INFLAMMATION AND THE BRAIN BARRIERS

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Introduction

The blood-brain barrier (BBB), here defined broadly to include the endothelial, epithelial/ependymal, and tanycytic barriers, is involved in a wide variety of neuroinflammatory processes and most aspects of BBB pathophysiology are affected by neuroinflammation. Similar events likely occur at the blood-retinal, blood-spinal cord, and other specialized barriers. BBB-neuroinflammation interactions are so diverse that this area constitutes one of the unifying concepts in BBB, as exemplified by their involvement in neurodegenerative diseases, CNS injury, neuroendocrine secretions, and drug delivery. Important examples of BBB-neuroinflammation interactions are alterations in BBB permeability (e.g., disruption and dysregulation of transporters, alterations of tight junction architecture), trafficking of immune cells and transport of cytokines across the BBB, secretion of neuroimmune substances by the cells constituting the various BBBs, developmental changes in BBB induced by perinatal inflammatory events, and trafficking of pathogens (viruses, bacteria, parasites) across the BBB. More subtle changes leading to pro-inflammatory interactions with the BBB as observed in Alzheimer's disease also occur. BBB-neuroinflammation interactions are fundamentally involved in a wide variety of diseases; a short sampling of which includes multiple sclerosis, posterior uveitis, CNS vasculitides, stroke, Alzheimer's disease, neuroAIDS, insulin resistance/obesity, and cerebral malaria. The concept of the neurovascular unit (NVU), which emphasizes the continuous cross talk of cellular elements of the BBB with cells within the neuroparenchyma (e.g., neurons, pericytes, microglia, and astrocytes) is easily expanded to include cross talk with cells on the blood side of the BBB (e.g., immune cells, tissue macrophages, dendritic cells); much of this cross talk is mediated by substances with neuroimmune activity (Fig. 1).

Leukocyte Trafficking

Progress:

Since the late 1980's, it has been known that immune cells gain access to the CNS and that immune responses can thus be mounted within the CNS.[1] However, the unique CNS microenvironment strictly controls these immune reactions starting with tightly controlled immune

cell entry into the CNS at the endothelial BBB and, though with different characteristics, at the epithelial, or blood-cerebrospinal fluid (CSF), barrier. Recruitment of circulating immune cells into the CNS depends on the sequential interaction of different adhesion and signaling molecules on the surface of the leukocyte and endothelial cell. Most studies have been performed in experimental autoimmune encephalomyelitis as a model for autoimmune CNS inflammation and have focused on $CD4^+$ T cells.[2] It is now established that the process by which immune cells cross the brain endothelial barrier, termed diapedesis, is transcellular rather than paracellular for some cell types, whereas evidence suggests other cell types may use a paracellular route.[3] The molecular mechanisms involved in diapedesis remain to be investigated. It is clear that leukocyte interactions differ for non-BBB endothelial cells and that large vessel, embryonic, or umbilical endothelial cells cannot be used as substitutes for brain endothelial cells. It is also clear that many of the chemokines on endothelial cells are translocated from the abluminal to luminal surface and probably even from other cells of the NVU by unknown mechanisms.[4]

The type of leukocytes that traverse the blood-CSF barrier and the mechanisms they use are different than at the vascular barrier. For example, terminally committed effector memory cells cross the vascular BBB, while moderately activated central memory cells cross into the CSF.[5] Current in vitro experimental paradigms are insufficient to address questions about the specific molecular mechanisms involved in leukocyte migration across the BBB. The recent development of intravital fluorescence videomicrosopy on the brain and spinal cord, which allows direct visualization of the CNS microcirculation in real time, will aid the study of the molecular mechanisms involved in the multi-step recruitment cascade of immune cells across the healthy and the inflamed BBB in vivo.[6,7] Today, we know that recruitment of CD4+ T cells across the BBB is unique due to the predominant involvement of $\alpha 4$-integrins. This knowledge has been translated into the clinic, where multiple sclerosis patients can now be successfully treated with an anti-$\alpha 4$-integrin antibody Natalizumab (Tysabri). Interdicting leukocyte trafficking across the BBB is now the best-validated treatment for MS.

Unresolved and New Questions:

The molecular mechanisms of brain endothelial cell diapedesis need to be worked out. Better in vitro models based on brain endothelial cells and emphasis on in vivo models is key for this work. Models need to consider and determine the relevance of physiological factors such as blood flow, shear force, and other rheological characteristics on both leukocyte adhesion and diapedesis. The mechanisms by which chemokines are produced and secreted, transported and localized, and taken up at both luminal and abluminal surfaces needs investigation. Of special interest is the abluminal to luminal translocation that some chemokines apparently undergo and how chemokines made by other elements of the NVU translocate to the vessel lumen. These questions pertain to all vascular beds, but are most compelling for BBB studies, because of the unique nature of the cerebrovascular endothelium, and because of the multi-compartment nature of the extravasation process from vessel lumen to perivascular space, across the glia limitans to the parenchyma. This type of navigation mandates particularly intricate chemokine distribution and function. The mechanisms by which leukocytes enter the CSF, which differ from the mechanisms used to traverse the vascular barrier, need to be elucidated as do how different types of leukocytes (T-cells, B-cells, macrophages, PMNs, etc) target each of these arms of the barrier.

