

Inhibition of β 2 Integrin-Mediated Leukocyte Adhesion
Attenuates the Inflammatory Response and is
Neuroprotective Following Transient Global Cerebral
Ischemia

By

Ryan Paul Francis Salewski

A thesis submitted in conformity with the requirements
for the degree of Master's of Science

Institute of Medical Science
University of Toronto

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Ryan Salewski
Master of Science
Institute of Medical Science
University of Toronto

2009

Abstract

Leukocyte adhesion to cerebral endothelial cells plays a critical role in the inflammatory response following transient global cerebral ischemia but its contribution to delayed neuronal cell death is not completely understood. We compared ischemic mice treated with a monoclonal antibody to β 2-integrin adhesion receptors (anti-CD18) or a non-binding control antibody following ischemia. Inflammation was characterized by increased CD18 expression on leukocytes and inflammatory mediators in the peripheral blood and brain tissue. Notably, interleukin-1 β , which has been shown to mediate cell death in neurons, was elevated in the blood and brain. Anti-CD18 blocked leukocyte adhesion as well as the inflammatory responses, including interleukin-1 β expression in neurons. Blocking leukocyte adhesion protected the structural integrity of the hippocampus, cerebral cortex and thalamus, and preserved spatial. Leukocytes adhesion to endothelial cells plays an important role in the evolution of neurological deficit in global cerebral ischemia despite the lack of transmigration of leukocytes across blood-brain-barrier.

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LIST OF ABBREVIATIONS

2 vessel occlusion (2VO)

4', 6-diamidino-2-phenylindole (DAPI)

4 vessel occlusion (4VO)

9-aminomethyl-9,10-dihydroanthracene (AMDA)

Adenosine Tri-Phosphate (ATP)

Blood Brain Barrier (BBB)

Calcium (Ca)

Centimeter (cm)

Cerebrospinal fluid (CSF)

Cerebral Blood Flow (CBF)

Cornu Ammonis (CA)

Cluster of differentiation (CD)

Cyanine-3 (Cy3)

Degrees Celsius (°C)

Dentate Gyrus (DG)

Deoxyribonucleic Acid (DNA)

Endothelium Selectin (E-selectin)

Fetal Bovine Serum (FBS)

Fluorescein Isothiocyanate (FITC)

Grams (g)

Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF)

Hour (h)

Hydrogen chloride (HCl)

Immunoglobulin (Ig)

Inducible Nitrite Oxide Synthase (iNOS)

Intercellular adhesion molecule (ICAM)

Interleukin (IL)

Interleukin-1 receptor antagonist (IL-1ra)

Intra-peritoneal (IP)

Keratinocyte derived chemokine (KC)

Kilogram (kg)

Liter (L)

Leukocyte factor antigen (LFA)

Leukocyte-selectin (L-selectin)

Macrophage-1 antigen (Mac-1)

Macrophage inflammatory protein (MIP)

Matrix Metalloprotease (MMP)

Myeloperoxidase (MPO)

Major Histocompatibility complex (MHC)

Microgram (μg)

Microlitre (μl)

Milligram (mg)

Milliliter (ml)

Molar (M)

Monocyte chemoattractant protein (MCP)

Mucosal Vascular Addressin Cell Adhesion Molecule (MadCAM)

N-methyl-D-aspartic acid (NMDA)

Nanomolar (nM)

Neuronal Nuclei (NeuN)

Nuclear Factor- κ B (NF- κ B)

Paraformaldehyde (PFA)

Phycoerythrin (PE)

per Hydrogen (pH)

Phosphate Buffered Saline (PBS)

Plasminogen Activator inhibitor (PAI)

Platelet-endothelial cell adhesion molecule (PECAM)

Platelet Selectin (P-selectin)

P-selectin sialoglycoprotein ligand (PSGL)

Revolutions per minute (rpm)

Seconds (sec)

Sialyl Lewis X (sLeX)

Sodium Azide (NaN₃)

Soluble Endothelium Selectin (sE-Selectin)

Soluble Intercellular Adhesion Molecule (sICAM)

Soluble Vascular Cell Adhesion Molecule (sVCAM)

T-Cell helper (Th)

Tissue Plasminogen Activator (t-PA)

Tumor Necrosis Factor (TNF)

Vascular cell adhesion molecule (VCAM)

Chapter 1

Introduction

1.1 Cerebral Ischemia/Reperfusion.

Cerebral ischemia is a leading cause of death and morbidity in western societies. (R. Berti et al., 2002) It occurs when cerebral blood flow (CBF) becomes insufficient to deliver oxygen and nutrients to maintain the metabolism and function of the brain. If the ischemia were to persist indefinitely, without restoration of blood flow, there would be severe and irreversible damage. However, reperfusion, although necessary, is associated with the pathogenesis of cerebral ischemia/reperfusion injury. Cerebral ischemic injury may be categorized as either focal or global depending upon the extent of blood flow reduction.

Focal cerebral ischemia, commonly referred to as stroke, is the result of a highly localized reduction in CBF to a specific area of the brain, most often caused by thrombosis or embolism. In contrast, global cerebral ischemia is caused by the reduction in blood flow to all or most of the brain. This may be the result of cardiac arrest, near drowning or severe hypotension. (T. Back et al., 2004) The extent of neurological injury among survivors of cardiac arrest depends upon the duration of global ischemia. Approximately 80% of patients with cerebral damage from cardiac arrest die or exhibit a persistent vegetative state. (C. Bassetti et al., 1996) Embolism or severe hypotension are well-documented complications of cardiopulmonary surgeries and may result in a significant amount of focal or global ischemic injury causing the survivors to exhibit

neurological and neuropsychological morbidity. (P. J. Shaw et al., 1987) Cerebral ischemia/reperfusion injury represents a significant cause of impairment and death and is, therefore, an important area of research. An increased understanding of the mechanism of cerebral ischemia/reperfusion may lead to new therapies that could reduce this morbidity and mortality.

1.2 Mechanisms of Ischemic brain Injury

Cerebral ischemia and reperfusion initiates a complex cascade of pathological events such as excitotoxicity, neuro-inflammation and apoptosis, which ultimately result in ischemic brain damage. This pathophysiological cascade begins within minutes of the ischemic event and may continue for days to weeks following restoration of blood flow. (F. Block, 1999) The majority of injury may not occur immediately following insult but rather develop gradually over many hours and days after the ischemic event. (T. Back et al., 2004)

1.2.1 Excitotoxicity, Calcium Accumulation and Oxidative Stress

The failure of central nervous system (CNS) homeostasis occurs rapidly following the onset of ischemia. Within 4 minutes of complete occlusion of blood flow, the ATP supply in the brain approaches zero. (G. S. Krause et al., 1988) Subsequently, during the ischemic insult and early in the reperfusion period, there is a substantial release of neurotransmitters, including the excitatory amino acid glutamate, in vulnerable areas of

the brain. (H. Benveniste et al., 1984; M. Y. Globus et al., 1991) Under normal condition glutamate, the main excitatory amino acid of the brain, stimulates ligand-gated ion channels on receptors, such as NMDA and AMDA, only transiently. With excitotoxic conditions, the glutamate activity is prolonged. This release is mediated through ischemia-induced depolarization of presynaptic vesicles. (G. S. Krause et al., 1988) Inhibition of neurotransmitter reuptake and energy failure adds to the excess glutamate and further exacerbates the excitotoxicity. (B. K. Siesjo and P. Siesjo, 1996)

In addition, the considerable increase of excitatory amino acids leads to a massive increase of intracellular calcium ions (Ca^{2+}). (M. Erecinska and I. A. Silver, 1992) The activation of both receptor-gated and voltage-gated Ca^{2+} channels and the release of intracellular Ca^{2+} stores into the cytosol contribute to the increased concentration. With ischemic energy failure, Ca^{2+} /ATPase transporters are unable to remove the excess ions. (N. Sakamoto et al., 1986; D. M. Hartley et al., 1993) The ischemia-induced influx of calcium results in the activation of various phospholipases and proteases, including protein C, calpain I, xanthine oxidase and nitric oxide. (D. W. Choi, 1988; B. K. Siesjo and P. Siesjo, 1996) For example, calpain is a Ca^{2+} activated protease that found in the greater concentrations in selectively vulnerable neurons of brain compared to the more resistant areas. Upon Ca^{2+} activation of calpain there is proteolysis of its substrates, activation of NOS, the pro-apoptotic protein Bad, and the accumulation of free arachidonic acid. (B. C. White et al., 2000) This can lead to the degradation of cytoskeletal and membrane proteins. Furthermore, there is evidence that excitotoxicity and the increased intracellular Ca^{2+} concentration are associated with the generation of reactive oxygen species and free radicals. (T. Kristian and B. K. Siesjo, 1998) Reactive

oxygen species have been implicated in ischemic damage once the oxygen deprived brain tissue becomes re-oxygenated with reperfusion. Free radicals and reactive oxygen species can be harmful by a variety of pathogenic processes including DNA and protein damage, as well as lipid peroxidation. (B. K. Siesjo et al., 1989) Free radicals can lead to cell death by necrotic and apoptotic pathways, (C. N. Oliver et al., 1990; F. M. Yakes and B. Van Houten, 1997; R. P. McDonald et al., 1999) as well as the activation of neutrophil extracellular traps (V. Brinkmann et al., 2004). They may, also, cause cerebral edema through disruption of the blood-brain barrier (BBB). (P. H. Chan et al., 1984)

1.2.2 Neuro-inflammation

Neuro-inflammation induced by cerebral ischemia and reperfusion was initially thought to be primarily a repair process in response to the primary injury. Inflammatory mediators, such as cytokines and chemokines, are not directly neurotoxic to neurons in a healthy, uninjured brain. (D. Piani et al., 1992) Furthermore, in an injured brain an inflammatory environment has been shown to promote plasticity and function recovery. (B. B. Johansson and M. Grabowski, 1994; T. Kawamata et al., 1997) However, in contrary to this beneficial role, there is growing evidence that indicates that inflammatory pathways can contribute to cell death following ischemia/reperfusion, ie. secondary brain injury. (K. J. Becker, 1998) This theory is supported by multiple experiments demonstrating neuroprotection through the inhibition of inflammatory mediators following ischemia/perfusion injury. (A. L. Betz et al., 1995; J. K. Relton et al., 1996; H. Nawashiro et al., 1997; F. C. Barone et al., 1998) Current evidence suggests that the

acute inflammatory response is involved in furthering neuronal damage while the later, delayed inflammation may be responsible for facilitating remodeling and neurogenesis. (F. C. Barone and G. Z. Feuerstein, 1999) Spatial and temporal parameters must be taken into consideration when evaluating the neuroprotective and neuropathogenic effects of neuro-inflammation.

1.2.2.1 Inflammatory Cells

1.2.2.1.1 Leukocytes

The leukocyte population primarily consists of neutrophils, monocytes and lymphocytes, each of which can contribute to inflammation following ischemia. Leukocytes, primarily neutrophils, are recruited to sites of injury and interact with the endothelium within minutes of the restoration of blood flow following the ischemic insult. (I. T. Sutcliffe et al., 2001) (Leukocyte-endothelial interactions will be discussed in greater detail in a subsequent section) In general, neutrophils are the first leukocyte subtype to be activated during ischemia, and can contribute to potentiating injury and influencing neighbouring cells by the release of cytotoxic substances and other inflammatory mediators. (J. M. Hallenbeck, 1996) Their association to ischemic damage relies on the general observation that leukocytes, particularly neutrophils, are often found in ischemia damaged tissue. (D. F. Emerich et al., 2002) Conflicting information exists in the literature regarding the roles of the other leukocyte subtypes, such as monocytes and lymphocytes, with regards to ischemia induced neuro-inflammation. (K. Becker et al., 2001; Z. G. Nadareishvili et al., 2004) Some debate still continues as to whether leukocytes, specifically neutrophils are

in fact an important pathogenic factor or simply a bystander during injury progression. (D. F. Emerich et al., 2002)

1.2.2.1.2 Microglia/Macrophages

Microglia are the resident macrophages of the CNS. Their activation is characterized by morphologic and phenotypic changes including the expression of specific surface antigens. Once activated these cells are indistinguishable from circulating macrophages. (M. B. Graeber et al., 1988) Areas of the brain that are vulnerable to ischemia, including the hippocampal CA-1 region, show characteristics of activated microglia before evidence of delayed neuronal death. (T. Morioka et al., 1991; J. Gehrmann et al., 1992) The activation of microglia following ischemia has both neuroprotective and neuropathogenic properties. (P. L. Wood, 1995; H. Watanabe et al., 2000; N. Zhang et al., 2005) Activated microglia release a variety of inflammatory mediators and regulators that modulate the activity of other cells including neurons. Proteases, lipases and glutamate are released by microglia and have been shown to induce apoptosis. (M. P. Flavin et al., 1997; M. P. Flavin and L. T. Ho, 1999) *In vitro* models have shown microglial activation to be associated with damage to the BBB. (M. A. Yenari et al., 2006) Also, Minocycline, an inhibitor of microglial activation, protected the brain following focal (J. Yrjanheikki et al., 1999) and global ischemia (J. Yrjanheikki et al., 1998). These findings suggest that microglia play a detrimental role in ischemic injury, however, pretreatment of cultured neuronal cells with microglial culture medium conveyed an early protection against excitotoxicity. (H. Watanabe et al., 2000) Lastly,

studies have shown that brain macrophages are involved in propagating inflammation and pathogenesis rather than circulating macrophages since the depletion of peripheral blood macrophages did not effect injury following cerebral ischemia/reperfusion. (M. Schroeter et al., 1997)

1.2.2.1.3 Astrocytes

Astrocytes are found in the brain and are the most numerous of the neuronal glial cells. They are irregularly shaped cells with long processes that extent to the BBB. The astrocytes' primary role in the CNS is to aid in neuron maintenance and function. In response to injury, including ischemic insult, these cells become activated resulting in the expression of glial fibrillary acidic protein. This process is termed "reactive astrogliosis". These cells can modulate the inflammatory response by expressing MHC and facilitating the anti-inflammatory Th-2 response. (Y. Dong and E. N. Benveniste, 2001) Astrocytes, also, participate in brain inflammation by the release of cytokines, chemokines and inducible nitric oxide synthase (iNOS). (Y. Dong and E. N. Benveniste, 2001) Specifically, astrocytic iNOS production after global ischemia was found in areas of brain exhibiting delayed neuronal death. (M. Endoh et al., 1994) These findings suggest that astrocytic activation represents a potentially damaging mechanism following cerebral ischemia.

1.2.2.2 Leukocyte-Endothelial Interactions

Leukocyte-endothelial interactions are a fundamental component of the inflammatory response to ischemia. Endothelial cells become activated during reperfusion following the initial cerebral ischemic event. This is followed by the recruitment of leukocytes to the area of injury. (M. Ishikawa et al., 2003; F. Gavins et al., 2007) The endothelium is essential for the trafficking of leukocyte by regulating leukocyte chemoattraction, adhesion and transmigration from the peripheral blood. (E. S. Connolly, Jr. et al., 1996) The rolling and adhesion of leukocytes on the endothelium is mediated by cellular adhesion molecules. (D. N. Granger and P. Kubes, 1994) There are three main families of cell adhesion molecules: (i) The selectins, (ii) The immunoglobulin (Ig) superfamily and (iii) The integrins. (T. J. DeGraba, 1998; H. C. Emsley and P. J. Tyrrell, 2002)

1.2.2.2.1 Selectins

Selectins are cell adhesion molecules that are required for the initial capture and rolling of leukocytes on the vessel wall during inflammation. (R. P. McEver, 1997) There are three types of selectin molecules, Leukocyte-selectin (L-selectin, or CD62L), Endothelium-selectin (E-selectin, or CD62E) and Platelet-selectin (P-selectin, CD62P). Selectins are named for the cell type that expresses them on their surface; however, P-Selectin is expressed on endothelial cells in addition to its expression on platelets. These molecules are membrane-anchored Ca^{2+} dependant (C-type) lectins. (R. P. McEver, 1997) They interact with Sialyl Lewis X (sLeX) on glycoproteins and P-selectin sialoglycoprotein ligand-1 (PSGL-1). (R. P. McEver, 1997)

L-selectin is constitutively expressed on leukocytes and can recognize ligands expressed on the cytokine activated endothelial cells as well as on other leukocytes. (O. Abbassi et al., 1991; T. M. Carlos and J. M. Harlan, 1994) L-selectin is involved in the capture of un-stimulated leukocytes from the bloodstream. (R. F. Bargatze et al., 1994) L-selectin is rapidly shed from the surface of leukocytes upon activation, including following chemokine stimulation. (M. A. Jutila et al., 1989; T. K. Kishimoto et al., 1989) Although L-selectins mediates the initial rolling of leukocytes, it does not appear to play a critical role in the development of ischemic injury. Antibody inhibition of L-selectin did not lessen the extent of leukocyte adhesion and transmigration at the areas of damage in a rabbit model of focal cerebral ischemia. (M. A. Yenari et al., 2001) However, shed L-selectin may be a relevant factor in recruitments of neutrophils from the bone marrow (S. F. van Eeden et al., 1997) as well as functioning as a regulator of leukocyte rolling velocities (J. B. Huppa and H. L. Ploegh, 1997).

P- and E-selectin have been more extensively examined than L-selectin and appear to play a critical role in inflammatory damage. Under endogenous conditions E-selectin is minimally expressed (S. I. Simon and C. E. Green, 2005), however, upon activation by cytokines, thrombin or histamine stimulation it is rapidly up-regulated. (D. A. Jones et al., 1993; M. B. Lawrence and T. A. Springer, 1993) P-selectin is preloaded in the cell and rapidly shuttle to the cell surface in response to ischemia. (S. I. Simon and C. E. Green, 2005) The slow rolling of leukocytes has traditionally been attributed to solely E-selectin (S. I. Simon et al., 2000; C. E. Green et al., 2004) however, recent evidence in a shear stress cell culture model of leukocyte endothelial interactions demonstrated a P-selectin mediated slow rolling. (J. A. DiVietro et al., 2001) The expression of endothelial

P- and E-Selectin is increased following cerebral ischemia, and blocking their function has neuroprotective effects in certain stroke models. (J. Huang et al., 2000; J. Mocco et al., 2002) However, attenuating neutrophil rolling by pre-injury treatment with anti-P-selectin antibody resulted in an increased mortality rate in a gerbil model of global ischemia. (J. Lehmberg et al., 2006)

The rolling of the leukocyte along the endothelium signals for the activation of other cell adhesion molecules, such as integrins, and the initiation of leukocyte adhesion. Firm adhesion is, also, supported by chemokine activation of β 2-integrins. (M. Yoshida et al., 1996; Y. Hu et al., 2000; C. E. Green et al., 2004)

1.2.2.2.2 Immunoglobulin (Ig) Superfamily

The immunoglobulin superfamily class of cell adhesion molecules are required for the adhesion of leukocytes to endothelial cells. They are expressed on the endothelial cells and interact with the integrin class of adhesion molecules. This interaction is required for the arrest and firm adhesion of leukocytes on the vessel wall. (S. I. Simon and C. E. Green, 2005) In terms of leukocyte-endothelial interactions, the Ig superfamily consists of five molecules: Intercellular adhesion molecule-1 and -2 (ICAM-1 or CD54, ICAM-2 or CD102), vascular cell adhesion molecule-1 (VCAM-1 or CD106), platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and muscosal vacular addressin cell adhesion molecule-1 (MAdCAM-1). Of these 5 members of the Ig superfamily, ICAM-1 and VCAM-1 are the most extensively investigated with regards to cerebral ischemia-induced inflammation.

Intercellular adhesion molecule-1 is constitutively expressed at low levels on the cell membrane of endothelial cells. Upon cytokine stimulation, its expression is increased. Under hypoxic conditions the endothelial cells release inflammatory mediators, such as interleukin-1 (IL-1), which signal ICAM-1's up-regulation. (E. S. Connolly, Jr. et al., 1996) Transient cerebral ischemia induces expression of ICAM-1 as a homodimer on the membrane of inflamed cerebral endothelial cells, (D. E. Staunton et al., 1988) reaching peak levels between 12 and 24 hour post-injury. (X. Wang and G. Z. Feuerstein, 1995) Studies have shown that ICAM-1 deficient mice have smaller infarcts compared to wild-type mice following cerebral ischemia. (E. S. Connolly, Jr. et al., 1996; K. Kitagawa et al., 1998) These findings help emphasize the critical role of leukocyte adhesion in furthering inflammatory injury following ischemia.

Vascular cell adhesion molecule-1 is up-regulated following stimulation by cytokines (eg. IL-1 and tumor necrosis factor- α (TNF α)). (K. Yonekawa and J. M. Harlan, 2005) However, VCAM-1's role in inflammatory injury is not completely understood. Inhibition of VCAM-1 expression was neuroprotective in a model of transient global cerebral ischemia (L. H. Zhang and E. Q. Wei, 2003), while inhibition of VCAM-1 was not neuroprotective with a focal cerebral ischemia model. (C. Justicia et al., 2006)

Lastly, increased plasma and cerebral spinal fluid (CSF) concentrations of soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) was measureable in patients shortly following cerebral ischemic events and these concentrations correlated with the severity of injury. (A. M. Simundic et al., 2004; E. Ehrensperger et al., 2005)

1.2.2.2.3 Integrins

The integrins are a family of adhesion molecules that consist of a common β -subunit dimerized with a variable α -subunit. (S. M. Albelda, 1991) They are transmembrane surface proteins that respond to a variety of inflammatory mediators, including cytokines, chemokines and chemoattractants. (C. W. Smith, 1993) Leukocyte adhesion to the endothelium is dependant on integrins binding to endothelial cell adhesion molecules. The β_2 -integrin subunit, CD18, can dimerizes with the α_L (CD11a) and α_M (CD11b) subunits as well as other CD11 α -subunits. The CD11a/CD18 integrin is referred to as LFA-1 (Leukocyte function associated antigen-1), and CD11b/CD18 is referred as Mac-1 (Macrophage-1 antigen). Both LFA-1 and Mac-1 are expressed on neutrophils, monocytes and lymphocytes. Although, their primary ligand is ICAM-1, these molecules can bind to a wide variety of molecules including myeloperoxidase, elastase, heparin, complement fragment C3_{bi}, fibronectin and albumin. (G. D. Ross and J. D. Lambris, 1982; M. S. Diamond and T. A. Springer, 1994; M. S. Diamond et al., 1995; T. Q. Cai and S. D. Wright, 1996; M. W. Johansson et al., 1997). Leukocyte factor antigen-1 bonds have been shown support slow rolling, while Mac-1 maintains stable adhesive bonds (E. R. Hentzen et al., 2000) demonstrating the overlap with the selectins and integrins during the capture of leukocytes in the microvasculature to their firm arrest. Vascular cell adhesion molecule-1 bind to the $\alpha_4\beta_1$ intergin, also known as very late antigen-4, which is primarily expressed on lymphocytes and monocytes. (C. J. Frijns and L. J. Kappelle, 2002)

Beta-2-integrins mediate leukocyte adhesion, from the slow rolling of leukocytes, to their firm arrest. (R. O. Hynes, 1992; A. D. Taylor et al., 1996) Beta-2-integrins are constitutively expressed on leukocytes in an un-activated, low avidity state. Their affinity and avidity for ICAM-1 is rapidly, within seconds, induced with a conformation change to the active state through selectin mediated rolling (S. I. Simon and C. E. Green, 2005) or by stimulation with chemotactic agents. The factors that induce a high avidity and affinity state of β 2-integrins for their ligand include the chemokines, interleukin-8 (IL-8) (T. W. Kuijpers et al., 1992), monocyte chemoattractant protein-1 (MCP-1) (A. G. Kumar et al., 1997), and macrophage inflammatory protein-1 (MIP-1) (P. A. Tessier et al., 1997) and the growth factor cytokine, granulocyte/macrophage colony stimulating factor (GM-CSF) (A. R. Huber et al., 1991). The cytokine stimulated endothelial cells can release IL-8, which activates β 2-integrins. (A. R. Huber et al., 1991; G. A. Zimmerman et al., 1996) Furthermore, the binding of β 2-integrins to ICAM-1 further promotes leukocyte activation and induces a conformational change to the active state of these molecules. (M. E. Labadia et al., 1998)

Beta-2-integrins have been shown to be important in focal ischemic damage. Knockout mice to CD18 have reduced leukocyte trafficking and less brain injury following experimental stroke. (C. J. Prestigiacomo et al., 1999)

1.2.2.3 Inflammatory Mediators

1.2.2.3.1 Cytokines

Cytokines are inflammatory mediators produced in response to a diverse range of

injuries. Following cerebral ischemia, cytokines are up-regulated in the brain. They are produced by leukocytes, macrophages, endothelial cells and the resident cells within the CNS, including microglia, astrocytes and neurons. (T. Liu et al., 1994; T. Sairanen et al., 2001) Key cytokines shown to be involved with ischemia induced inflammation are IL-1, TNF α , interleukin-6 (IL-6) and interleukin-10 (IL-10), (S. M. Allan and N. J. Rothwell, 2001) as well as the growth factor cytokines, granulocyte colony stimulating factor (G-CSF) and GM-CSF. (E. Schneeloch et al., 2004; G. A. Matchett et al., 2007)

Interleukin-1 is a major mediator of the inflammatory response following ischemia, with potentially neurotoxic effects. It exists in two isoforms, the IL-1 α and IL-1 β , and has an endogenous inhibitor, IL-1 receptor antagonist (IL-1ra). (W. P. Arend, 1991) These molecules interact with two types of IL-1 receptors that are expressed by a variety of cell types throughout the CNS, including both neuronal and glial cells. (O. Touzani et al., 1999) Interleukin-1 binding to IL-1 receptor Type I leads to a pro-inflammatory response, while IL-1 binding to IL-1 receptor Type II does not propagate a signal. Since IL-1 receptor Type II competes for IL-1 with IL-1 receptor Type I, over expression of this receptor can reduce IL-1 pro-inflammatory signaling. (K. Kuno and K. Matsushima, 1994; A. Basu et al., 2005) Of the isoforms, IL-1 β has been implicated in the inflammatory response to cerebral ischemia. (C. A. Davies et al., 1999) Interleukin-1 β expression is biphasic following both global and focal cerebral ischemia, with significant increases in the brain within hours of injury and a secondary peak at approximately 24 hours post-ischemia. (A. S. Haqqani et al., 2005) The administration of exogenous IL-1 β has been shown to exacerbate ischemia injury (Y. Yamasaki et al., 1995) while mice receiving IL-1 antagonism had smaller infarcts (G. Yang et al., 1997;

N. J. Mulcahy et al., 2003) in rodent models of focal ischemia. The exact mechanism of IL-1 β mediated cellular injury is unknown, however, it has been shown to induce neurotoxic molecules. These include phospholipase A2, cyclooxygenase, nitric oxide and presumably other unidentified mediators. (N. J. Rothwell and G. N. Luheshi, 2000) Lastly, IL-1 and IL-1 receptor type I have been shown to be essential for advancing ischemic pathology. (A. Basu et al., 2005)

Tumor necrosis factor- α is another major player in the inflammatory response, however there is debate with regards to its consequences on brain cell survival. Its expression pattern following ischemia is similar to that of IL-1 β , in that it is biphasic with an increase in the brain within the first few hours of reperfusion (T. Liu et al., 1994) and with a second peak at between 24 and 36 hours post-injury. (Y. Murakami et al., 2005) Tumor necrosis factor- α is expressed by both neurons (T. Liu et al., 1994) and glial cells (H. Uno et al., 1997). Studies have shown that the inhibition of TNF α reduces ischemic brain injury. (G. Y. Yang et al., 1998) However, TNF α deficient mice exhibit larger infarcts, possibly due to a lack of TNF α -mediated tolerance to ischemic damage. (I. Ginis et al., 2002) Other studies have shown that TNF α does not have any effects, either positive or negative, on the outcome following ischemia. (Y. Murakami et al., 2005) The variable outcomes with regards to the effects of blocking or knocking out TNF α in different models of ischemia could be due to differential TNF α receptor expression. (J. M. Hallenbeck, 2002)

Interleukin-6 is generally considered to be a pro-inflammatory cytokine, but its role in cerebral ischemia induced inflammation is unclear. Similar to TNF α , IL-6 has

been shown to be beneficial (O. Herrmann et al., 2003) and harmful (C. J. Smith et al., 2004) following ischemic insults. Interleukin-6 inhibits neuronal damage caused by NMDA infusion (S. Toulmond et al., 1992) and following focal cerebral ischemia (S. A. Loddick et al., 1998). However, transgenic mice over-expressing IL-6 demonstrates a sensitivity to neurodegeneration, (I. L. Campbell et al., 1993) Also, serum levels of IL-6 was the strongest single predictor of in-hospital mortality in patients that had suffered an acute ischemic stroke. (L. S. Rallidis et al., 2006)

Interleukin-10 is an anti-inflammatory cytokine that acts by inhibiting the function of IL-1 and TNF α . Interleukin-10 is released by the CNS in response to cerebral ischemia. In general, IL-10 can be viewed as a “classic” anti-inflammatory cytokine and is considered to be neuroprotective following cerebral ischemia insult. Exogenous IL-10 injection following global cerebral ischemia increased the survival of hippocampal CA-1 neuron in rodents. (W. D. Dietrich et al., 1999) It suppresses cytokine release as well as the expression and activity of the cytokine receptors. (K. Strle et al., 2001)

The growth factor cytokines, G-CSF and GM-CSF play an important role during inflammation. In addition to their aforementioned function in stimulating leukocyte trafficking, these factors promote leukocyte differentiation in the bone marrow. However, G-CSF pre-treatment has been shown to be neuroprotective in global cerebral ischemia. (G. A. Matchett et al., 2007) Granulocyte-colony stimulating factor expression in the brain has been shown to activate anti-apoptotic pathways, promote neurogenesis and decrease infarct volume following experimental stroke. (C. L. Gibson et al., 2005; A. Schneider et al., 2005) Granulocyte-colony stimulating factor and GM-CSF share similar expression patterns as well as neuroprotective properties. (W. R. Schabitz et al., 2008)

1.2.2.3.2 Chemokines

Chemokines are regulatory proteins that are involved in cell communication and recruitment during inflammation. They are categorized by the position of cystine residues and are members of the G-protein coupled receptor superfamily. (A. Bajetto et al., 2001) These molecules are up-regulated following ischemia and signal leukocytes to traffic on the inflamed cerebral endothelium. (Y. Chen et al., 2003) Key chemokines that have been shown to be important in ischemia/reperfusion injury are MCP-1, MIP-1 α and IL-8, of which keratinocyte derived chemokine (KC) is the mouse homologue.

