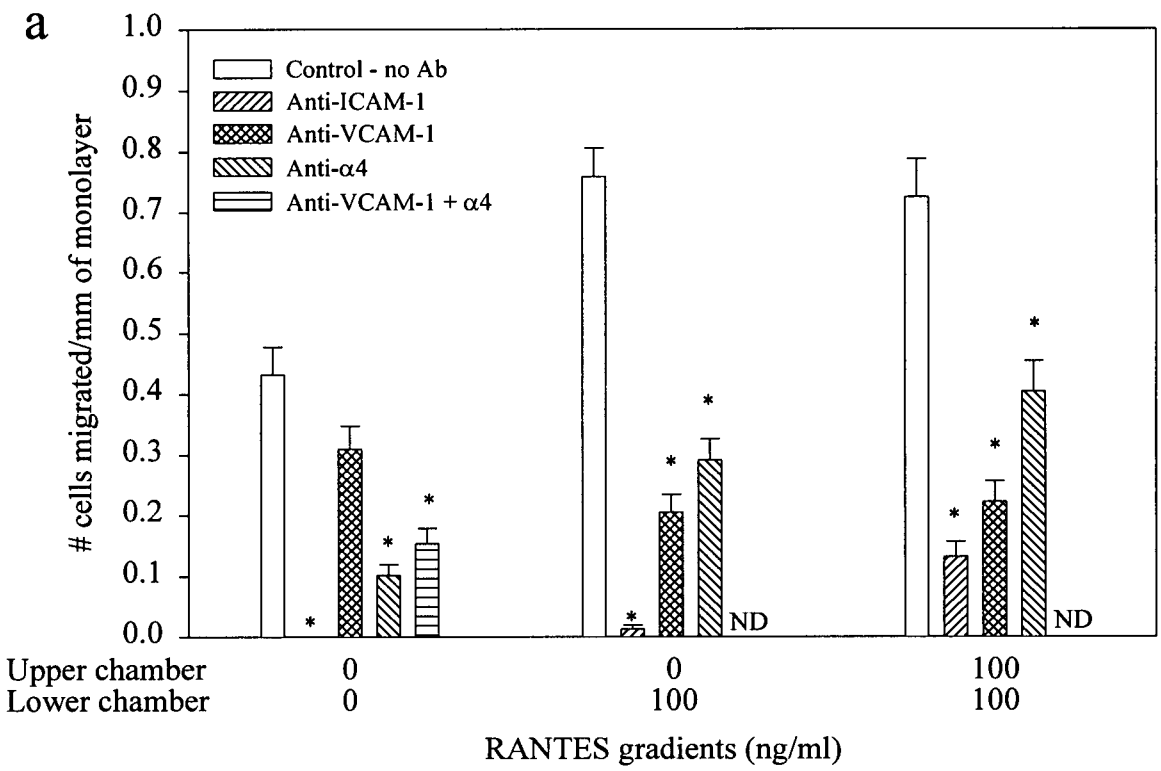


Fig. 39 Effects of cell adhesion molecule blocking on chemokine-enhanced migration of activated CD4+ T cells across cytokine-treated HBMEC. Adhesion was allowed to proceed uninhibited for 30 minutes before the addition of blocking mAbs. Blocking adhesion molecules reduced the migration of (a) RANTES enhanced and (b) MIP-1β enhanced migration of activated CD4+ T cells to activated HBMEC in the absence or presence of concentration gradients. 0.75×10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment. * = $p < 0.05$. ND = not determined in the case of combination of mAbs to VCAM-1 and α4 when RANTES or MIP-1β were present.

Fig. 39 Effects of cell adhesion molecule blocking on chemokine-enhanced migration of activated CD4+ T cells across cytokine-treated HBMEC.



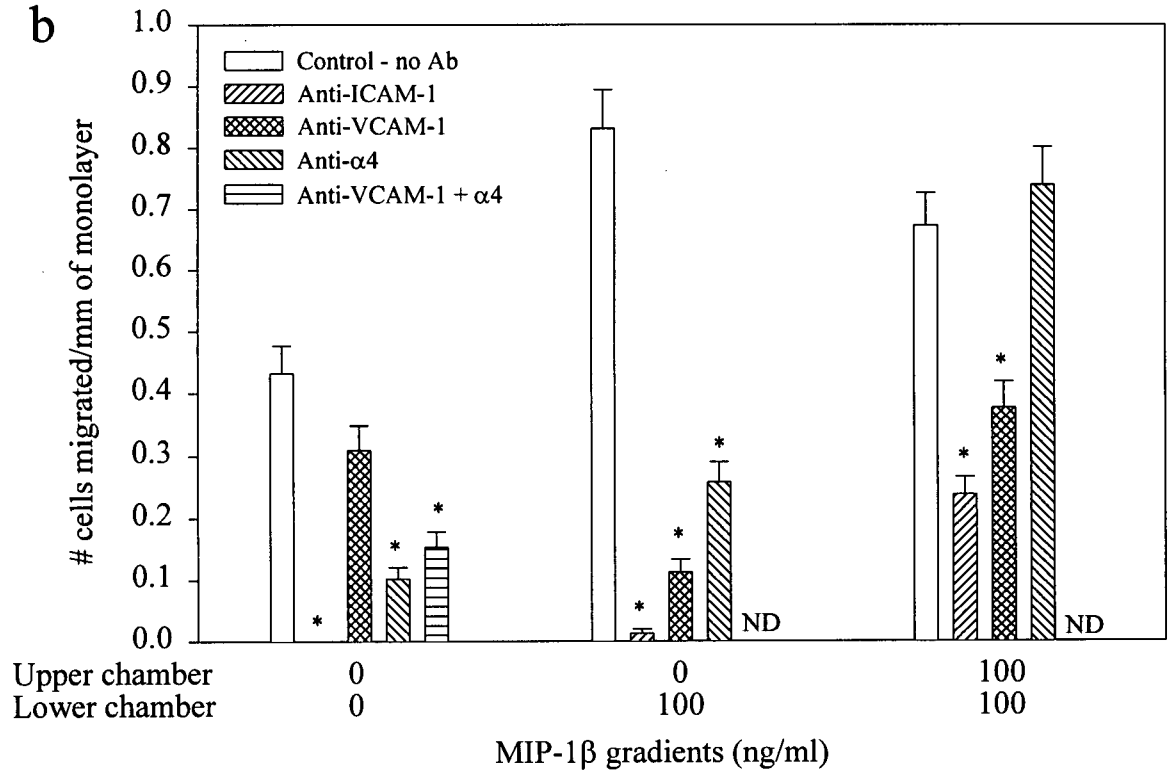


Fig. 40 Effects of (a) RANTES and (b) MIP-1β upon memory CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers. Black bars and white bars represent migration across resting and cytokine-treated HBMEC monolayers respectively. Neither chemokine affected migration across untreated HBMEC monolayers, whereas migration increased significantly in response to chemokine gradients.0.8 x 10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment.* = $p < 0.05$.

Fig. 40 Effects of RANTES and MIP-1β upon memory CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers.

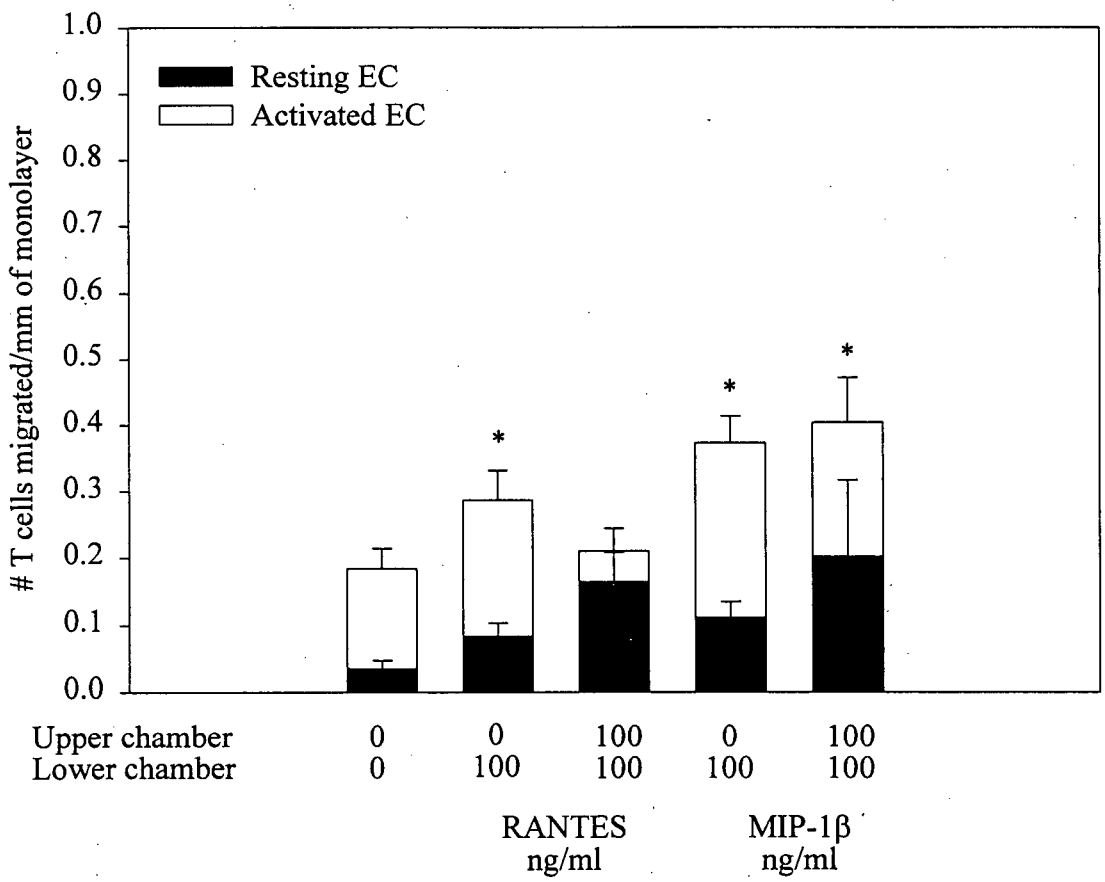


Fig. 41 Light micrograph of memory CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue. (a) Memory CD4+ T cell migration was minimal across untreated HBMEC monolayers and was enhanced by cytokine-treatment of HBMEC (b; one migrated T cell shown). The presence of (c) RANTES and (d) MIP-1β (100 ng/ml) in the lower chamber enhanced migration across activated HBMEC; two migrated cells are seen for each treatment.

Fig. 41 Light micrographs of memory CD4+ T cell migration across HBMEC monolayers in 1μm thick plastic sections stained with toluidine blue.

Fig. 42 Effects of (a) RANTES and (b) MIP-1β upon naive CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers. Black bars and white bars represent migration across resting and cytokine-treated HBMEC monolayers respectively. Neither chemokine affected migration across untreated or cytokine-treated HBMEC monolayers. 1×10^5 T cells were added per well. Migration is expressed as # of cells migrated per mm of monolayer. Values are expressed as means ± SEM of 100 sections per treatment.* = $p < 0.05$.

Fig. 42 Effects of RANTES and MIP-1β upon naive CD4+ T cell migration across untreated and cytokine-treated HBMEC monolayers.