Neurovascular Unit

Progress:

It is well recognized that maintenance of BBB characteristics in brain endothelial cells depends on the microenvironment, more precisely on the cells of the neurovascular unit.[8] The composition of this unit is best described by following a circulating leukocyte across the BBB. After having migrated across the endothelial BBB, it has not reached the CNS parenchyma yet. Rather it will first enter a perivascular space, which is delineated by the 2 molecularly distinct basement membranes produced by the endothelium and the astrocytes, respectively. In EAE models using gene-targeted mice, it has been shown that clinical disease only starts when leukocytes have penetrated the basement membrane produced by astrocytes and have thus entered the CNS

parenchyma.[9] Therefore, when investigating leukocyte recruitment across the BBB, we need to consider the distinct steps of leukocyte migration across the entire neurovascular unit, which is composed of endothelial cells, the extracellular matrix, pericytes, perivascular dendritic cells, macrophages, and astrocytes.

Furthermore, differential behavior of vascular endothelial cells regarding their position in the vascular tree as well as their organ of origin is now well recognized, although the reasons for these differences remain largely unknown at the molecular level.[10,11] Therefore, we have to consider distinct BBB characteristics at the capillary versus the microvascular level, i.e. at the level of post-capillary venules where leukocytes extravasate.

Unresolved and New Questions:

Differentiate the requirements for leukocyte diapedesis across the endothelial cells of the BBB versus their diapedesis through the extracellular matrix within the neurovascular unit and across the glia limitans.

Define the precise role of the individual cells of the neurovascular unit, i.e. pericyte versus astrocyte, in maintaining BBB integrity and in contributing to disease.

BBB Integrity: Tight Junctions

Progress:

During the last years, we have gained tremendous knowledge in the molecular composition of tight junctions including those of the BBB and the blood-CSF barrier.[12] Especially the protein family of tight junction proteins including claudins has been characterized. Despite this knowledge, we know little about the regulation of tight junction integrity. The first hint about tight junction regulation is derived from claudin-5 deficient mice, which die perinatally due to BBB leakiness to small but not large molecules.[13] Additionally, the molecular composition of BBB tight junctions is changed during CNS inflammation, which is generally accompanied by BBB leakiness.[14] Thus, it has become clear that individual tight junction proteins play different roles in the maintenance of the BBB and in BBB leakiness during CNS inflammation. Presently, the

majority of research on tight junction function is, however, performed in epithelial cells rather than endothelial cells. For the reasons already discussed above, this might not be the correct cellular environment to study tight junction regulation of the BBB.

Unresolved and New Questions:

Understand the mechanisms of regulation of TJ permeability in brain endothelial cells: identity of the components of TJ-associated multi-protein complexes, post-translational modifications of these components, and the signaling pathways upstream of these modifications. These areas are especially important in terms of development and aging.

Cytokines and Immune Modulators

Progress:

Cytokines and other immune modulators (e.g., prostaglandins, lipopolysaccharide, opiates) have numerous effects on BBB functions. The interactions of the BBB with many cytokines, including a number of interleukins, interferons, neurotrophic factors, smaller neurotrophic peptides, and adipokines, have been studied.[15] Cytokines and immune modulators can disrupt the BBB, but can also selectively modulate BBB saturable transport systems. Injuries to the CNS modulate transporters, probably through immune mechanisms, including those which carry immune-active and neurotrophic substances. Modulation of efflux (brain-to-blood) transport systems, such as P-glycoprotein, by neuroimmune substances and events can affect drug delivery and the brain's microenvironment.[16]

Many peripherally produced cytokines, including those with neurotrophic activity, are transported across the BBB in amounts that affect CNS function. These transporters undergo complex regional and temporal changes in response to CNS injury.[17] The studies not only addressed the pharmacokinetics of transport in animals and cellular models of the BBB, but also dealt with effects of cytokines on cerebral microvessel endothelia, which relay secondary mediators to the

CNS. Key scientific advances in this field have been[1] characterization of the CNS effects of peripheral cytokines in physiological processes, including feeding, sleep, memory, and mood;[2] identification of regulatory changes in pathological conditions, such as trauma, inflammation, autoimmunity, ischemia, hypoxia, tumor, and other diseases altering cerebral blood flow and metabolism (e.g., diabetes and hypertension);[3] mechanistic studies of the trafficking process inside the cells;[4] amplification of the original cytokine signal by de novo synthesis of other cytokines and soluble factors.