Inhibition of chemokines during ischemic injury is linked with improved outcomes (A. Garau et al., 2005), while an over-expression of chemokines has been shown to exacerbate injury through increased recruitment of inflammatory cells. (Y. Chen et al., 2003) For example, a pharmacological inhibitor of IL-8, Repartaxin, is neuroprotective in a rodent model of transient brain ischemia and its beneficial effects have attributed to the inhibition of neutrophil recruitment and decreased secondary injury. (A. Garau et al., 2005) Monocyte chemoattractant protein-1 and MIP-1 α 's deleterious actions following cerebral ischemic insult have also been attributed to their role in neutrophil recruitment. (Z. Zhang & M. Chopp, 1998) Following global cerebral ischemia, there is an increase in the expression of MCP-1 in the astrocytes of the hippocampus, a selectively vulnerable area of the brain that exhibits delayed neuronal cell death. (Y. Sakurai-Yamashita et al., 2006)

In addition to its role in recruiting inflammatory cells, MCP-1 may also disrupt the

BBB. Blood-brain barrier permeability was significantly increased following MCP-1 administration in an *in vitro* model. (S. M. Stamatovic et al., 2005)

1.2.2.3.3 Other Mediators

Other mediators that may play important roles in furthering secondary injury following cerebral ischemia/reperfusion include nitric oxide, matrix metalloproteinase-9 (MMP-9) and plasminogen activator inhibitor-1 (PAI-1).

Nitric oxide (NO) is an important component of the CNS through its involvement in a diverse range of processes such as neural transmission, host defense and regulation of vascular tone. (Q. Wang et al., 2007) It is a stable gas that can diffuse across cell membrane to propagate its effects. The nitric oxide synthases (NOS) family of enzymes can generate NO. Nitric oxide can form peroxynitrite and evoke DNA damage following cerebral ischemia. (J. Cui et al., 2000; D. Huang et al., 2000) There are 3 type of NOS, neural NOS, endothelial NOS and inducible NOS (iNOS). The later can to be expressed by the immune cells, leukocytes, astrocytes, and microglia as well as endothelial cells and is thought to be involved in secondary injury following ischemia. Inducible NOS null mice have smaller infracts following experimental stroke. (X. Zhao et al.) Also, pharmacological approaches that modulated post-ischemic iNOS expression conferred protection (T. Coughlan et al.; E. M. Park et al.)

Matrix metalloproteinase-9 is a member of a family of proteins that breakdown extracellular components and are involved in matrix remodeling. Matrix

metalloproteinase-9 can disrupt the BBB. (M. A. Kelly et al., 2006) The activation of glial cells is required for pro-MMP-9 to be cleaved to the active form of MMP-9. (G. A. Rosenberg, 1999) Both gene knockout and enzyme inhibition of MMP-9 following focal cerebral ischemia in mice showed decreases MMP-9 concentration in the brain and a significant reduction in lesion size. (M. Asahi et al., 2000)

Plasminogen activator inhibitor-1 acts with tissue plasminogen activator (t-Pa) to maintain a balance between the formation and breakdown of fibrin in clotting. Tissue plasminogen activator is used to treat acute stroke patients due to its ability to improve reperfusion. (W. Hacke et al., 1995)(N. Nagai et al., 2003) In contrary, excess PAI-1 is pro-thrombotic and can exacerbate injury following transient cerebral ischemia. (J. H. Jansson et al., 1991; C. Held et al., 1997) Over-expression of PAI-1 in a mouse model transient focal cerebral ischemia resulted in larger infarct volumes. (N. Nagai et al., 2003) The neuropathological action of PAI-1 is observed in ischemia/reperfusion injury but not in permanent ischemia injury models. (N. Nagai et al., 2003)

1.2.3 Apoptosis

Apoptosis is the process of programmed cell death that acts as a defense mechanism to remove damaged, unwanted or potentially harmful cells. This process is present in all higher eukaryotic cells and can be evoked by a variety of stimuli. In general, apoptosis is regulated by a family of proteins known as the caspases, which ultimately results in the fragmentation of cells into apoptotic bodies and cellular death. (A. J. Hale et al., 1996) A key component of apoptosis that differentiates it from other types of cell death, such as

necrosis, is that it involves active protein synthesis as well as requires intact mitochondria. (D. D. Newmeyer et al., 1994; G. Kroemer et al., 1995; N. Zamzami et al., 1996; L. J. Martin et al., 1998) Also, apoptotic cell death pathways do not trigger an acute inflammatory response whereas necrosis acts as a potent generator of the inflammatory response. (A. H. Wyllie et al., 1980; L. J. Martin et al., 1998) Apoptosis is a highly controlled and organized cellular dismantling while necrotic death is characterized by a rapid deregulation and dysfunction of normal cellular physiology. (L. J. Martin et al., 1998)

Even though apoptosis has a critical role in injury repair, this process can be inappropriately induced furthering damage during brain injury or stroke. (M. D. Linnik et al., 1993; H. Steller, 1995) Intracellular Ca^{2+} concentrations (J. T. Coyle and P. Puttfarcken, 1993), excess glutamate (J. T. Coyle and P. Puttfarcken, 1993; C. B. Thompson, 1995), and free radical release (Y. Kiyota et al., 1993) have all been shown to initiate apoptosis and are associated with ischemia/reperfusion injury progression. Furthermore, specific cytokines can modulate cellular apoptosis. Pro-inflammatory cytokines can increase leukocyte half-life by the inhibition of apoptosis. (J. P. McNamee et al., 2005) However, within tissues, specifically the brain, $\text{IL-1}\beta$ and $\text{TNF}\alpha$ promote apoptosis by inducing pro-apoptotic caspase-3. (M. Kajta et al., 2006)

Apoptosis plays an important role in the pathogenesis of delayed neuronal cell death following cerebral ischemia. In focal ischemia, apoptosis occurs in the vulnerable penumbra around the core of the infarct. (Y. Li et al., 1995) Apoptosis is also observed in vulnerable areas of the brain following transient global cerebral ischemia, (J. P. MacManus et al., 1993) peaking between 3 and 4 days post-ischemia. (S. Kihara et al.,

1994; T. Nitatori et al., 1995) During global ischemia apoptosis has been shown to be the predominant mechanism of cell death. (M. D. Linnik et al., 1993; J. P. MacManus et al., 1993; T. Nitatori et al., 1995) The delayed neuronal death was reduced with apoptosis antagonism. (K. Goto et al., 1990; N. Nakata et al., 1993)

1.3 Anti-Leukocyte Therapies for Cerebral Ischemia

1.3.1 Neutrophil Depletion

Previous investigators have depleted neutrophils prior to ischemia by administering anti-neutrophil serums or anti-neoplastic agents with the aim of reducing damage from global or focal cerebral ischemia. Neutrophil depletion by anti-neutrophil serum has had conflicting results with ischemia studies. Treatment with anti-neutrophil serum reduces neutrophils by 70% to 95%. Anti-neutrophil treatment prior to experimental global ischemia improved regional CBF (B. Groggaard et al., 1989; L. Schurer et al., 1990), but had no neuroprotective effects. (R. J. Schott et al., 1989; L. Schurer et al., 1991)

Another method of neutrophil depletion is the use of anti-neoplastic agents, originally used in cancer treatment. Anti-neoplastic drugs cause myelosuppression and severely reduce circulating red blood cells, platelets and, most importantly, leukocytes. This treatment has serious adverse effects including vomiting, that may cause significant dehydration, cardiovascular collapse and metabolic acidosis. (A. J. Dutka et al., 1989) Nevertheless, anti-neoplastic agents, such as Vinblastine and Cyclophosphamide, improved regional CBF and evoked potentials (B. S. Aspey et al., 1989; U. S. Vasthare et al., 1990) and reduced infarct size in rodent models of global ischemia. (L. A. Heinel et al., 1994)

Whether the potential neuroprotective effects of these anti-neoplastic drugs are related to induced leucopenia or some unidentified mechanisms remains unclear.

Lack of consistent neuroprotection from neutrophil depletion may be because the depletion, although substantial, is incomplete and a small number of neutrophils may be sufficient to evoke neuro-inflammation. (R. Engler and J. W. Covell, 1987) Alternatively, selective depletion of neutrophils misses the possibility that mononuclear leukocytes, such as lymphocytes, are important in the propagation of ischemic injury (R. D. Strachan et al., 1992) The negative results of the early neutrophil depletion studies demonstrate the need for a more targeted approach to anti-leukocyte therapies.

1.3.2 Leukocyte-Endothelial-Interaction Inhibitors

The majority of therapeutic interventions targeting leukocyte-endothelial interactions focus on LFA-1, Mac-1 and ICAM-1 modulation in focal models of cerebral ischemia. Monoclonal antibodies that inhibit leukocyte adhesion have been shown to be neuroprotective in experimental stroke models. (M. P. Bowes et al., 1993; H. Chen et al., 1994; M. Chopp et al., 1994) Even though these therapies did not improve cerebral blood flow, they reduced infarct size and occurrence of apoptosis. (M. Chopp et al., 1994; S. G. Soriano et al., 1999)

Although there is increasing evidence that inflammation plays a crucial role in the pathogenesis of global ischemic injury, only a few studies have investigated the role of anti-leukocyte adhesion in a global cerebral ischemia model. This may be due to the

previous belief that the majority of global ischemic damage is mediated by the calcium-triggered pathways. (B. K. Siesjo et al., 1995) However, activation of adherent leukocytes may amplify the inflammatory response leading to neuronal cell death and neurological impairment. (W. G. Mayhan, 1998, 2002) Inhibition of leukocyte adhesion has been associated with neuroprotection in global cerebral ischemia. (H. L. Xu et al., 2006; T. Altay et al., 2007; E. F. Hauck et al., 2007) In contrast, inhibiting selectin-mediated leukocyte rolling appears to have a worsening effect on survival following global ischemia in a rodent model. (J. Lehmberg et al., 2006) Further investigation into inhibition of leukocyte adhesion to endothelial cells as a neuroprotective strategy is warranted following global cerebral ischemia.

1.4 Clinical Trials of Leukocyte Anti-Adhesion Therapies

There are three clinical trials that have examined the efficacy of modulating leukocyte-endothelial interactions following stroke in humans. They are: (i) Enlimomab Acute Stroke Trial (EAST), (ii) Hu23F2G (LeukArrest) Anti-adhesion to Limit cyto-Toxic injury (HALT), and (iii) Neutrophil inhibitory factor (UK-279,276).

Enlimomab Acute Stroke Trial was a 62 center trial that evaluated the efficacy of Enlimomab, a mouse monoclonal antibody to human ICAM-1. Treatment with Enlimomab or placebo was administered within 6 hours of the onset of stroke symptoms. This trial reached phase III testing and involved 625 patients. The EAST study was ultimately terminated due to adverse reactions, such as fever and infection, along with an overall increase in mortality with the treatment. (2001) A possible examination for the

worsening of outcomes with Enlimomab was the activation of an immune response when the patient was exposed to the mouse monoclonal antibody. (D. Schneider et al., 1998; K. J. Becker, 2002)

The Hu23F2G Anti-adhesion to Limit cyto-Toxic injury trial attempted to avoid some of the problems that the EAST study encountered by using a humanized monoclonal antibody. Hu23F2G, or LeukArrest, is an antibody to CD11/CD18. This trial, also, reached phase III testing and involved 310 patients. The therapy was administered within 12 hours of the onset of stroke symptoms. Although there were few adverse effects, the trial was terminated due to a lack of clinical benefit in neurological outcomes. (L. Seachrist, 2000; D. F. Emerich et al., 2002)

Lastly, the neutrophil inhibitory factor study, also referred as the Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN), is examining the UK-279,276 compound, which is a CD11b/CD18 antagonist, following stroke. The UK-279,276 compound was shown to be well tolerated by stroke patients with no adverse effects. (K. R. Lees et al., 2003) This trial reached phase IIb testing and involved 966 stroke patients who received the therapy within 6 hours of the onset of stroke symptoms. Unfortunately, this trial was also terminated due to lack of observed neurological benefit. (M. Krams et al., 2003) However, further analysis of a subset of patient noted modest neuroprotection when UK-279,276 was co-administrated with t-Pa within 3 hours of the symptoms. (M. E. Sughrue and E. S. Connolly, Jr., 2004)

There are many possible explanations for the lack of positive results with these clinical trials that would not rule out leukocyte adhesion as a promising area of

neuroprotection following ischemia/reperfusion injury. There could have been an insufficient dose to elicit the protective effects. Stroke intervention may have a narrow therapeutic window and these treatments were administered too late to effectively protect against secondary damage. (D. F. Emerich et al., 2002) Also, it has been suggested that leukocyte adhesion is not a component permanent ischemia injury or reperfusion injury when the ischemia is more prolonged (J. M. Harlan and R. K. Winn, 2002). These studies did not differentiate between these types of injury and an ischemia/reperfusion injury. Further work is necessary and warranted in models of global cerebral ischemia to determine if anti-adhesion therapy remains effective with prolonged ischemia.

1.5 Animal Models of Transient Global Cerebral Ischemia

Cerebral ischemia/reperfusion is a complex injury with multiple molecular pathways that lead to cell death. To ensure successful translation of the research finding into clinical practice, it is important to choose an appropriate model.

One of the earliest models of global ischemia was developed in 1964 by Lowry and colleagues. The animal was decapitated and ischemia was stopped by the rapid freezing of the brain. (O. Lowry et al., 1964) Other models of global ischemia include the use of neck tourniquets (E. Siemkowicz and A. J. Hansen, 1978) or neck cuffs (H. Kabat et al., 1942), however, these techniques produced inconsistencies in the duration of cerebral ischemia and did not allow for long term recovery. Cardiac arrest achieved through ventricular fibrillation has also been used to produce global cerebral ischemia. (J. R. Michael et al., 1984) Although the clinical relevance of cardiac arrest is favorable, with

an entire body ischemia it would be difficult to delineate the effects of the cerebral ischemia alone.

The first rodent model of reversible global cerebral ischemia that was relatively simple and allowed for long-term recovery was the four vessel occlusion (4VO) model in rats developed by Pulsinelli and Brierley. (W. A. Pulsinelli and J. B. Brierley, 1979) In this model the vertebral arteries were permanently occluded and then the common carotid arteries are transiently occluded for a specific duration of ischemia. Cerebral blood flow is reduced to less than 3% of normal. This model has been simplified to a two vessel occlusion (2VO) model in the rat where only the common carotid arteries required occlusion. (B. Eklof and B. K. Siesjo, 1972) In order to achieve global ischemia in this model hypotension was induced to prevent forebrain perfusion via the vertebral arteries. The selectively vulnerable areas have been described in this model. The vulnerable areas of the brain include the hippocampus, particularly the CA-1 region, thalamus and cortex. (M. L. Smith et al., 1984)

The model of global cerebral ischemia that we use is a modified version of the 2-vessel occlusion model in rats. We used bilateral common carotid artery occlusion in C57Bl/6 mice. Although occlusion of the vertebral arteries or hypotension is not required in this model there is reliable and reproducible global cerebral ischemia with a low mortality rate. This is possible since C57Bl/6 mice have hypoplasia of the posterior communicating arteries. (T. Olsson et al., 2003) The pattern of hippocampal, cortical and thalamic selective damage is reasonably consistent in this model. The C57Bl/6 strain mice were shown to be the most susceptible to this normotensive 2 vessel occlusion when compared to ICR, BALB/c, C3H, CBA, ddY and DBA/2 strains, based on neurological

tests and histological evaluation. (G. Yang et al., 1997)

1.6 The Hippocampus and Spatial Memory

The hippocampus, specifically the CA-1 region, is the most vulnerable regions of the brain following global cerebral ischemia. Ischemia periods as brief as 2 minutes can significantly damage the hippocampal CA-1 region. (M. L. Smith et al., 1984) The hippocampus plays a critical role in the spatial memory and learning of rodents (R. Cammalleri et al., 1996; N. Burgess et al., 2002) Spatial memory is an animal's ability to learn the location of a reward, such as food resources, and is an essential feature of higher cognitive processing. (A. J. Silva et al., 1998) It refers to a system of reference knowledge about the environment, making it possible to distinguish one location in a familiar environment from another. (R. G. Morris et al., 1982) Legions in the rat and mouse hippocampus have been associated with an inability learn or recall spatial memory tasks.

Neurological impairment such as decreased intellectual capacity, apraxia, spatial disorientation and memory deficits have been shown in patients who have suffered from global ischemic injury. (O. Godefroy et al., 1994; H. Bokura and R. G. Robinson, 1997) The hippocampus and related structures are involved spatial memory in humans as well as other function higher cognitive functions, such as episodic memory. (G. K. Aguirre et al., 1996; N. Burgess et al., 2002) This reiterates the relevance of analysis of this neurological outcome following cerebral ischemia injury.

1.6.1 Morris Water Maze

The Morris water maze was first described almost 30 years ago and remains one of the most commonly used devices to test spatial memory and learning in neuroscience. Due to the variability of infraction following ischemia injury, it is difficult to reliably assess behavioural outcomes. (P. D. Lyden et al., 1992) The relative simplicity, diverse applicability and high sensitivity make the Morris water maze an ideal test for ischemia/reperfusion injury. Originally described in by Morris in 1981, it was found that rodents can rapidly learn to locate a submerged platform in a large circular tank provided it remains in a fixed spatial location relative to distal room cues by employing spatial memory. (R. G. Morris et al., 1981)

Rats with a damaged hippocampus are unable to learn the platform location in a Morris water maze. (R. G. Morris et al., 1982; R. J. Sutherland et al., 1982) This is also observed in mice with hippocampal lesions. (Y. H. Cho et al., 1999) Hippocampal damage did not impair the rodents ability to find the platform with visual cues demonstrating spatial learning and memory impairment rather than visual or motor cortex involvement. The Morris water maze has been shown to be sensitive to global cerebral ischemia in a number of rodent studies. (Brandeis et al., 1989) There is a direct relationship between the hippocampus CA-1 damage and observed performance in spatial learning and memory; however, this is only present when most (greater than 80%) of this region has been damaged. (A. Nelson et al., 1997)

1.7 Experimental Rationale, Hypothesis and Objectives

1.7.1 Experimental Rationale

The basis for our experimental design is the premise that neuro-inflammation is important in the pathology of global cerebral ischemia/reperfusion injury and that leukocyte adhesion to endothelial cells is a critical step in this inflammatory response. Therefore modulating leukocyte adhesion is a potential target for therapies to improve global cerebral ischemia and reperfusion outcomes, possibly by anti-inflammatory pathways.

A direct correlation between leukocyte infiltration and ischemic damage has not been well established. On this basis, we hypothesis that the paradigm that leukocytes must transmigrate the BBB to elicit their damage following ischemia/reperfusion injury is fundamentally flawed. Numerous studies of transient ischemia/reperfusion injury reviewed by Emerich and colleagues, demonstrated the increase of infiltrating leukocytes occurs after the appearance of the infarct. (D. F. Emerich et al., 2002) Also, a dose/response relationship, where a greater number of infiltrating neutrophils resulted in greater damage, is also poorly defined in the literature. (D. F. Emerich et al., 2002) These observations are often used to support the “bystander” theory of leukocytes in ischemic damage. However, combining these observations with the various studies showing the neuroprotective effects of inhibition of leukocyte-endothelial cell adhesion warrants a shift in the current paradigm.

We believe that leukocyte adhesion to endothelial cells, which occurs within minutes following the insult, is sufficient to propagate the inflammatory response following transient ischemia/reperfusion injury. Since the leukocytes themselves do not

enter the parenchyma in our model, the process of firm adhesion of the leukocyte is potentially sufficient to induce the leukocyte or endothelial cells to initiating the inflammatory cascade in the brain parenchyma. (Fig. 1)

Our laboratory has previously shown that our model of global ischemia in C57Bl/6 mice, CBF is reduced to less than 5% of baseline level in the forebrain. (Supplementary Figure 1) and, that treatment with a monoclonal antibody to CD18 inhibits leukocyte rolling and adhesion following IL-1 β stimulation using intravital microscopy. (Supplementary Figure 2) Also, our laboratory has shown that leukocytes do not transmigrate across the BBB following induction of our model of global ischemia. (Supplementary Figure 3) Even though leukocyte transmigration does not occur, we hypothesize that the adhesion of leukocytes to the endothelial cells in the microcirculation is sufficient to propagate systemic and cerebral inflammation. Furthermore, this inflammatory response may have detrimental effects on brain cells following global ischemia.

1.7.2 Hypothesis

β 2 integrin-mediated leukocyte adhesion is sufficient to propagate an inflammatory response and induce secondary injury following global cerebral ischemia. Also, inhibition of leukocyte endothelial adhesion events will be anti-inflammatory as well as neuroprotective following transient global cerebral ischemia.

1.7.3 Objectives

We aim to test this hypothesis following 10 minutes of bilateral common carotid artery occlusion and reperfusion by:

- i) Determining the temporal profile of CD18 expression on leukocytes and thereby identifying the likely effective duration required for anti-adhesion therapy;
- ii) Demonstrating that the anti-CD18 monoclonal antibody binds to leukocytes to support our theory that anti-CD18 interaction with leukocytes are the cause of inhibition of adhesion events;
- iii) Confirming leukocyte endothelial cell adhesion events are induced with our model of transient global ischemia and that anti-CD18 treatment blocks this adhesion;
- iv) Determining the systemic inflammatory response following global cerebral ischemia and the effect of anti-CD18 on this inflammation;
- v) Determining the cerebral inflammatory response following global cerebral ischemia and the effect of anti-CD18 on this inflammation;
- vi) Determining brain cell death in the selectively vulnerable areas at 3 days and 4 weeks following ischemia and the effect of anti-CD18 therapy on the cell death, and

- vii) Determining the effect of anti-CD18 therapy on spatial memory following global cerebral ischemia using a series of hippocampal depend spatial learning and memory analysis.

Chapter 2:

Material and Methods

2.1 Animals

Male C57Bl/6 mice (Charles River, Montreal, QC), weighing between 19-22g before experimentation, were housed in cages in groups of five. The light/dark cycle was 12:12 with lights on at 06:00, and all treatments were performed between 08:00 and 11:00. The animals were randomized into either global ischemia or sham procedure. All animal protocols used were approved by Laboratory Animal Service at the Hospital for Sick Children, Toronto, Canada.

2.2 Murine Global Ischemia Model.

C57/Bl6 mice were anesthetized by intra-peritoneal (IP) injection of xylazine (10 mg/kg) / ketamine hydrochloride (135 mg/kg) mixture. Rectal temperature was recorded continuously during ischemia and reperfusion, and kept at $37.0 \pm 0.3^\circ\text{C}$ throughout the experimental protocol until recovery from anesthetic, using a heated water mat and an overhead-heating lamp until the mouse had fully recovered. Animals in the ischemic injury group were subjected to a 10-minute bilateral common carotid artery occlusion (BCCAO) as previously described. (G. Yang et al., 1997; K. Murakami et al., 1998; T. Olsson et al., 2003) Cessation of cephalad blood flow was produced by gently retracting the carotid arteries with the ligature for 10 minutes. After the 10 minutes of bilateral common carotid artery occlusion the ligature was released and blood was observed to

reflow. For the sham-operated animals, the carotid arteries were exposed for 10- minutes but no occlusion was performed. The mice had their wound closed with interrupted 5/0 non-absorbable sutures.