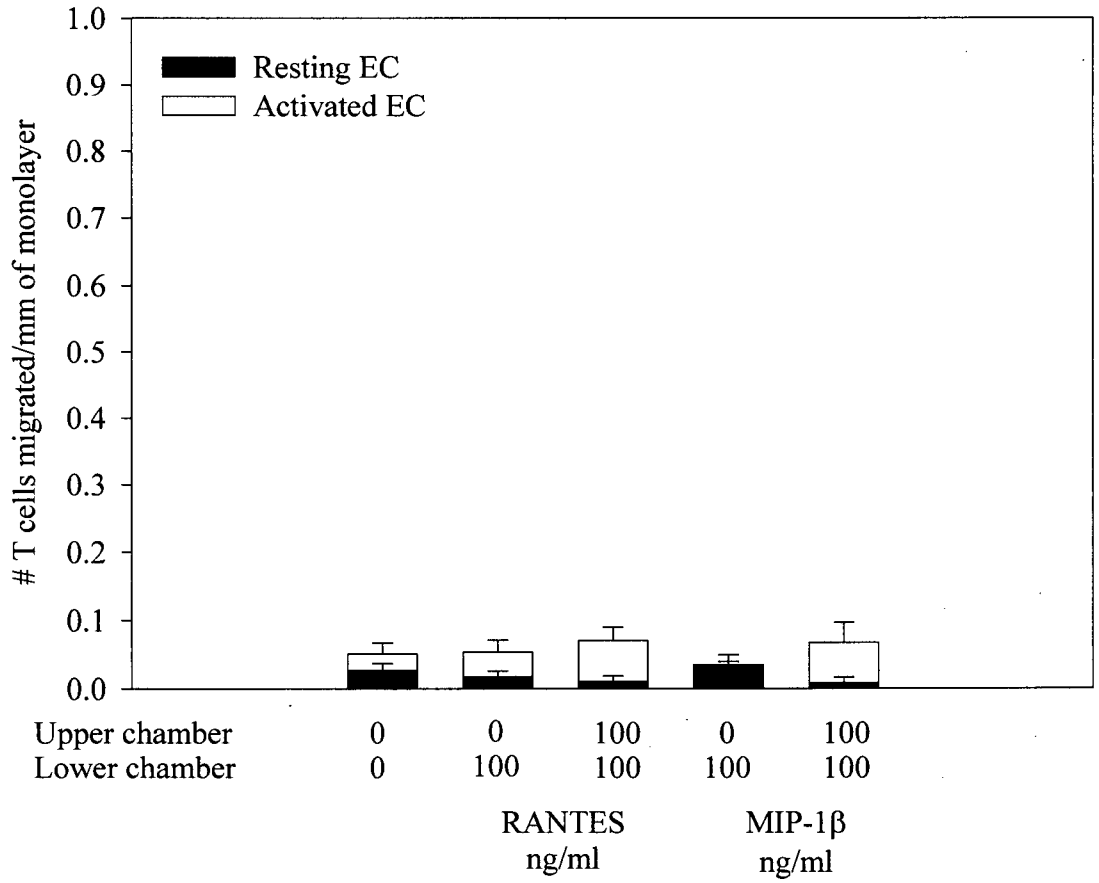


Fig. 43 Electron micrographs of HBMEC co-incubated with TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 24 hrs. EC are elongated and focally overlap (a) X 11,600. Cytokine-treated HBMEC display prominent finger-like projections extending from their apical surface (b) X 18,200. Rough endoplasmic reticulum (ER) is prominent. There is no increase in the number of pinocytotic vesicles. (c) A tight junction (arrow) binds two adjacent EC. Discontinuous basement material (*) is present between the EC and the collagen membrane. X 64,300.

Fig. 43 Electron micrographs of HBMEC co-incubated with TNF-α and IFN-γ for 24 hours.

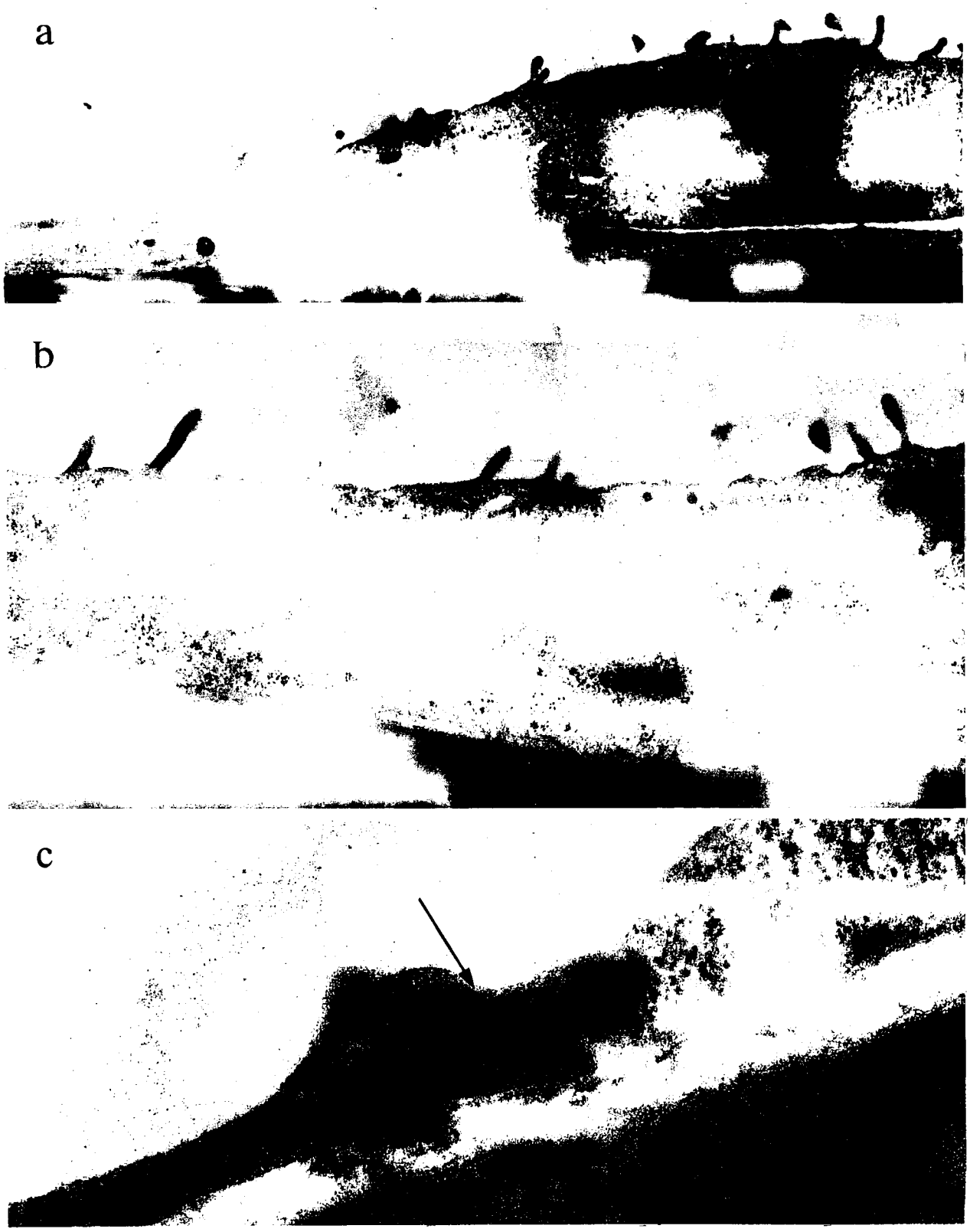


Fig. 44 Adhesion and migration of resting CD4+ T cells to activated HBMEC in the absence of chemokine gradients. (a) A T cell extends pseudopodia towards the EC. X 32,600. (b) This T cell is firmly adherent to the apical surface of the EC. The cytoplasmic membranes of the two cells are closely apposed. X 18,600. (c) Following firm adhesion, this lymphocyte begins its transendothelial migration by inserting a long cytoplasmic process (arrow) between the processes of two EC (arrow heads) that have separated, but are in close contact with the migrating T cell. X 26,300.

Fig. 44 Adhesion and migration of resting CD4+ T cells to activated HBMEC in the absence of chemokine gradients.

Fig. 44

Fig. 44

Fig. 45 Adhesion and migration of resting CD4+ T cells across activated HBMEC in the presence of chemokine gradients proceeds in a similar way as in Fig. 43, however, after 3 hrs of HBMEC/T cell incubation, a greater number of T cells has crossed the monolayers. (a) In the presence of RANTES gradients an adhering lymphocyte has become elongated and is partly firmly adherent to the endothelium. X 17,100. In the presence of MIP-1β in both chambers, lymphocytes first adhere firmly to the endothelium (b) (X 15,400) and then migrate across the monolayer (c) X 21,000. At the end of the migration period, the monolayers resume their continuity and inter-endothelial tight junctions appear intact (arrow head).

Fig. 45 Adhesion and migration of resting CD4+ T cells across activated HBMEC in the presence of chemokine gradients.

a

Fig. 45

Fig. 45

Fig. 46 T lymphocyte migration across HBMEC monolayers. (a) In the presence of RANTES in both chambers, a lymphocyte is firmly adherent to EC. The arrow head points to two processes of adjacent EC that seem to separate under an advancing cytoplasmic process of the lymphocyte. X 13,000. (b) Migrated T cells become elongated and remain in close contact to the overlying EC and the underlying collagen. X 10,000.

Fig. 46 T lymphocyte migration across HBMEC monolayers.

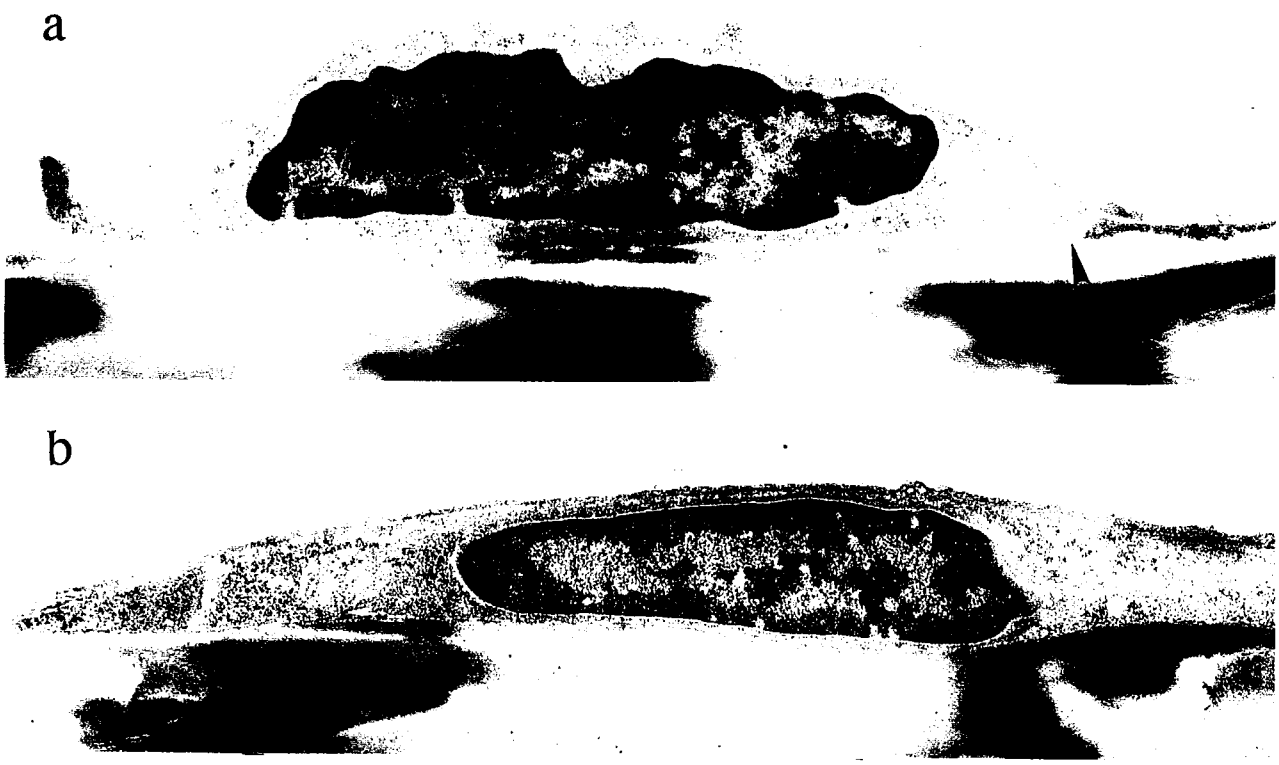


Fig. 47 Adhesion and migration of activated CD4+T cells across cytokine-treated HBMEC monolayers. (a) In the presence of MIP-1β gradients a lymphocyte adhered to the endothelium. X 32,600. The long cytoplasmic process is a uropod that protrudes from the opposite, non-adhering part of the cell. (b) This activated T cell starts migrating by inserting a long cytoplasmic process (arrow) between two adjacent EC processes (arrow heads) that remain in close contact with the lymphocyte. X 36,200. (c) Two lymphocytes have migrated in the presence of MIP-1β gradients. The cell to the left has just migrated through the monolayer and a small cytoplasmic projection is caught between two EC processes (arrow). X 16,600. (d) Similar to (c), a greater number of activated T cells cross treated HBMEC cultures in the presence of RANTES gradients. The monolayer appears intact over the migrated lymphocytes. X 16,200.