The endothelial and epithelial cells which comprise the blood-brain/CSF barriers, respectively, secrete a host of neuroimmune substances, including cytokines, chemokines, nitric oxide, and prostaglandins.[18,19] Secretion of these substances can be constitutive or induced by substances such as lipopolysaccharide. Because the BBB provides a polarized interface between blood and the CNS, polarized responses can occur.[20] That is, the BBB can respond to neuroimmune stimuli received from one compartment by secreting into the other, thus forming a communication pathway between the peripheral and CNS tissues.

Unresolved and New Questions:

Are cytokines and immune modulators physiological regulators of paracellular (i.e., tight junction) and transcellular (i.e., transcytotic) BBB permeability? What effects do immune modulation of BBB properties have on drug delivery? What role does transport of cytokines across the BBB play in physiology (e.g., regulation of physiologic sleep) and pathology (e.g., mediation of sickness behaviors)? How and by what mechanisms are these transporters altered in disease states and by drugs? How is plasticity of BBB transporters regulated and what are the pathophysiological implications of immune modulation of BBB saturable transporters? Can these transporters be used to deliver therapeutic doses of neurotrophins? Can immune-mediated modifications of the transporters of substances with neuroprotective and neurotrophic properties be harnessed to deliver therapeutic amounts of their ligands? What substances are secreted by BBB cells? How do the secretions affect other aspects of BBB function and the cross talk with other elements of

the neurovascular unit, circulating elements, and peripheral tissues? What regulates that secretion, to what degree is secretion and its control polarized (that is, luminal vs abluminal or basal vs apical surfaces)? What are the implications of secretions for CNS-peripheral tissue communication?

Pathogen Transport Across the BBB

Progress:

Parasites, bacteria, and viruses invading the CNS must cross the BBB and they utilize a variety of strategies to enter the brain and bypass or disrupt the biological barriers. Some pathogens enter as free agents – probably by transcytosis, whereas others enter inside infected immune cells through a so called "Trojan horse" mechanism.[21] This model has been best described for HIV-1 entry into the brain. HIV-1-infected leukocytes (CD4+ T-lymphocyte and/or circulating monocytes) have an enhanced ability to pass the endothelial and epithelial BBB.[22] It appears that part of this ability may be mediated by released HIV-1 proteins, such as Tat.[23] Alternatively, pathogen produced proteins have been shown to directly interact with tight junction proteins. For example, claudin-3 and claudin-4 act as high affinity receptors for *Clostridium perfringens* enterotoxin (CPE), and the C-terminal half of CPE (C-CPE) can selectively remove claudins from reconstituted tight junction strands. Indeed, treatment with C-CPE results in fragmentation of claudin-3 into spot-like structures, followed by its gradual disappearance.[24] Interesting approaches for entry into brain endothelial cells were developed by *Neisseria meningitidis*. Adhering bacteria stimulate phopshorylation of cortactin in endothelial cells via Rho-dependent mechanisms. *N. meningitidis* strains also induce the formation of membrane protrusions, which then participate in bacteria uptake, and actively recruit ezrin, moesin, and ezrin binding adhesion molecules. These mechanisms are important, because they impair leukocyte-endothelial cell interaction and thus, diminish normal inflammatory responses in host cells.[25] Several of the strategies for cellular entry of pathogens were described for the epithelial barrier and it remains yet to be determined if pathogens can enter the brain using similar mechanisms. Group B coxsackieviruses (CVBs) can enter endothelial cells via interaction with the coxsackievirus and

adenovirus receptor (CAR), which is a transmembrane protein and a component of tight junctions. However, CVBs can also utilize another cellular receptor, namely, the glycosylphosphatidylinositol-anchored decay-accelerating factor (DAF). Interaction of CVBs with DAF activates intrinsic signaling in host cells, such as stimulation of Fyn kinase and induction of phosphorylation of caveolin, with subsequent transport of the viruses into the cells.[26] To enter the cells, mammalian reoviruses use junctional adhesion molecule-A (JAM-A), another tight junction component, as a cellular receptor. Reovirus attachment, infection, and replication are mediated by the amino-terminal D1 domain of JAM-A and sialic acid can serve as a co-receptor. Following binding to the receptor and co-receptor, reoviruses are internalized by endocytosis.[27]

Unresolved and New Questions:

How do pathogens bind to and cross the BBB? What are the cellular mechanisms used for transcytosis, and what events induce and regulate pathogen permeability? How do pathogens alter BBB physiology?

Effect of Systemic Inflammation on the developing BBB

Progress:

Only a few laboratories have addressed the effects of systemic inflammation on the developing blood-brain barrier and the possible implications of inflammation-induced changes in barrier function for subsequent neurological and behavioural development.[28,29] Perry and Anthony used intracerebral injections of inflammatory agents.[30] Nico reported barrier breakdown to horseradish peroxidase after induction of inflammation in E20 chicks.[31] There are several groups in Europe and North America working on effects of inflammation induced in immature animals and subsequent brain development behavior in the adult. None are working on barrier permeability or barrier transport processes, i.e. alterations in influx or efflux mechanisms.