2.3 Postoperative recovery and antibody therapy.

Ischemic mice received IP injections of either 2 mg/kg anti-CD18 (IgG2a, kappa light chain Rat-anti-Mouse, M18/2.a.12.7 Developmental Hybridoma Study Bank, University of Iowa, Iowa City, USA), or Isotype matched non-binding control antibody (Purified Rat IgG2a Immunoglobulin Isotype Control, Santa Cruz Inc., California, USA). Surgery, followed immediately by the initial injection of anti-CD18 or control antibody, was done in the morning with the second dose of antibody administered 8 hours later. Two injections of antibody a day (08:00 and 17:00 hours) continued until 3 days post-injury. Animals subject to either sham surgery or cerebral ischemia were then given buprenorphine 0.05 mg/kg (Schering Plough, Pointe-Claire PQ) every 6 to 8 hours for 24 hours beginning just prior to emergence from anesthesia. Animals were then housed, one per cage, in a temperature-controlled environment with a 12-h light/dark cycle and access to standard laboratory chow and water ad libitum. The mortality in the long term studies (survival to 4 weeks) was approximately 20 percent.

Twice daily dosing of 2 mg/kg of anti-CD18 blocked leukocyte adhesion for at least 24 hours whereas once daily dosing did not (data not shown). We injected monoclonal antibody to CD18 into the peritoneal cavity of mice twice a day for 3 days to block maximal CD18 expression following cerebral ischemia.

2.4 Anti-CD18 Antibody Binding Analysis Using Flow Cytometry.

Heparinized blood samples were taken from animals 3 hours following sham or ischemia surgery with an injection of either anti-CD18 or non-binding control. Samples were incubated with FITC labeled anti-Rat antibody (Jackson Laboratories) that recognizes the anti-CD18 antibody following red blood cell lysing using BD PharmLyse (BD bioscience, Canada). The cells were washed and re-suspended in cold wash buffer (PBS/1%FBS/0.1%NaN₃). The samples were analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson LSRII, Canada).

2.5 Flow Cytometric Analysis of CD18 on leukocytes

Leukocytes and subpopulations (polymorphic nuclear cells and mononuclear cells) were identified using flow cytometry (BD FACS Calibur, BD Bioscience, Mississauga, Canada) by their forward and side light scatter characteristics. The percentage of leukocyte populations and mean fluorescence intensity of integrin receptors were determined using monoclonal antibodies against neutrophil specific marker (FITC conjugated rat anti-neutrophil [7/4], Abcam, Cambridge, USA) and CD18 (PE conjugated rat anti-CD18 IgG2a, BD Pharmagen, Mississauga, Canada). Immediately following peripheral blood collection, red blood cells were removed using a commercial lysing solution (BD Pharmlyse, BD Bioscience, Mississauga, Canada) and centrifugation. A 1 µg/million cells concentration of monoclonal antibody was added to the samples and incubated for 30 minutes at 4°C in the dark. Propidium iodide staining solution (BD

Pharmagen, Mississauga, Canada) was used to evaluate cellular viability during flow cytometry. 10,000 viable leukocytes were analyzed in each sample. FlowJo software (v8.6.1, Treestar Inc. Ashland, USA) was used to analyze the data.

2.6 Intra-vital microscopy and assessment of leukocyte-endothelial interactions.

Using an operating microscope (Leitz, Germany), and a high-speed drill (Fine science Tools Inc., Vancouver, British Columbia, Canada), an open cranial window was made in the right parietal bone. A fluorescent intra-vital video-microscope was used to focus on pial venules using a 20X long working distance objective as previously described (I. T. Sutcliffe et al., 2001). Leukocyte trafficking was observed after injection of acridine orange (17 µg/kg, Sigma, St Louis, Missouri, USA) via the tail vein with the microcirculation illuminated using a mercury vapor lamp through a 495/535 nm excitation/emission filter. Real time video recordings were made of 2-4 venules per mouse for 60 seconds each. Image analysis software was used for measuring micro-vessel diameter and distances in the micro-vessels following capture of an image to a personal computer using a frame-grabber. The number of leukocytes rolling and adhering to pial venules was determined in a blinded fashion during playback of the videotapes. Rolling leukocytes were quantified as the number of cells rolling past a reference line in the pial venules over 30 seconds. Adhering leukocytes were quantified as those stationary along a 100 µm length of the pial venule wall for longer than 30 seconds (adhering) over the 60 second recording.

2.7 Serum Sample Preparation

Blood samples were collected via decapitation at 24 hours and 72 hours following global cerebral ischemia with twice daily injections of anti-CD18 or control antibody therapy and sham surgery. Blood samples were allowed to clot for 30 minutes at room temperature and then were centrifuged for 10 minutes at 5000 rpm. The supernatant was collected and stored at -80°C.

2.8 Brain Homogenate Sample Preparation

Animals were randomized into sham or global cerebral ischemia groups with twice daily injections of anti-CD18 or control antibody therapy and sacrificed following 24 hours. The hippocampus was dissected from the brain and manually homogenized in an ice cold solution of 0.32M sucrose in 50mM Tris-HCl, at pH 7.4, containing complete mini protease inhibitors cocktail (Roche Applied Sciences, Laval, QC Canada). The brain homogenate was centrifuged at 14,000 rpm for 12 minutes at 4°C and the supernatant was collected and store at -80°C.

2.9 Cytokine/Chemokine Microbead Array

Serum and brain homogenate samples were analyzed using Millipore multiplex techniques. Interleukin-1beta, IL-6, IL-10, G-CSF, GM-CSF, KC, TNF α , MIP-1 α , and

MCP-1 were analyzed using a Milliplex Map Kit Mouse Cytokine/Chemokine Panel (MPXMCYTO-70K-09, Millipore, Billerica, MA, USA) Median fluorescence intensity was read using a Luminex 100SI apparatus and software (Luminex Corp. Austin, TX, USA). The kits include samples for generation of a 5-parameter logimetric standard curve as well as, quality control samples. The total protein concentration was determined using a standard Branford protein assay. (Bio-Rad Laboratories, Mississauga, ON, Canada)

2.10 Soluble Adhesion Molecule/MMP-9/PAI-1 Microbead Array

Serum samples were analyzed using Millipore Milliplex technology. Soluble E-selectin, sVCAM-1, sICAM-1, MMP-9 and PAI-1 were analyzed using a Milliplex Map Kit Mouse CVD1 Panel (MPXMCVD1-77AK-05, Millipore, Billerica, MA, USA). Median fluorescent intensity was read using a Luminex 100SI apparatus and software (Luminex Corp. Austin, TX, USA). The kits include samples for generation of a 5-parameter logimetric standard curve as well as, quality control samples. The total protein concentration was determined using a standard Branford protein assay. (Bio-Rad Laboratories, Mississauga, ON, Canada)

2.11 Cytokine Immunofluorescent Staining

Animals were randomized into sham or BCCAO groups with twice daily injections of anti-CD18 or control antibody therapy and sacrificed following 24 hours or 72 hours of reperfusion. Frozen brain tissue was sectioned (10 μ m) and mounted on Superfrost/Plus

slides (Fischer Scientific, Ottawa, ON). Tissue was fixed for 30 minutes with 4% paraformaldehyde. Non-specific binding was blocked by incubation with 3% normal donkey serum (Jackson Labs, West Grove, PA) for 1 hour. The slides were incubated overnight at 4°C with either goat anti-mouse TNF α /TNFSF1A (R&D System Inc. Minneapolis, MN) or goat anti-mouse IL-1 β /IL-1F2 (R&D System Inc. Minneapolis, MN) at concentration of 15 ug/ml. Slides were incubated for 30 minutes with Cy3 AffiniPure donkey anti-goat secondary antibody (1:300, Jackson Labs, West Grove, PA). Lastly, slides were mounted with Vectashield hardest mounting medium with DAPI (Vector Labs, Burlingame CA). Areas of the hippocampus, cerebral cortex and thalamus from 2 coronal sections from bregma -1.96 mm were examined using Zeiss Axiovert 200 inverted fluorescence microscope with two separate diode pumped solid state laser line (488m, 561m. Spectral Applied Research, Richmond Hill, ON) and a back-thinned EM-CCD camera (Hamamatsu, Bridgewater, NJ). Volocity Imaging software (v. 4.2.1, Imaging and Vision Company Ltd. Waltam, MA) was used for acquisition and analysis.

Double labeling for neuronal specificity was performed as above, at 24 hours following ischemia, with the addition of incubation with mouse anti-neuronal nuclei (NeuN) Alexa fluor 488 conjugated monoclonal antibody (1:500, MAB377X, Millipore, Billerica, MA, USA). Image acquisition and analysis was also performed as above.

2.12 Histological Analysis: 72 hours Post-ischemia

At 72 hours after sham surgery or ischemia, brains were harvested for histology. After induction of anesthesia, the mice were perfused with normal saline via the left ventricle

for 10 minutes at a pressure of 160 mmHg. Mice were then perfused with 20 mls of 4% paraformaldehyde (PFA) in 1% phosphate buffer solution. The mouse was then decapitated, and the brain extracted by careful removal of the cranium. The brain was then placed in 4% PFA, dehydrated in ethanol, and embedded in paraffin. Coronal slices of 5 μ m were mounted and stained using a standard hematoxylin and eosin protocol. Hippocampal regions CA-1, CA-2, CA-3 and the dentate gyrus (DG) along with the barrel field cortex and the posterior lateral thalamus were examined in 6 sections, at 6 specific locations, ranging from -1.53 mm to -2.80 mm from the bregma and live and dead cells were counted. Live cells were defined as cells showing normal cell architecture. Dead or dying cells were defined as cells showing eosinophilia, pyknosis or vacuolization. The cells were counted by a researcher, who was blinded to the experimental group of the mice, using Image-J software. (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2007)

2.13 Histological Analysis: 4 weeks Post-ischemia

At 4 weeks after sham surgery or ischemia, brains were harvested for histology. The tissue was prepared as above. The total number of live cells (dead or dying cells were not seen by 28 days post-ischemia) in the hippocampal regions CA-1, CA-2 and CA-3 were counted from 2 adjacent coronal sections at bregma -1.96 mm. The cells were again counted by a researcher, who was blinded to the experimental group of the mice, using Image-J software. (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2007)

2.14 Water Maze Training Procedures and Spatial Memory Analysis

All behavioural testing was done in a circular water maze (diameter 120 cm, depth 50 cm) in a dimly lit room. The water maze tank was filled with water to a depth of 40 cm and the water temperature was maintained at $28^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ by a heating mat. Non-toxic white paint was added to make the water opaque and the escape platform (diameter 10 cm) was submerged 0.5 cm below the water level to ensure that there were no visual cues in the tank. Surrounding the tank were white curtains with distinctive visual cues painted on them. All training and probe tests were video recorded and data were analyzed using an automated tracking system (Actimetrics, Wilmette, Illinois, USA). The mice were handled twice a day, with each session lasting 2 minutes, for one week prior to water maze training. For each trial, the mouse was placed facing the wall of the pool at one of the four start locations, which were determined pseudo-randomly. The trial was completed when the mouse either found the platform and remained there for at least 2 seconds, or the 60 second time limit elapsed. If the mouse was unable to find the platform, the water maze operator guided the mouse to the platform. The water maze training day consisted of 6 trials (presented in 2 blocks of 3 trials; inter-block interval was 3 hours; inter-trial interval was approximately 15 seconds). The mice received 5 days of training prior to ischemia or sham surgery. During training the time required to reach the platform was recorded; improved performance was seen throughout the training period. The mice were then randomized to 3 experimental groups: 1) sham surgery; 2) 10 minutes of global cerebral ischemia with twice-daily injections of anti-CD18 antibody or 3) ischemia with twice-daily injections of isotype matched non-binding control antibody.

On the morning of the 7th day following ischemia spatial memory was assessed in a 60 second probe test in which the platform had been removed from the pool. The mice were placed in the pool at the same location and allowed to search for the platform. During the probe test a number of parameters were recorded. The time spent in the each zone, swim speed and time spent within 5 cm of the edge of the pool was recorded. The target or trained zone was an area in the southwest quadrant of the water maze with a 20 cm radius, centered on the former location of the platform and represented 11% of the total surface area of the tank. The time spent in zones of equal size and location in the other 3 quadrants of the pool was also recorded.

To assess if the animals could relearn to find the platform, training procedures were recommenced from day 7 to 11 following ischemia or sham surgery. The water maze training day, again, consisted of 6 trials (presented in 2 blocks of 3 trials; inter-block interval was 3 hours; inter-trial interval was approximately 15 seconds). On the morning of the 21st day following ischemia, spatial memory was assessed in another 60-second probe test. All recordings in the Morris water maze were done by a researcher who was blinded to the experimental group of the mice.

2.15 Statistical Analysis

A Student's unpaired t-test or one-way analysis of variance was done with post-hoc Student-Newman-Keuls method for pairwise comparisons of subgroups when data followed a normal distribution. A Mann-Whitney U test or Kruskal-Wallis test with post-hoc Mann-Whitney U test method for pairwise comparison of subgroups with Bonferroni

correction was done when non-parametric distribution of data was observed. Normality was determined by the Kolmogorov-Smirnov test. Results were considered to be statistically significant if $p < 0.05$. All statistics were performed using SPSS (v16.0.1, SPSS Inc. Chicago, Illinois, USA, 2007).

CHAPTER 3: RESULTS

3.1 CD18 expression was increased on leukocytes in the peripheral blood following global cerebral ischemia.

Neutrophil and mononuclear leukocyte subpopulations in the peripheral blood were identified and quantified using flow cytometry gating for forward and side scatter characteristics and using antibody markers. A rapid increase in neutrophil CD18 mean fluorescence intensity was observed at 4 hours post-ischemia and this reached a maximum at 48 hours post-ischemia (Fig. 2A). A delayed increase in CD18 mean fluorescence intensity was observed on mononuclear leukocytes with a significant increase at 24, 48 and 72 hours following ischemia (Fig. 2B). There was a predominance of neutrophils in peripheral blood at 4 hours post-ischemia and a predominance of mononuclear cells at 24, 48 and 72 hour. (Fig. 2C)

3.2 Monoclonal antibody to CD18 bound to leukocytes.

The binding of the monoclonal antibody to CD18 on the surface of leukocytes in the peripheral blood was demonstrated using flow cytometry following an injection of the anti-CD18 monoclonal antibody into the peritoneal cavity of C57Bl/6 mice. We observed a significantly higher mean fluorescence of antibody to CD18 on leukocytes at 3 hours following cerebral ischemia compared to sham surgery and control Ig treatment. (Fig. 3)

3.3 Leukocyte adhesion to endothelial cells was inhibited by a monoclonal antibody to CD18.

We confirmed that leukocyte rolling and adhesion to endothelial cells was increased at 3 hours following 10 minutes of bilateral carotid artery occlusion and reperfusion using fluorescent intravital microscopy through a cranial window. (Fig. 4A) Anti-CD18 treatment reduced leukocyte rolling and blocked adhesion to endothelial cells, in pial venules at 3 hours following ischemia, compared to mice that were injected with the control antibody. (Fig. 4B)

3.4 Anti-CD18 blocked the systemic inflammatory response after global cerebral ischemia.

The cytokine/chemokine concentrations in the peripheral blood serum following ischemia were evaluated using a microbead protein array. There was an increase in a number of inflammatory mediators at 24 and 72 hours following global cerebral ischemia, and this inflammatory response markedly inhibited by anti-CD18 treatment. (Fig. 5)

At 24 hours post-ischemia with non-binding control antibody therapy there was a significant increase in IL-1 β , IL-6 and G-CSF concentrations, as well as, a trend towards an increased MIP-1 α concentration. (Fig 5A,B,E,H; left) Also, at 72h post-ischemia with control antibody therapy, a more potent inflammatory was observed with significant increases in IL-1 β , IL-6, TNF α , IL-10, and MIP-1 α and a trend towards increased in

MCP-1 concentration. (Fig. 5A,B,C,D,G,H; right) Inhibiting leukocyte adhesion to endothelial cells blocked or attenuated the IL- β , IL-6, TNF α , IL-10, MIP-1 α and MCP-1 inflammatory responses. (Fig. 5A,B,C,D,G,H) Anti-CD18 treatment did not effect the concentration of G-CSF in the peripheral blood following ischemia. (Fig. 5E) There were no significant changes in GM-CSF and KC concentration in peripheral blood following ischemia. (Fig 7A,B)

3.5 Anti-CD18 augmented systemic concentrations of soluble adhesion molecules following global cerebral ischemia.

There was a significant reduction in sICAM-1 and sVCAM-1 following global cerebral ischemia with control antibody treatment. Following ischemia, levels of sICAM-1 and sVCAM-1 were similar to sham when leukocyte adhesion was blocked with anti-CD18 treatment. (Fig. 6A,B) Anti-CD18 treatment caused a significant increase in soluble E-selectin. (Fig. 6C) There were no significant changes in the concentrations of MMP-9 and PAI-1 in peripheral blood following ischemia. (Fig. 7D,E)

3.6 Anti-CD18 inhibited interleukin-1 β expression in the brain following global cerebral ischemia.

Two key pro-inflammatory cytokines, IL-1 β and TNF α were expressed in the brain following global cerebral ischemia. (Fig. 8) Microscopic images showing the immunofluorescence of IL-1 β and TNF α , in sections of mouse cerebral cortex, are shown

at 72 hours following sham or ischemia surgery in figure 8A-F. Cells expressing IL-1 β were confirmed to be neurons using double staining for IL-1 β and NeuN (Fig. 9). Blocking CD18 significantly attenuated this increase in IL-1 β in the hippocampus by 24 hours post-ischemia (Fig. 8G) and in the cerebral cortex and thalamus by 72 hours post-ischemia (Fig. 8H) compared to control antibody treatment. There was also a significant increase in the number of cells expressing TNF α following ischemia. However, blocking CD18 had no effect on the number of cells expressing TNF α compared to control antibody treatment. (Fig. 8I,J)

3.7 Anti-CD18 blocked the cerebral inflammatory response after global cerebral ischemia.

The hippocampus is selectively vulnerable to cell death following global ischemia. At 24 hours post-ischemia immunofluorescence staining showed an increase in IL-1 β expression that was attenuated with anti-adhesion therapy. A cytokine/chemokine multiplex array was used to examine the concentration of IL-1 β and 8 other inflammatory mediators in the hippocampus at 24 hours post-ischemia. There was a marked increase in the hippocampal expression of IL-1 β , IL-6, G-CSF, MCP-1 and KC following ischemia with control antibody treatment. Anti-adhesion therapy attenuated the expression of these inflammatory mediators. (Fig. 10A,B,E,I) There were no significant changes in hippocampal concentrations of IL-10, MIP-1 α , TNF α and GM-CSF. (Fig. 10C,D,F,H)

3.8 Anti-CD18 inhibited cell death after global cerebral ischemia.

We examined dead and dying cells in brain sections stained with hematoxylin and eosin in the areas previously described to be vulnerable in this model; the hippocampus, cerebral cortex and thalamus. Dead and dying cells were evaluated in these regions following sham or ischemic surgery with anti-CD18 antibody or control antibody therapy (Fig. 11A,B). Animals injected with anti-CD18 treatment following ischemia had increased cell survival in the CA-1, CA-2, and CA-3 regions of the hippocampus and the barrel field cortex and thalamus at 3 days following injury compared to ischemic control mice. The dentate gyrus (DG) was resistant to ischemia damage. (Fig. 11C) There was no cellular death seen in the sham surgery group.

By 4 weeks after ischemia dead cells had been removed and therefore damage was assessed by a decrease in total hippocampal pyramidal cells in brain sections stained with hematoxylin and eosin. There was a significant increase in cellular survival, in the hippocampal pyramidal cell layer following cerebral ischemia, in the anti-CD18 therapy group compared to the non-binding control antibody group (Fig. 12).

3.9 Anti-CD18 therapy preserved spatial memory after global cerebral ischemia.

To evaluate whether anti-CD18 treatment rescued memory deficits induced by ischemia a Morris water maze test was used. Mice were randomized to the sham surgery or ischemia

with anti-CD18 or non-binding control antibody treatments. The mice were trained to find a submerged platform in a pool for 5 days and then subjected to ischemia or sham surgery (Fig. 13A). Memory was subsequently tested in a probe test at 7 days following ischemia or sham operation. The platform was removed from the pool and the mice were allowed to search for it for 60 seconds. The mice in the anti-CD18 treated ischemic group and sham surgery group spent more time searching for the platform in the target zone, where the platform was previously located, compared to the ischemic control group which spent a similar amount of time in each of the 4 quadrants of the pool (Fig. 13B,C). The swim speeds were similar between all experimental groups. (Fig. 13D) The animals that received the control antibody following global cerebral ischemia spent significantly more time in the area close to the pool wall compare to sham and anti-CD18 groups. (Fig. 13E)

To assess if the animals could re-learn to find the platform mice were trained again for 5 days from day 7 to 11 post-ischemia (Fig. 14A). On day 21, following ischemia or sham surgery, spatial memory was re-assessed in another 60-second probe test. Although there was some recovery of spatial learning in the ischemic control group, the mice in the ischemic anti-CD18 group continued to demonstrate improved performance compared to the ischemic control group. The ischemic anti-CD18 and sham groups spent significantly more time in the target zone compared to other zones, where the ischemia control Ig group spent a similar amount of time in the target and adjacent zones (Fig. 14B,C). Similar to the 7 day probe test, the swim speeds were similar between all groups. (Fig. 14D) and the animals that received the control antibody following cerebral ischemia spent significantly more time near the tank walls. (Fig. 14E)

CHAPTER 4

DISCUSSION

4.1 Major Findings:

We have shown that transient global ischemia leads to increased leukocyte adhesion and a systemic inflammatory response characterized by a marked increase in cytokines, chemokines and other inflammatory proteins in peripheral blood. Also, there was an increase in the expression of inflammatory proteins in the hippocampus, an area selectively vulnerable to ischemic injury. One of the pro-inflammatory cytokines, IL-1 β , was expressed in neurons following ischemia. Lastly, we have demonstrated that by blocking adhesion of leukocytes to endothelial cells the systemic inflammatory response and cerebral inflammatory proteins, specifically IL-1 β expression, were reduced. Anti-leukocyte adhesion therapy, also, preserved the cellular integrity of the hippocampus, cerebral cortex and thalamus following ischemia. These findings were supported by a robust series of water maze experiments designed to test spatial learning and memory. At 7 days following ischemia, anti-CD18 treated animals had improved spatial memory compared to the ischemic control antibody group. Although there was some recovery in the ischemic control group, the anti-CD18 treatment groups continued to demonstrate better spatial learning and memory in the second probe test at 21 days following cerebral ischemia. These results indicate that leukocyte adhesion plays a vital role in the global ischemic inflammatory response, and the mechanism leading to cells death in our model of global cerebral ischemia.

4.2 Neuroprotective Effects of Inhibition of Leukocyte Adhesion

Our findings are supported by a study by Xu and colleagues. They investigated the effect of inhibiting vascular adhesion protein-1-dependent leukocyte adhesion following transient forebrain ischemia in diabetic ovariectomized female rats, a model that promotes inflammation. They found that the increased inflammation and infiltration of leukocytes in the brain post-ischemic was decreased with anti-adhesion therapy. (H. L. Xu et al., 2006) In our model we demonstrated leukocyte adhesion in the microcirculation, but without infiltration of leukocytes into the brain, following the transient forebrain ischemia. This suggests a novel finding in our research that the adhesion of leukocytes to endothelial cells without transmigration is sufficient to promote cell death following ischemia.

Inhibiting leukocyte adhesion to endothelial cells has been associated with improved neuronal survival in other models of global cerebral ischemia. In a gerbil global ischemia model, Hauck and colleagues demonstrated that blocking endothelin_A receptors achieved inhibition of leukocyte-endothelial adhesion and was associated with improved survival of cortical neurons. (E. F. Hauck et al., 2007) Endothelin receptors are expressed in the cerebral microvasculature receptors and have been shown to play a critical in modulating leukocyte activity. (G. M. Rubanyi and M. A. Polokoff, 1994) A single dose of Endothelin_A receptor antagonist was administered at the onset of reperfusion. These researchers demonstrated a dose-depend decrease in both rolling and adherent neutrophils following insult. In another recent paper, Atlay and colleagues reported that Slit protein inhibits leukocyte adhesion and is neuroprotective in a mouse model of global ischemia. (T. Altay et al., 2007) The Slit family of secreted proteins that act to repel leukocyte

recruitment in response to cytokine and chemokine activation and thereby inhibit the interaction of leukocytes on the endothelium. The anti-leukocyte therapy was given continuously for 24 hours begin at onset of reperfusion and blocked leukocyte adherence for duration of treatment. There was significant survival of hippocampus region CA-1 neurons seven days following global ischemia. These results support our findings that leukocyte adhesion is a critical step in global ischemia/reperfusion injury but do not address specific changes in inflammatory mediators or demonstrate long-term (greater than 7 days) effects of anti-adhesion therapies.

Previous investigators have shown that anti-adhesion therapy following global cerebral ischemia prevents breakdown of the BBB. Leukocyte adherence following global ischemia was examined in a piglet birth asphyxia vascular injury model. Gidday and colleagues demonstrated that an anti-CD18 antibody therapy decreased leukocyte adhesion to the endothelium maintained normal BBB vascular permeability. (J. M. Gidday et al., 1997) These finding support our hypothesis that leukocyte adhesion plays a key role in pathology following global ischemia. However, the investigators were unable correlate the protection of endothelial integrity with neuroprotection in vulnerable areas of the brain. Both systemic and cerebral inflammation can influence the permeability of the BBB. Gidday and colleagues' findings may support an inflammatory mechanism for the breakdown of the BBB following global cerebral ischemia that the anti-CD18 treatment inhibited.

Therapies inhibiting leukocyte rolling appear to be associated with poorer outcome after global ischemia. In contrast to our finding, Lehmberg and colleagues found that anti-P-selectin therapy decreased survival in a gerbil model of global ischemia. (J.

Lehmberg et al., 2006) Possible differences between this study and our experimental design that may account for the contrasting conclusions include the use of a different species and models of ischemia (gerbil versus mouse), as well as differences in the target and duration of leukocyte-endothelial interaction blockade. These investigators administered a single pre-ischemia dose of anti-P-selectin, that decreased rolling leukocytes during the first 3 hours post-ischemia, however, there was no significant decrease in firmly adherent leukocyte during the same time period. In contrast, we inhibited firmly adherent leukocytes, and maintained the anti-adhesion therapy for 3 days post-ischemia. The requirement for 3 days of treatment is supported by our findings that CD18 expression begins early and is sustained on neutrophil and mononuclear leukocytes.