Fig. 47 Adhesion and migration of activated CD4+ T cells across cytokine-treated HBMEC monolayers.

a

Fig. 47

Fig. 47

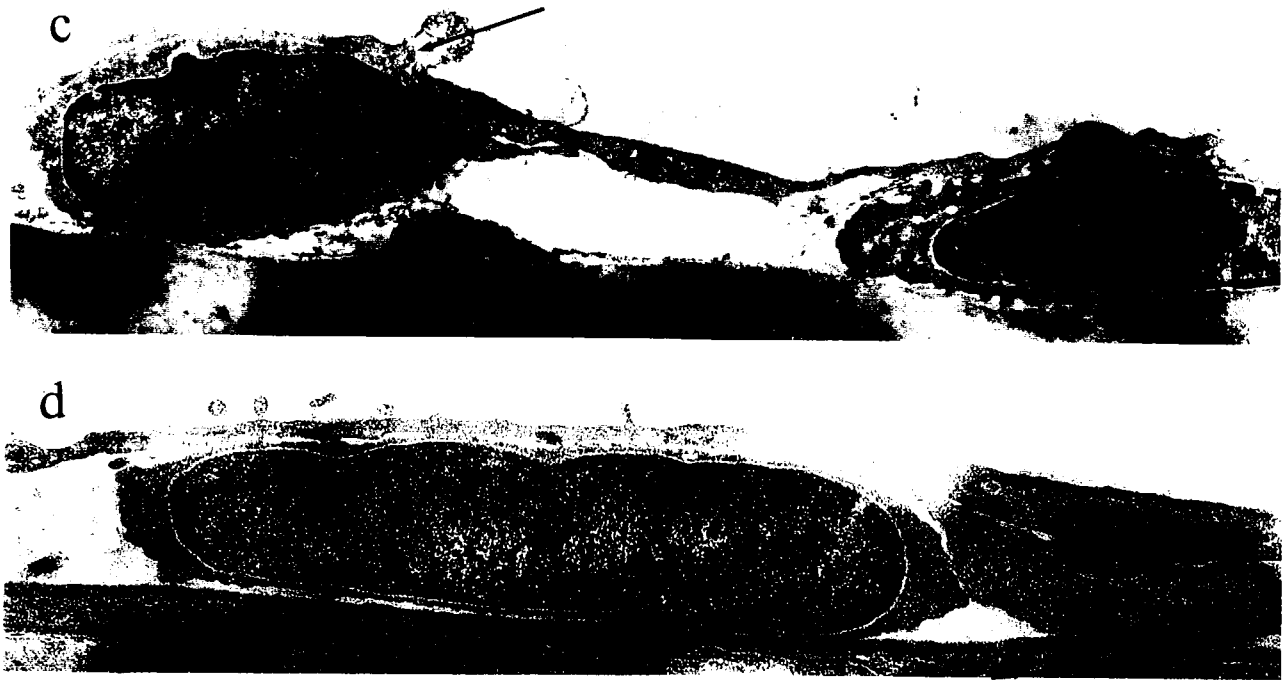
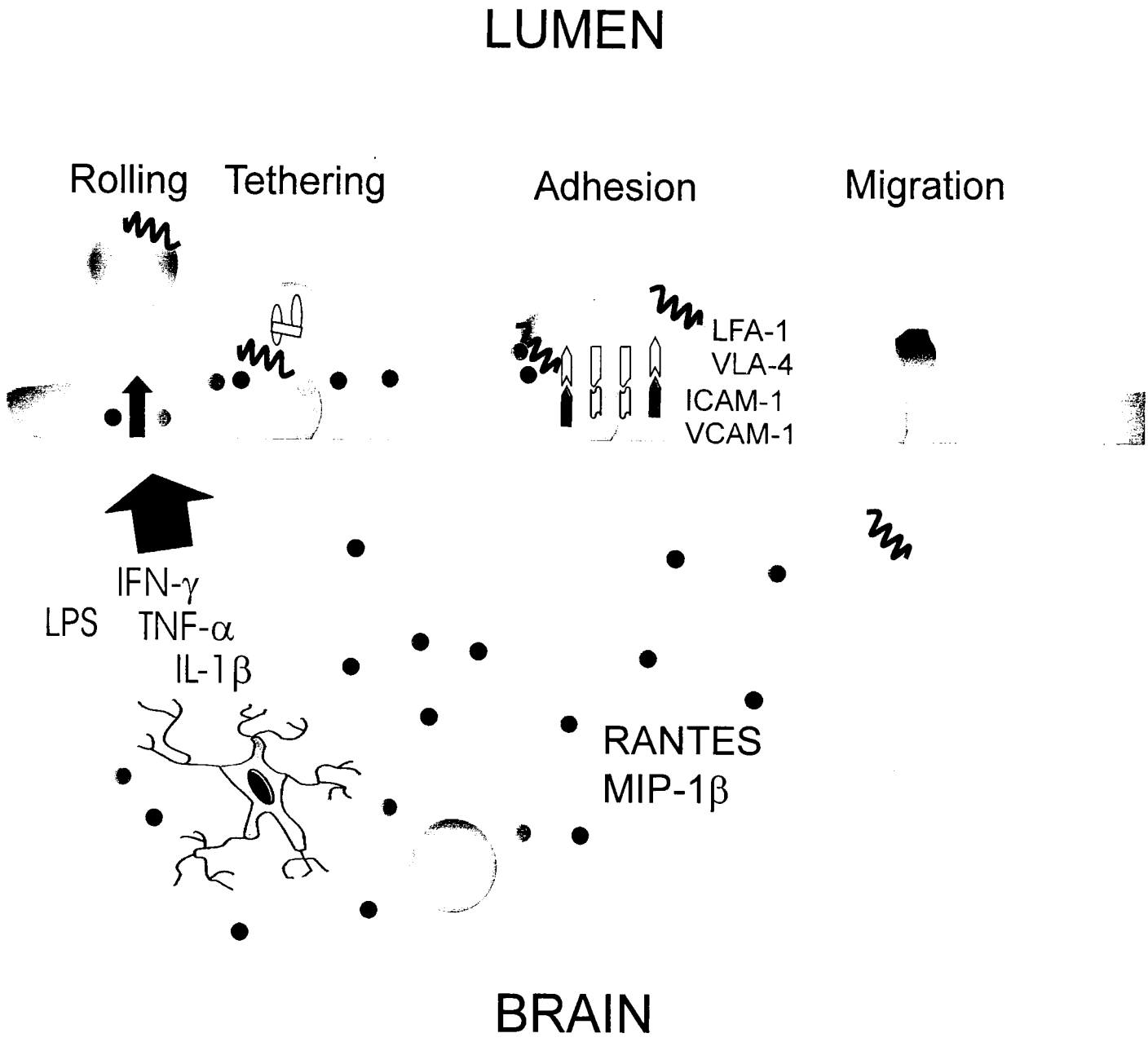


Fig. 48 Proposed hypothesis for lymphocyte/chemokine interactions at the BBB. Following initial insult or antigen recognition by T cells in the CNS, a variety of cytokines are produced by microglia, astrocytes, as well as infiltrating leukocytes, which serve to activate the BBB EC. This enhances cell adhesion molecule expression, chemokine expression, and increases permeability. At the EC surface, T cells roll along, independent of their activation status. Once EC have been activated, chemokines are released and presented to T cells by glycosaminoglycans at the cell surface. Signaling through the chemokine receptor on the T cell results in increased affinity of integrins for their ligands on the endothelium, leading to firm adhesion. Lymphocytes then migrate across the BBB, following the haptotactic gradient established by the chemokines RANTES and MIP-1β. Thus, chemokines appear to play a significant role in mediating the adhesion and migration of distinct lymphocyte subsets across the BBB during CNS inflammation through their role as both activators and chemoattractants.

Fig.48 Proposed hypothesis for lymphocyte/chemokine interactions at the blood-brain barrier.



REFERENCES

Adams DH, Harvath L, Bottaro DP, Interrante R, Catalano G, Tanaka Y, Strain A, Hubscher S and Shaw S. Hepatocyte growth factor and macrophage inflammatory protein 1β: Structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. Proc. Natl. Acad. Sci USA 91:7144-7148, 1994.

Adams DH, Hubscher S, Fear J, Johnston J, Shaw S and Afford S. Hepatic expression of macrophage inflammatory protein-1α and macrophage inflammatory protein-1β after liver transplantation. Transplantation 61:817-825, 1996.

Afford SC, Fisher NC, Neil DAH, Fear J, Brun P, Hubscher SG and Adams DH. Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. J. Pathol. 186:82-89, 1998.

Akira S, Hirano T, Taga T and Kishimoto T. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J. 4:2860-7, 1990.

Akiyama H. Inflammatory response in Alzheimer's disease. Tohoku J. Exp. Med. 174:295-303, 1994.

Albayrak S, Zhao Q, Siesjo BK and Smith M-L. Effect of transient focal ischemia on blood-brain barrier permeability in the rat: correlation to cell injury. Acta Neuropathol. 94:158-163, 1997.

Allouche M, Sahruoui Y, Augerty-Bourget Y, Perrakis M, Jasmin C and Georgoulias V. Interleukin-2 receptors. Leukemia Res. 14:699-703, 1990.

Archelos JJ and Hartung H. The role of adhesion molecules in multiple sclerosis: biology, pathogenesis and therapeutic implications. Mol. Med. Today 10:310-321, 1997.

Asensio VC, Campbell B. Chemokine gene expression in the brains of mice with lymphocytic choriomeningitis. J. Virol. 71:7832-40, 1997.

Bacon KB, Premack BA, Gardner P and Schall TJ. Activation of dual T cell signaling pathways by the chemokine RANTES. Science 269:1727-1730, 1995.

Baggiolini M, Dewald B, and Moser B. IL-8 and related chemotactic cytokines-CXC and CC chemokines. Annu. Immunol. Rev. 55:97-179, 1994.

Balabanov R and Dore-Duffy P. Role of the CNS microvascular pericyte in the blood-brain barrier. J. Neurosci. Res. 53:637-644, 1998.

Barnes DA, Huston M, Holmes R, Benveniste EN, Yong VW, Scholz P and Perez HD. Induction of RANTES expression by astrocytes and astrocytoma cell lines. J. Neuroimmunol. 71:207-214, 1996.

Baron JL, Madri JA, Ruddle NH, Hasim G and Janeway CA. Surface expression of alpha 4 integrin by CD4+ T cells is required for their entry in to brain parenchyma. J. Exp. Med. 177: 57-68, 1993.

Baron S, Tyring SK, Fleischmann WR Jr, Coppenhaver DH, Niesel DW, Klimpel GR, Stanton GJ and Hughes TK. The interferons. Mechanisms of action and clinical applications. JAMA 266:1375-1383, 1991.

Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rosal D, Greaves DR, Zlotnik A and Schall TJ. A new class of membrane-bound chemokine with a CX_3C motif. Nature 385:640-644, 1997.

Bebo BF and Linthicum DS. Expression of mRNA for 55-kDa and 75-kDa tumor necrosis factor (TNF) receptors in mouse cerebrovascular endothelium: effects of interleukin-1β, interferon-γ and TNF-α on cultured cells. J. Neuroimmunol. 62:161-167, 1995.

Beck J, Rondot P, Catinot L, Falcoff E, Kirchner H and Wietzerbin J. Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: do cytokines trigger off exacerbations? Acta Neurol. Scand. 78:318-323, 1988.

Bell MD and Perry VH. Adhesion molecule expression on murine cerebral endothelium following the injection of a proinflammagen or during acute neuronal degeneration. J. Neurocytol. 24:695-710, 1995.

Bell MD, Taub DD and Perry VH. Overriding the brain's intrinsic resistance to leukocyte recruitment with intraparenchymal injections of recombinant chemokines. Neuroscience 74:283-292, 1996.

Bellamy AS, Calder VL, Feldmann M and Davison AN. The distribution of interleukin-2 receptor bearing lymphocytes in multiple sclerosis: evidence for a key role of activated lymphocytes. Clin. Exp. Immunol. 61:248-256, 1985.

Benveniste EN, Sparacio SM and Bethea JR. Tumor necrosis factor-alpha enhances interferon-gamma mediated class II antigen expression on astrocytes. J. Neuroimmunol. 25:209-219, 1989.

Benveniste EM. Human cytokines: Their role in research and therapy. Aggarawal B and Puri R, Eds. Blackwell Scientific Publications. 195-216, 1995.

Benveniste E. Cytokine expression in the nervous system. In: Immunology of the nervous system. Ed. RW Keane, WF Hickey. Oxford Press, New York, 419-459, 1997.

Berman JW, Guida MP, Warren J, Amat J and Brosnan CF. Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in rat. J. Immunol. 156:3017-3023, 1996.

Beuckmann C, Hellwig S and Galla H-J. Induction of the blood/brain barrier-associated enzyme alkaline phosphatase in endothelial cells from cerebral capillaries is mediated via cAMP. Eur. J. Biochem. 229:641-644, 1995.

Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YC, Mathison J, Ulevitch R, Cerami A. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature 316:552-4, 1985.

Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu. Rev. Immunol. 11:767-804, 1993.

Billiau A. Interferon-γ in autoimmunity. Cytokine Growth Factor Rev. 7:25-34, 1996.

Bjorklund A and Stenevi U. Intracerebral neural grafting: a historical perspective. In: Neural grafting in the Mammalian CNS. Eds. A Bjorklun, U Stenevi. Elsevier, Amsterdam, 3-14, 1985.

Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood. 86:1649-1660, 1995.

Bolin LM, Murray R, Lukacs NW, Strieter RM, Kunkel SL, Schall TJ and Bacon KB. Primary sensory neurons migrate in response to the chemokine RANTES. J. Neuroimmunol. 81:49-57, 1998.

Bonate PL. Animal models for studying transport across the blood-brain barrier. J. Neurosci. Meth. 56:1-15, 1995.

Borthwick NJ, Akbar AN, MacCormac LP, Lowdell M, Craigen JL, Hassan L, Grundy JE, Salmon M and Yong KL. Selective migration of highly differentiated primed T cells, defined by low expression of CD45RB, across human umbilical vein endothelial cells: effects of viral infection on transmigration. Immunology 90:272-280, 1997.

Bostik P, Villinger F, Brice GT, Chikkala NF, Brar SS, Cruikshank WW, Adams JW, Hillyer CD and Ansar AA. Expression and in vitro evaluation of rhesus macaque wild type and modified CC chemokines. J. Med. Primatol. 27:113-120, 1998.

Bradbury MWB and Cserr HF. Drainage of cerebral interstitial fluid and of cerebrospinal fluid into lymphatics. In: Experimental Biology of the Lymphatic Circulation. Ed. MG Johnston. Elsevier, Amsterdam, 355-394, 1985.

Bradbury MWB. The blood-brain barrier. Exp. Physiol. 78:453-472, 1993.

Brandtzaeg P, Kierulf P, Gaustad P, Skulberg A, Bruun JN, Halvorsen S and Sorensen E. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J. Infect. Dis. 159:195-204, 1989.

Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G and Stern D. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J. Exp. Med. 169:1977-1991, 1989.

Brightman MW and Reese TS. Junctions between intimately apposed cell membranes in the vertebrate brain. J. Cell Biol. 40:648-677, 1969

Brightman MW, Hori M, Rapoport SI, Reese TS and Westergard E. Osmotic opening of tight junctions in cerebral endothelium. J. Comp. Neurol. 152:317-325, 1973.

Budd RC, Cerottini JC and MacDonald HR. Phenotypic identification of memory cytolytic T lymphocytes in a subset of Lyt-2+ cells. J. Immunol. 138:1009-1013, 1987.

Burke-Gaffney A and Keenan AK. Modulation of human endothelial cell permeability by combinations of the cytokines interleukin-1 α/β, tumor necrosis factor-α and interferon-γ. Immunopharmacology 25:1-9, 1993.

Butt AM. Effect of inflammatory agents on electrical resistance across the blood-brain barrier in pial microvessels of anaesthetized rats. Brain Res. 696:145-150, 1995.

Cabirac GF, Murray RS, McLaughlin LB, Skolnick DM, Hogue B, Dorovini-Zis and Didier PJ. *In vitro* interaction of coronaviruses with primate and human brain microvascular endothelial cells. Corona and Related Viruses. Eds. PJ Talbot and GA Levy. Plenum Press, New York, 1995.

Calvo CF, Yoshimura T, Gelman M and Mallat M. Production of monocyte chemotactic protein-1 by rat brain macrophages. Eur. J. Neurosci. 8:1725-34, 1996.

Campanero MR, del Pozo MA, Arroyoo AG. ICAM-3 regulates lymphocyte morphology and integrin-mediated T cell interaction with endothelial cell and extracellular matrix ligands. J. Cell Biol. 123:1007-1017, 1993.

Cannella B, Cross AH and Raine CS. Upregulation and co-expression of adhesion molecules correlate with relapsing autoimmune demyelination in the central nervous system. J. Exp. Med. 172:1521-1524, 1990.

Cannella B and Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. Ann. Neurol. 37:424-434, 1995.

Chandler S, Miller KM, Clements JM. Lury J, Corkill D, Anthony DCC, Adams SE and Gearing AJH. Matrix methalloproteinases, tumor necrosis factor and multiple sclerosis: an overview. J. Neuroimmunol. 72:155-161, 1997.

Chopp W, Zhang RL, Chen H, Li Y, Jiang N and Rusche JR. Postischemic administration of an anti-MAC-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in rats. Stroke 25:869-875, 1994.

Chuluyan HE, Schall TJ, Toshimura T, Issekutz AC. IL-1 activation of endothelium supports VLA-4 (CD49d/CD29)-mediated monocyte transendothelial migration to C5a, MIP-1 alpha, RANTES, and PAF but inhibits migration to MCP-1: a regulatory role for endothelium-derived MCP-1. J. Leukocyte Biol. 58:71-79, 1995.

Clark WM, Madden KP, Rothelin R and Zivin JA. Reduction of central nervous system ischemic injury by monoconal antibody to intercellular adhesion molecule. J. Neurosurg. 75:623-627, 1991.

Claudio L, Raine CS and Brosnan CF. Evidence of persistent blood-brain barrier abnormalities in chronic-progressive multiple sclerosis. Acta Neuropathol. 90:228-238, 1995.

Cossins JA, Clements JM, Ford J, Miller KM, Pigott R, Vos W. Van der Valk P and De Groot CJA. Enhanced expression of MMP-7 and MMP-9 in demyelinating multiple sclerosis lesions. Acta Neuropathol. 94:590-598, 1997.

Cotran RS. New roles for the endothelium in inflammation and immunity. American Association of Pathologists president's address. Am. J. Path. 129:401-413, 1987.

Covell DG, Smythers GW, Gronenbron AM and Clore GM. Analysis of hydrophobicity in the α and β chemokine families and its relevance to dimerization. Protein Sci. 3:2064-2072, 1994.

Crone C and Olesen SP. Electrical resistance of brain microvascular endothelium. Brain Res. 241:49-55, 1982.

Cross AH, Cannella B, Brosnan CF and Raine CS. Homing to central nervous system vasculature by antigen specific lymphocytes. I. Localization of ^{14}C-labeled cells during acute, chronic and relapsing experimental allergic encephalomyelitis. Lab. Invest. 63:162-170, 1990.

Damle NK, Aruffo A. VCAM-1 induces T cell antigen receptor dependent activation of human CD4+ T lymphocytes. Proc. Natl. Acad. Sci. USA 88:6403-6407, 1991.

Damle NK, Klussman K, Aruffo A. ICAM-2, a second ligand for CD11a/CD18 (LFA-1), provides a co-stimulatory signal for T cell receptor initiated activation of human T cells. J. Immunol. 148:665-671, 1992.

Defazio G, Ribatti BN, Ricchiuti F, De Salvia R, Roncali L and Livrea P. Endocytosis of horseradish peroxidase by brain microvascular and umbilical vein endothelial cells in culture: an ultrastructural and morphometric study. Brain Res. Bull. 43:467-472, 1997.

Deli MA and Joo F. Cultured vascular endothelial cells of the brain. Keio J. Med. 45:183-199, 1996.

del Pozo MA, Sanchez-Mateos P, Nieto M and Sanchez-Madrid F. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. J. Cell Biol. 131:495-508, 1995.

del Pozo MA, Sanchez-Mateos P and Sanchez-Madrid F. Cellular polarization induced by chemokines: a mechanism for leukocyte recruitment? Immunol. Today 17:127-135, 1996.

del Pozo MA, Cabanas C, Montoya MC, Ager A, Sanchez-Mateos P and Sanchez-Madrid F. ICAMs redistributed by chemokines to cellular uropods as a mechanism for recruitment of T lymphocytes. J. Cell Biol. 137:493-508, 1997.

Del Prete G. The concept of type-1 and type-2 helper T cells and their cytokines in humans. Int. Rev. Immunol. 16:427-55, 1998.

Desbaillets I, Sisereens A-C, de Tribolet N, Hamou M-F and Van Meir EG. Upregulation of Interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis. J. Exp. Med. 186:1201-1212, 1997.

Devergne O, Marfaing-Koka A, Schall TJ, Leger-Ravet M-B, Sadick M, Peuchmaur M, Crevon M-C, Kim T, Glanaud P and Emilie D. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J. Exp. Med. 179:1689-1694, 1994.

Diamond MS and Springer TA. The dynamic regulation of integrin adhesiveness. Curr. Biol. 4:506-517, 1994.

Dinarello CA. Interleukin-1. Cytokine Growth Factor Rev. 8:253-265, 1997.

Dorovini-Zis K, Sato M, Goping G, Rapoport S and Brightman M. Ionic lanthanum passage across cerebral endothelium exposed to hyperosmotic arabinose. Acta Neuropathol. 60:49-60, 1983.

Dorovini-Zis K, Bowman PD, Betz AL and Goldstein GW. Hyperosmotic arabinose solutions open the tight junctions between brain capillary endothelial cells in tissue culture. Brain Res. 302:383-386, 1984.

Dorovini-Zis K, Bowman PD, Betz AL, Goldstein GW. Hyperosmotic urea reversibly opens the tight junctions between brain capillary endothelial cells in cell culture. J. Neuropath. Exp. Neurol. 46:130-147, 1987.