Unresolved and New Questions:

What effects do perinatal inflammatory events have on BBB permeability? What role does the BBB play in the development of neurological diseases associated with perinatal infection and inflammatory events? Which influx and efflux mechanisms are active during BBB development and altered by perinatal infection and inflammation?

Overall Concepts that Need to be Developed

In general, the concept of the brain barrier cells as passive elements receiving input from the periphery, (i.e. the blood stream or from within the CNS) needs to change. Brain barrier cells are active components of the NVU, which integrate the molecular signals from the periphery and the CNS to maintain barrier function and to actively orchestrate neuroimmune inflammation and repair mechanisms. The wider scientific community needs to be informed that the functions of the BBB are not simply that of a barrier, but of an active, regulated and regulatory interface, with transport, secretory, and enzymatic activities. Finally, translational models and clinical studies need to be advanced. The BBB as a seat of disease, and so itself a therapeutic target, has been largely ignored. Poor dissemination of knowledge about the BBB has impeded drug delivery programs.

The BBB in Different Areas of the Brain

Despite the many years of work on inflammation and the BBB, there remain some important and fundamental questions that have yet to be resolved. Many of these questions are confounded by different studies carried out in different species, on different regions of the neuraxis, and in the main are often qualitative rather than quantitative. Although many are aware of the very important distinction to be made between the different regions (e.g., cortical, hippocampal, hypothalamic, spinal cord, retinal) and compartments of the brain (parenchyma, meninges, ventricles, CVOs, grey and white matter), the differential inflammatory responses and permeabilities in these compartments and regions and the different endothelial cell structure and

function is all too often lost in reviews of the topic. The confusion about the different barrier interfaces extends to the assumption that changes in CSF concentrations of a molecule necessarily reflect changes in the vascular BBB, rather than changes at the choroid plexus interface, which is rarely considered. Thus a concept of compartmentalization of BBBs needs to be developed. In this context, it would additionally be important to identify at the molecular level markers of brain capillaries, post-capillary venules, and arterioles which would help understand the mechanisms of cell-cell interactions between brain endothelium and blood-borne cells, immune cells, bacteria, viruses, as well as permeability to drugs.

Leukocyte Trafficking Across the BBB

As described above, most leukocyte trafficking studies across the BBB have been performed in the EAE model and on $CD4^+$T cells. Based on the successful translation from studies in the EAE model to the clinic, it is obvious that investigating the molecular mechanisms involved in immune cell recruitment across the BBB need to be extended to other CNS inflammatory disease models, i.e. stroke, and to additional cell populations such as neutrophils, CD8+ T cells, Th17 cells, T_{regs}, dendritic cells and monocytes to improve our understanding of immunosurveillance of the CNS and to obtain novel therapeutic targets for inflammatory CNS diseases. Different barriers need to be considered in this regard (vascular, blood-CSF barrier, blood-retinal barrier, parenchymal versus meningeal) which are already anatomically distinct. Work on modeling the choroid plexus in vitro would move the research forward by allowing comparative analysis of transmigration at the various BBBs.

Traffic of cells across the BBB in response to an inflammatory challenge is also highly compartmentalized with different responses in meninges/pia versus the parenchyma and probably between CNS grey and white matter and also between brain and spinal cord. Within the parenchyma, there also appears to be both age specific responses (a window of susceptibility – identified for different agents such as IL-1 and LPS),[30] and also neuraxis differences with the spinal cord being more “permissive” than the forebrain.[32] The aspects of BBB function that

regulate these differences - i.e. what is the molecular and morphological basis of the effect of inflammation (systemic LPS) on permeability of white matter blood vessels versus grey matter blood vessels to proteins - are not known and need to be investigated in more detail. Therefore, concepts for leukocyte migration into the different CNS compartments need to be developed.

BBB In Vitro Models

Reliable and validated *in vitro* BBB models forming true tight junctions are necessary to study questions relevant to CNS inflammation. At present, research results are difficult to integrate across labs, because of technical variation.[33-35] A number of consensus *in vitro* BBB models are highly desirable, so that the field can advance by combining data from multiple groups working in parallel. There is a specific need for a validated *in vitro* model of human BBB (or ultimately models of brain capillaries, post-capillary venules, arterioles), which could be used by Industry as a predictive model for drug permeability and by basic scientists as a working model for investigating the molecular mechanisms of cell-cell interactions mentioned above. However, this should not be construed that a single construct is desirable. Flexibility in the use of monocultures, co-cultures, or tri-cultures of endothelial cells with pericytes, neurons, macrophages, astrocytes, etc as dictated by the specific scientific question is one of the great strengths of current in vitro models. Optional features such as models that incorporate flow and other rheological parameters would also be helpful. *In vitro* models of the choroid plexus and tanycytic barrier are also needed.