4.3 The Systemic Inflammatory Response in the Peripheral Blood

Our findings that the increase in cytokines and chemokines in the peripheral is delayed, generally reaching levels significantly greater than sham concentrations by 72 hours post-ischemia, may be explained movement of inflammatory mediators or their stimuli from the brain and CSF to the peripheral blood. In a study of global ischemia/reperfusion injury, serum cytokines concentrations did not correlate strongly with cerebral expression of cytokines or with injury. (M. Wender et al., 2005) In another study, CSF concentrations of cytokines did correlate with the severity of the brain lesion. (J. S. Kim, 1996) The delayed increase in serum inflammatory mediators coincides with the breakdown of the BBB following ischemia. Global ischemia/reperfusion injury can open

the BBB to the leakage to both low and high molecular weight marker. (Y. Nakagawa et al., 1990; W. D. Dietrich et al., 1991) It is possible that the cytokines from the brain and CSF could cross into the blood stream or inflammatory triggers are released as a result of the BBB breakdown that cause the peripheral system to unregulated inflammatory mediators. In preliminary studies in our laboratory, we have shown a loss of BBB integrity at 72 hours post-ischemia and protection of BBB integrity with anti-adhesion therapy in our model. (Data not shown) Disruption of the BBB has been shown to occur following global cerebral ischemia in a number of studies. (M. J. Mossakowski et al., 1994; M. Shinnou et al., 1998) Therefore, a possible mechanism of neuroprotection by anti-leukocyte adhesion therapy is via the attenuation of serum cytokines and chemokines along with the preservation of the BBB.

4.4 Effects of Ischemia on Soluble Adhesion Molecules

Soluble ICAM-1 and soluble VCAM-1 have been shown to be increase in the serum following an inflammatory insult, as well as acting as a marker for stroke severity in humans. (A. Blann et al., 1999) We, however, observed the opposite finding with a significant decrease in serum concentration of sICAM-1 and sVCAM-1 following global ischemia. The reduction below sham control concentrations is consistent with finding presented by Clark and colleagues. (W. M. Clark et al., 1993) They observed a reduction in circulating ICAM-1, even though neutrophil adhesion was significantly increased. They postulated that since the cleaved ICAM-1 retained most of the structure and function of the membrane bound protein, it could bind to CD18 on leukocytes. With the

increase in number and activity of CD18 on leukocytes, supported by our data, there may be a sequestration of the sICAM-1.

4.5 The Cerebral Inflammatory Response

Blocking leukocyte adhesion resulted in a reduction in the magnitude of several cerebral inflammatory cytokines and chemokines. This directly supports our hypothesis that leukocyte adhesion is a critical step in the cerebral inflammatory response following global ischemia even though leukocytes are not observed to transmigrate across the microvessel wall. The attenuation of IL-1 β expression by anti-adhesion therapy represents a promising mechanism of neuroprotection since IL-1 β has been shown to exacerbate ischemic damage. (C. A. Davies et al., 1999; O. Touzani et al., 2002) There is increasing evidence that IL-1 β is a critical mediator of neuronal cell death following cerebral ischemia in both rodent models of focal ischemia and *in vitro* models of neuronal hypoxia. (E. Pinteaux et al., 2006; B. Fogal et al., 2007; B. W. McColl et al., 2007) Interleukin-1 has been shown to be essential for the progression of neurodegeneration following hypoxic/ischemic injury. (A. Basu et al., 2005) Fogal and colleagues have recently shown that the enhancement of ischemia-induced neuronal cell death by IL-1 β may be dependant on astrocyte-mediated alterations in the cystine/glutamate antiporter, which promotes excitotoxicity. (B. Fogal et al., 2007) Interleukin-1 β has also been shown to influence ischemic injury independent of IL-1 receptor Type I receptors, suggesting multiple pathways of IL-1 β mediated damage. (O. Touzani et al., 2002) Therefore, further studies are necessary to determine the mechanisms by which leukocyte adhesion

to endothelial cells contributes to IL-1 β -mediated cell death in neurons following transient forebrain ischemia.

We observed that IL-1 β was exclusively found in neurons during the initial days post-ischemia. In addition to neuronal expression, IL-1 β expression in the CNS has been reported in glial cells following ischemia injury. (N. J. Rothwell and G. N. Luheshi, 2000; C. X. Wang and A. Shuaib, 2002) However, during our model of global cerebral ischemia microglial and astrocytic cells do not reach maximal activation until as late as 7 days post-insult. (M. B. Jorgensen et al., 1993; M. Endoh et al., 1994) This suggests that IL-1 β may exhibit an expression profile similar to TNF α following global ischemia with a neuronal expression acutely (T. Liu et al., 1994) and glial expression later on. (H. Uno et al., 1997)

In addition to IL-1 β , many other cytokines and chemokines examined also decrease with anti-leukocyte adhesion therapy. This is consistent with the interconnected nature of the cerebral inflammatory response. A possible mechanism that may account for a number of anti-leukocyte adhesion anti-inflammatory effects are via the Nuclear factor- κ B (NF- κ B) activated pathways. Nuclear Factor promoter sites are found in many inflammatory cytokine and chemokine genes including IL-1 β , TNF α , MCP-1, MIP-1 α and IL-8/KC (P. J. Nelson et al., 1993; N. Mukaida et al., 1994; U. Siebenlist et al., 1994; A. Ueda et al., 1994) Nuclear factor- κ B also influence the expression of adhesion factors such as ICAM-1, VCAM-1 and E-selectin (A. van de Stolpe et al., 1994; M. F. Iademaro et al., 1995). Also, leukocyte adhesion has been demonstrated to be required for NF- κ B activation. (I. S. Bhullar et al., 1998; S. Klein et al., 2002; R. Kettritz et al.,

2004) Nuclear Factor- κ B has been shown to partially mediate free radicals (R. Schreck et al., 1991), cause glutamate excitotoxicity (M. Grilli et al., 1996), up-regulation of iNOS and COX-2 (K. Schulze-Osthoff et al., 1997) and activation of CD95 in FAS mediated apoptosis (M. Vogt et al., 1998). Also, NF- κ B decoy therapy in neurons post-global cerebral ischemia was shown to attenuate IL-1 β and ICAM-1 expression in the hippocampus during the initial reperfusion and promoted neuronal survival at 7 days following injury. (T. Ueno et al., 2001)

4.6 Spatial Memory Assessment

Our findings of impairment of spatial memory measured using the Morris water maze are consistent with previous findings examining memory following global cerebral ischemia. In general, the spatial memory deficit is directly related to the hippocampal damage following ischemia, specifically CA-1 injury. (A. Nelson et al., 1997; F. Block, 1999) The probe test, that we used, has been shown to be sensitive to global ischemia induced spatial memory deficits. (J. W. Wright et al., 1996; F. Block and M. Schwarz, 1997; A. Nelson et al., 1997)

Since the swim speeds were similar across experimental groups and treatments, it is likely that the hippocampal injury rather than motor performance impairment accounts for the neurological deficit in our model. Consistent swim speeds have been reported in multiple studies with global cerebral ischemia. (A. Nelson et al., 1997; F. Block and M. Schwarz, 1998)

Thigmotaxis is the natural tendency of the animal to spend time close to the walls of the pool. It is a behaviour that does not have a benefit in successful searching for the submerged platform and is routinely observed in untrained animals. A return to this behaviour, consistent with our observations, has been shown to be associated with hippocampal injury. (F. Block and M. Schwarz, 1998)

Our model of ischemia was shown to induce hippocampal injury along with a spatial memory deficit, both of which were reduced with anti-adhesion therapy. During the platform training sessions between day 7 and day 11 post-ischemia working memory was evaluated. Although a greater latency time was observed with the ischemia without anti-adhesion therapy in the early sessions, during the final sessions all mice able to find the platform with continued training demonstrating an intact working memory system. This has also been observed in other rodent models of global ischemia (F. Block and M. Schwarz, 1998) and suggests that the areas of the hippocampus damaged with global ischemia plays less of a role in working memory compared to its role spatial memory referencing. There was, also, some improvement of the mice subjected to ischemia with the control antibody at the day 21 probe test compared to its day 7 probe test performance. This presents the possibility that the global ischemia induced spatial memory impairment is only transient. However, studies have shown that the behavioural deficits evaluated using a Morris water-maze can be detected up to 20 weeks post injury. (A. Nelson et al., 1997) A possible explanation for the improved memory retention and recall test results could be due neurogenesis in the hippocampus. This possibility was examined by Bueters and colleagues in a rat model of global ischemia. They noted that beginning 21 days post-injury and continuing for 90 days, there was evidence of newly

formed neurons. This neurogenesis was associated with improved performance in the Morris water maze. (T. Bueters et al., 2008) However, this neurogenesis and cognitive improvement was only temporary with all newly derived neurons and improved spatial disappearing by approximately 150 days post injury. (T. Bueters et al., 2008)

4.7 Relevance to Clinical Trials

Modulation of leukocytes endothelial interactions has been a popular target for clinical trials as a therapy to decrease the mortality and morbidity following stroke. These trials have all failed to show any observable benefit with the inhibition of the adhesion molecules involved in the firm adhesion of leukocytes. (M. E. Sughrue et al., 2004) One possible examination for this in the large therapy window used, with therapy administrated in one trial as late as 12 hours following the onset of stroke symptoms. Also, they did not focus specifically on ischemia/reperfusion injury with the inclusion of permanent and prolonged ischemia injuries for which these therapies are unlikely to be beneficial.

Our results support leukocyte adhesion to the endothelial as the critical stage during neuroinflammation and secondary injury during moderate global ischemia/reperfusion injury. Since these events can begin almost immediately following reperfusion, we show evidence that the therapeutic timing of the onset of treatment used may be too late. Although the timing of anti-leukocyte therapies appears to precede the infiltration of neutrophils into damage area, it would be insufficient to block the potential signaling initiated from leukocyte adhesion. Also, reperfusion is key in the initiation of

neuroinflammation and secondary damage. It has been proposed that with permanent or prolonged ischemia neuronal death pathways are primary Ca^{2+} mediated (B. K. Siesjo, 1988) and thereby would not represent an opportunities for anti-inflammatory interventions. The neuroprotection we observed was during a moderate ischemia duration followed by reperfusion reiterating the value of these therapies when treating this particular type of injury.

A modest benefit with the neutrophil inhibitory factor, UK-279,276, was observed when the therapy was co-administered with t-Pa. Tissue plasminogen activator is a used to promote reperfusion following embolic stroke and has a very narrow therapy window with a maximum treatment commencement time of 3 hours post-stroke. (M. E. Sughrue et al., 2004) Therefore, an antagonist to CD18/CD11b showed slight benefits when administered early (within 3 hours) with likely reperfusion. Further examination of these therapies are warranted and a greater understanding of the mechanism and differences between types of stroke is also important area research need before a effective clinical can be developed.

4.8 Limitations

This study has several limitations. The bilateral common carotid artery occlusion model of global cerebral ischemia is a widely used and extensively studied model, however, in some ways it lacks direct clinical relevance. The abrupt onset of ischemia and abrupt complete restoration of blood flow does not reflect an unrealistic reperfusion paradigm.

Also, since we only used male mice, we may not have accounted for sexual dimorphic differences in the response to global cerebral insults.

Also, the anti-leukocyte treatment schedule following ischemia is unlikely to be achievable in a “real world” situation. We commenced anti-CD18 treatment immediately following ischemia since leukocytes are shown to interact with microvasculature within seconds of reperfusion. However, under clinical condition this would be impossible. Varying the onset antibody injections following ischemia would be needed to establish the therapeutic window of anti-leukocyte adhesion therapy.

Lastly, there is the possibility that anti-CD18 antibody effects following ischemia are not propagated via the inhibition of leukocyte adhesion to the endothelial cells but by some unknown mechanism. Even though we showed that CD18 is up-regulated on the leukocytes for three days following injury and that the anti-CD18 monoclonal antibody binds to the surface of leukocytes, it does not preclude the possibility that the antibody treatment generates multiple effects that appear to happen simultaneously with these observations. Perhaps, a lower dose of anti-CD18 may continue to inhibit leukocyte adhesion but lose the therapeutic efficacy. Further dose/response experiments are warranted

4.9 Conclusions

In conclusion, we reported that leukocyte anti-adhesion therapy blocked inflammation and provided histological and neurobehavioral protection after transient global forebrain

ischemia in mice. Anti-inflammatory mechanisms may be responsible for the improvement in both histological and neurobehavioural outcome observed in this model of global cerebral ischemia. Furthermore the short term protective effect were maintained for at least one month following injury suggesting that the neuroprotection mediated by anti-adhesion therapy during the first three days post-injury is lasting rather than transient. We hypothesize that the neuroprotective mechanism is by inhibiting IL-1 β – mediated neuronal cell death following global cerebral ischemia.

CHAPTER 5

FUTURE DIRECTIONS

We recommend further research into the mechanisms by which leukocyte adhesion to cerebral endothelial cells leads to neuronal cell death following global ischemia/reperfusion injury as well as address some concerns brought up in the limitations section.

To confirm that the neuroprotective and anti-inflammatory effects can be attributed to the inhibition of leukocyte adhesion and not an allosteric effect of the treatment, a series of pharmacodynamic experiments should be performed. These will focus on demonstrating a cause and effect relationship between leukocyte adhesion and inflammatory mediator concentrations and neuronal damage. Also, receptor occupancy, a dose/response curve and the use of CD18 anti-sense experiments will further strengthen the support of our hypothesis. Next, determination of the effective therapeutic window is also required to formulate a realistic treatment schedule.

The future directions for this project include investigating the inflammatory mechanisms leading to neuronal cell death and impairment of neurological function following global cerebral ischemia, with a focus on the role of IL-1 β mediated neurological injury. We plan to investigate how inflammatory proteins, specifically IL-1 β , are released during leukocyte-endothelial cell adhesion, what mediators across the BBB leading to cerebral inflammation and mechanisms leading to neuronal cell death in rodent *in vivo* and *in vitro* models of cerebral ischemia. Extending our finding from the

global cerebral ischemia model to a cardiac arrest model of neurological injury may also increase the clinical relevance of our studies.

Specific experiments that would examine the mechanism of how leukocyte adhesion to the endothelium propagates inflammation and secondary injury could include mass spectrometry, laser capture and *in vitro* co-culture. Mass spectrometry of peripheral blood serum may identify a mediator that is released during ischemia induced leukocyte adhesion. Proteomic analysis of endothelial cells may also show differential protein up regulation as a result of the leukocyte adhesion. Lastly, using an *in vitro* co-culture system may be helpful in recreating the cell-cell interaction in a more controlled setting. Endothelial cells cultured on an ultra thin membrane with the cells of the CNS can construct an *in vitro* blood brain barrier. Using this model we could induce hypoxic/ischemic conditions and with leukocytes on the surface of endothelial and delineate the mechanism and signaling pathways.

Overall, we hope to identify how leukocyte adhesion to the endothelial cells, without the transmigration across the blood brain barrier, can signal secondary damage following cerebral ischemia/reperfusion injury.

REFERENCES

- (2001) Use of anti-ICAM-1 therapy in ischemic stroke: results of the Enlimomab Acute Stroke Trial. *Neurology* 57:1428-1434.
- Abbassi O, Lane CL, Krater S, Kishimoto TK, Anderson DC, McIntire LV, Smith CW (1991) Canine neutrophil margination mediated by lectin adhesion molecule-1 in vitro. *J Immunol* 147:2107-2115.
- Aguirre GK, Detre JA, Alsop DC, D'Esposito M (1996) The parahippocampus subserves topographical learning in man. *Cereb Cortex* 6:823-829.
- Albelda SM (1991) Endothelial and epithelial cell adhesion molecules. *Am J Respir Cell Mol Biol* 4:195-203.
- Allan SM, Rothwell NJ (2001) Cytokines and acute neurodegeneration. *Nat Rev Neurosci* 2:734-744.
- Altay T, McLaughlin B, Wu JY, Park TS, Gidday JM (2007) Slit modulates cerebrovascular inflammation and mediates neuroprotection against global cerebral ischemia. *Exp Neurol* 207:186-194.
- Arend WP (1991) Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *J Clin Invest* 88:1445-1451.
- Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH (2000) Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. *J Cereb Blood Flow Metab* 20:1681-1689.
- Aspey BS, Jessimer C, Pereira S, Harrison MJ (1989) Do leukocytes have a role in the cerebral no-reflow phenomenon? *J Neurol Neurosurg Psychiatry* 52:526-528.
- Back T, Hemmen T, Schuler OG (2004) Lesion evolution in cerebral ischemia. *J Neurol* 251:388-397.
- Bajetto A, Bonavia R, Barbero S, Florio T, Schettini G (2001) Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol* 22:147-184.
- Bargatze RF, Kurk S, Butcher EC, Jutila MA (1994) Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J Exp Med* 180:1785-1792.
- Barone FC, Feuerstein GZ (1999) Inflammatory mediators and stroke: new opportunities for novel therapeutics. *J Cereb Blood Flow Metab* 19:819-834.
- Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ (1998) Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 29:1937-1950; discussion 1950-1931.
- Bassetti C, Bomio F, Mathis J, Hess CW (1996) Early prognosis in coma after cardiac arrest: a prospective clinical, electrophysiological, and biochemical study of 60 patients. *J Neurol Neurosurg Psychiatry* 61:610-615.
- Basu A, Lazovic J, Krady JK, Mauer DT, Rothstein RP, Smith MB, Levison SW (2005) Interleukin-1 and the interleukin-1 type 1 receptor are essential for the progressive neurodegeneration that ensues subsequent to a mild hypoxic/ischemic injury. *J Cereb Blood Flow Metab* 25:17-29.

- Becker K, Kindrick D, Relton J, Harlan J, Winn R (2001) Antibody to the alpha4 integrin decreases infarct size in transient focal cerebral ischemia in rats. *Stroke* 32:206-211.
- Becker KJ (1998) Inflammation and acute stroke. *Curr Opin Neurol* 11:45-49.
- Becker KJ (2002) Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke. *Curr Med Res Opin* 18 Suppl 2:s18-22.
- Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* 43:1369-1374.
- Berti R, Williams AJ, Moffett JR, Hale SL, Velarde LC, Elliott PJ, Yao C, Dave JR, Tortella FC (2002) Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. *J Cereb Blood Flow Metab* 22:1068-1079.
- Betz AL, Yang GY, Davidson BL (1995) Attenuation of stroke size in rats using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist in brain. *J Cereb Blood Flow Metab* 15:547-551.
- Bhullar IS, Li YS, Miao H, Zandi E, Kim M, Shyy JY, Chien S (1998) Fluid shear stress activation of IkappaB kinase is integrin-dependent. *J Biol Chem* 273:30544-30549.
- Blann A, Kumar P, Krupinski J, McCollum C, Beevers DG, Lip GY (1999) Soluble intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1 and von Willebrand factor in stroke. *Blood Coagul Fibrinolysis* 10:277-284.
- Block F (1999) Global ischemia and behavioural deficits. *Prog Neurobiol* 58:279-295.
- Block F, Schwarz M (1997) Correlation between hippocampal neuronal damage and spatial learning deficit due to global ischemia. *Pharmacol Biochem Behav* 56:755-761.
- Block F, Schwarz M (1998) Global ischemic neuronal damage relates to behavioural deficits: a pharmacological approach. *Neuroscience* 82:791-803.
- Block F, Schwarz M (1998) Global ischemic neuronal damage relates to behavioural deficits: a pharmacological approach. *Neuroscience* 82:791-803.
- Bokura H, Robinson RG (1997) Long-term cognitive impairment associated with caudate stroke. *Stroke* 28:970-975.
- Bowes MP, Zivin JA, Rothlein R (1993) Monoclonal antibody to the ICAM-1 adhesion site reduces neurological damage in a rabbit cerebral embolism stroke model. *Exp Neurol* 119:215-219.
- Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. *Science* 303:1532-1535.
- Bueters T, von Euler M, Bendel O, von Euler G (2008) Degeneration of newly formed CA1 neurons following global ischemia in the rat. *Exp Neurol* 209:114-124.
- Burgess N, Maguire EA, O'Keefe J (2002) The human hippocampus and spatial and episodic memory. *Neuron* 35:625-641.
- Cai TQ, Wright SD (1996) Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. *J Exp Med* 184:1213-1223.

- Cammalleri R, Gangitano M, D'Amelio M, Raieli V, Raimondo D, Camarda R (1996) Transient topographical amnesia and cingulate cortex damage: a case report. *Neuropsychologia* 34:321-326.
- Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L (1993) Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc Natl Acad Sci U S A* 90:10061-10065.
- Carlos TM, Harlan JM (1994) Leukocyte-endothelial adhesion molecules. *Blood* 84:2068-2101.
- Chan PH, Schmidley JW, Fishman RA, Longar SM (1984) Brain injury, edema, and vascular permeability changes induced by oxygen-derived free radicals. *Neurology* 34:315-320.
- Chen H, Chopp M, Zhang RL, Bodzin G, Chen Q, Rusche JR, Todd RF, 3rd (1994) Anti-CD11b monoclonal antibody reduces ischemic cell damage after transient focal cerebral ischemia in rat. *Ann Neurol* 35:458-463.
- Chen Y, Hallenbeck JM, Ruetzler C, Bol D, Thomas K, Berman NE, Vogel SN (2003) Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. *J Cereb Blood Flow Metab* 23:748-755.
- Cho YH, Friedman E, Silva AJ (1999) Ibotenate lesions of the hippocampus impair spatial learning but not contextual fear conditioning in mice. *Behav Brain Res* 98:77-87.
- Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1:623-634.
- Chopp M, Zhang RL, Chen H, Li Y, Jiang N, Rusche JR (1994) Postischemic administration of an anti-Mac-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in rats. *Stroke* 25:869-875; discussion 875-866.
- Clark WM, Coull BM, Briley DP, Mainolfi E, Rothlein R (1993) Circulating intercellular adhesion molecule-1 levels and neutrophil adhesion in stroke. *J Neuroimmunol* 44:123-125.
- Connolly ES, Jr., Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ (1996) Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *J Clin Invest* 97:209-216.
- Coughlan T, Gibson C, Murphy S (2005) Modulatory effects of progesterone on inducible nitric oxide synthase expression in vivo and in vitro. *J Neurochem* 93:932-942.
- Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689-695.
- Cui J, Holmes EH, Greene TG, Liu PK (2000) Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. *FASEB J* 14:955-967.
- Davies CA, Loddick SA, Toulmond S, Stroemer RP, Hunt J, Rothwell NJ (1999) The progression and topographic distribution of interleukin-1beta expression after permanent middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 19:87-98.

- DeGraba TJ (1998) The role of inflammation after acute stroke: utility of pursuing anti-adhesion molecule therapy. *Neurology* 51:S62-68.
- Diamond MS, Springer TA (1994) The dynamic regulation of integrin adhesiveness. *Curr Biol* 4:506-517.
- Diamond MS, Alon R, Parkos CA, Quinn MT, Springer TA (1995) Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD1). *J Cell Biol* 130:1473-1482.
- Dietrich WD, Busto R, Bethea JR (1999) Postischemic hypothermia and IL-10 treatment provide long-lasting neuroprotection of CA1 hippocampus following transient global ischemia in rats. *Exp Neurol* 158:444-450.
- Dietrich WD, Halley M, Valdes I, Busto R (1991) Interrelationships between increased vascular permeability and acute neuronal damage following temperature-controlled brain ischemia in rats. *Acta Neuropathol* 81:615-625.
- DiVietro JA, Smith MJ, Smith BR, Petruzzelli L, Larson RS, Lawrence MB (2001) Immobilized IL-8 triggers progressive activation of neutrophils rolling in vitro on P-selectin and intercellular adhesion molecule-1. *J Immunol* 167:4017-4025.
- Dong Y, Benveniste EN (2001) Immune function of astrocytes. *Glia* 36:180-190.
- Dutka AJ, Kochanek PM, Hallenbeck JM (1989) Influence of granulocytopenia on canine cerebral ischemia induced by air embolism. *Stroke* 20:390-395.
- Ehrensperger E, Minuk J, Durcan L, Mackey A, Wolfson C, Fontaine AM, Cote R (2005) Predictive value of soluble intercellular adhesion molecule-1 for risk of ischemic events in individuals with cerebrovascular disease. *Cerebrovasc Dis* 20:456-462.
- Eklof B, Siesjo BK (1972) The effect of bilateral carotid artery ligation upon acid-base parameters and substrate levels in the rat brain. *Acta Physiol Scand* 86:528-538.
- Emerich DF, Dean RL, 3rd, Bartus RT (2002) The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct? *Exp Neurol* 173:168-181.
- Emsley HC, Tyrrell PJ (2002) Inflammation and infection in clinical stroke. *J Cereb Blood Flow Metab* 22:1399-1419.
- Endoh M, Maiese K, Wagner J (1994) Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. *Brain Res* 651:92-100.
- Engler R, Covell JW (1987) Granulocytes cause reperfusion ventricular dysfunction after 15-minute ischemia in the dog. *Circ Res* 61:20-28.
- Erecinska M, Silver IA (1992) Relationship between ions and energy metabolism: cerebral calcium movements during ischaemia and subsequent recovery. *Can J Physiol Pharmacol* 70 Suppl:S190-193.
- Flavin MP, Ho LT (1999) Propentofylline protects neurons in culture from death triggered by macrophage or microglial secretory products. *J Neurosci Res* 56:54-59.
- Flavin MP, Coughlin K, Ho LT (1997) Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. *Neuroscience* 80:437-448.
- Fogal B, Li J, Lobner D, McCullough LD, Hewett SJ (2007) System x(c)- activity and astrocytes are necessary for interleukin-1 beta-mediated hypoxic neuronal injury. *J Neurosci* 27:10094-10105.

- Frijns CJ, Kappelle LJ (2002) Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. *Stroke* 33:2115-2122.
- Garau A, Bertini R, Colotta F, Casilli F, Bigini P, Cagnotto A, Mennini T, Ghezzi P, Villa P (2005) Neuroprotection with the CXCL8 inhibitor repertaxin in transient brain ischemia. *Cytokine* 30:125-131.
- Gavins F, Yilmaz G, Granger DN (2007) The evolving paradigm for blood cell-endothelial cell interactions in the cerebral microcirculation. *Microcirculation* 14:667-681.
- Gehrmann J, Bonnekoh P, Miyazawa T, Hossmann KA, Kreutzberg GW (1992) Immunocytochemical study of an early microglial activation in ischemia. *J Cereb Blood Flow Metab* 12:257-269.
- Gibson CL, Bath PM, Murphy SP (2005) G-CSF reduces infarct volume and improves functional outcome after transient focal cerebral ischemia in mice. *J Cereb Blood Flow Metab* 25:431-439.
- Gidday JM, Park TS, Gonzales ER, Beetsch JW (1997) CD18-dependent leukocyte adherence and vascular injury in pig cerebral circulation after ischemia. *Am J Physiol* 272:H2622-2629.
- Ginis I, Jaiswal R, Klimanis D, Liu J, Greenspon J, Hallenbeck JM (2002) TNF-alpha-induced tolerance to ischemic injury involves differential control of NF-kappaB transactivation: the role of NF-kappaB association with p300 adaptor. *J Cereb Blood Flow Metab* 22:142-152.
- Globus MY, Busto R, Martinez E, Valdes I, Dietrich WD, Ginsberg MD (1991) Comparative effect of transient global ischemia on extracellular levels of glutamate, glycine, and gamma-aminobutyric acid in vulnerable and nonvulnerable brain regions in the rat. *J Neurochem* 57:470-478.
- Godefroy O, Rousseaux M, Pruvo JP, Cabaret M, Leys D (1994) Neuropsychological changes related to unilateral lenticulostriate infarcts. *J Neurol Neurosurg Psychiatry* 57:480-485.
- Goto K, Ishige A, Sekiguchi K, Iizuka S, Sugimoto A, Yuzurihara M, Aburada M, Hosoya E, Kogure K (1990) Effects of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res* 534:299-302.
- Graeber MB, Streit WJ, Kreutzberg GW (1988) Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. *J Neurosci Res* 21:18-24.
- Granger DN, Kubes P (1994) The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. *J Leukoc Biol* 55:662-675.
- Green CE, Pearson DN, Camphausen RT, Staunton DE, Simon SI (2004) Shear-dependent capping of L-selectin and P-selectin glycoprotein ligand 1 by E-selectin signals activation of high-avidity beta2-integrin on neutrophils. *J Immunol* 172:7780-7790.
- Grilli M, Pizzi M, Memo M, Spano P (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* 274:1383-1385.
- Grogaard B, Schurer L, Gerdin B, Arfors KE (1989) Delayed hypoperfusion after incomplete forebrain ischemia in the rat. The role of polymorphonuclear leukocytes. *J Cereb Blood Flow Metab* 9:500-505.