Dorovini-Zis K, Prameya R and Bowman PD. Culture and characterization of microvascular endothelial cells derived from human brain. Lab. Invest. 64:425-435, 1991.

Dorovini-Zis K, Wong D and Prameya R. Transendothelial migration of leukocytes and tumor necrosis factor alter the permeability of human brain microvessel endothelial cell monolayers. J. Neuropath. Exp. Neurol. 54:440, 1995.

dos Santos WLC, Tahman J, Klein N and Male DK. Control of lymphocyte adhesion to brain and aortic endothelium: ICAM-1, VCAM-1 and negative charge. J. Neuroimmunol. 66:125-134, 1996.

Doukas J and Pober JS. IFN-γ enhances endothelial activation induced by tumor necrosis factor but not IL-1. J. Immunol. 145:1727-1733, 1990.

Dustin ML and Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. 107:321-331, 1988.

Ehrlich P. Das Sauerstoff-Bedürfnis des Organismus: eine farbenanalytische Studie. Hirschwald, Berlin. 1885.

Elenius K, Vainio S, Laato M, Salmivirta M, Thesleff I, and Jalkanen M. Induced expression of syndecan in healing wounds. J. Cell Biol. 114:585-595,1991.

Engelhardt B, Conley FK, Kilshaw PJ and Butcher EC. Lymphocytes infiltrating the CNS during inflammation display a distinctive phenotype and bind to VCAM-1 but not to MadCAM-1. Int. Immunol. 7:481-491, 1995.

Fabry Z, Sandor M, Gajewski TF, Herlein JA, Waldschmidt MM, Lynch RG and Hart MN. Differential activation of Th1 and Th2 CD4+ cells by murine brain microvessel endothelial cells and smooth muscle/pericytes. J. Immunol. 151:38-47,1993a.

Fabry Z, Fitzsimmons KM, Herlein JA, Moninger TO, Dobbs MB, and Hart MN. Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes. J. Neuroimmunol. 47:23-34, 1993b.

Fabry Z, Raine CS and Hart MN. Nervous tissue as an immune compartment: the dialect of the immune response in the CNS. Immunol. Today 15:218-224, 1994.

Fabry Z, Tophan DJ, Fee D, Herlein J, Carlino JA, Hart MN and Sriram S. TGF-β2 decreases migration of lymphocytes *in vitro* and homing of cells into the central nervous system *in vivo*. J. Immunol. 155:325-332, 1995.

Fawcett J, Hoiness CLL, Needham LA, Turley H, Gatter IC, Mason DY and Simmon DL. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature 360:481-484, 1992.

Feinman R, Henriksen-DeStefano M, Tsujimoto M and Vilcek J. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. J. Immunol. 138:635-640, 1987.

Fiala M, Zhang L, Gan X, Sherry B, Taub D, Graves MC, Hama S, Way D, Weinand M, Witte M, Lorton D, Kuo Y-M and Roher AE. Amyloid-β induces chemokine secretion and monocyte migration across a human blood-brain barrier model. Mol. Med. 4:480-489, 1998.

Fisher SN, Vanguri P, Shin HS and Shin ML. Regulatory mechanisms of MuRantes and CRG-2 chemokine gene induction in central nervous system glial cells by virus. Brain Behav. Immun. 9:331-344, 1995.

Frigerio S, Gelati M, Cuisani E, Corsini E, DufourA, Massa G and Salmaggi A. Immunocompetence of human microvascular brain endothelial cells: cytokine regulation of IL-1β, MCP-1, IL-10, sICAM-1 and sVCAM-1. J. Neurol. 245:727-730, 1998.

Frohman EM, Frohman TC, Dustin ML, Vayuvegula B, Choi B, Gupta A, van den Noort S and Gupta S. The induction of intercellular adhesion molecule 1 (ICAM-1) expression on human fetal astrocytes by interferon-gamma, tumor necrosis factor alpha, lymphotoxin and interleukin-1: relevance to intracerebral antigen presentation. J. Neuroimmunol. 23:117-124, 1989.

Furie MB, Cramer EB, Naprstek BL and Silverstein SC. Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. J. Cell Biol. 98:1033-1041, 1984.

Gause WC, Mitro V, Via C, Linsley P, Urban JF and Greenwald RJ. Do effector and memory T helper cells also need B7 ligand costimulatory signals? J. Immunol. 159:1055-1058, 1997.

Gearing AJH. Processing of tumour necrosis factor alpha precursor by metalloproteinases. Nature 370:555-557, 1994.

Gehrmann J, Bonnekoh P, Miyazawa T, Hossmann KA and Kreutzberg GW. Immunocytochemical study of an early microglial activation in ischemia. J. Cerebr. Blood Flow Metab. 12:257-269, 1992.

Gehrmann J, Banati RB, Wiessner C, Hossmann K-A and Kreutzberg GW. Reactive microglia in cerebral ischemia: an early mediator of tissue damage? Neuropath. Appl. Neuro. 21:277-289, 1995.

Gendelman HE, Lipton SA, Tardieu M, Bukrinsky MI and Nottet HS. The neuropathogenesis of HIV-1 infection. J. Leukocyte Biol. 56:389-398, 1994.

Ghirnikar RS, Lee YL, He TR and Eng LF. Chemokine expression in rat stab wound brain injury. J. Neurosci. Res. 46:727-33, 1996.

Gilat D, Hershkoviz R, Mekori YA, Vlodabsky I and Lider O. Regulation of adhesion of CD4+ T lymphocytes to intact or heparinase-treated subendothelial extracellular matrix by diffusible of anchored RANTES and MIP-1β. J. Immunol. 153:4899-4906, 1994.

Giulian D and Lachman B. Interleukin-1 stimulation of astroglial proliferation after brain injury. Science. 228:497-499, 1985.

Giulian D, Woodward J, Young DG, Krebs JF and Lachmann LB. Interleukin-1 injected into mammalian brain stimulated astrogliosis and neovascularization. J. Neurosci. 8:2488-2490, 1988.

Glabinski AR, Tani M, Tuohy VK, Tuthill RJ and Ransohoff RM. Central nervous system chemokine mRNA accumulation follows initial leukocyte entry at the onset of acute murine experimental autoimmune encephalomyelitis. Brain, Behavior, Immun. 9:315-330, 1995.

Glabinski AR, Balasingam V, Tani M, Kunkel SL, Strieter RM, Yong VW and Ransohoff RM. Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain J. Immunol. 156:4363-8, 1996.

Goebeler M, Yoshimura T, Toksoy A, Ritter U, Bröcker E-B, and Gillitzer R. The chemokine repertoire of human dermal microvascular endothelial cells and its regulation by inflammatory cytokines. J. Invest. Dermatol. 108:445-451, 1997.

Goetzl EG, Banda MH and Leppert D. Matrix metalloproteinases in immunity. J. Immunol. 156:1-4, 1996.

Gordon EL, Danielsson PE, Nguyen R-S and Winn HR. A comparison of primary cultures of rat cerebral microvascular endothelial cells to rat aortic endothelial cells. In Vitro Cell. Dev. Biol. 27A:312-326,1991.

Gordon EJ, Myers KJ, Dougherty JP, Rosen H and Ron Y. Both anti-CD11a (LFA-1) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 62:153-160, 1995

Gourmala NG, Buttini M, Limonta S, Sauter A and Boddeke HW. Differential and time-dependent expression of monocyte chemottractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration. J. Neuroimmunol. 74:35-44, 1997.

Graber N, Venkat Gopal T, Wilson D, Beall LD, Polte T and Newman W. T cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotein and endothelial leukocyte adhesion molecule-1. J. Immunol. 145:819-819-830, 1990

Greenwood J and Calder VL. Lymphocyte migration through cultured endothelial cell monolayers derived from the blood-retinal barrier. Immunology 80:401-406, 1993.

Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, and Stadel JM. Chemokine receptors in human endothelial cells. J. Biol. Chem. 273:4282-4287, 1998.

Hannet I, Erkeller-Yuksel F, Lydyard P, Deneezs V and DeBruyere M. Developmental and maturational changes in human blood lymphocyte subpopulations. Immunol. Today 13:215-218, 1992.

Hauser SL, Bhan AK, Gilles FH, Hoban CJ, Reinherz EL, Schlossman SF and Weiner HL. Immunohistochemical staining of human brain with monoclonal antibodies that identify lymphocyte, monocytes, and the Ia antigen. J. Neuroimmunol. 5:197-205, 1983.

Hauser SL, Bhan AK, Gilles FF, Kemp M, Kerr C and Weiner HL. Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. Ann. Neurol. 19:578-587, 1986.

Hausmann EHS, Berman NEJ, Wang Y-Y, Meara JB, Wood GW and Klein RM. Selective chemokine mRNA expression following brain injury. Brain Res. 788:49-59, 1998.

He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J and Gabuzda D. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature 385:645-649, 1997.

Heller RA and Kronke M. Tumor necrosis factor receptor-mediated signaling pathways. J. Cell Biol. 126:5-9, 1994.

Hernandez-Casselles T, Rubio G, Campanero MR, del Pozo MA, Muro M, Sanchez-Madrid F and Aparicio P. ICAM-3, the third LFA-1 counter-receptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. Eur. J. Immunol. 23:2799-2806, 1993.

Hess DC, Bhutwala T, Sheppard JC, Zhao W and Smith J. ICAM-1 expression on human brain microvascular endothelial cells. Neurosci. Lett. 168:201-204, 1994.

Hesselgesser J, Halks-Miller M, DelVecchio V, Peiper SC, Hoxie J, Kolson DL, Taub D and Horuk R. CD4-independent association between HIV-1 gp120 and CXCR4:functional chemokine receptors are expressed in human neurons. Curr. Biol. 7:112-121, 1997.

Hickey WF and Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen *in vivo*. Science 239:290-292, 1988.

Hickey WF, Hsu, BL and Kimura H. T lymphocyte entry into the central nervous system. J. Neurosci. Res. 28:254-260, 1991.

Hinrichs DJ, Wgmann KW and Dietsch GN. Transfer of experimental allergic encephalomyelitis to bone marrow chimeras. Endothelial cells are not a restricting element. J. Exp. Med. 166:1906-1917, 1987.

Hofman FM, Hinton DR, Baemayr J, Weil M and Merrill JE. Lymphokines and immunoregulatory molecules in subacute sclerosing panencephalitis. Clin. Immunol. Immunopathol. 58:331-342, 1991.

Hohki G, Terada N, Hamano N, Kitaura M, Nakajima T, Yoshie O, Ikeda T, Kimura S and Konno A. The effects of eotaxin on the surface adhesion molecules of endothelial cells and on eosinophil adhesion to microvascular endothelial cells. Biochem. Biophys. Res. Commun. 241:136-141, 1997.

Holmin S, Mathieson R, Shetye J and Biberfeld P. Intracerebral inflammatory response to experimental brain contusion. Acta Neurochir. 132:110-119, 1995.

Holmin S, Soderlund J, Biberfeld P and Mathiesen T. Intracerebral inflammation after human brain contusion. Neurosurgery 42:291-298, 1998.

Hoogewerf AJ, Kuscher GSV, Proudfoot AEI, Borlat F, Clark-Lewis I, Power CA and Wells TNF. Glycosaminoglycans mediate cell surface oligomerization of chemokines. Biochemistry 36:13570-13578, 1997.

Hopkins SJ and Rothwell NJ. Cytokines and the nervous system I: expression and recognition. Trends Neurosci. 18:83-88, 1995.

Horuk R, Martin A, Hesselgesser J, Hadley T, Lu A, Wang Z and Peiper SC. The Duffy antigen receptor for chemokines: structural analysis and expression in the brain. J. Leukocyte Biol. 59:29-38, 1996.

Horuk R, Martin AW, Wang Z, Schweitzer L, Gerassimides A, Guo H, Lu Z, Hesselgesser J, Perez HD, Kim J, Parker J, Hadley TJ and Peiper SC. Expression of chemokine receptors by subsets of neurons in the central nervous system. J. Immunol. 158:2882-2890, 1997.