Intracellular signaling in brain endothelium

Brain endothelial-leukocyte interactions lead to functional changes in the endothelium.[36-38] It is likely that endothelial interactions with other cell types of the NVU also have similar effects. Interactions trigger cascades of signaling events in brain endothelial cells, modifying the cytoskeleton, tightness of the BBB, transcription factors, expression of adhesion molecules, transporters, etc. Distinct intracellular signaling in the brain endothelium initiated by different types of leukocytes (neutrophils, lymphocytes, monocytes) and other cell types and physiological

states (e.g., activated vs non-activated) require more attention. The concept of endothelial cell reactions as a major contributor to extend, modify, and magnifyneuro-inflammation will allow development of new therapeutic strategies to treat stroke, multiple sclerosis, encephalitides, and post-treatment reactions of brain tumors among others.

BBB Integrity – Concept of Neurovascular Unit during Inflammation

The functions of the BBB that have received most attention are the integrity of the barrier function and the traffic of leukocytes. However, BBB function is not solely reflected by tight junction alterations or leukocyte interaction. Given the importance of the BBB in the regulation of CNS homeostasis, it is remarkable how few studies have been carried out on other functions of the CNS endothelium during disease states. It is common knowledge that brain endothelium is in a continuous cross-talk with the cellular elements of the NVU in order to maintain homeostasis within the CNS. Thus, a dynamic brain endothelium continuously "senses" changes in the blood-milieu. Astrogliosis and activated microglia/macrophages, which are uniformly present during neuroinflammation caused by any cause, have an impact on the brain endothelium. Nevertheless, changes in basement membrane/composition of extracellular matrix, astrocyte polarization, glucose transport, amino acid transport, cytokine transport, efflux transporters, and so forth are poorly documented in many human pathological states. In models of acute and chronic neurodegenerative disease there is even less or no information on these important parameters, which might lead to a pro-inflammatory stage of the brain barrier cells.

There is evidence that in HIV-1 associated neuroinflammation, astrocytes could amplify inflammatory responses (chemokine/cytokines production, MMPs activation).[39] Thus, effects of non-endothelial cells within the NVU should be the potential focus of future studies by investigating cellular polarity, i.e. understanding the changes in the subcellular distribution of ion channels, water channels,[40] extracellular matrix receptors, plus the specific profiles of secretory molecules and other factors. These studies will contribute to our understanding of the regulation of BBB integrity during inflammation.

BBB Permeability – Tight Junctions

Although the molecular composition of BBB tight junctions seems to be known, little is known about the regulation of BBB or blood-CSF-barrier tight junctions. In vitro studies using brain endothelial cells or choroid plexus epithelial cells are required to understand the specific regulation of these junctions. Additionally, gene targeted mice with inducible expression of tight junction proteins will provide further insight into the regulation of tight junctions. In this context, it needs to be defined what would be the most appropriate in vivo and in vitro models and methods that should be used in asking questions about barrier effects in inflammatory states and whether these could these be standardized. Which molecules are the appropriate tracers to study tight junction alteration and thus BBB breakdown in CNS inflammation? What is the link between barrier leakage to proteins and CNS damage?

Noninvasive BBB Imaging

There is a need to develop high resolution, non-invasive imaging techniques for studies of BBB function during inflammation in humans but also in small animals (mice, rats). Methods include magnetic resonance imaging (MRI), high-resolution ultrasound, synchrotron imaging, SPECT, PET, and spectroscopic methods. Interdisciplinary approaches will be necessary to accomplish this task. There is also a need to develop rheological imaging and for better contrast reagents that would “mark” activated endothelium. Established imaging techniques of the retina should be distributed.

Experimental/Invasive BBB Imaging

Modern (multiphoton) intravital microscopy allows observation of leukocyte-BBB interaction in real time or in time lapse studies over an extended period.[41,42] This technology permits individual leukocytes to be followed on their way across the BBB and study of the development of BBB leakiness after a defined challenge. Use of these advanced imaging techniques in combination

with novel models of gene targeted mice expressing fluorescent proteins within the NVU will produce important information of protein-protein and protein-lipid interactions. In vivo cellular imaging using 2-photon microscopy or similar techniques of the brain intraparenchymal microvasculature, probably by using transgenic mouse models, are needed to investigate molecular changes at the level of the BBB during inflammation. Again, available protocols for imaging the blood-retina barrier should be disseminated and applied.

Developing BBB

There is a lack of understanding of the complex nature and multiplicity of barrier mechanisms and their level of function in developing brain. BBB mechanisms tend to be considered as a single entity, the integrity of which can be measured by markers such as dye-binding proteins or HRP, which only test general integrity of the barrier and not specific efflux or influx mechanisms.

Ageing BBB

Although we are well aware from human pathological material that the vasculature is different from that of the younger brain, the current literature leaves many gaps in our knowledge. Study of the BBB in young rodents is unlikely to reflect these changes and studies in aged mice or mouse models of chronic neurodegeneration have in the main been limited to BBB leakage with only about a dozen transport systems having been studied. Understanding which receptors, transporters and so forth are changed, what mechanisms control these changes, and what implications these changes have for aging is important. The long-term consequences of inflammation-induced changes in BBB permeability on brain development and behavior are unknown.