- Hacke W, Kaste M, Fieschi C, Toni D, Lesaffre E, von Kummer R, Boysen G, Bluhmki E, Hoxter G, Mahagne MH, et al. (1995) Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke. The European Cooperative Acute Stroke Study (ECASS). *JAMA* 274:1017-1025.
- Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC, Williams GT (1996) Apoptosis: molecular regulation of cell death. *Eur J Biochem* 236:1-26.
- Hallenbeck JM (1996) Significance of the inflammatory response in brain ischemia. *Acta Neurochir Suppl* 66:27-31.
- Hallenbeck JM (2002) The many faces of tumor necrosis factor in stroke. *Nat Med* 8:1363-1368.
- Haqqani AS, Nesic M, Preston E, Baumann E, Kelly J, Stanimirovic D (2005) Characterization of vascular protein expression patterns in cerebral ischemia/reperfusion using laser capture microdissection and ICAT-nanoLC-MS/MS. *FASEB J* 19:1809-1821.
- Harlan JM, Winn RK (2002) Leukocyte-endothelial interactions: clinical trials of anti-adhesion therapy. *Crit Care Med* 30:S214-219.
- Hartley DM, Kurth MC, Bjerkness L, Weiss JH, Choi DW (1993) Glutamate receptor-induced 45Ca^{2+} accumulation in cortical cell culture correlates with subsequent neuronal degeneration. *J Neurosci* 13:1993-2000.
- Hauck EF, Hoffmann JF, Heimann A, Kempfski O (2007) EndothelinA receptor antagonist BSF-208075 causes immune modulation and neuroprotection after stroke in gerbils. *Brain Res* 1157:138-145.
- Heinel LA, Rubin S, Rosenwasser RH, Vasthare US, Tuma RF (1994) Leukocyte involvement in cerebral infarct generation after ischemia and reperfusion. *Brain Res Bull* 34:137-141.
- Held C, Hjemdahl P, Rehnqvist N, Wallen NH, Bjorkander I, Eriksson SV, Forslund L, Wiman B (1997) Fibrinolytic variables and cardiovascular prognosis in patients with stable angina pectoris treated with verapamil or metoprolol. Results from the Angina Prognosis study in Stockholm. *Circulation* 95:2380-2386.
- Hentzen ER, Neelamegham S, Kansas GS, Benanti JA, McIntire LV, Smith CW, Simon SI (2000) Sequential binding of CD11a/CD18 and CD11b/CD18 defines neutrophil capture and stable adhesion to intercellular adhesion molecule-1. *Blood* 95:911-920.
- Herrmann O, Tarabin V, Suzuki S, Attigah N, Coserea I, Schneider A, Vogel J, Prinz S, Schwab S, Monyer H, Brombacher F, Schwaninger M (2003) Regulation of body temperature and neuroprotection by endogenous interleukin-6 in cerebral ischemia. *J Cereb Blood Flow Metab* 23:406-415.
- Hu Y, Kiely JM, Szente BE, Rosenzweig A, Gimbrone MA, Jr. (2000) E-selectin-dependent signaling via the mitogen-activated protein kinase pathway in vascular endothelial cells. *J Immunol* 165:2142-2148.
- Huang D, Shenoy A, Cui J, Huang W, Liu PK (2000) In situ detection of AP sites and DNA strand breaks bearing 3'-phosphate termini in ischemic mouse brain. *FASEB J* 14:407-417.
- Huang J, Choudhri TF, Winfree CJ, McTaggart RA, Kiss S, Mocco J, Kim LJ, Protosaltis TS, Zhang Y, Pinsky DJ, Connolly ES, Jr. (2000) Postischemic

- cerebrovascular E-selectin expression mediates tissue injury in murine stroke. *Stroke* 31:3047-3053.
- Huber AR, Kunkel SL, Todd RF, 3rd, Weiss SJ (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* 254:99-102.
- Huppa JB, Ploegh HL (1997) In vitro translation and assembly of a complete T cell receptor-CD3 complex. *J Exp Med* 186:393-403.
- Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.
- Iademarco MF, Barks JL, Dean DC (1995) Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF-alpha in cultured endothelial cells. *J Clin Invest* 95:264-271.
- Ishikawa M, Cooper D, Russell J, Salter JW, Zhang JH, Nanda A, Granger DN (2003) Molecular determinants of the prothrombotic and inflammatory phenotype assumed by the postischemic cerebral microcirculation. *Stroke* 34:1777-1782.
- Jansson JH, Nilsson TK, Olofsson BO (1991) Tissue plasminogen activator and other risk factors as predictors of cardiovascular events in patients with severe angina pectoris. *Eur Heart J* 12:157-161.
- Johansson BB, Grabowski M (1994) Functional recovery after brain infarction: plasticity and neural transplantation. *Brain Pathol* 4:85-95.
- Johansson MW, Patarroyo M, Oberg F, Siegbahn A, Nilsson K (1997) Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). *J Cell Sci* 110 (Pt 9):1133-1139.
- Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW (1993) P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys J* 65:1560-1569.
- Jorgensen MB, Finsen BR, Jensen MB, Castellano B, Diemer NH, Zimmer J (1993) Microglial and astroglial reactions to ischemic and kainic acid-induced lesions of the adult rat hippocampus. *Exp Neurol* 120:70-88.
- Justicia C, Martin A, Rojas S, Gironella M, Cervera A, Panes J, Chamorro A, Planas AM (2006) Anti-VCAM-1 antibodies did not protect against ischemic damage either in rats or in mice. *J Cereb Blood Flow Metab* 26:421-432.
- Jutila MA, Rott L, Berg EL, Butcher EC (1989) Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. *J Immunol* 143:3318-3324.
- Kabat H, Dennis C, Baker A (1942) Recovery of function following arrest of the brain circulation. *Am J Physiol* 132:737-747.
- Kajta M, Trotter A, Lason W, Beyer C (2006) Impact of 17beta-estradiol on cytokine-mediated apoptotic effects in primary hippocampal and neocortical cell cultures. *Brain Res* 1116:64-74.
- Kawamata T, Dietrich WD, Schallert T, Gotts JE, Cocke RR, Benowitz LI, Finklestein SP (1997) Intracisternal basic fibroblast growth factor enhances functional recovery and up-regulates the expression of a molecular marker of neuronal sprouting following focal cerebral infarction. *Proc Natl Acad Sci U S A* 94:8179-8184.
- Kelly MA, Shuaib A, Todd KG (2006) Matrix metalloproteinase activation and blood-brain barrier breakdown following thrombolysis. *Exp Neurol* 200:38-49.

- Kettritz R, Choi M, Rolle S, Wellner M, Luft FC (2004) Integrins and cytokines activate nuclear transcription factor-kappaB in human neutrophils. *J Biol Chem* 279:2657-2665.
- Kihara S, Shiraishi T, Nakagawa S, Toda K, Tabuchi K (1994) Visualization of DNA double strand breaks in the gerbil hippocampal CA1 following transient ischemia. *Neurosci Lett* 175:133-136.
- Kim JS (1996) Cytokines and adhesion molecules in stroke and related diseases. *J Neurol Sci* 137:69-78.
- Kishimoto TK, Jutila MA, Berg EL, Butcher EC (1989) Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 245:1238-1241.
- Kitagawa K, Matsumoto M, Mabuchi T, Yagita Y, Ohtsuki T, Hori M, Yanagihara T (1998) Deficiency of intercellular adhesion molecule 1 attenuates microcirculatory disturbance and infarction size in focal cerebral ischemia. *J Cereb Blood Flow Metab* 18:1336-1345.
- Kiyota Y, Pahlmark K, Memezawa H, Smith ML, Siesjo BK (1993) Free radicals and brain damage due to transient middle cerebral artery occlusion: the effect of dimethylthiourea. *Exp Brain Res* 95:388-396.
- Klein S, de Fougères AR, Blaikie P, Khan L, Pepe A, Green CD, Kotliansky V, Giancotti FG (2002) Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. *Mol Cell Biol* 22:5912-5922.
- Krause GS, White BC, Aust SD, Nayini NR, Kumar K (1988) Brain cell death following ischemia and reperfusion: a proposed biochemical sequence. *Crit Care Med* 16:714-726.
- Krams M, Lees KR, Hacke W, Grieve AP, Orgogozo JM, Ford GA (2003) Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN): an adaptive dose-response study of UK-279,276 in acute ischemic stroke. *Stroke* 34:2543-2548.
- Kroemer G, Petit P, Zamzami N, Vayssière JL, Mignotte B (1995) The biochemistry of programmed cell death. *FASEB J* 9:1277-1287.
- Kristian T, Siesjo BK (1998) Calcium in ischemic cell death. *Stroke* 29:705-718.
- Kuijpers TW, Hoogerwerf M, Roos D (1992) Neutrophil migration across monolayers of resting or cytokine-activated endothelial cells. Role of intracellular calcium changes and fusion of specific granules with the plasma membrane. *J Immunol* 148:72-77.
- Kumar AG, Ballantyne CM, Michael LH, Kukielka GL, Youker KA, Lindsey ML, Hawkins HK, Birdsall HH, MacKay CR, LaRosa GJ, Rossen RD, Smith CW, Entman ML (1997) Induction of monocyte chemoattractant protein-1 in the small veins of the ischemic and reperfused canine myocardium. *Circulation* 95:693-700.
- Kuno K, Matsushima K (1994) The IL-1 receptor signaling pathway. *J Leukoc Biol* 56:542-547.
- Kusterer K, Bojunga J, Enghofer M, Heidenthal E, Usadel KH, Kolb H, Martin S (1998) Soluble ICAM-1 reduces leukocyte adhesion to vascular endothelium in ischemia-reperfusion injury in mice. *Am J Physiol* 275:G377-380.

- Labadia ME, Jeanfavre DD, Caviness GO, Morelock MM (1998) Molecular regulation of the interaction between leukocyte function-associated antigen-1 and soluble ICAM-1 by divalent metal cations. *J Immunol* 161:836-842.
- Lawrence MB, Springer TA (1993) Neutrophils roll on E-selectin. *J Immunol* 151:6338-6346.
- Lees KR, Diener HC, Asplund K, Krams M (2003) UK-279,276, a neutrophil inhibitory glycoprotein, in acute stroke: tolerability and pharmacokinetics. *Stroke* 34:1704-1709.
- Lehmberg J, Beck J, Baethmann A, Uhl E (2006) Effect of P-selectin inhibition on leukocyte-endothelium interaction and survival after global cerebral ischemia. *J Neurol* 253:357-363.
- Li Y, Chopp M, Jiang N, Yao F, Zaloga C (1995) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 15:389-397.
- Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24:2002-2008; discussion 2008-2009.
- Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC, Feuerstein GZ (1994) Tumor necrosis factor-alpha expression in ischemic neurons. *Stroke* 25:1481-1488.
- Loddick SA, Turnbull AV, Rothwell NJ (1998) Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab* 18:176-179.
- Lowry O, Passonneau J, Hasselberger F, Schulz D (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathways. *J Biol Chem* 249:18-30.
- Lyden PD, Zivin JA, Chabolla DR, Jacobs MA, Gage FH (1992) Quantitative effects of cerebral infarction on spatial learning in rats. *Exp Neurol* 116:122-132.
- MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 164:89-92.
- Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. *Brain Res Bull* 46:281-309.
- Matchett GA, Calinisan JB, Matchett GC, Martin RD, Zhang JH (2007) The effect of granulocyte-colony stimulating factor in global cerebral ischemia in rats. *Brain Res* 1136:200-207.
- Mayhan WG (1998) Effect of lipopolysaccharide on the permeability and reactivity of the cerebral microcirculation: role of inducible nitric oxide synthase. *Brain Res* 792:353-357.
- Mayhan WG (2002) Cellular mechanisms by which tumor necrosis factor-alpha produces disruption of the blood-brain barrier. *Brain Res* 927:144-152.
- McCull BW, Rothwell NJ, Allan SM (2007) Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. *J Neurosci* 27:4403-4412.

- McDonald RP, Horsburgh KJ, Graham DI, Nicoll JA (1999) Mitochondrial DNA deletions in acute brain injury. *Neuroreport* 10:1875-1878.
- McEver RP (1997) Selectin-carbohydrate interactions during inflammation and metastasis. *Glycoconj J* 14:585-591.
- McNamee JP, Bellier PV, Kutzner BC, Wilkins RC (2005) Effect of pro-inflammatory cytokines on spontaneous apoptosis in leukocyte sub-sets within a whole blood culture. *Cytokine* 31:161-167.
- Michael JR, Guerci AD, Koehler RC, Shi AY, Tsitlik J, Chandra N, Niedermeyer E, Rogers MC, Traystman RJ, Weisfeldt ML (1984) Mechanisms by which epinephrine augments cerebral and myocardial perfusion during cardiopulmonary resuscitation in dogs. *Circulation* 69:822-835.
- Mocco J, Choudhri T, Huang J, Harfeldt E, Efros L, Klingbeil C, Vexler V, Hall W, Zhang Y, Mack W, Popilskis S, Pinsky DJ, Connolly ES, Jr. (2002) HuEP5C7 as a humanized monoclonal anti-E/P-selectin neurovascular protective strategy in a blinded placebo-controlled trial of nonhuman primate stroke. *Circ Res* 91:907-914.
- Morioka T, Kalehua AN, Streit WJ (1991) The microglial reaction in the rat dorsal hippocampus following transient forebrain ischemia. *J Cereb Blood Flow Metab* 11:966-973.
- Mossakowski MJ, Lossinsky AS, Pluta R, Wisniewski HM (1994) Abnormalities of the blood-brain barrier in global cerebral ischemia in rats due to experimental cardiac arrest. *Acta Neurochir Suppl (Wien)* 60:274-276.
- Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. *Nature* 297:681-683.
- Mulcahy NJ, Ross J, Rothwell NJ, Loddick SA (2003) Delayed administration of interleukin-1 receptor antagonist protects against transient cerebral ischaemia in the rat. *Br J Pharmacol* 140:471-476.
- Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto S, Kasahara T, Matsushima K (1994) Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* 269:13289-13295.
- Murakami K, Kondo T, Kawase M, Chan PH (1998) The development of a new mouse model of global ischemia: focus on the relationships between ischemia duration, anesthesia, cerebral vasculature, and neuronal injury following global ischemia in mice. *Brain Res* 780:304-310.
- Murakami Y, Saito K, Hara A, Zhu Y, Sudo K, Niwa M, Fujii H, Wada H, Ishiguro H, Mori H, Seishima M (2005) Increases in tumor necrosis factor-alpha following transient global cerebral ischemia do not contribute to neuron death in mouse hippocampus. *J Neurochem* 93:1616-1622.
- Nadareishvili ZG, Li H, Wright V, Maric D, Warach S, Hallenbeck JM, Dambrosia J, Barker JL, Baird AE (2004) Elevated pro-inflammatory CD4+CD28- lymphocytes and stroke recurrence and death. *Neurology* 63:1446-1451.
- Nagai N, Demarsin E, Van Hoef B, Wouters S, Cingolani D, Laroche Y, Collen D (2003) Recombinant human microplasmin: production and potential therapeutic properties. *J Thromb Haemost* 1:307-313.

- Nakagawa Y, Fujimoto N, Matsumoto K, Cervos-Navarro J (1990) Morphological changes in acute cerebral ischemia after occlusion and reperfusion in the rat. *Adv Neurol* 52:21-27.
- Nakata N, Kato H, Kogure K (1993) Protective effects of basic fibroblast growth factor against hippocampal neuronal damage following cerebral ischemia in the gerbil. *Brain Res* 605:354-356.
- Nawashiro H, Martin D, Hallenbeck JM (1997) Inhibition of tumor necrosis factor and amelioration of brain infarction in mice. *J Cereb Blood Flow Metab* 17:229-232.
- Nelson A, Lebessi A, Sowinski P, Hodges H (1997) Comparison of effects of global cerebral ischaemia on spatial learning in the standard and radial water maze: relationship of hippocampal damage to performance. *Behav Brain Res* 85:93-115.
- Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM (1993) Genomic organization and transcriptional regulation of the RANTES chemokine gene. *J Immunol* 151:2601-2612.
- Newmeyer DD, Farschon DM, Reed JC (1994) Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79:353-364.
- Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibana K, Kominami E, Uchiyama Y (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci* 15:1001-1011.
- Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA (1990) Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc Natl Acad Sci U S A* 87:5144-5147.
- Olsson T, Wieloch T, Smith ML (2003) Brain damage in a mouse model of global cerebral ischemia. Effect of NMDA receptor blockade. *Brain Res* 982:260-269.
- Park EM, Cho S, Frys KA, Glickstein SB, Zhou P, Anrather J, Ross ME, Iadecola C (2006) Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *J Cereb Blood Flow Metab* 26:392-401.
- Piani D, Spranger M, Frei K, Schaffner A, Fontana A (1992) Macrophage-induced cytotoxicity of N-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines. *Eur J Immunol* 22:2429-2436.
- Pinteaux E, Rothwell NJ, Boutin H (2006) Neuroprotective actions of endogenous interleukin-1 receptor antagonist (IL-1ra) are mediated by glia. *Glia* 53:551-556.
- Prestigiacomo CJ, Kim SC, Connolly ES, Jr., Liao H, Yan SF, Pinsky DJ (1999) CD18-mediated neutrophil recruitment contributes to the pathogenesis of reperfused but not nonreperfused stroke. *Stroke* 30:1110-1117.
- Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke* 10:267-272.
- Rallidis LS, Vikelis M, Panagiotakos DB, Rizos I, Zolindaki MG, Kaliva K, Kremastinos DT (2006) Inflammatory markers and in-hospital mortality in acute ischaemic stroke. *Atherosclerosis* 189:193-197.
- Relton JK, Martin D, Thompson RC, Russell DA (1996) Peripheral administration of Interleukin-1 Receptor antagonist inhibits brain damage after focal cerebral ischemia in the rat. *Exp Neurol* 138:206-213.

- Rosenberg GA (1999) Ischemic brain edema. *Prog Cardiovasc Dis* 42:209-216.
- Ross GD, Lambris JD (1982) Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils, and erythrocytes. *J Exp Med* 155:96-110.
- Rothwell NJ, Luheshi GN (2000) Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci* 23:618-625.
- Rubanyi GM, Polokoff MA (1994) Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 46:325-415.
- Sairanen T, Carpen O, Karjalainen-Lindsberg ML, Paetau A, Turpeinen U, Kaste M, Lindsberg PJ (2001) Evolution of cerebral tumor necrosis factor-alpha production during human ischemic stroke. *Stroke* 32:1750-1758.
- Sakamoto N, Kogure K, Kato H, Ohtomo H (1986) Disturbed Ca²⁺ homeostasis in the gerbil hippocampus following brief transient ischemia. *Brain Res* 364:372-376.
- Sakurai-Yamashita Y, Shigematsu K, Yamashita K, Niwa M (2006) Expression of MCP-1 in the hippocampus of SHRSP with ischemia-related delayed neuronal death. *Cell Mol Neurobiol* 26:823-831.
- Schabitz WR, Kruger C, Pitzer C, Weber D, Laage R, Gassler N, Aronowski J, Mier W, Kirsch F, Dittgen T, Bach A, Sommer C, Schneider A (2008) A neuroprotective function for the hematopoietic protein granulocyte-macrophage colony stimulating factor (GM-CSF). *J Cereb Blood Flow Metab* 28:29-43.
- Schneeloch E, Mies G, Busch HJ, Buschmann IR, Hossmann KA (2004) Granulocyte-macrophage colony-stimulating factor-induced arteriogenesis reduces energy failure in hemodynamic stroke. *Proc Natl Acad Sci U S A* 101:12730-12735.
- Schneider A, Kruger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schabitz WR (2005) The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest* 115:2083-2098.
- Schneider D, Berrouschot J, Brandt T, Hacke W, Ferbert A, Norris SH, Polmar SH, Schafer E (1998) Safety, pharmacokinetics and biological activity of enlimomab (anti-ICAM-1 antibody): an open-label, dose escalation study in patients hospitalized for acute stroke. *Eur Neurol* 40:78-83.
- Schott RJ, Natale JE, Ressler SW, Burney RE, D'Alecy LG (1989) Neutrophil depletion fails to improve neurologic outcome after cardiac arrest in dogs. *Ann Emerg Med* 18:517-522.
- Schreck R, Rieber P, Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10:2247-2258.
- Schroeter M, Jander S, Huitinga I, Witte OW, Stoll G (1997) Phagocytic response in photochemically induced infarction of rat cerebral cortex. The role of resident microglia. *Stroke* 28:382-386.
- Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S (1997) Regulation of NF-kappa B activation by MAP kinase cascades. *Immunobiology* 198:35-49.
- Schurer L, Groggaard B, Gerdin B, Arfors KE (1990) Effects of neutrophil depletion and superoxide dismutase on postischemic hypoperfusion of rat brain. *Adv Neurol* 52:57-62.

- Schurer L, Groggaard B, Gerdin B, Kempinski O, Arfors KE (1991) Leucocyte depletion does not affect post-ischaemic nerve cell damage in the rat. *Acta Neurochir (Wien)* 111:54-60.
- Seachrist L (2000) ICOS ends LeukArrest program, plans to move pafase forward. In: *Bioworlds Online*.
- Shaw PJ, Bates D, Cartlidge NE, French JM, Heaviside D, Julian DG, Shaw DA (1987) Neurologic and neuropsychological morbidity following major surgery: comparison of coronary artery bypass and peripheral vascular surgery. *Stroke* 18:700-707.
- Shinnou M, Ueno M, Sakamoto H, Ide M (1998) Blood-brain barrier damage in reperfusion following ischemia in the hippocampus of the Mongolian gerbil brain. *Acta Neurol Scand* 98:406-411.
- Siebenlist U, Franzoso G, Brown K (1994) Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 10:405-455.
- Siemkowicz E, Hansen AJ (1978) Clinical restitution following cerebral ischemia in hypo-, normo- and hyperglycemic rats. *Acta Neurol Scand* 58:1-8.
- Siesjo BK, Siesjo P (1996) Mechanisms of secondary brain injury. *Eur J Anaesthesiol* 13:247-268.
- Siesjo BK, Agardh CD, Bengtsson F (1989) Free radicals and brain damage. *Cerebrovasc Brain Metab Rev* 1:165-211.
- Siesjo BK, Katsura K, Zhao Q, Folbergrova J, Pahlmark K, Siesjo P, Smith ML (1995) Mechanisms of secondary brain damage in global and focal ischemia: a speculative synthesis. *J Neurotrauma* 12:943-956.
- Siesjo BK (1988) Mechanisms of ischemic brain damage. *Crit Care Med* 16:954-963.
- Silva AJ, Giese KP, Fedorov NB, Frankland PW, Kogan JH (1998) Molecular, cellular, and neuroanatomical substrates of place learning. *Neurobiol Learn Mem* 70:44-61.
- Simon SI, Green CE (2005) Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annu Rev Biomed Eng* 7:151-185.
- Simon SI, Hu Y, Vestweber D, Smith CW (2000) Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. *J Immunol* 164:4348-4358.
- Simundic AM, Basic V, Topic E, Demarin V, Vrkcic N, Kunovic B, Stefanovic M, Begonja A (2004) Soluble adhesion molecules in acute ischemic stroke. *Clin Invest Med* 27:86-92.
- Smith CJ, Emsley HC, Gavin CM, Georgiou RF, Vail A, Barberan EM, del Zoppo GJ, Hallenbeck JM, Rothwell NJ, Hopkins SJ, Tyrrell PJ (2004) Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. *BMC Neurol* 4:2.
- Smith CW (1993) Leukocyte-endothelial cell interactions. *Semin Hematol* 30:45-53; discussion 54-45.
- Smith ML, Auer RN, Siesjo BK (1984) The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. *Acta Neuropathol* 64:319-332.

- Soriano SG, Coxon A, Wang YF, Frosch MP, Lipton SA, Hickey PR, Mayadas TN (1999) Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. *Stroke* 30:134-139.
- Stamatovic SM, Shakui P, Keep RF, Moore BB, Kunkel SL, Van Rooijen N, Andjelkovic AV (2005) Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. *J Cereb Blood Flow Metab* 25:593-606.
- Staunton DE, Marlin SD, Stratowa C, Dustin ML, Springer TA (1988) Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell* 52:925-933.
- Steller H (1995) Mechanisms and genes of cellular suicide. *Science* 267:1445-1449.
- Strachan RD, Kane PJ, Cook S, Chambers IR, Clayton CB, Mendelow AD (1992) Immunosuppression by whole-body irradiation and its effect on oedema in experimental cerebral ischaemia. *Acta Neurol Scand* 86:256-259.
- Strle K, Zhou JH, Shen WH, Broussard SR, Johnson RW, Freund GG, Dantzer R, Kelley KW (2001) Interleukin-10 in the brain. *Crit Rev Immunol* 21:427-449.
- Sughrue ME, Connolly ES, Jr. (2004) Effectively bridging the preclinical/clinical gap: the results of the ASTIN trial. *Stroke* 35:e81-82; author reply e81-82.
- Sughrue ME, Mehra A, Connolly ES, Jr., D'Ambrosio AL (2004) Anti-adhesion molecule strategies as potential neuroprotective agents in cerebral ischemia: a critical review of the literature. *Inflamm Res* 53:497-508.
- Sutcliffe IT, Smith HA, Stanimirovic D, Hutchison JS (2001) Effects of moderate hypothermia on IL-1 beta-induced leukocyte rolling and adhesion in pial microcirculation of mice and on proinflammatory gene expression in human cerebral endothelial cells. *J Cereb Blood Flow Metab* 21:1310-1319.
- Sutherland RJ, Kolb B, Whishaw IQ (1982) Spatial mapping: definitive disruption by hippocampal or medial frontal cortical damage in the rat. *Neurosci Lett* 31:271-276.
- Taylor AD, Neelamegham S, Hellums JD, Smith CW, Simon SI (1996) Molecular dynamics of the transition from L-selectin- to beta 2-integrin-dependent neutrophil adhesion under defined hydrodynamic shear. *Biophys J* 71:3488-3500.
- Tessier PA, Naccache PH, Clark-Lewis I, Gladue RP, Neote KS, McColl SR (1997) Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-alpha. *J Immunol* 159:3595-3602.
- Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456-1462.
- Toulmond S, Vige X, Fage D, Benavides J (1992) Local infusion of interleukin-6 attenuates the neurotoxic effects of NMDA on rat striatal cholinergic neurons. *Neurosci Lett* 144:49-52.
- Touzani O, Boutin H, Chuquet J, Rothwell N (1999) Potential mechanisms of interleukin-1 involvement in cerebral ischaemia. *J Neuroimmunol* 100:203-215.
- Touzani O, Boutin H, LeFeuvre R, Parker L, Miller A, Luheshi G, Rothwell N (2002) Interleukin-1 influences ischemic brain damage in the mouse independently of the interleukin-1 type I receptor. *J Neurosci* 22:38-43.

- Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T (1994) NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153:2052-2063.
- Ueno T, Sawa Y, Kitagawa-Sakakida S, Nishimura M, Morishita R, Kaneda Y, Kohmura E, Yoshimine T, Matsuda H (2001) Nuclear factor-kappa B decoy attenuates neuronal damage after global brain ischemia: a future strategy for brain protection during circulatory arrest. *J Thorac Cardiovasc Surg* 122:720-727.
- Uno H, Matsuyama T, Akita H, Nishimura H, Sugita M (1997) Induction of tumor necrosis factor-alpha in the mouse hippocampus following transient forebrain ischemia. *J Cereb Blood Flow Metab* 17:491-499.
- van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, van der Saag PT (1994) 12-O-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. *J Biol Chem* 269:6185-6192.
- van Eeden SF, Miyagashima R, Haley L, Hogg JC (1997) A possible role for L-selectin in the release of polymorphonuclear leukocytes from bone marrow. *Am J Physiol* 272:H1717-1724.
- Vasthare US, Heinel LA, Rosenwasser RH, Tuma RF (1990) Leukocyte involvement in cerebral ischemia and reperfusion injury. *Surg Neurol* 33:261-265.
- Vogt M, Bauer MK, Ferrari D, Schulze-Osthoff K (1998) Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells. *FEBS Lett* 429:67-72.
- Wang X, Feuerstein GZ (1995) Induced expression of adhesion molecules following focal brain ischemia. *J Neurotrauma* 12:825-832.
- Wang CX, Shuaib A (2002) Involvement of inflammatory cytokines in central nervous system injury. *Prog Neurobiol* 67:161-172.
- Wang Q, Tang XN, Yenari MA (2007) The inflammatory response in stroke. *J Neuroimmunol* 184:53-68.
- Watanabe H, Abe H, Takeuchi S, Tanaka R (2000) Protective effect of microglial conditioning medium on neuronal damage induced by glutamate. *Neurosci Lett* 289:53-56.
- Wender M, Michalowska-Wender G, Szczech J (2005) Minimal changes of TNF-alpha and MCP-1 expression in blood serum of rats subjected to experimental cardiac arrest. *Folia Neuropathol* 43:109-111.
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* 179:1-33.
- Wood PL (1995) Microglia as a unique cellular target in the treatment of stroke: potential neurotoxic mediators produced by activated microglia. *Neurol Res* 17:242-248.
- Wright JW, Clemens JA, Panetta JA, Smalstig EB, Weatherly LA, Kramar EA, Pederson ES, Mungall BH, Harding JW (1996) Effects of LY231617 and angiotensin IV on ischemia-induced deficits in circular water maze and passive avoidance performance in rats. *Brain Res* 717:1-11.
- Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251-306.

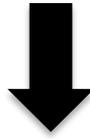
- Xu HL, Salter-Cid L, Linnik MD, Wang EY, Paisansathan C, Pelligrino DA (2006) Vascular adhesion protein-1 plays an important role in postischemic inflammation and neuropathology in diabetic, estrogen-treated ovariectomized female rats subjected to transient forebrain ischemia. *J Pharmacol Exp Ther* 317:19-29.
- Yakes FM, Van Houten B (1997) Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A* 94:514-519.
- Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K (1995) Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke* 26:676-680; discussion 681.
- Yang G, Kitagawa K, Matsushita K, Mabuchi T, Yagita Y, Yanagihara T, Matsumoto M (1997) C57BL/6 strain is most susceptible to cerebral ischemia following bilateral common carotid occlusion among seven mouse strains: selective neuronal death in the murine transient forebrain ischemia. *Brain Res* 752:209-218.
- Yang GY, Gong C, Qin Z, Ye W, Mao Y, Bertz AL (1998) Inhibition of TNFalpha attenuates infarct volume and ICAM-1 expression in ischemic mouse brain. *Neuroreport* 9:2131-2134.
- Yenari MA, Sun GH, Kunis DM, Onley D, Vexler V (2001) L-selectin inhibition does not reduce injury in a rabbit model of transient focal cerebral ischemia. *Neurol Res* 23:72-78.
- Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG (2006) Microglia potentiate damage to blood-brain barrier constituents: improvement by minocycline in vivo and in vitro. *Stroke* 37:1087-1093.
- Yonekawa K, Harlan JM (2005) Targeting leukocyte integrins in human diseases. *J Leukoc Biol* 77:129-140.
- Yoshida M, Westlin WF, Wang N, Ingber DE, Rosenzweig A, Resnick N, Gimbrone MA, Jr. (1996) Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. *J Cell Biol* 133:445-455.
- Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J (1998) Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci U S A* 95:15769-15774.
- Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J (1999) A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci U S A* 96:13496-13500.
- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G (1996) Mitochondrial control of nuclear apoptosis. *J Exp Med* 183:1533-1544.
- Zhang LH, Wei EQ (2003) Neuroprotective effect of ONO-1078, a leukotriene receptor antagonist, on transient global cerebral ischemia in rats. *Acta Pharmacol Sin* 24:1241-1247.
- Zhang N, Komine-Kobayashi M, Tanaka R, Liu M, Mizuno Y, Urabe T (2005) Edaravone reduces early accumulation of oxidative products and sequential inflammatory responses after transient focal ischemia in mice brain. *Stroke* 36:2220-2225.

- Zhao X, Haensel C, Araki E, Ross ME, Iadecola C (2000) Gene-dosing effect and persistence of reduction in ischemic brain injury in mice lacking inducible nitric oxide synthase. *Brain Res* 872:215-218.
- Zimmerman GA, McIntyre TM, Prescott SM (1996) Adhesion and signaling in vascular cell-cell interactions. *J Clin Invest* 98:1699-1702.

FIGURES

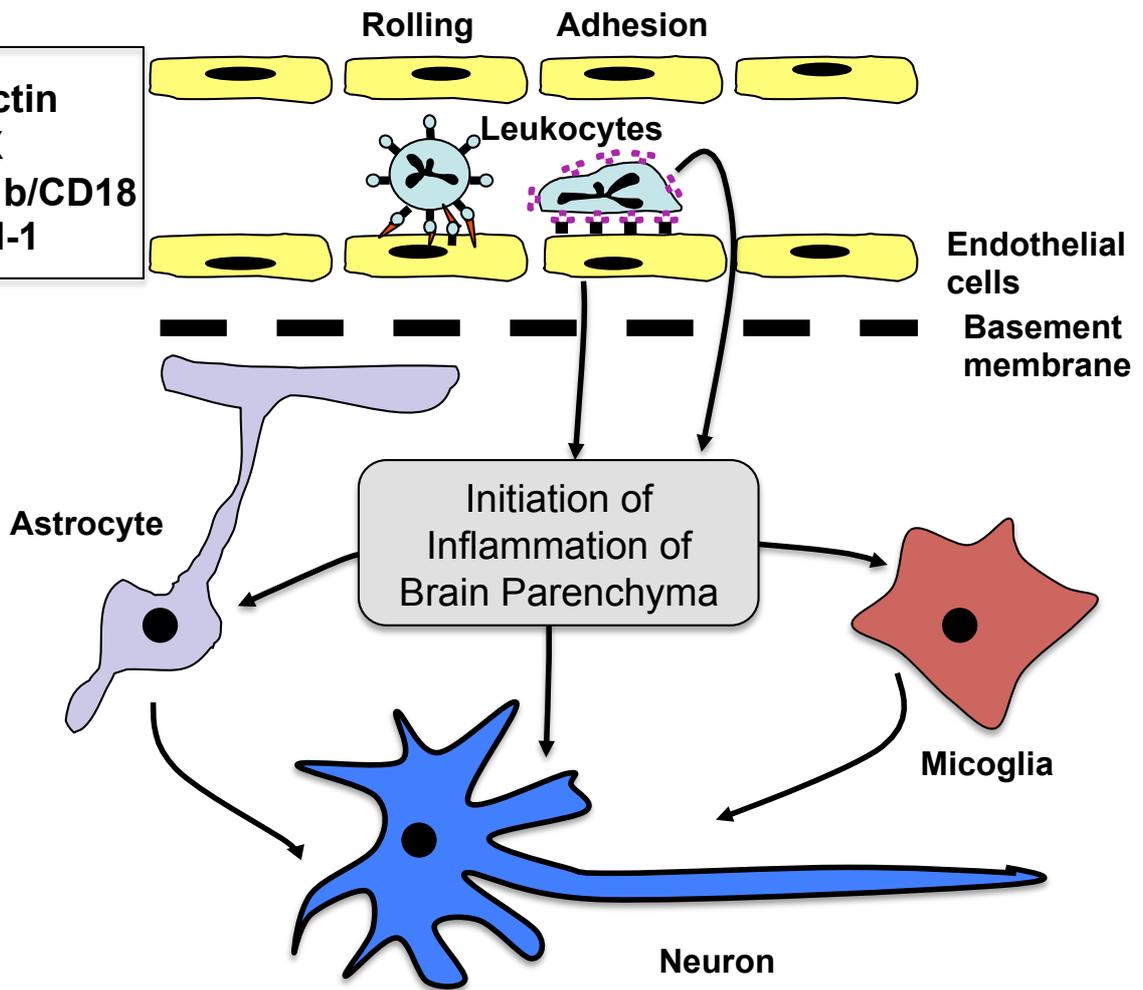
Figure 1. Hypothetical schematic for leukocyte adhesion dependant induction of the inflammatory response following global cerebral ischemia. Leukocytes first roll along and then adhere to endothelium in the cerebral microcirculation following global cerebral ischemia. Inflammatory mediators released from leukocytes and/or endothelial cells may cross the blood-brain barrier and initiate an inflammatory cascade in the brain parenchyma. These inflammatory mediators may activate glial cells and neurons to further propagate the inflammatory response. The result of the inflammation is neuronal cell death, cerebral edema and neurological dysfunction.

Ischemia



Leukocyte-endothelial cell interactions

- = selectin
- = SLeX
- = CD11b/CD18
- = ICAM-1

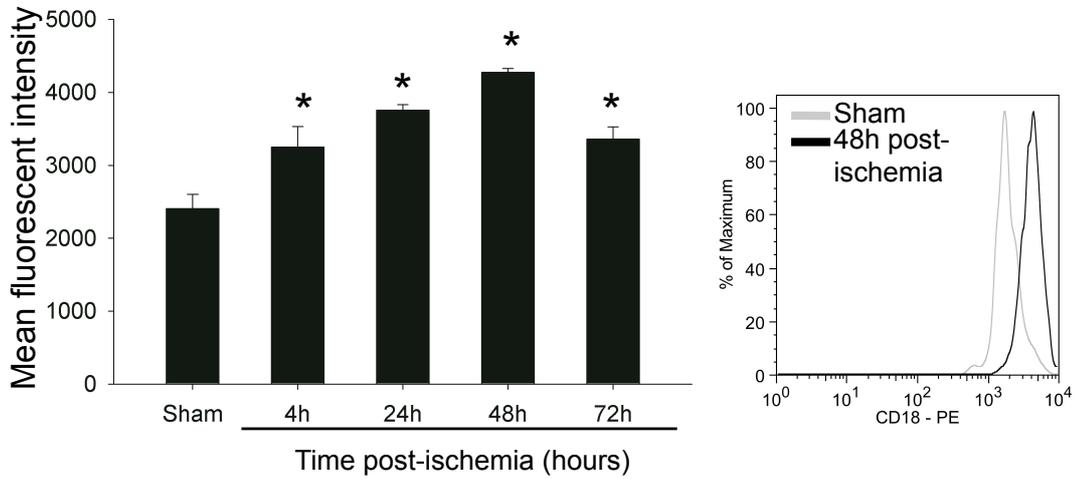


Neuronal cell death, cerebral edema and neurological dysfunction

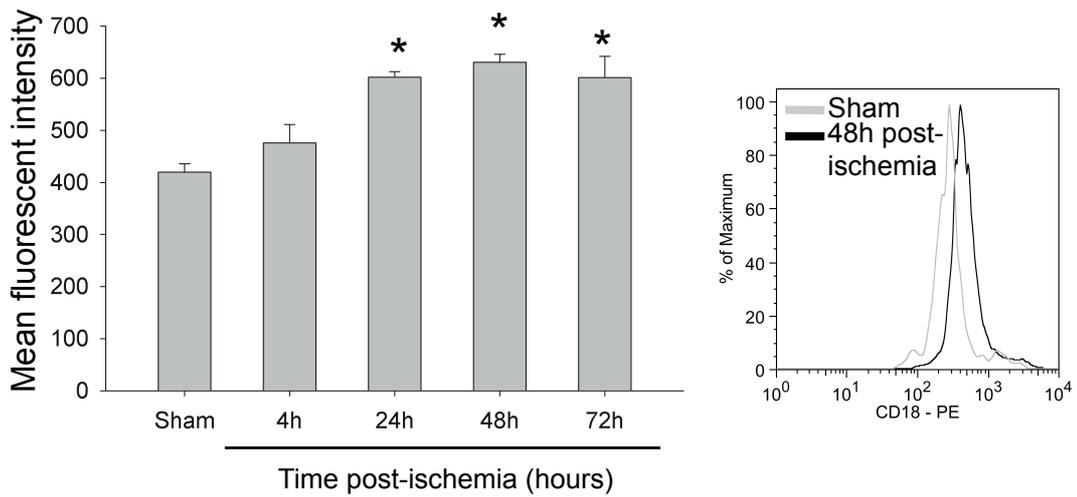
Figure 2. CD18 expression on leukocytes following global cerebral ischemia.

Expression of CD18 was measured on neutrophils and mononuclear leukocytes using flow cytometry. (A, B) Mean fluorescence intensity are shown in the panels on the left and representative histograms are shown on the right. CD18 expression on neutrophils (A) was increased by 4 hours and continued to increase to a maximum at 48 hours post-ischemia. At 72 hours post-ischemia the expression of CD18 on neutrophils began to decrease. CD18 expression on mononuclear cells (B) was increased by 24 hours and this level was maintained through 72 hours post-ischemia. (C) Differential leukocyte counts are shown in sham mice and at 4, 24, 48 and 72 hours following cerebral ischemia. There is a rapid increase in the proportion of neutrophils in the blood 4 hours following ischemia, while at 24, 48 and 72 hours post-ischemia mononuclear cell are the predominate leukocyte population. All data are displayed as mean \pm s.e.m. (n=4 mice in each group and time point). *p<0.05 compared to sham.

A



B



C

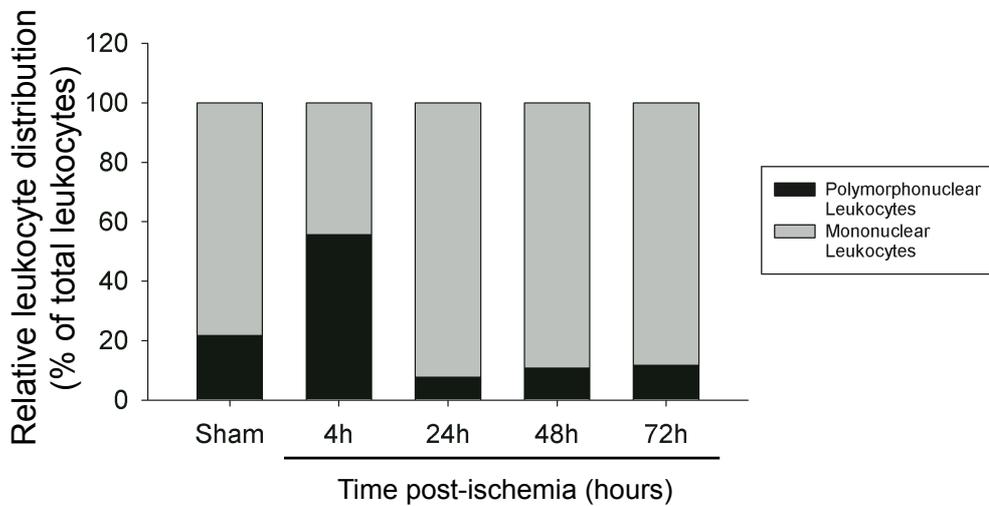


Figure 3. Anti-CD18 monoclonal antibody bound leukocytes. (A) Flow cytometric analysis of peripheral blood incubated with FITC labeled antibody to CD18 blocking antibody demonstrated binding of anti-CD18 (aCD18) three hours following ischemia compared to sham surgery. M1 = Margin 1 (unlabeled) and M2 = Margin 2 (labeled). (B) There was significantly greater mean fluorescence of the aCD18 detection antibody following ischemia compare to sham surgery and control antibody (Control Ig) therapy. (n=4 in each group). All data displayed as mean \pm s.e.m. *p<0.05 compared to all other groups.

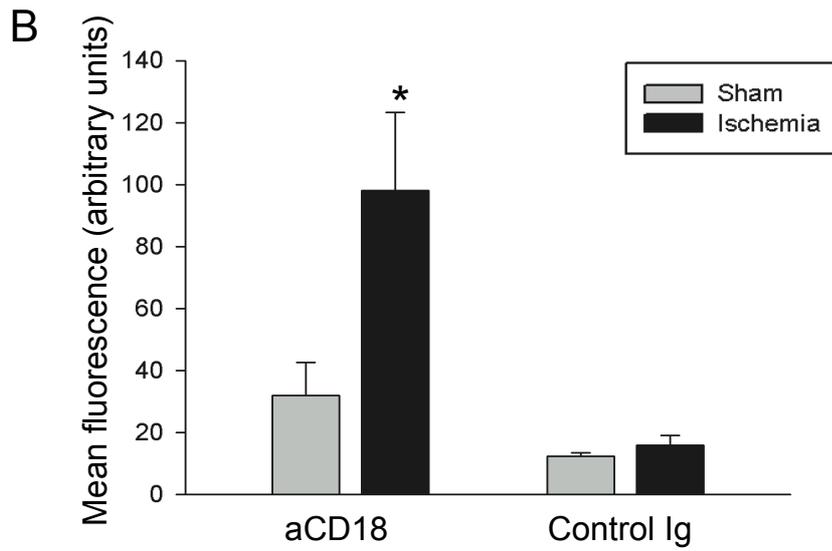
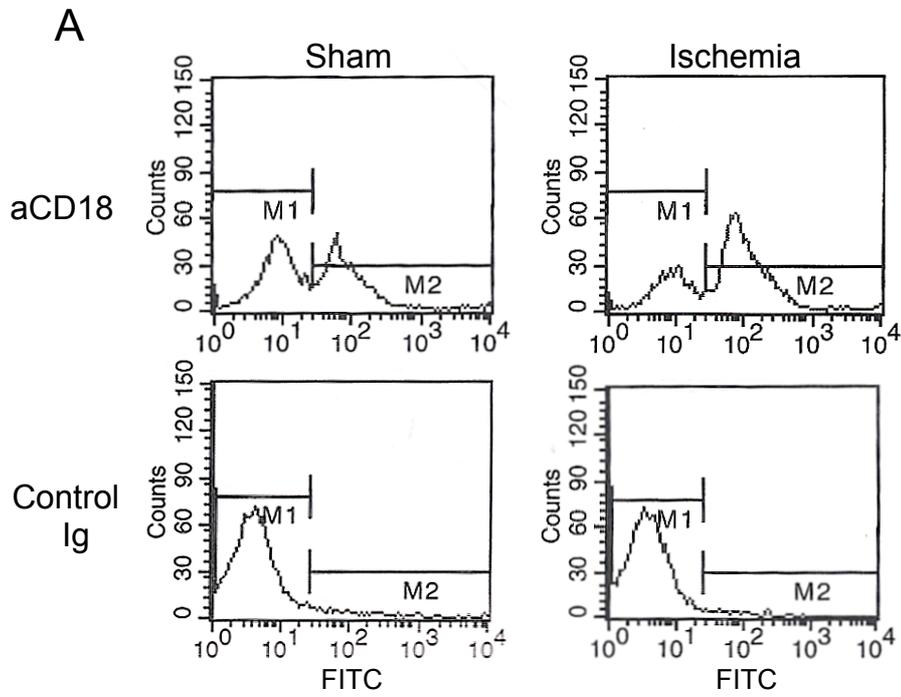


Figure 4. The effect of anti-CD18 on leukocyte-endothelial cell interactions following global cerebral ischemia. Leukocyte rolling and adhesion was counted in pial venules using intravital microscopy through a cranial window. **(A)** Three hours following cerebral ischemia there was significantly more leukocytes rolling and adhering compared to mice injected with sham surgery. **(B)** Three hours following ischemia, anti-CD18 treatment significantly reduced adherent leukocytes compared to control antibody treatment. Rolling events were reduced with anti-CD18 treatment compared to control antibody (Control Ig) treatment, however results were not statistically significant. (n=5 in all groups) All data are displayed as mean \pm s.e.m. *p<0.05 compared to other group.

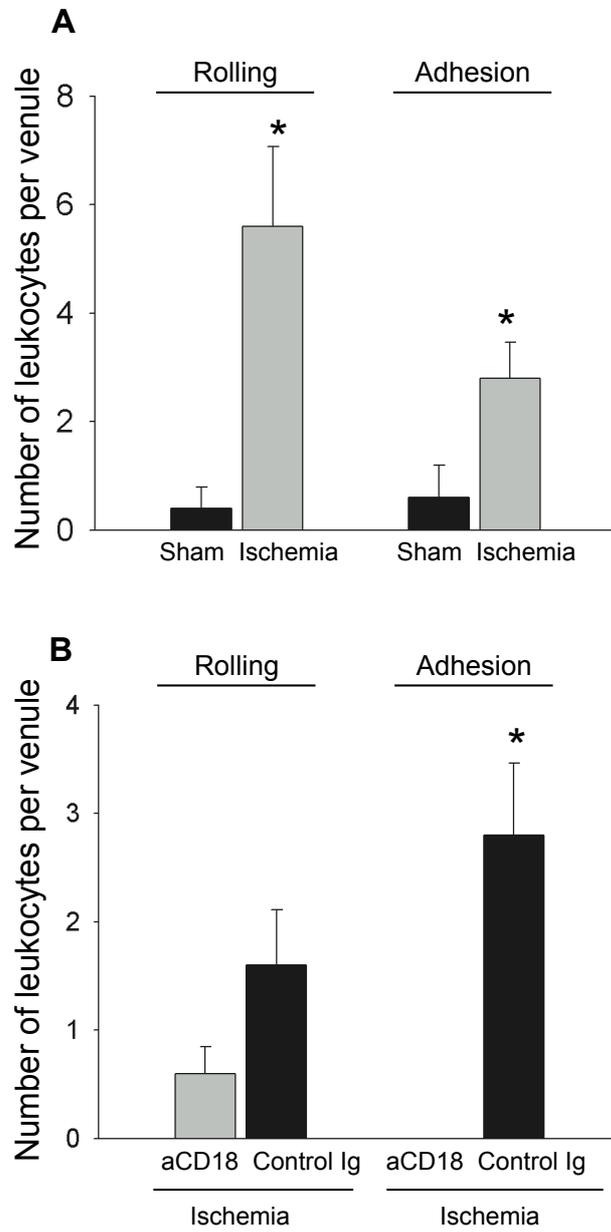


Figure 5. Anti-CD18 blocked the systemic inflammatory response following global cerebral ischemia. There was an increase in the pro-inflammatory cytokines (**A-C**), anti-inflammatory cytokines (**D**), growth factor cytokines (**E**) and chemokines (**F-G**) after global cerebral ischemia. At 24h post-ischemia in mice treated with control antibody (Control Ig) therapy there were significant increases in IL-1 β (**A**, left), IL-6 (**B**, left), and G-CSF (**E**, left) concentrations compared to sham mice. The MIP-1 α (**G**, left) concentrations were increased at 24h compared to sham although not significantly. The increase in IL-1 β , IL-6 and MIP-1 α induced by ischemia was blocked or attenuated by anti-CD18 (aCD18) therapy. Anti-CD18 treatment had no effect on the increase in G-CSF induced by ischemia. At 72h post-ischemia in mice treated with control Ig therapy, IL-1 β (**A**, right), IL-6 (**B**, right), TNF α (**C**, right), IL-10 (**D**, right), MCP-1 (**F**, right) and MIP-1a (**G**, right) showed a significant increase in concentration compared to sham mice. The MCP-1 (**F**, right) concentration was also increased at 72h compared to sham although not significantly. This increased in IL-1 β , IL-6, TNF α , IL-10, MCP-1 and MIP-1 α induced by ischemia were, again, blocked or attenuated by anti-CD18 therapy. (n=4-6 mice per experimental group and time point) Data shown as mean \pm s.e.m. *p<0.05 compared to sham. #p<0.05 compared to aCD18.