Hsieh CS, Heimberger AB, Gold JS, O'Garra A and Murphy KM. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. Proc. Natl. Acad. Sci. 89:6065-6069, 1992.

Huang K, Geoffrey JS, Singer MS, and Rosen SD. A lymphocyte homing receptor (L-selectin) mediates the *in vitro* attachment of lymphocytes to myelinated tracts of the central nervous system. J. Clin. Invest. 88:1778-1783, 1991.

Hub E and Rot A. Binding of RANTES, MCP-1, MCP-3 and MIP-1α to cells in human skin. Am. J. Pathol. 152:749-757, 1998.

Hughes CCW, Male DK and Lantos PL. Adhesion of lymphocytes to cerebral microvascular cells: effects of interferon-γ, tumor necrosis factor and interleukin-1. Immunology 64:677-681, 1988.

Huynh H, Oger J and Dorovini-Zis K. Interferon-β downregulated interferon-γ-induced class II MHC molecule expression and morphological changes in primary cultures of human brain microvessel endothelial cells. J. Neuroimmunol. 60:63-72, 1995.

Huynh H and Dorovini-Zis K. Effects of interferon-gamma on primary cultures of human brain microvessel endothelial cells. Am. J. Pathol. 142: 1265-1278, 1993.

Hvas J, McLean C, Justesen J, Kannourkakis G, Steinman L, Oksenberg JR and Bernard CCA. Perivascular T cells express the pro-inflammatory chemokine RANTES mRNA in multiple sclerosis lesions. Scand. J. Immunol. 46:195-203, 1997.

Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M Kakizaki M, Takagi S, Moniyama H, Schall TJ and Yoshie O. Identification and molecular characterization of fractalkine receptor. Cell 91:521-530, 1997.

Imamura K, Spriggs D and Kufe D. Expression of tumor necrosis factor receptors on human monocytes and internalization of receptor bound ligand. J. Immunol. 139:2989-2992, 1987.

Ishizuka K, Igata-YR, Kimura T, Hieshima K, Kukita T, Kin Y, Misumi Y, Yamamoto M, Nomiyama H, Miura R, Takamatsu J, .Katsuragi T and Miyakawa T. Expression and distribution of CC chemokine macrophage inflammatory protein-1 alpha/LD78 in the human brain. Neuroreport. 8:1215-8, 1997.

Jaffe EA, Nachman RL, Becker CG and Minick CR. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. 52:2745-2756, 1973.

Jensen MA, Arneson BG, Toscas A, Noronha A. Preferential increase of IL-2+ CD4+ T cells and CD45RB- CD4+ T cells in the central nervous system in experimental allergic encephalomyelitis. J. Neuroimmmunol. 38:255-261, 1992.

Jeynes B. Reactions of granular pericytes in a rabbit cerebrovascular ischemia model. Stroke 16:121-5, 1985.

Jiang Y, Salagranca MN, Adhikari S, Xia Y, Feng L, Sonntag MK, deFiebre CM, Pennell NA, Streit WJ and Harrison JK. Chemokine receptor expression in cultured glia and rat experimental allergic encephalomyelitis. J. Neuroimmunol. 86:1-12, 1998.

Jinno A, Shimizu N, Soda Y, Haraguchi Y, Kitamura T and Hoshino H. Identification of the chemokine receptor TER1/CCR8 expressed in brain-derived cells and T cells as a new coreceptor for HIV-1 infection. Biochem. Biophys. Res. Commun. 243(2):497-502, 1998

John M, Hirst SJ, Jose PJ, Robichaud A, Berkaman N, Witt C, Twort CHC, Barnes P and Chung KF. Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines. J. Immunol. 158:1841-1847, 1997.

Johnatty RN, Taub DD, Reeder SP, Turcovski-Corales SM, Cottam DW, Stephenson TJ and Rees RC. Cytokine and chemokine regulation of proMMP-9 and TIMP-1 production by human peripheral blood lymphocytes. J. Immunol. 158:2327-2333, 1997.

Johnson DR and Pober JS. Tumor necrosis factor and immune interferon synergistically increase transcription of HLA class I heavy and light-chain genes in vascular endothelium. Proc. Natl. Acad. Sci. USA 87:5183-5187, 1990.

Johnson RT, Griffin DE, Hirsch RL, Wolinsky JS, Roedenbeck S, Lindo de Soriano I and Vaisberg A. Measles encephalomyelitis-clinical and immunologic studies. N. Engl. J. Med. 310:137-141, 1984.

Kalaria RN. The immunopathology of Alzheimer's disease and some related disorders. Brain Pathol. 3:333-347, 1993.

Kalman B, Alder H and Lublin FD. Characteristics of the T lymphocytes involved in experimental allergic encephalomyelitis. J. Neuroimmunol. 61:107-116, 1995.

Karpus WJ, Lukacs NW, McRae BL, Strieter RM, Kunkel SL and Miller SD. An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of T-cell mediated autoimmune disease, experimental autoimmune encephalomyelitis. J. Immunol. 155:5003-5010, 1995.

Kedl RM and Mescher MF. Qualitative differences between naïve and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response. J. Immunol. 161:674-683, 1998.

Kennedy MK, Clatch RJ, Dal Canto MC, Trotter JL and Miller SD. Monoclonal antibody-induced inhibition of relapsing EAE in SJL/J mice correlates with inhibition of neuroantigen-specific cell-mediated immune responses. J. Neuroimmunol. 16:345-364, 1987.

Keszthelyi E, Karlik S, Hyduk S, Rice GPA, Gordon G, Yednock T and Horner H. Evidence for a prolonged role of $\alpha 4$ integrin throughout active experimental allergic encephalomyelitis. Neurology 47:1053-1059, 1996.

Kilger G, Needham LA, Neirlsen PH, Clements J, Vestweber D and Holzmann B. Differential regulation of alpha 4-integrin-dependent binding to domains 1 and 4 of vascular cell adhesion molecule-1. J. Biol. Chem. 270:5979-5986, 1995.

Kjellen L and Lindahl U. Proteoglycans: structures and intractions. Annu. Rev. Biochem. 60:443-475, 1991.

Klein CL, Bittinger F, Kohler H, Wagner M, Otto M, Hermanns I, Kirkpatrick CJ. Comparative studies on vascular endothelium *in vitro*. Pathobiol. 63:83-92, 1995.

Klinger MHF, Wilhelm D, Bubel S, Sticherling M, Schröder J-M and Kuehnel W. Immunocytochemical localization of the chemokines RANTES and MIP-1α within human platelets and their release during storage. Int. Arch. Allergy Immunol. 107:541-546, 1995.

Kuchroo VK, Martin CA, Greer JM, Ju ST, Sobel RA, Dorf ME. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. J. Immunol. 151:4371-82, 1993.

Kurodo Y and Matsui M. Cerebrospinal fluid interferon-gamma is increased in HTLV-1 associated myelopathy. J. Neuroimmunol. 42:223-226, 1993.

Lahrtz F, Piali L, Spanaus KS, Seebach J and Fontana A. Chemokines and chemotaxis of leukocytes in infectious meningitis. J. Neuroimmunol. 85:33-43, 1998.

Lavi E. Siziira A, Soverberg DH and Weiss SR. Tumor necrosis factor induces expression of MHC class I antigens on mouse. J. Neuroimmunol. 18:245-253, 1988.

Lavi E, Strizki JM, Ulrich AM, Zhang W, Fu L, Wang Q, O'Connor M, Hoxie JA and Gonzalez-Scarano F. CXCR-4 (Fusin), a co-receptor for the Type 1 Human Immunodeficiency Virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. Am. J. Pathol. 151:1035-1042, 1997.

Lavi E, Kolson DL, Ulrich AM, Fu L and Gonzalez-Scarano F. Chemokine receptors in the human brain and their relationship to HIV infection. J. Neurovirol. 4:301-311, 1998.

Lee K-H, Chung K-Y and Koh Y-J. Memory T lymphocytes' adherence to interferon gamma-activated human dermal microvascular endothelial cells via E-selectin. J. Dermatol. Res. 10:166-175, 1995.

Li H, Ireland GW, Farthing PM and Thornhill MH. Epidermal and oral keratinocytes are induced to produce RANTES and IL-8 by cytokine stimulation. J. Invest. Dermatol. 106:661-666, 1996.

Libby P, Ordovas JM, Auger KR, Robbins AH, Birinyi LK and Dinarello CA. Endotoxin and Tumor Necrosis Factor induce Interleukin-1 gene expression in adult human vascular endothelial cells. Am. J. Pathol. 124:179-186, 1986.

Liew FY, Li Y and Millorr S. Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. 145:4306-4310, 1990.

Lloyd AR, Oppenheim JJ, Kelvin DJ and Taub DD. Chemokines regulate T cell adherence to recombinant adhesion molecules and extracellular matrix proteins. J. Immunol. 156:932-938, 1996.

Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M and Moser B. Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. FASEB J. 8:1055-1060, 1994.

Loetscher P, Seitz M, Baggiolini M and Moser B. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. J. Exp. Med. 184:569-577, 1996.

Loetscher P, Uguccioni M, Bordoli L, Baggiolini M and Moser B. CCR5 is characteristic of Th1 lymphocytes. Nature 391:344-345, 1998.

Lou J, Dayer J-M, Grau GE and Burger D. Direct cell/cell contact with stimulated T lymphocytes induces the expression of cell adhesion molecules and cytokines by human brain microvascular endothelial cells. Eur. J. Immunol. 26:3107-3113, 1996.

Lowenthal JS, Ballard DW, Bohnlein E and Greene WC. Tumor necrosis factor alpha induces proteins that bind specifically to kappa B-like elements and regulate interleukin-2 receptor alpha-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA 86:2331-2335, 1989.

Lu C-Z, Jensen MA and Arnason BGW. Interferon-γ and interleukin-4-secreting cells in multiple sclerosis. J. Neuroimmunol. 46:123-128, 1993.

Lucchinetti CF, Bruck W, Rodriguez M and Lassmann H. Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis. Brain Pathol. 6:259-274, 1996.

Mackay CR, Marson WL, Dudler L, Spertini O, Tedder TF and Hein WR. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. Eur. J. Immunol. 22:887-895, 1992.

Mackay CR. Homing of naïve, memory and effector lymphocytes. Curr. Opin. Immunol. 5:423-427, 1993.

Male DK, Pryce G and Huges CC. Antigen presentation in brain: MHC induction on brain endothelium and astrocytes compared. Immunology 60:453-459, 1987.

Male D, Pryce G and Rahman J. Comparison of the immunological properties of rat cerebral and aortic endothelium. J. Neuroimmunol. 30:161-168, 1990.

Male D, Rahman J, Pryce G, Tamatami T and Miyasaka M. Lymphocyte migration into the CNS modelled *in vitro*: roles of LFA-1, ICAM-1 and VLA-4. Immunol. 81:366-372, 1994.

Male D. The blood-brain barrier-no barrier to a determined lymphocyte. New Concepts of a Blood-Brain Barrier. Ed. By J. Greenwood et al., Plenum Press, New York, 311-314, 1995.

Male D, Rahman J, Linke A, Zhao W, Hickey W. An interferon-inducible molecule on brain endothelium which controls lymphocyte adhesion mediated by integrins. Immunology 84:453-460, 1995.

Mantovani A and Dejana E. Cytokines as communication sigals between leukocytes and endothelial cells. Immunol. Today 10:370-375, 1989.

Marfaing-Koka A, Devergne O, Gorgone G, Portier A, Schall TJ, Glanaud P and Emilie D. Regulation of the production of the RANTES chemokines by endothelial cells. J. Immunol. 154:1870-1878, 1995.