Cytokine Transport Across the BBB in Inflammation

These include determination of how cytokine transport across the BBB fits into the overall picture of CNS development, synaptogenesis, and neuroregeneration and how the transport system for cytokines can be targeted for CNS drug delivery.

Summary Points

Key Scientific Advances Since Stroke Progress Review Group (SPRG) 2001

1. Establishment of intravital (multiphoton) microscopy techniques for both brain and spinal cord that allow the direct observation of leukocyte interaction with the BBB in vivo
2. It has been recognized that endothelial cells actively participate in leukocyte diapedesis across the BBB and that this is affected by neuroimmune events
3. Characterization of the neurovascular unit as relevant to maintain BBB characteristics
4. The role key role of claudins in maintaining tight junction integrity has been recognized
5. Understanding that cytokines and other neuroimmune substances interact directly with the BBB to modulate barrier, transporter, enzymatic, and secretory functions
6. Understanding that the BBB transports and secretes cytokines and other neuroimmune-related functions and that secretion and transport are altered by neuroimmune events

Unresolved Questions

1. The mechanisms by which chemokines contribute to leukocyte recruitment across the BBB in vivo

2. The effect of shear stress in leukocyte recruitment across the BBB

3. Endothelial signaling cascades involved in leukocyte diapedesis across the BBB

4. Precise molecular contribution of each cellular element within the neurovascular unit to BBB maintenance or BBB breakdown

5. Regulation of tight junction integrity

6. What substances are secreted by BBB cells, what controls that secretion, how is it regulated and affected by disease states, and how does it contribute to physiology and disease

7. What are the mechanisms of cytokine transport and how and by what mechanisms do alterations in physiology and disease occur.

What Needs to Be Done?

1. Establishment of resources specifically available to BBB research:

- Funding mechanism for international (cross continent) research projects (to include full costs) at the level of 2-3 laboratories within the same discipline but complementary knowledge (HFSP only supports interdisciplinary collaborations, i.e. chemists with biologist, physicists with clinicians etc.) without any direct objective of clinical application
- Increased targeted funding opportunities with specified set-asides

2. Establishment of a BBB rescource center:

- Set up of a general database listing BBB specific genes, reagents, antibodies, primers, siRNA etc.
- Set up of a BBB-Map similar to the brain-map published in Nature January 2007 – needs funding!

3. Teaching

- Establish annual courses, i.e. "International Summer School of BBB research"

- Establish a graduate student program specifically designed to attract high quality students to work on the BBB with the opportunity to spend time in another laboratory in the USA or abroad

4. People:

- Not enough groups investigating BBB inflammation at the mechanistic level (driven traditionally by physiologists and pharmacologists). More cell and molecular biologists, neurobiologists and immunologists are needed investigating inflammation and the BBB.

- A critical mass of BBB researchers is lacking. This limits the intellectual advance of the field and does not give scope for replication of key findings or even dissemination to peripheral fields.

- BBB research needs to get a profile – until today it is hidden in many different disciplines! i.e. research on immune cell trafficking is mostly associated with MS research.

- Cell biologists working on tight junction and isolation of transporter biology need to be attracted into the BBB field. Most research by this group of researchers is performed in epithelial cells, which are distinct from endothelial cells.

- Better behavioral methods are required for evaluating effects of barrier dysfunction during development. This will probably require attracting behavioral scientists into the field.

5. Key Research Areas

1. Investigation of the molecular mechanisms of the diapedesis of different leukocyte subpopulations across the BBB in vivo and in vitro using intravital microscopy and validated in vitro BBB models respectively

2. Investigate the molecular regulation of **endothelial** tight junctions in **brain endothelial** cells in vitro or in transgenic mouse models in vivo
3. Definition of the secretory aspects of all types of BBB cells
4. Characterization of transporters modulated by and transporters of neuroimmune-related substances and the mechanisms by which they are modified under physiological and pathological conditions
5. Characterization of chemokines produced within the neurovascular unit and definition of their function
6. Characterization of the BBB in different brain compartments and at different levels of the vascular tree

Conclusion and Single Most Important Issue

The central topic for investigation is the **"Role of activated BBB cells in orchestrating the neuroinflammatory axis"**. BBB cells, like other elements of the neurovascular unit (NVU), become activated with neuroimmune stimulation. BBB cells are never in a quiescent state, but in continuous cross-talk with the cellular elements surrounding them to maintain barrier properties (Fig. 2). Thus, activation by neuroimmune events forms a spectrum and is more like a volume control than an on-off switch. In fact, neuroimmune interactions with the BBB are part of normal physiology, i.e. during immunosurveillance, and not just a phenomenon of disease. As such, a proper understanding of BBB activation depends on an understanding of normal BBB function. All BBB cell types (vascular, ependymal, retinal, tanycytic, and the other "specialized" barrier cells) can be activated, although they do not necessarily respond in the same way. For example, some barriers are more relevant in specific disease processes, as exemplified by the blood-retinal barrier in type II diabetes mellitus or the vascular BBB in multiple sclerosis. Activation may occur with minimal input and early on the BBB cell responses can be adaptive and act to limit response of itself and surrounding tissues. This means BBB activation can take radically different forms