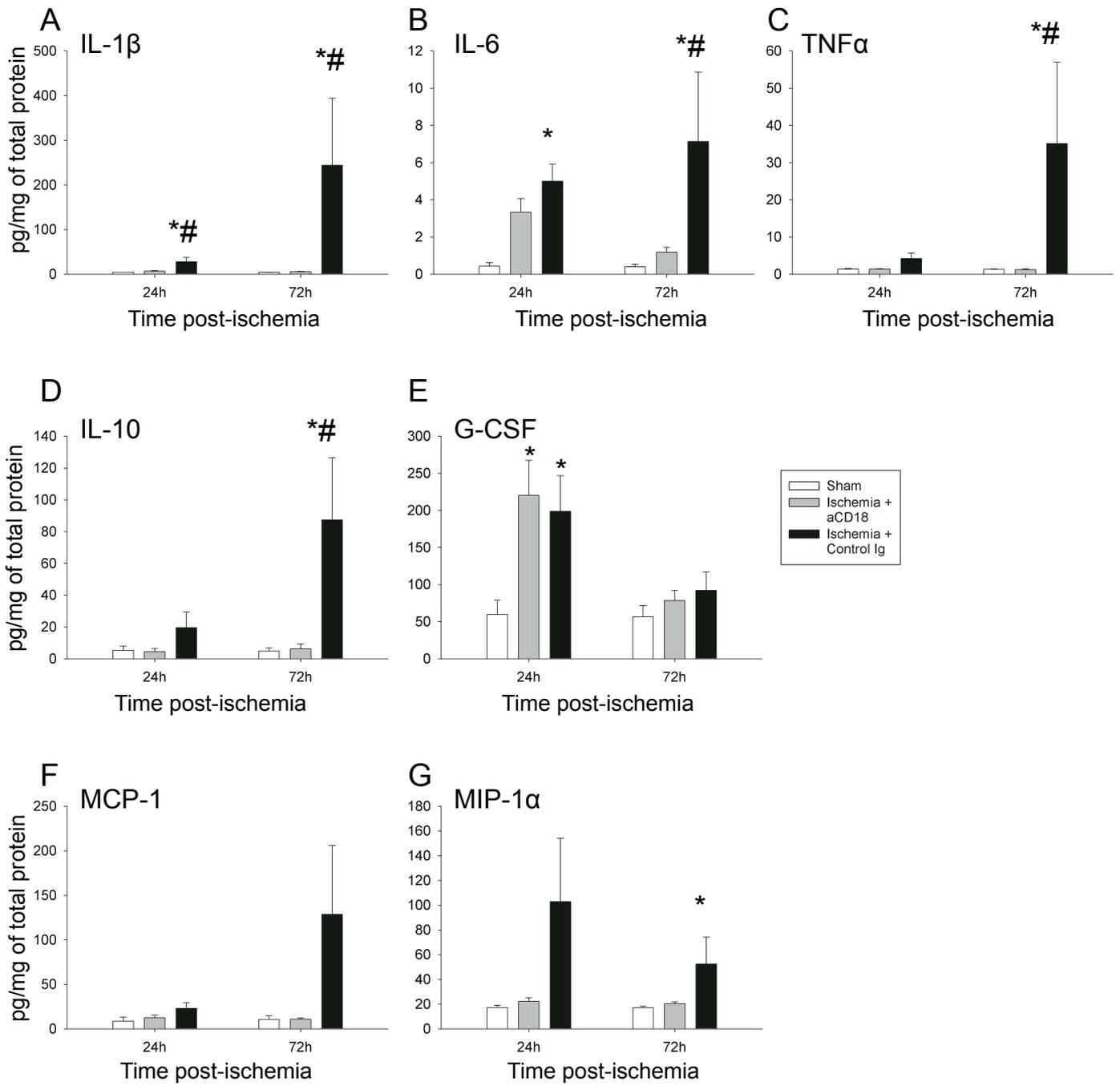


Figure 6. Anti-CD18 augmented systemic concentrations of soluble adhesion molecules following global cerebral ischemia. (A) Soluble ICAM-1 (sICAM-1) concentrations were significantly lower than sham at both 24h and 72h post-ischemia in the control antibody (Control Ig) treatment group, while concentrations of sICAM-1 were similar to sham in anti-CD18 (aCD18) group. (B) Soluble VCAM-1 (sVCAM-1) concentration was significantly lower than sham at 24h post ischemia in control Ig. However, the both experimental treatment groups exhibit a trend toward less than sham levels of sVCAM-1. (C) Anti-CD18 treatment group showed an increase in the peripheral blood concentration of soluble E-selectin (sE-Selectin) at 24 hours post-ischemia, but returned to sham levels by 72 hours post-ischemia. There was no significant difference in sE-Selectin concentration in the control Ig group. (n=4-6 mice per experimental group and time point) Data shown as mean \pm s.e.m. *p<0.05 compared to sham, #p<0.05 compared to aCD18.

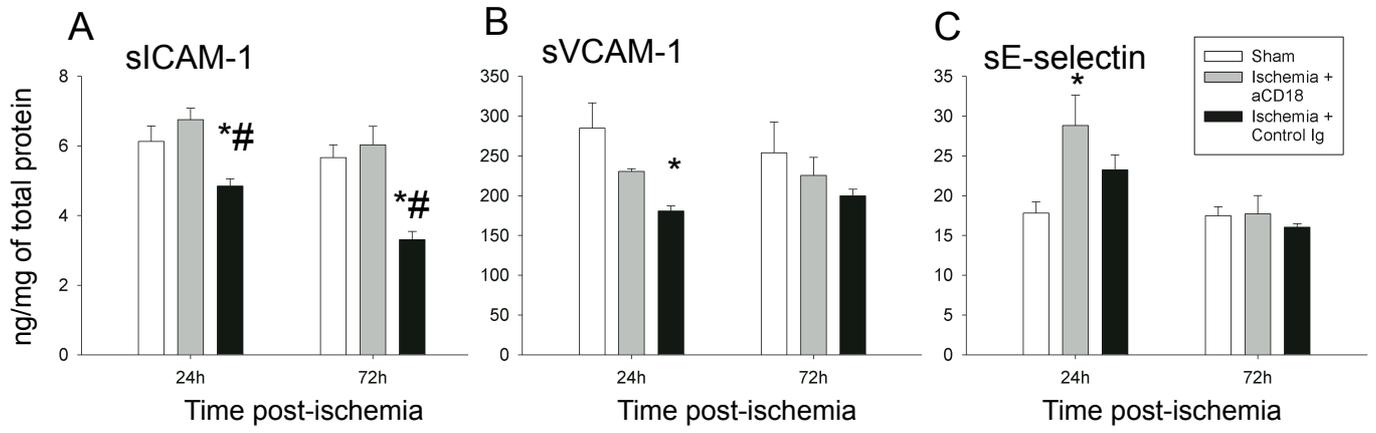


Figure 7. Other inflammatory proteins in the peripheral blood following cerebral ischemia. Other inflammatory proteins measured in serum, GM-CSF (**A**), KC (**B**), PAI-1 (**C**), and MMP-9 (**D**), exhibited minimal or no response to cerebral ischemia. Blocking adhesion did not affect the serum concentrations of these proteins. Data are shown as mean \pm s.e.m.

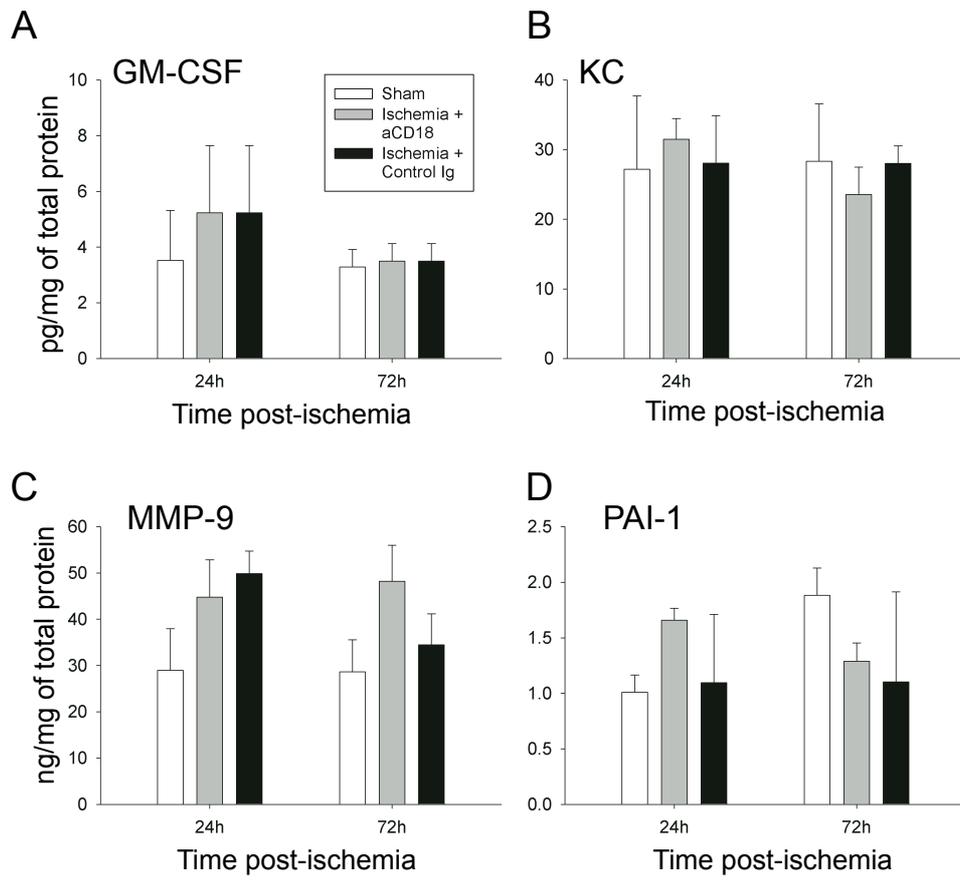


Figure 8. Interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α) expression in the brain are increased following global cerebral ischemia. Anti-CD18 treatment inhibited IL-1 β expression. (A-F), Representative images of IL-1 β (A-C) or TNF α (D-F) immunofluorescence (red) in the cerebral cortex 72 hours following sham surgery or ischemia with anti-CD18 and control antibody (Control Ig) treatment. The nuclei of all cells in the tissue were labeled with DAPI (blue). (G-J) IL-1 β and TNF α expressing cells along with total number of cells were counted in the CA1, CA2 and CA3 regions of the hippocampus, the cerebral cortex and thalamus following sham surgery or ischemia with anti-CD18 (aCD18) or control antibody (Control Ig) treatment. Anti-CD18 treatment significantly attenuated the number of cells expressing IL-1 β in the hippocampus (CA1 and CA3) at 24 hours (G) and in the cerebral cortex and thalamus at 72 hours (H) following ischemia compared to control antibody. Anti-CD18 treatment had no effect on the number of cells expressing TNF α , in all brain regions examined at 24 and 72 hours following ischemia, compared to control therapy. Scale bar: (in A) represents 50 μ m. All data shown as \pm s.e.m. * p <0.05 compared to aCD18. n=6/group.

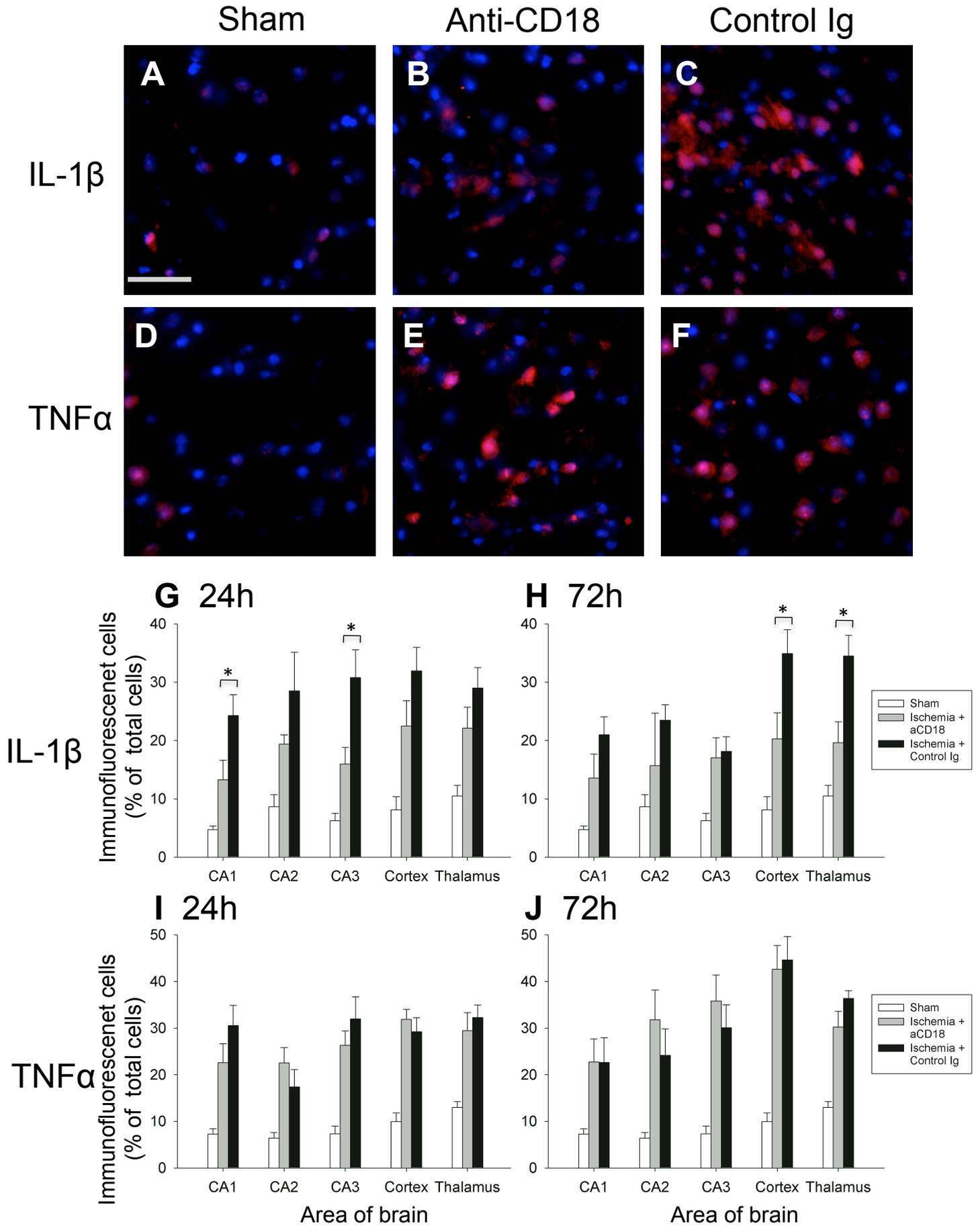


Figure 9. Cells expressing IL-1 β following cerebral ischemia are neurons. Double immunofluorescent labeling for IL-1 β (red) and NeuN (green) and merged images are shown in representative sections from the (A) hippocampus, (B) cerebral cortex and (C) thalamus. Scale bar: 50 μ m.

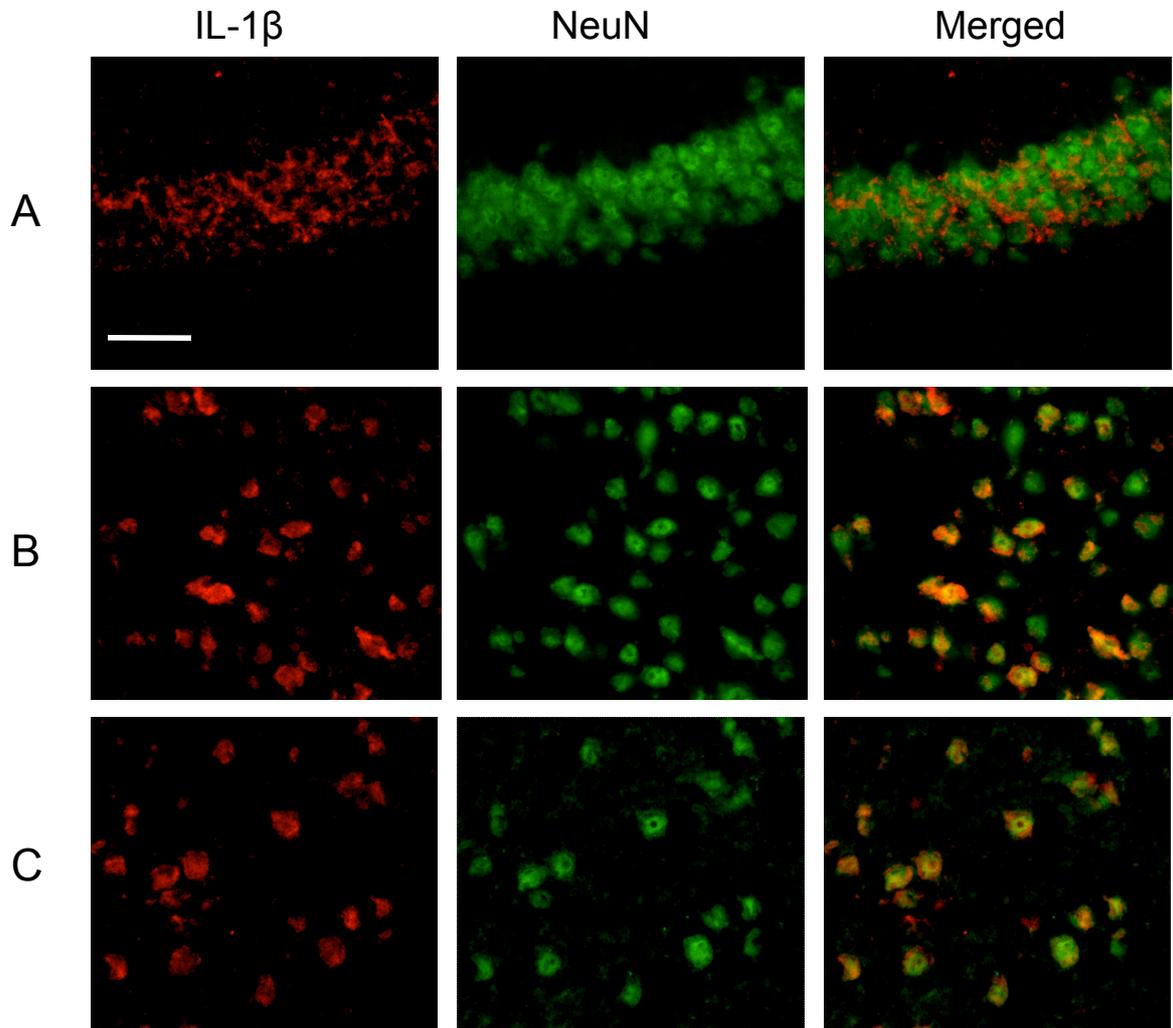


Figure 10. Anti-CD18 blocks inflammatory mediators in the hippocampus 24 hours following cerebral ischemia. There was a marked increase in the pro-inflammatory cytokines (**A-C**), anti-inflammatory (**D**), growth factor cytokines (**E-F**) and chemokines (**G-I**) after global cerebral ischemia. At 24h following ischemia with control antibody (Control Ig) therapy IL-1 β (**A**), IL-6 (**B**), G-CSF (**E**), and MCP-1 (**G**) in the hippocampus showed significant increases compared to sham tissue. This inflammatory response was blocked by anti-CD18 (aCD18) therapy. There was no significant changes in the hippocampal concentrations of TNF α (**C**), IL-10 (**D**), GM-CSF (**F**) and MIP-1 α (**H**) with ischemic insult. (n=8 mice per experimental group) Data shown as mean \pm s.e.m. *p<0.05 compared to sham; # p<0.05 compared to aCD18.

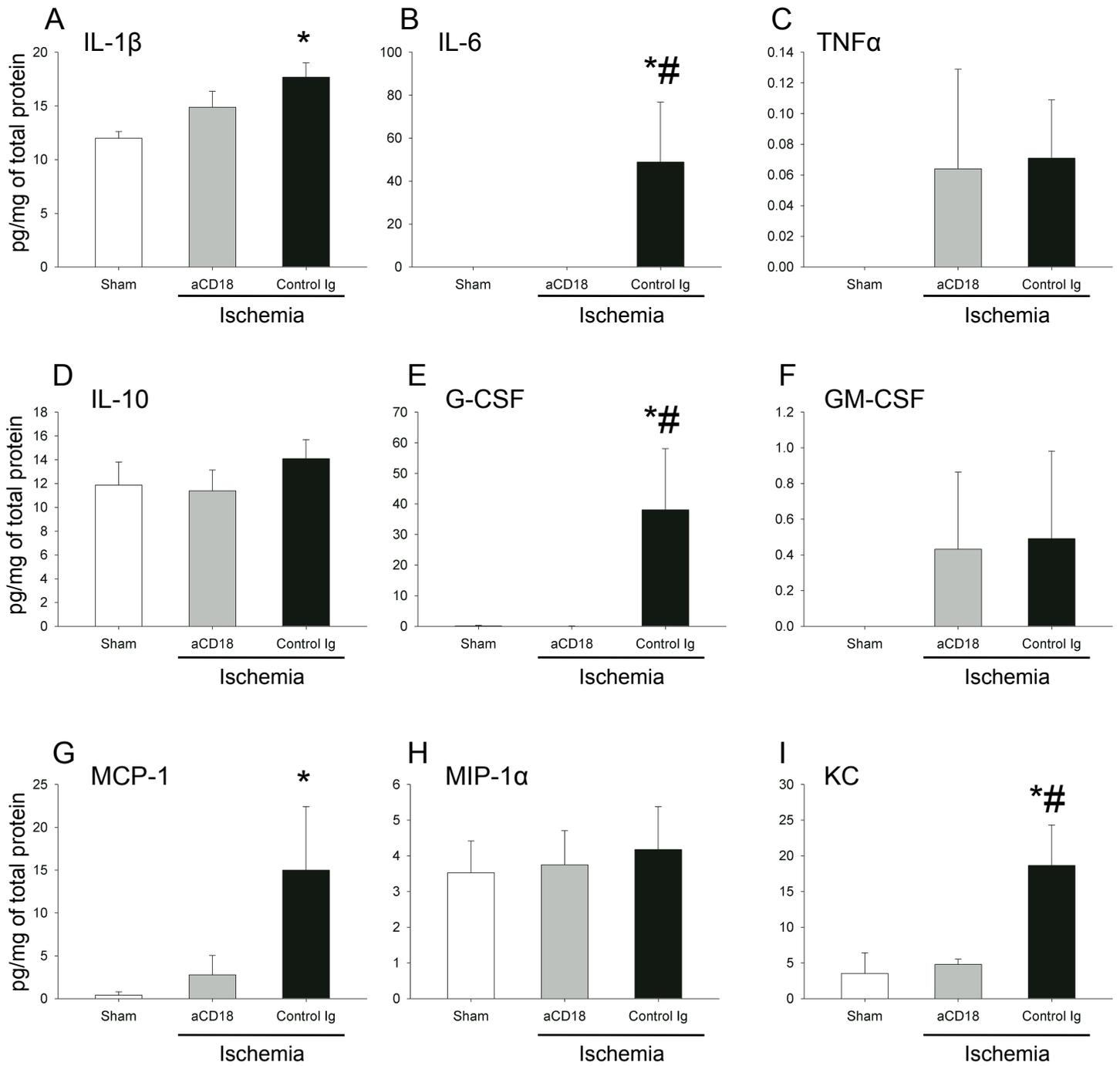


Figure 11. Anti-CD18 improved survival of brain cells 3 days post-ischemia. (A,B)

The hippocampus, dentate gyrus, cerebral cortex and thalamus were examined in coronal sections stained using hematoxylin and eosin 72 hours following ischemia with anti-CD18 therapy (aCD18, **A**) or control therapy (Control Ig, **B**). Live cells have normal architecture while dead or dying cells exhibit eosinophilia (pink cytoplasm), pyknosis (dark, condensed nuclei) or vacuolization. (**C**) The percent of live cells was evaluated 72 hours following ischemia. Anti-CD18 (n=9) therapy significantly increased the percent of viable cells following ischemia in the regions CA-1, CA-2 and CA-3 of the hippocampus, in the cerebral cortex and thalamus, but not the dentate gyrus (DG), compared to control Ig (n=6) therapy. Scale bar: (in CA-1 in **A, B**) all brain regions, 50 μm . Data shown as mean \pm s.e.m. * $p < 0.05$ compared to aCD18 group.

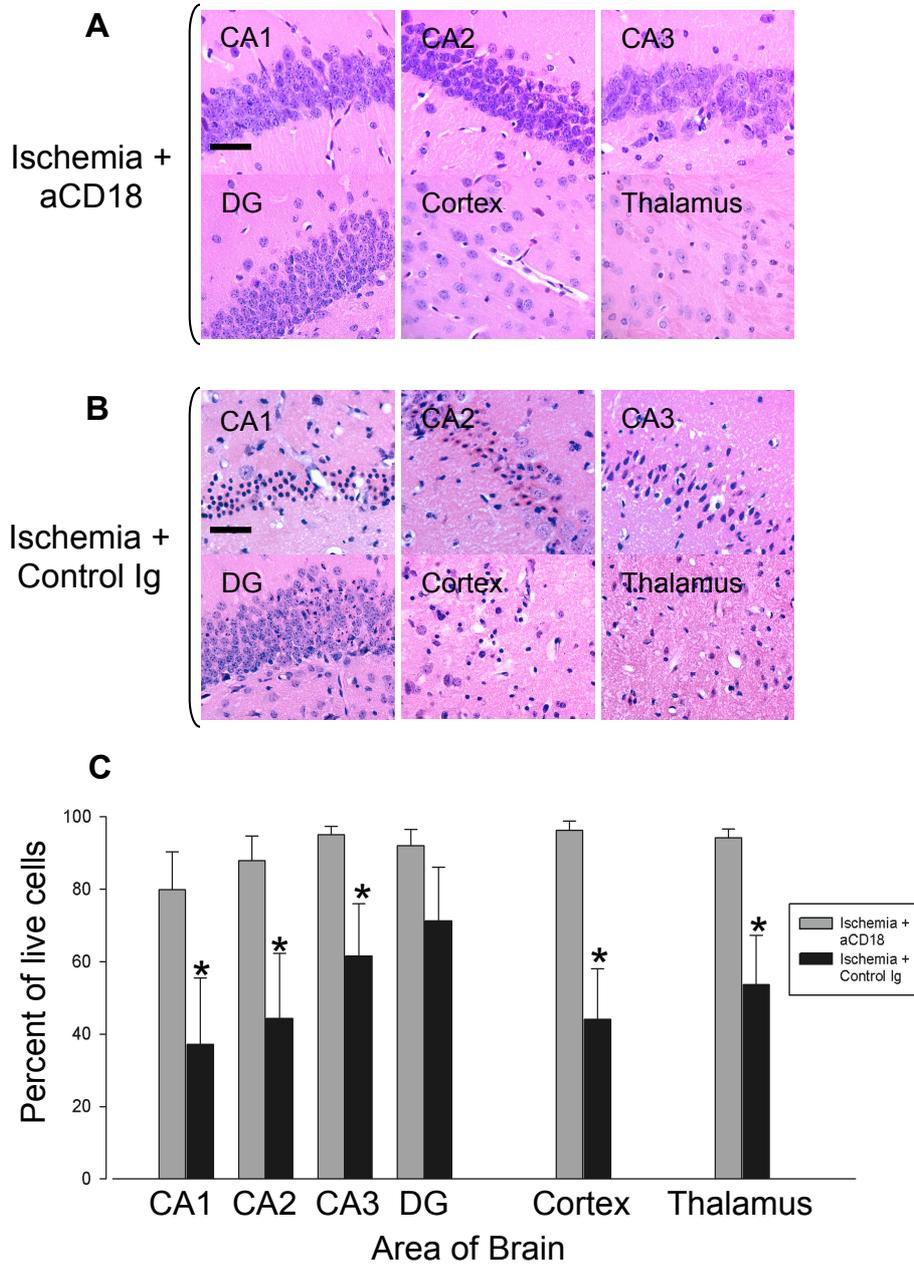


Figure 12. Anti-CD18 improved survival of hippocampal cells at 4 weeks post-ischemia. Representative microscopic images of the hippocampus show dying cells in CA-1 following ischemia in a mouse injected with non-binding control antibody (Control Ig) and a normal cellular pattern in a mouse injected with anti-CD18 (aCD18). The graph shows that mice with ischemia treated with anti-CD18 (n=9) had significant preservation of cells in the hippocampus pyramidal cell layer compared to the control Ig (n=8) and sham groups (n=11). Scale bar: (sham) all 3 experimental groups, 50 μ m. Data shown as mean \pm s.e.m. *p<0.05 compared to control group and aCD18 group.