Masuyama J, Berman JS, Cruikshank WW, Morimoto C and Center DM. Evidence for recent as well as long term activation of T cells migrating through endothelial cell monolayers *in vitro*. J. Immunol. 148:1367-1374, 1992.

Matsubara T, Furukawa S and Yabuta K. Serum levels of tumor necrosis factor, interleukin 2 receptor and interferon-gamma in Kawasaki disease involved coronary-artery-lesions. Clin. Immunol. Immunopathol. 56:29-36, 1990.

McCarron RM, Wang L, Racke MK, McFarlin DE and Spatz M. Cytokine-regulated adhesion between encephalitogenic T lymphocytes and cerebrovascular endothelial cells. J. Neuroimmunol. 43:23-30, 1993.

McGeer PL, Itagaki S and McGeer EG. Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. Acta Neuropathol. 76:550-557, 1988.McManus CM, Brosnan CF and Berman JW. Cytokine induction of MIP-1α and MIP-1β in human fetal microglia. J. Immunol. 160:1449-1455, 1998.

Medawar PB. Immunity of homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to sub-cutaneous tissue and to the anterior chamber of the eye. Br. J. Exp. Pathol. 29:58-69, 1948.

Merrill JE, Stron SR, Ellison GW and Myers LW. *In vitro* study of mediators of inflammation in multiple sclerosis. J. Clin. Immunol. 9:84-96, 1989.

Meyer J, Mischeck U, Veyhl M, Henzel K and Galla HJ. Blood-brain barrier characteristic enzymatic properties in cultured brain capillary endothelial cells. Brain Res. 514:305-309, 1990.

Miyagishi R. Kikuchi S, Takayama C, Inoue Y and Tashiro K. Identification of cell types producing RANTES, MIP-1α and MIP-1β in rat experimental autoimmune encephalomyelitis by in situ hybridization. J. Neuroimmunol. 77:17-26, 1997.

Miyazato M, Fukuda M and Iwamasa T. Growth characteristics of retinal capillary endothelial cells compared with pulmonary vein endothelial cells in culture. Acta Pathol. Japon. 41:133-142, 1991.

Mo H, Monard S, Pollack H, Ip J, Rochford G, Wu L, Hoxie J, Borkowsky W, Ho DD and Moore JP. Expression patterns of the HIV Type 1 coreceptors CCR5 and CXCR4 on CD4+ T cells and monocytes for cord and adult blood. Aids Res. Human Retrovir. 14:607-617, 1998.

Moriuchi H, Moriuchi M and Fauci AS. Nuclear factor-κB potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J. Immunol. 158:3483-3491, 1997.

Murphy WJ, Taub DD, Anver M, Conlon K, Oppenheim JJ, Kelvin DJ and Longo DL. Human RANTES induces the migration of human T lymphocytes into the peripheral tissues of mice with severe combined immune deficiency. Eur. J. Immunol. 24:1823-1827, 1994.

Nag S, Takahashi JL and Kilty DW. Role of vascular endothelial growth factor in blood-brain barrier breakdown and angiogenesis in brain trauma. J. Neuropath. Exp. Neurol. 56:912-921, 1997.

Nagelkerken. Role of Th1 and Th2 cells in autoimmune demyelinating disease. Braz. J. Med. Biol. Res. 31:55-60, 1998.

Navikas V, He B, Link J, Haglund M, Soderstron M, Fredrikson S, Ljundahl, A, Hojeberg B, Qiao J, Olsson T and Link H. Augmented expression of tumor necrosis factor-α and lymphotoxin in mononuclear cells in multiple sclerosis and optic neuritis. Brain 119:213-223, 1996.

Needham LA, Van Dijk S, Pigott R, Edwards RM, Shepherd M, Hemingway I, Jack L and Clements JM. Activation dependent and independent VLA-4 binding sites on vascular cell adhesion molecule-1. Cell Adh. Commun. 2:87-89, 1994.

Nelson PH, Kim HT, Manning WC, Goralski TJ and Krensky AM. Genomic organization and transcriptional regulation of the RANTES chemokine gene. J. Immunol. 151:2601-2612, 1993.

Neote K, Mak JY, Kolakowski LF and Schall TJ. Functional and biochemical analysis of the cloned duffy antigen: identity with the red blood cell chemokine receptor. Blood 84:44-52, 1994.

Nieto M, Frade JMR, Sancho D, Mellado M, Martinez-A and Sanchez-Madrid F. Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. J. Exp. Med. 186:153-158, 1997.

Nishiyori A, Minami M, Ohtani Y, Takami S, Yamamoto J, Kawaguchi N, Kume T, Akaike A, Satoh M. Localization of fractalkine and CX_3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia? FEBS Lett. 429:167-172, 1998.

O'Garra A, McEvoy LM and Zlotnik A. T-cell subsets: Chemokine receptors guide the way. Curr. Biol. 8:R646-649, 1998.

Ohmori Y, Schrieber RD and Hamilton TA. Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. J. Biol. Chem. 272:14899-14907), 1997.

Olsson T, Diener P, Ljungdahl A, Hojeberg B van der Medie PH and Kristensson K. Facial nerve transection causes expansion of myelin autoreactive T cells in regional lymph nodes and T cell homing to the facial nucleus. Autoimmunity 13:117-126, 1992.

Oppenheimer-Marks N and Ziff M. Migration of lymphocytes through endothelial cell monolayers: augmentation by interferon-γ. Cell. Immunol. 114:307-323, 1988.

Oppenheimer-Marks N, Davis LS and Lipsky PE. Human T lymphocyte adhesion to endothelial cells and transendothelial migration. J. Immunol. 145:140-148, 1990.

Oppenheimer-Marks N, Davis LS, Bogne DT, Ramberg J and Lipsky PE. Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J. Immunol. 147:2913-2921, 1993a.

Oppenheimer-Marks N and Lipsky PE. The adhesion and transendothelial migration of human T lymphocyte subsets. Behring. Inst. Mitt. 92:44-50, 1993b.

Page C, Rose M, Yacoub M and Pigott R. Antigenic heterogeneity of vascular endothelium. Am. J. Pathol. 141:673-683, 1992.

Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC, Gearing D. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature. 387:611-7, 1997.

Panitch HS, Hirsch R, Schindler J and Johnson KP. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology 37:1097-1102, 1987.

Pardridge WM and Oldendorf WH. Kinetic analysis of blood-brain barrier transport of amino acids. Biochim. Biophys. Acta. 401:128-136, 1975.

Pardridge WM and Oldendorf WH. Transport of metabolic substrates through the blood-brain barrier. J. Neurochem. 28:5-12, 1977.

Perry VH, Bell MD, Brown HC and Matyszak K. Inflammation in the nervous system. Curr. Biol. 5:636-641, 1995.

Peterson PK, Hu S, Salak-Johnson J, Molitor TW and Chao CC. Differential production of and migratory response to β chemokines by human microglia and astrocytes. J. Infect. Dis. 175:478-481, 1997.

Pietschmann P, Cush JJ, Lipsky PE and Oppenheimer-Marks N. Identification of subsets of human T cells capable of enhanced transendothelial migration. J. Immunol. 149:1170-1178, 1992.

Pitzalis C, Kingsley G, Haskard D, Panayi G. The preferential accumulation of helper-inducer T lymphocytes in inflammatory lesions: evidence for regulation by selective endothelial and homotypic adhesion. Eur. J. Immunol. 18:1397-1404, 1988.

Pober JS and Cotran RS. The role of endothelial cells in inflammation. Transplantation 50:537-44, 1990.

Pober JS, Gimbrone MA Jr, Cotran RS, Reiss CS, Burakoff SJ, Fiers W and Ault KA. Ia expression by vascular endothelium is inducible by activated T cells and by human gamma inteferon. J. Exp. Med. 157:1339-1353, 1983.

Poubelle PE, Grassi J, Pradelles P and Marceau F. Pharmacological modulation of interleukin 1 production by cultured endothelial cells from human umbilical veins. Immunopharmacology 19:121-130, 1990.

Premack BA and Schall TJ. Chemokine receptors: gateways to inflammation and infection. Nature Med. 2:1174-1178, 1996.

Pryce G, Male D, Campbell I and Greenwood J. Factors controlling T-cell migration across rat cerebral endothelium *in vitro*. J. Neuroimmunol. 75:84-94, 1997.

Raine C. Demyelinating diseases. In Textbook of Neuropathology. Davis RL and Robertson DM [Eds.] Williamand Wilkins, Baltimore. 2nd edition, 1991.

Ransohoff RM. Chemokines in neurological disease models: correlation between chemokine expression patterns and inflammatory pathology. J. Leuk. Biol. 62:645-652, 1997.

Reese TS and Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J. Cell Biol. 34:207-217, 1967.

Rogers J. Inflammation as a pathogenic mechanism in Alzheimer's disease. Arzneim.-Forsch./Drug Res. 45:439-442, 1995.

Romanic AM and Madri JA. The induction of 72-kDa gelatinase in T cells upon adhesion to endothelial cells is VCAM-1 dependent. J. Cell Biol. 125:1165-1178, 1994.

Rot A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. Immunol. Today 13:291-294, 1992.

Roth SJ, Woldemar Carr M and Springer TA. C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-γ inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. Eur. J. Immunol. 25:3482-3488, 1995.

Roth SJ, Diacovo TH, Brenner MB, Rosat J-P, Buccola J, Morita CT and Springer TA. Transendothelial chemotaxis of human α/β and γ/δ T lymphocytes to chemokines. Eur. J. Immunol. 28:104-113, 1998.

Rothlein R, Dustin ML, Marlin SD and Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J. Immunol. 137:1270-1274, 1986.

Rottman JB, Ganley KP, Williams K, Wu L, Mackay CR and Ringler DJ. Cellular localization of the chemokine receptor CCR5. Am. J. Pathol. 151:1341-1351, 1997.

Rutten MN, Hoover RL and Karnovsky MJ. Electrical resistance and macromolecular permeability of brain endothelial monolayer cultures. Brain Res. 425:301-310, 1987.

Sakai K, Tabira T, Endo M and Steinman L. Ia expression in chronic relapsing experimental allergic encephlomyelitis induced by long-term cultured T cell lines in mice. Lab. Invest. 54:345-362, 1986.

Sallusto F, Lenig D, Mackay DR and Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J. Exp. Med 187:875-883, 1998.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA and Shaw S. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-1, CD2 and LFA-1) and three other molecules (UCHL-1, CDw29 and Pgp-1) and have enhanced IFN-gamma production. J. Immunol. 1401-1407, 1988.

Sasseville VG, Smith MM, Mackay CR, Pauley DR, Mansfield KG, Ringler DJ and Lackner AA. Chemokine expression in Simian Immunodeficiency Virus-induced AIDS encephalitis. Am. J. Pathol. 149:1459-1467, 1996.

Schall TJ, Bacon K, Toy KJ and Goeddel DB. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347:669-671, 1990.

Schall TJ, Bacon K, Camp RDR, Kaspari JW and Goeddel DV. Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes. J. Exp. Med. 177:1821-1825, 1993.

Scheinberg LC, Kotsilimbas DG, Karpf R and Mayer N. Is the brain an "immunologically privileged site"? Arch. Neurol. 15:62-67, 1966.

Schmidtmayerova H, Sherry B and Bukrinsky M. Chemokines and HIV replication. Nature 382:767, 1996.

Schmitt E, Hoehn P, Huels C, Goedert S, Palm N, Rude E and Germann T. T helper type 1 development of naïve CD4+ T cells requires the coordinate action of interleukin-12 and interferon-gamma and is inhibited by transforming growth factor-beta. Eur. J. Immunol. 24:793-798, 1994.

Schobitz B, de Kloet ER and Holsboer F. Gene expression and function of interleukin-1, interleukin-6 and tumor necrosis factor in the brain. Prog. Neurobiol. 44:397-432, 1994.

Seder RA and Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12:635-643, 1994.