depending on disease state and occur with proinflammatory conditions as radically different as multiple sclerosis, stroke, Alzheimer's disease, or obesity. The activated BBB cell interacts with other elements of the NVU, which must be broadly defined to include cells and molecules in the circulation in addition to those on the abluminal side of the BBB. Because the BBB is the only component of the neuroimmune axis, which resides simultaneously in both the CNS and periphery, it can be activated from either side and can directly act upon cells within either compartment. The response of the BBB can also be polarized; that is, it can respond to or interact with predominantly one side or the other. A special case of polarization is that of the BBB receiving input from one side but reacting primarily with the opposite side (e.g., luminal secretions in response to abluminal stimulations) and so forms a type of neuroimmune communication unique to the BBB. Activation affects all aspects of BBB structure and function: tight junction integrity, cytoskeletal arrangement, transcytosis, immune cell trafficking, saturable transporters (including those for neuroimmune active substances such as cytokines and those important for drug delivery such as p-glycoprotein), BBB cell secretions (including immune active substances such as nitric oxide, cytokines, and prostaglandins), susceptibility to pathogen invasion, etc. The specific responses of an activated BBB cell varies as a function of type, intensity, and duration of neuroimmune stimulus, type of BBB cell stimulated, environmental influences, age and developmental status of the organism, and the pathophysiological status of the other peripheral and central components of the NVU. The **activated BBB cell is a unifying concept for neuroimmunology** as it is the basis of varied and rich links between the CNS and immune cells. It also is a unifying concept throughout the field of the BBB, as the neuroimmune active BBB cell is central to understanding and treating CNS injuries, neurodegeneration, CNS development, CNS tumors, and influences drug delivery.

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Figure Legends

Fig. 1: The neurovascular unit (NVU) consists of a complex cellular system including circulating blood elements, highly specialized endothelial cells, a high number of pericytes embedded in the endothelial cell basement membrane, perivascular antigen presenting cells, astrocytic endfeet and associated parenchymal basement membrane and neurons. Although the endothelial cells form the blood-brain barrier (BBB), the continuous cross-talk of brain endothelium with the cellular elements of the NVU are pre-requisites for barrier function.

Fig. 2: The Activated BBB. BBB cells are never quiescent, but can be further activated by a host of neuroimmune stimuli (e.g, viruses, lipopolysaccharide (LPS), neurotrauma, neuroimmune insult, cytokines, and other immune active substances). Activation can occur from input from either brain or blood side (blood side only shown for simplicity). Every aspect of BBB function is affected by interactions with the neuroimmune axis. For example, immune input can modulate or induce cytokine release (A) or the release of other neuroimmune active substances, including nitric oxide, substance P and prostaglandins (B). Many cytokines (as well as many pathogens) can cross the BBB (C) to modulate function on the side opposite of origin. Neuroimmune stimuli also modulate BBB permeability (D), including barrier integrity, saturable transporters, and immune cell trafficking.