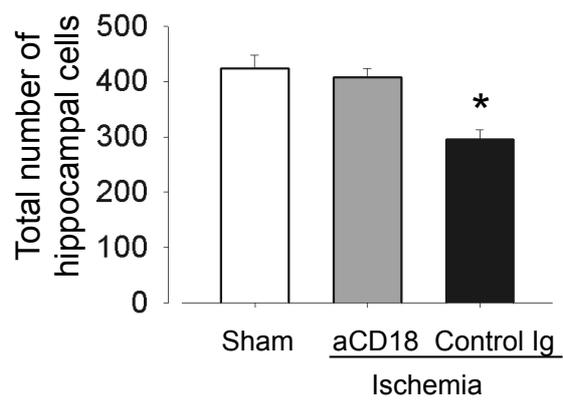
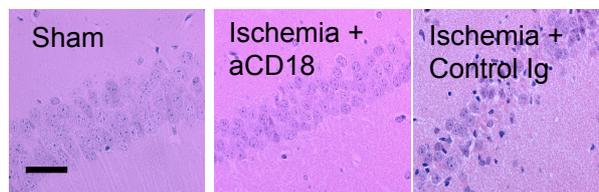


Figure 13. Anti-CD18 improves spatial memory 7 days following global ischemia.

(A) Prior to ischemia or sham operation the mice were trained, for 5 days with 2 sessions per day, to locate a submerged platform in a circular pool using spatial cues. The time required to reach the platform for each group of animals was recorded. Three days after training, mice were subject to sham surgery (Sham, n=11) or ischemia followed by 3 days of injections of anti-CD18 (aCD18, n=9) or control antibody (Control Ig, n=8). (B) At 7 days following ischemia or sham operation a single trial memory retention probe test was given, in which the platform was removed from the pool and the mouse was allowed to search for it, for 60 seconds. The percent of time spent in each platform zone (TZ: Trained zone, AdjR: Right adjacent zone, AdjL: Left adjacent zone, and Opp: opposite zone to trained zone) was evaluated during the probe test. Ischemic animals treated with anti-CD18 spent significantly more time searching for the platform in the trained zone, compared to all other zones, while ischemia animals treated with control antibody did not have a dominantly searched zone (# denotes zone of dominant searching, $p < 0.05$). (C) Composite swim patterns for each cohort shows greater searching in the trained zone for Sham and aCD18 animals compared to the NBC group. (Warmer colours denote greater density of swim paths). (D) The average swim speed of the animal was similar across experimental groups. (E) The percent of time spent in an area within 5 cm of the tank wall was recorded. Sham and aCD18 animals spent significantly less time in this zone than control Ig animals. All data displayed as mean \pm s.e.m. * $p < 0.05$ compared to sham.

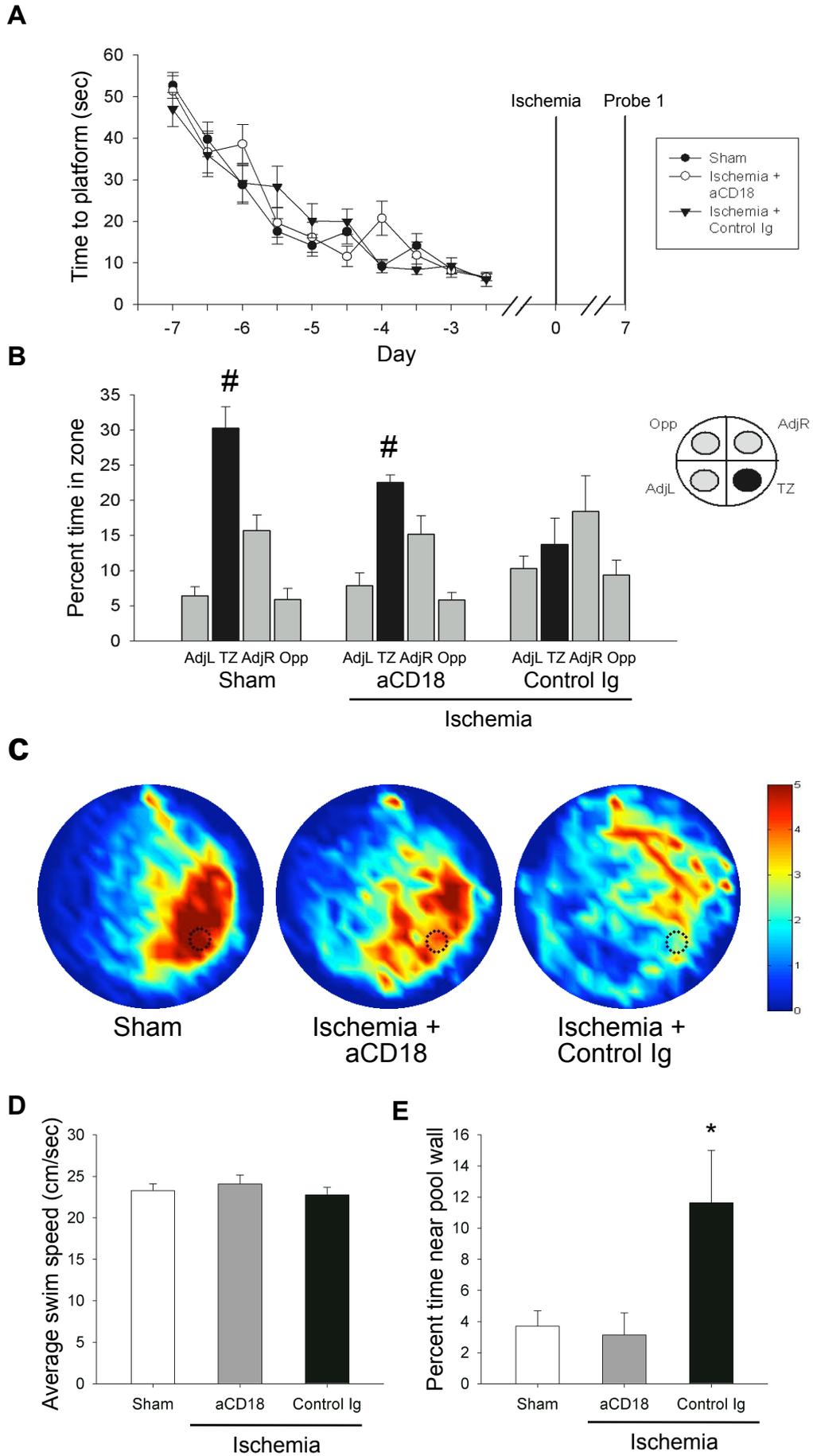
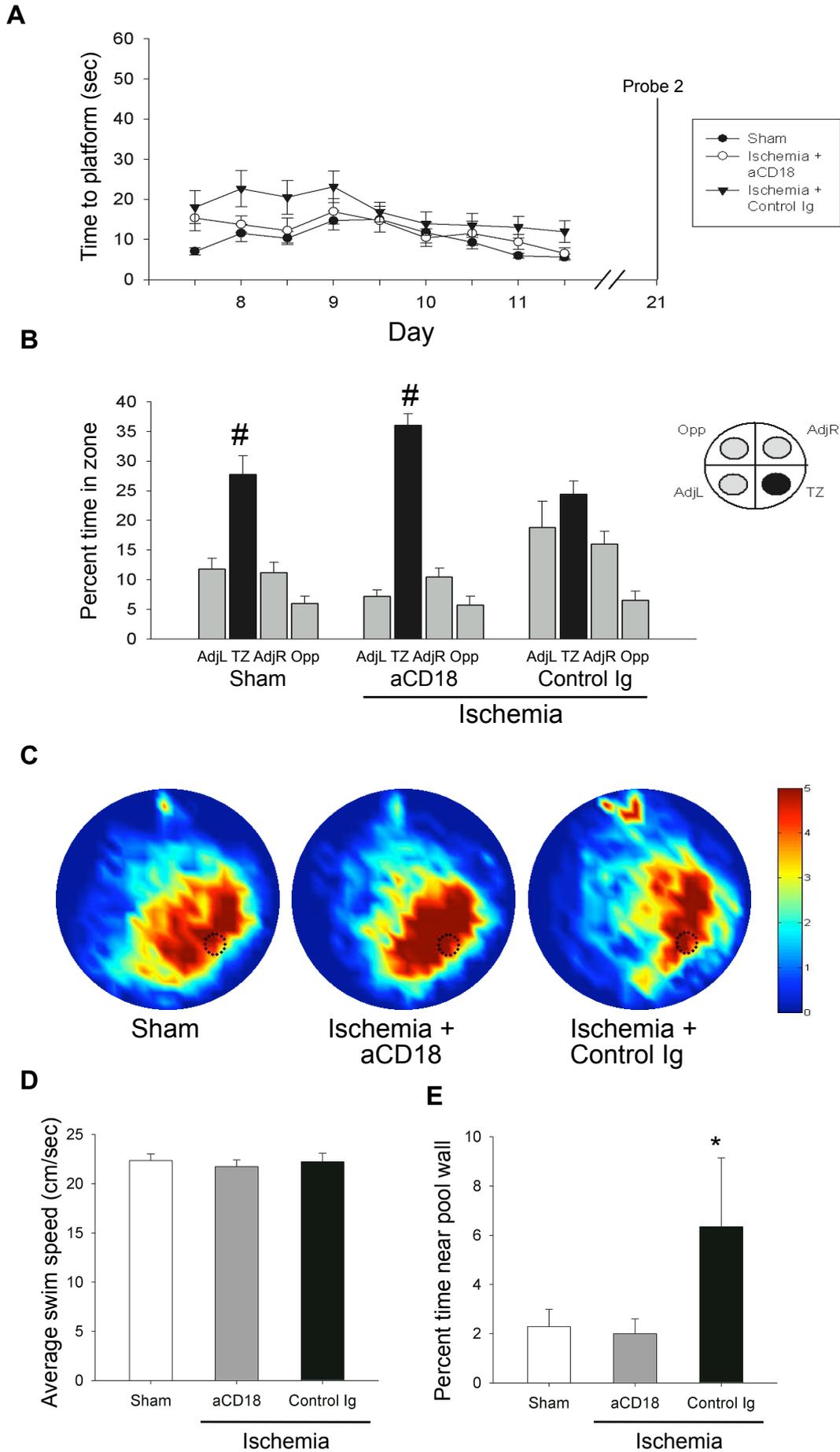


Figure 14. Anti-CD18 improves spatial 21 days following global ischemia. (A) Following the first probe test, platform training was resumed for 4.5 days. Another single trial memory retention probe test was administered one week following this training period. (B) During the second probe test the percent time spent swimming in each zone was determined (as in fig 12B). Animals injected with anti-CD18 therapy, following ischemia, spent significantly more time searching for the platform in the trained zone compared to all other zones while the control treatment group did not have a dominantly searched zone (# denotes zone of dominant searching, $p < 0.05$) (C) Composite swim patterns for each cohort shows greater searching in the trained zone for Sham and aCD18 animals compared to the control Ig group. (Warmer colours denote greater density of swim paths). (D) The average swim speed of the animal was similar across experimental groups. (E) The percent of time spent in an area within 5 cm of the tank wall was recorded. Sham and aCD18 animals spent significantly less time in this zone than control Ig animals. All data displayed as mean \pm s.e.m. * $p < 0.05$ compared to sham



Appendices

A1 Supplementary Methods:

A1.1 Laser Doppler Flowometry

Laser-Doppler flowometry (Model PF5000 with PR418-1 fiberoptic micro-probe, Perimed Inc., North Royalton, OH) placed 3 mm lateral and 6 mm posterior to the bregma and was used to record cerebral blood flow before, during and after induction of global cerebral ischemia.

A1.2 Micro computerized tomography (CT) scans

To confirm global forebrain ischemia in our model, mice received a subcutaneous injection of 500 units of heparin sulphate. A midline incision was made and the heart exposed by median sternotomy and sternal retraction. The left ventricle was then cannulated using a 25 gauge Butterfly® needle (Venisystems™, Abbott Laboratories, Sligo, Ireland). To allow free drainage of the perfusate, a right atriotomy was performed. The mice were then perfused with heparinised saline (0.9% NaCl with 0.1% Heparin Sulphate) for 10 minutes at a pressure of 160 mmHg at 37°C. Efficacy of perfusion was inferred by exsanguination of blood stained fluid followed by clearing of the solution exiting from the right atriotomy. Mice were then perfused for 10 minutes with 10 mls of a silicone rubber injection compound (Microfil™ MV 122, Flow-Tech Inc., Carver, MA, USA). This is a radio-opaque polymer that had been prepared according to the manufacturers instructions (a mixture of 4 mls of compound, 4 mls of diluent, and 0.45

mls of catalyst). The perfusate and the Microfil™ contrast were injected at a constant temperature and pressure by a perfusion apparatus. Confirmation of adequate systemic distribution of the Microfil™ was inferred by observing the vasculature in the splanchnic circulation and the femoral vessels turning the yellow color of the Microfil™. After the perfusion process was complete, the Butterfly® catheter was clamped and the mouse was left for 90 minutes to allow the Microfil™ to polymerize. The mouse head was decapitated and de-skinned and placed over night in 8% Formic Acid to facilitate bone decalcification. The cranium was then carefully removed, and placed in 10% formalin until computerized tomography scanning. Brains prepared with the Microfil™ contrast protocol were mounted on 10% gelatin with a small drop of 10% formalin, and then aligned on a scanning bed. A Locus SP Pre- Clinical Micro CT (GE Healthcare, Waukesha, WI, USA) was used to perform the scans. The scan protocol was for 720 views separated by 20 µm for 20 mm using a current of 80 µA (voltage 80 kV) with a 360° rotation for 2 hours. The angle of increment was 0.5° with an exposure time of 1700 ms.

A1.3 Intravital Microscopy

See the materials and methods in the manuscript.

A1.4 Neutrophil immunofluorescence and myeloperoxidase (MPO) assay

Mice were sacrificed at 24 hours (n=4/group) following ischemia. Mice were

transcardially perfused with 10 ml of 0.9% saline, followed by 10 ml 4% paraformaldehyde in 0.1 M phosphate buffer to wash out neutrophils in the microcirculation and thus quantify only infiltrating neutrophils. Perfused tissues were post-fixed in 4% paraformaldehyde overnight, then cryopreserved in a 10% sucrose phosphate buffered solution for 48 hours at 4°C. Brains were frozen using gaseous carbon dioxide, and coronally sectioned (10 µm) onto slides at bregma 1.1, -0.22, -1.94, and -3.16 mm for immunofluorescent staining. Neutrophil immunoreactivity was determined by labeling with a rat anti-mouse monoclonal neutrophil antibody (Cedarlane, Hornby, ON, Canada) and FITC labeled goat anti-rat IgG (1:200, Amersham Life Science Inc., Oakville, ON, Canada) and counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (1:1000, DAPI, Molecular Probes, Oregon, USA). The neutrophil antibody recognizes a polymorphic myelomonocytic antigen 7/4 found at high levels on neutrophil but absent from macrophages, and expressed at very low levels on monocytes.

Neutrophil mouse peritoneal exudate induced by intra-peritoneal injection of 2% glycogen (Sigma Chemicals Co, St. Louis, MO, USA) was used as a positive control. Two negative controls were also included: one processed as described above but lacking the addition of the neutrophil antibody, the other processed as described above but using a non-specific mouse IgG primary antibody (1:200, Sigma-Aldrich, Mississauga, ON, Canada) instead of the neutrophil antibody. Neutrophil and DAPI positive immunoreactivity was visualized using an upright epifluorescence microscope (Zeiss Axiophot microscope and Zeiss Plan-Neofluoar objectives, Germany).

Myeloperoxidase (MPO) is a heme lysosomal enzyme present in the azurophilic

granules of neutrophils. MPO activity was used as an indicator of tissue neutrophil accumulation in ischemic mouse brains. The assay is based on the ability of MPO to oxidize odianisidine (O-D) in the presence of H₂O₂ to generate a colored complex, which can be monitored spectrophotometrically. To eliminate intravascular blood, 24 h post-ischemic or sham-operated mice were intracardially perfused with 10 ml cold 0.9% NaCl (n=4/group). Brains were removed and frozen in 2-methylbutane (Sigma-Aldrich, Mississauga, ON) on dry ice, and stored at -80°C in an upright freezer. Brains were thawed, weighed in milligrams wet weight, and put on ice. Myeloperoxidase activity was quantified using the procedure described by Barone et al. (Barone et al., 1991). Aliquots of neutrophil rich peritoneal exudate were used as controls. Results were reported in U/gm wet weight, where 1 unit of MPO activity is defined as that degrading 1µmol peroxide/minute at 25°C.

A2 Supplementary Results:

A2.1 Validation of Global Forebrain Ischemia

Using laser Doppler flowmetry we confirmed severe incomplete forebrain ischemia (Supplementary Figure 1A). During sham surgery or bilateral common carotid artery occlusion, the brain was perfused with a radio-opaque silicone rubber based contrast agent via the left ventricle of the heart. Micro-computerized tomography scans showed a lack of perfusion of the radio-opaque polymer to the forebrain in the ischemia group (Supplementary Figure 1B).

A2.2 Leukocyte adhesion to endothelial cells is inhibited by monoclonal antibody to CD18

We confirmed leukocyte adhesion to endothelial cells in cerebral venules using fluorescent intravital microscopy through a cranial window following intra-peritoneal injection of the pro-inflammatory cytokine IL-1 β . (Supplemental Fig. 2A) The IL-1 β stimulated leukocyte rolling and adhesion was inhibited with anti-CD18 monoclonal antibody treatment. (Supplementary Figure 2B)

A2.3 Neutrophil immunofluorescence and MPO assay

To investigate neutrophil infiltration in this global cerebral ischemia model, sham-operated and ischemic mice were sacrificed at 24 hours of reperfusion. Dual immunofluorescence labeling with DAPI and a monoclonal neutrophil antibody was performed on coronally sectioned brain. Although numerous neutrophil/DAPI immunoreactive cells were found in the murine neutrophil positive control

(Supplementary Fig. 3A), no neutrophil positive immunoreactivity was found in any sham (Supplementary Fig. 3B). In the four ischemic mice, a total of two neutrophil/DAPI positive cells were found, both in the cerebral neocortex (Supplementary Fig. 3C). These findings suggest a negligible infiltration of neutrophils into cerebral parenchyma occurs 24 hours following global ischemia-reperfusion in this model.

Following 24 hours reperfusion, MPO activity was measured in brain tissue homogenates from sham-operated, and ischemic mice. Although neutrophil positive controls showed substantial MPO activity (1.03 ± 0.19 U), no significant MPO activity was found in sham or ischemia groups (Supplementary Fig. 3D). These findings suggest that no infiltration of neutrophils into cerebral parenchyma occurs 24 hours following global ischemia-reperfusion in this model.

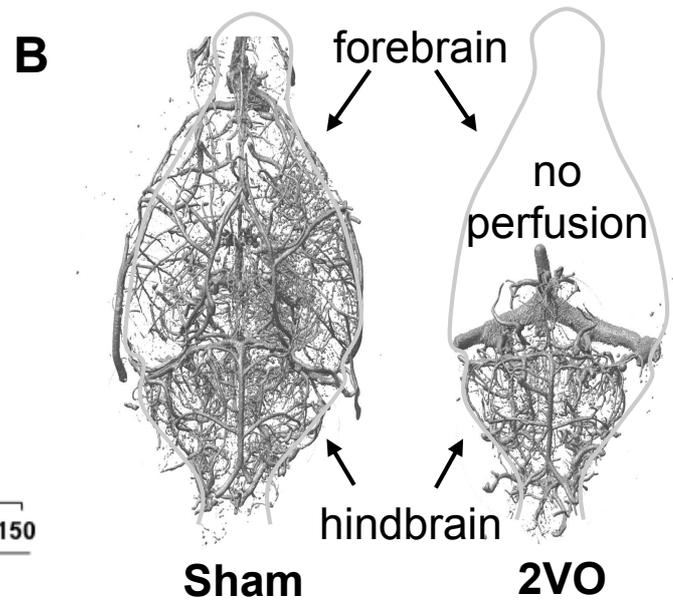
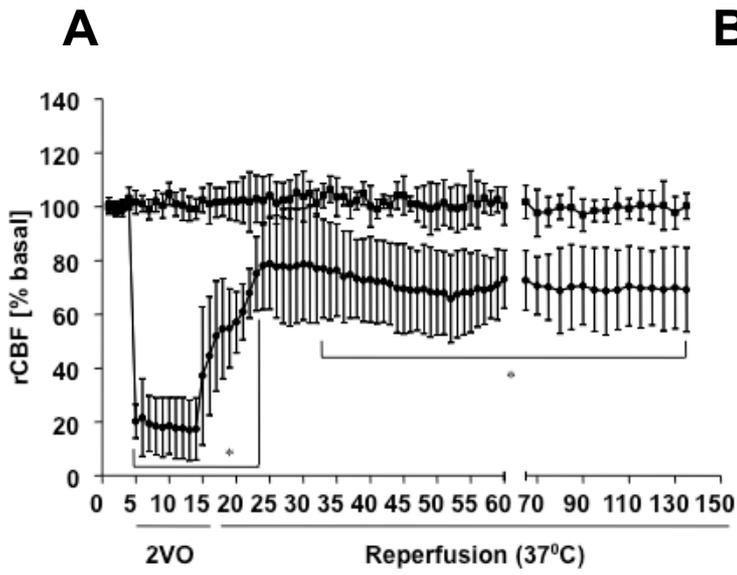
A3 Supplementary Figures:

Supplementary Figure 1: Validation of global cerebral ischemia animal model. (A)

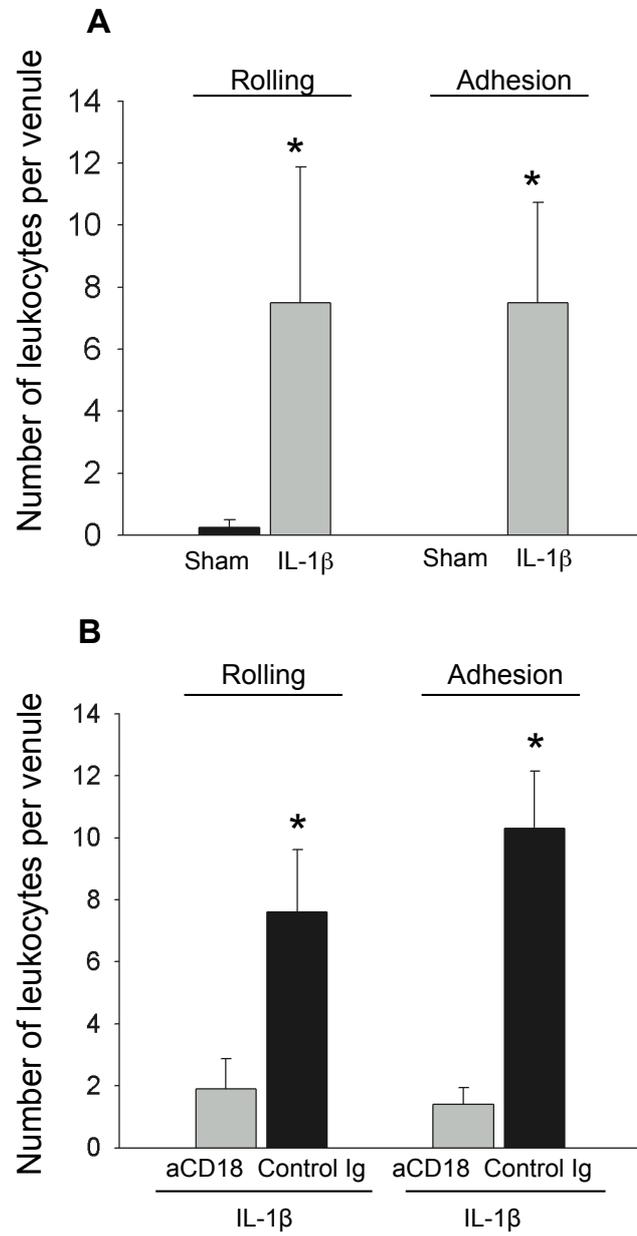
Regional cerebral blood flow measured using laser-Doppler flowmetry before, during and following sham (upper line no ischemia, n=5 mice) or ischemia (lower line, n=5 mice).

*denotes $P < 0.05$ compared to sham. **(B)** Three-dimensional iso-intensity surface

rendering of a microcomputed tomography scan of the mouse C57BL/6 brains during sham and cerebral ischemia illustrates bilateral common carotid artery occlusion [2 vessel occlusion (2VO)] as a forebrain global ischemic injury model.



Supplementary Figure 2. Leukocyte-endothelial cell interactions are increased following an injection of IL-1 β . Anti-CD18 inhibits leukocyte adhesion to endothelial cells. Numbers of leukocytes rolling and adhering were counted in pial venules using intravital microscopy through a cranial window. **(A)** Three hours following an injection of the IL-1 β (n=4) there was significantly more leukocytes rolling and adhering compared to mice injected with normal saline (sham, n=4). **(B)**, Three hours following an injection of IL-1 β , anti-CD18 treatment (aCD18, n=9) significantly reduced both rolling and adherent leukocytes compared to the non-binding control treatment (Control Ig, n=6) All data are displayed as mean \pm s.e.m. *p<0.05 compared to other group.



Supplementary Figure 3. Global cerebral ischemia did not induce infiltration of neutrophils into the mouse brain at 24 hours post-ischemia. After 24 hours survival, dual immunohistochemical labeling with a monoclonal mouse FITC-neutrophil antibody, and DAPI was performed on coronally sectioned brain, and sections were examined using a fluorescent microscope. Isolated murine neutrophil exudate was used as a positive control (**A**). Mice received sham-operation (**B**) or global cerebral ischemia (**C**). Arrows indicate positive immunoreactivity for both DAPI and mouse neutrophils (n = 4 mice/group). (**D**) Myeloperoxidase (MPO) assay was also used to determine neutrophil infiltration following cerebral ischemia in mice. Following 24 h reperfusion, MPO activity was measured in brain tissue homogenates from sham- operated, and ischemic mice. MPO activity was determined in isolated murine polymorphic nuclear cell peritoneal exudate from normal mice as a positive control. No significant difference ($p>0.05$) was found between the sham or ischemia groups. All data shown as \pm SD. n=4/group.