Seilhean D, Kogayashi K, He Y, Uchihara T, Rosenblum O, Katlama C, Bricaire F, Duyckaerts C and Hauw J-J. Tumor necrosis factor-α, microglia and astrocytes in AIDS dementia complex. Acta Neuropathol. 93:508-517, 1997.

Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. J. Clin. Invest. 87:949-954, 1991.

Serrador JM Alonso-Lebrero JL, del Pozo MA, Furthmayr H, Schwartz-Albiez R, Calvo J, Lozano F and Sanchez-Madrid F. Moesin interacts with the cytoplasmic region of intercellular adhesion molecule-3 and is redistributed to the uropod of T lymphocytes during cell polarization. J. Cell Biol. 138:1409-1423, 1997.

Sharief MK and Hentges R. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. N. Eng. J. Med. 325:467-472, 1991.

Sharief MK, McLean B and Thompson EJ. Elevated serum levels of tumor necrosis factor-alpha in Guillain-Barre sundrome. Ann. Neurol. 33:591-596, 1993.

Shimizu Y, Van Seventer FA, Horan KJ and Shaw S. Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature 345:250-253, 1990.

Shimizu Y, Newman W, Tanaka Y and Shaw S. Lymphocyte interactions with endothelial cells. Immunol. Today 13:106-112, 1992.

Simpson JE, Newcombe J, Cuzner M and Woodroofe MN. Expression of monocyte chemoattractant protein-1 and other β-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. J. Neuroimmunol. 84:238-249, 1998.

Slowik MR, DeLuca LG, Fiers W and Pober JS. Tumor necrosis factor activates human endothelial cells through the p55 tumor necrosis factor receptor but the p75 receptor contributes to activation at low tumor necrosis factor concentration. Am. J. Pathol. 143:1724-1730, 1993.

Spanaus KS, Nadal D, Pfister HW, Seebach J, Widmer U, Frei K, Gloor S and Fontana A. C-X-C and C-C chemokines are expressed in the cerebrospinal fluid in bacterial meningitis and mediate chemotactic activity on peripheral blood-derived polymorphonuclear and mononuclear cells in vitro. J. Immunol. 158:1956-1964, 1997.

Staunton DE, Dustin ML and Springer TA. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature 339:61-64, 1989.

Stellato C, Beck LA, Gorgone GZ, Proud D, Schall TJ, Ono SJ, Lichtenstein LM and Schleimer RP. Expression of the chemokine RANTES by a human bronchial epithelial cell line. J. Immunol. 155:410-418, 1995.

Stins MF, Gilles F and Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J. Neuroimmunol. 76:81-90, 1997.

Stolpen AH, Guinan EC, Fiers W and Pober JS. Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. Am. J. Pathol. 123:16-24, 1986.

Struyf S, De Meester I, Scharpe S, Lenaerts JP, Menten P, Wang J, Proost P and Van Damme J. Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. Eur. J. Immunol. 28:1262-1271, 1998.

Sun D, Lytle C and O'Donnell ME. Astroglial cell-induced expression of Na-K-Cl cotransporter in brain microvascular endothelial cells. Am. J. Physiol. 38:C1506-1512, 1995.

Szabo MC, Soo KS, Zlotnik A and Schall TJ. Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor. J. Biol. Chem. 270:25348-25351, 1995.

Szabo MC, Butcher EC, McIntyre BW, Schall TJ and Bacon KB. RANTES stimulation of T lymphocyte adhesion and activation: role for LFA-1 and ICAM-3. Eur. J. Immunol. 27:1061-1068, 1997.

Tagami M, Kubota A, Nara Y and Yamori Y. Detailed disease processes of cerebral pericytes and astrocytes in stroke-prone SHR. Clin. Exp. Hypertension. A13:1069-1075, 1991.

Tanabe S, Heesen M, Yoshizawa I, Berman MA, Luo Y, Bleul CC, Springer TA, Okuda K, Gerard N and Dorf ME. Functional expression of the CXC-chemokine receptor-4/fusin on mouse microglial cells and astrocytes. J. Immunol. 159:905-911, 1997.

Tanaka Y, Adams DH and Shaw S. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. Immunol. Today 14:111-115, 1993a.

Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U and Shaw S. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. Nature 361:79-82, 1993b.

Tanaka Y, Kimata K, Wake A, Mine S, Morimoto I, Yamakawa N, Habuchi H, Ashikari S, Yamamoto H, Saurai K, Yoshida K, Suzuki S and Eto S. Heparan sulfate proteoglycan on leukemic cells is primarily involved in integrin triggering and its mediated adhesion to endothelial cells. J. Exp. Med. 184:1987-1997, 1996.

Tanaka Y, Fujii K, Hübscher S, Aso M, Takazawa A, Saito K, Ota T and Eto S. Heparan sulfate proteoglycan on endothelium efficiently induces integrin-mediated T cell adhesion by immobilizing chemokines in patients with rheumatoid synovitis. Arthritis Rheum. 41:1365-1377, 1998.

Taub DD, Conlon K, Lloyd AR, Oppenheim JJ and Kelvin DJ. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1α and MIP-1β. Science 260:355-358, 1993a.

Taub DD, Lloyd AR, Wang J-M, Oppenhein JJ and Kelvin DJ. The effects of human recombinant MIP-1α, MIP-1β and RANTES on the chemotaxis and adhesion of T cell subsets. The Chemokines, Ed. IJD. Lindley et al., Plenum Press, New York 139-146, 1993b.

Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, Matsushima K, Kelvin DJ and Oppenheim JJ. Recombinant human Interferon-inducible Protein 10 is a chemoattractant for

human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J. Exp. Med. 177:1809-1814, 1993c.

Taupin V, Toulmond S, Serrano A, Benavides J and Zavala F. Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. J. Neuroimmunol. 42:177-186, 1993.

Theiler M. Spontaneous encephalomyelitis of mice, a new virus disease. J. Exp. Med. 65:705-719, 1937.

Traugott U, Reinherz EL and Raine CS. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. J. Neuroimmunol. 4:201-221, 1983.

Traugott U, Scheinberg LC and Raine CS. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. J. Neuroimmunol. 8:1-13, 1985.

Traugott U, McFarlin DE and Raine CS. Immunopathology of the lesion in chronic relapsing experimental autoimmune encephalomyelitis in the mouse. Cell. Immunol. 99:395-407, 1986.

Tsukada N, Matsuda M, Miyagi K and Yanagisawa N. Adhesion of cerebral endothelial cells to lymphocytes from patients with multiple sclerosis. Autoimmunity 14:329-333, 1993.

Tsukada N, Matsuda M, Miyahi K and Yanagisawa N. *In vitro* intercellular adhesion molecule-1 expression on brain endothelial cells in multiple sclerosis. J. Neuroimmunol. 49:181-187, 1994.

Vanguri P, Cho SY, Chi CM. Role of muIP-10 in interferon-gamma induction of Ia in rat astrocytes. Mol. Immunol. 33:1079-87, 1996.

VanSeventer GA, Shimizu Y, Hogan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor mediated activation of resting T cells. J. Immunol. 122:4579-4587, 1990.

Verbeek MM, Westphal JR, Ruiter DJ and de Waal RM. T lymphocyte adhesion to human brain pericytes is mediated via very late antigen-4/vascular cell adhesion molecule-1 interactions. J. Immunol. 154:5879-5884, 1995.

Ward SG and Westwick J. Chemokines: understanding their role in T-lymphocyte biology. Biochem. J. 333:457-470, 1998.

Westmoreland SV, Rottman JB, Williams KC, Lackner AA and Sasseville VG. Chemokine receptor expression on resident and inflammatory cells in the brain of macaques with Simian Immunodeficiency Virus encephalitis. Am. J. Pathol. 152:659-665, 1998.

Wight TN. Cell biology of arterial proteoglycans. Arteriosclerosis 9:1-20, 1989

Wilkinson PC and Higgins A. OKT3-activated locomotion of human blood lymphocytes: a phenomenon requiring contact of T cell with Fc receptor-bearing cells. Immunology 60:445-451, 1987.

Witt DP and Lander AD. Differential binding of chemokines to glycosaminoglycan subpopulations. Curr. Biol. 4:394-400, 1994.

Wolburg H, Neuhaus J, Kniesel U, Drauss B, Schmid E-M, Ocalan M, Farrell C and Risau W. Modulation of tight junction structure in blood-brain barrier endothelial cells. J. Cell Sci. 107:1347-1357, 1994.

Wong D and Dorovini-Zis K. Upregulation of intercellular adhesion molecule-1 (ICAM-1) expression in primary cultures of human brain microvessel endothelial cells by cytokines and lipopolysaccharide. J. Neuroimmunol. 39:11-21, 1992.

Wong D. Cell adhesion molecules on brain endothelium. The University of British Columbia, Ph.D. thesis, 1995.

Wong D and Dorovini-Zis K. Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture. Microvasc. Res. 49:325-339, 1995.

Wong D and Dorovini-Zis K. Regulation by cytokines and lipopolysaccharide of E-selectin exprsseion by human brain microvessel endothelial cells in primary culture. J. Neuropath. Exp. Neurol. 55:225-235, 1996a.

Wong D and Dorovini-Zis K. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) expression by human brain microvessel endothelial cells in primary culture. Brain Res. 731:217-220, 1996b.

Wong D, Prameya R and Dorovini-Zis K. *In vitro* adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1. J. Neuropath. Exp. Neurol. 58: In press, 1999.

Woodroofe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ, Tinker A and Cuzner ML. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J. Neuroimmunol. 33:227-236, 1991.

Wysocki J and Issekutz TB. Effect of T cell activation on lymphocyte-endothelial adherence and the role of VLA-4 in the rat. Cell. Immunol. 140:420-431, 1992.

Wysocki J and Wykretowicz. Adherence of blood T cells to rat microvascular endothelial cells. Comparative studies of T lymphocytes isolated from various tissues. Immunol. Invest. 22:53-62, 1993.

Xia M, Leppert D, Hauser SL, Sreedharan SP, Nelson P, Krensky AM and Goetzl EJ. Stimulus specificity of matrix metalloproteinase dependence of human T cell migration through a model basement membrane. J. Immunol. 156:160-167, 1996.

Xia M, Qin S, McNamara M, Mackay C and Hyman BT. Interleukin-8 receptor B immunoreactivity in brain and neuritic plaques of Alzheimer's disease. Am. J. Pathol. 150:1267-1274, 1997.

Yamada Y, Furumichi I, Furui H, Yokoi T, Ito T, Yamauchi K, Yokota M, Hayashi J, Saito H. Roles of calcium, cyclic nucleotides, and protein kinase C in regluation of endothelial permeability. Arteriosclerosis 10:410-420, 1990.

Yasumoto K, Okamoto SI, Mukaida N, Murakami S, Mai M and Matsushima K. Tumor necrosis factor α and interferon-γ synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-κB like binding sites of interleukin 8 gene. J. Biol. Chem. 267:22506-22517, 1992.

Yednock TA, Cannon C, Pritz LC, Sanchez-Madrid F, Steinman L and Karin N. Prevention of experimental aytoimmune encephalomyelitis by antibodies against α4β1 integrin. Nature 356:63-66, 1992.

Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. Annu. Rev. Immunol. 8:579-621, 1990.

Ziegler SF, Touch TW, Franklin TL, Armitage RJ and Alderson MR. Induction of macrophage inflammatory protein-1β gene expression in human monocytes by lipopolysaccharide and IL-7. J. Immunol. 147:2234-2239, 1991.

Ziegler SF, Ramsdell F and Alderson MR. The activation antigen CD69. Stem Cells 12:456-465, 1994.

Zoja C, Donadelli R, Colleoni S, Figliuzzi M, Bonazzola S, Morigi M and Remuzzi G. Protein overload stimulated RANTES production by proximal tubular cells depending on NF-κB activation. Kidney Int. 53:1608-1615, 1998.