Figure 1

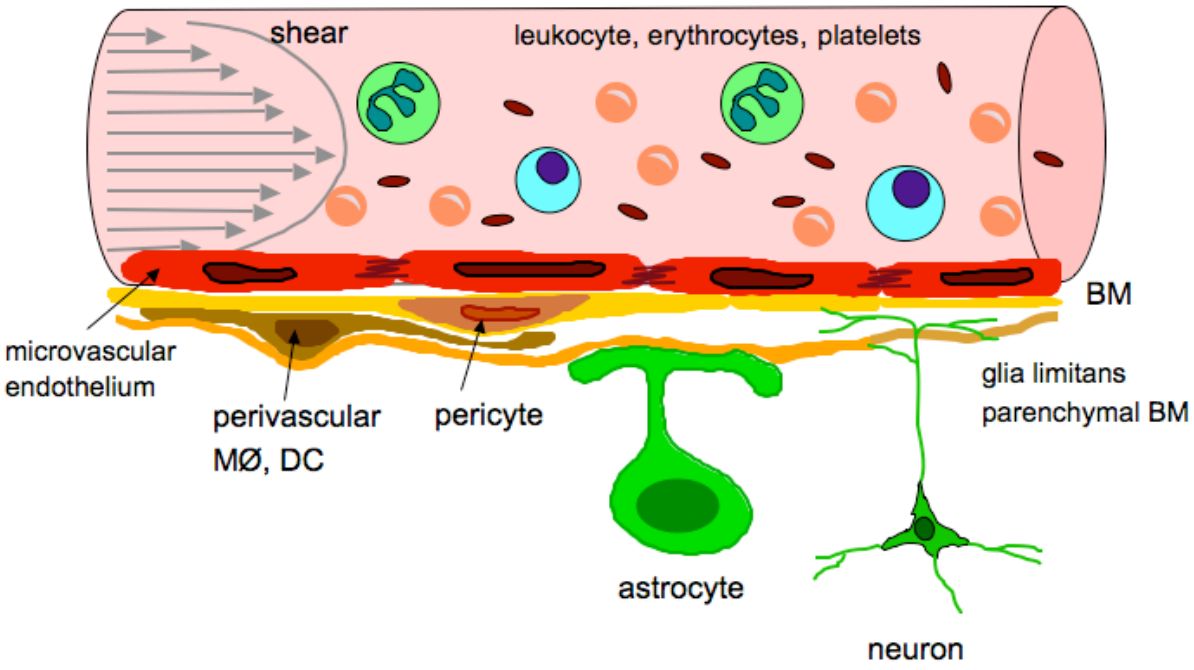
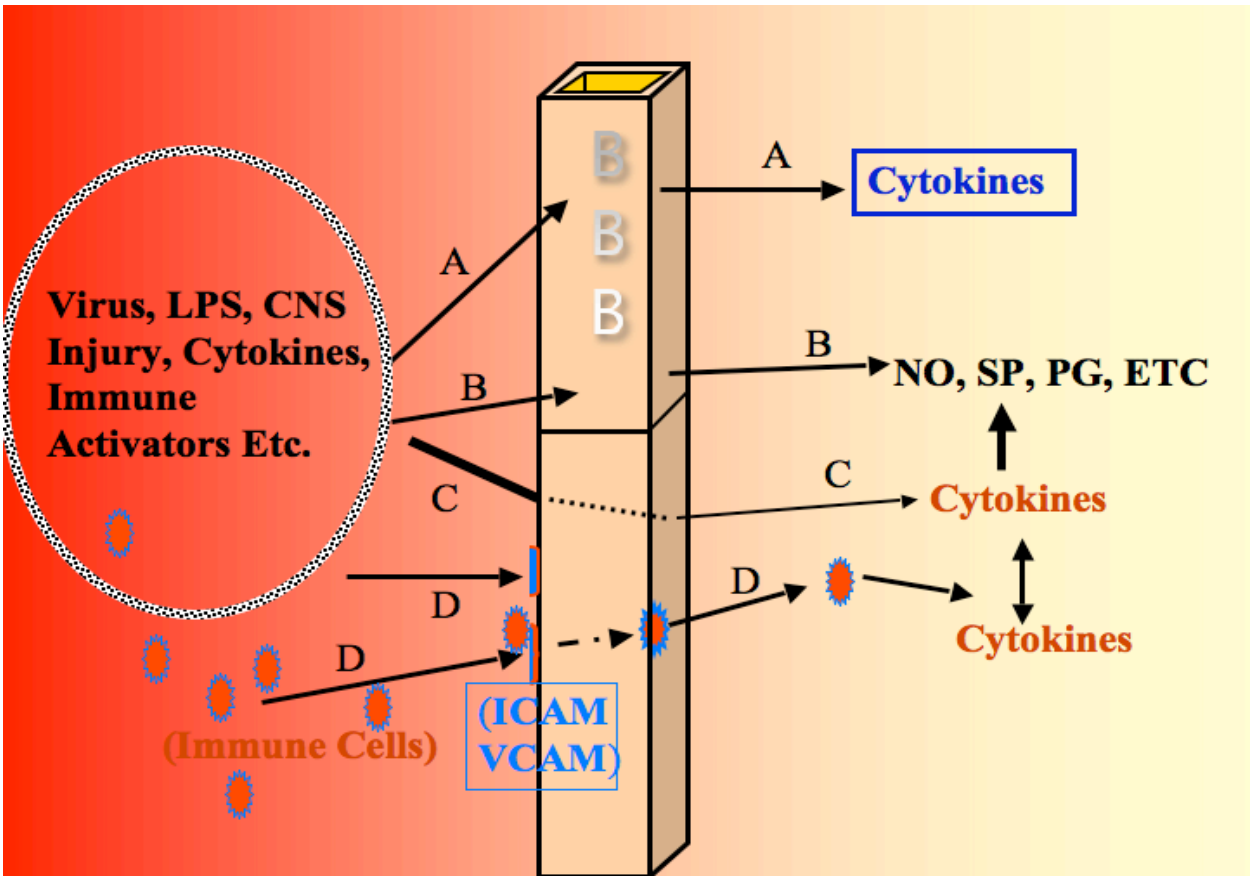

shear
leukocyte, erythrocytes, platelets
BM
microvascular
endothelium
perivascular
MØ, DC
pericyte
glia limitans
parenchymal BM
astrocyte
neuron

Figure 2


Virus, LPS, CNS Injury, Cytokines, Immune Activators Etc.
A
B
C
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D
(Immune Cells)
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NO, SP, PG, ETC
C
Cytokines
D
Cytokines