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Therapeutic Modalities Targeting Neuroinflammation After Neonatal Hypoxia-Ischemia

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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Therapeutic Modalities Targeting Neuroinflammation After Neonatal Hypoxia-Ischemia

by

Nancy Fathali

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Anatomy

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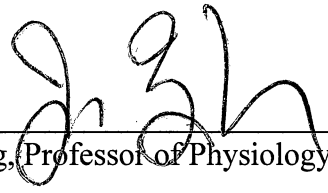

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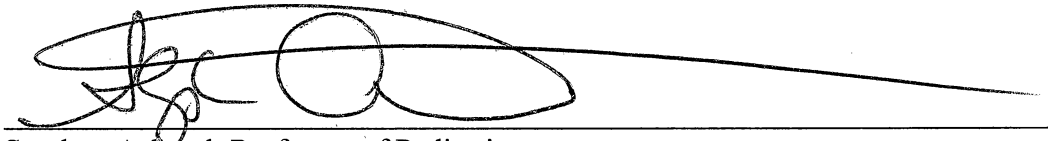

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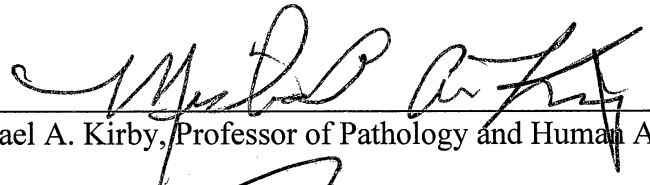

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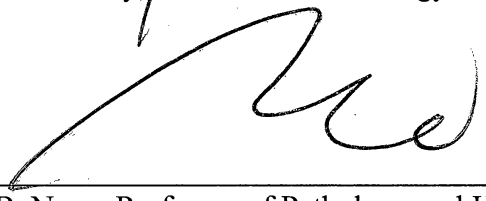

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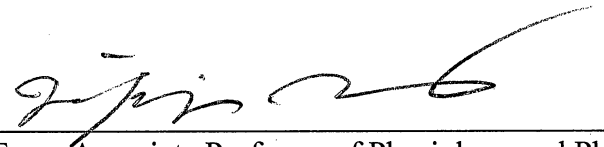

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Pedar va Madareh azizam, bah voo-joodeh-inkeh mee-do-nam heech kalah-meh-ee, yah johm-leh-hee barah-yeh tasha-kohr as sho-mah coffee-neest, valie-in-rah-beh-dah-neen, keh har-chee has-tam, va har-chee dah-ram, as sho-mahst. Va beh-do-neh sho-mah, har-gehz beh injah-ee keh ehm-rooz reh-seed-am, neh-me-reh-see-dan. Va in-rah-beh-dah-neen, keh ta roozie keh zehn-deh has-tam, boh-zohrg-ee-tahn-rah har-ghez fah-rah-moosh na-kha-ham-kard. Kheileeh doosteton daram!

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ABBREVIATIONS

4-HNE	COX-2 agonist
CD3	T cell marker
CD68	macrophage marker
CD161	natural killer cell marker
COX-2	cyclooxygenase-2
CNS	central nervous system
DAB	diaminobenzidine
G-CSF	granulocyte-colony stimulating factor
G-CSF(5d)	HI with G-CSF daily for 5 days
G-CSF(10d)	HI with G-CSF daily for 10 days
GFAP	glial fibrillary acidic protein
HI	hypoxia-ischemia
Iba1	microglia marker
IL	interleukin
iNOS	inducible nitric oxide synthase
INT	intact spleen
MPO	myeloperoxidase – neutrophil marker
NeuN	neuron marker
NK	natural killer
NS398	selective COX-2 inhibitor
NS-10	HI with 10 mg/kg NS398
NS-30	HI with 30 mg/kg NS398

PBS	phosphate-buffered saline
PG	prostaglandin
PI3K	phosphoinositide-3-kinase
RH/LH	right hemisphere to left hemisphere
RT-PCR	reverse transcriptase polymerase chain reaction
S.E.M.	standard error mean
siRNA	small interfering RNA
SPLN	splenectomy
TNF-α	tumor necrosis factor-alpha
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling

ABSTRACT OF THE DISSERTATION

Therapeutic Modalities Targeting Neuroinflammation After Neonatal Hypoxia-Ischemia

by

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Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, June 2010
Dr. John H. Zhang, Chairperson

Hypoxia-ischemia (HI) occurs in 1-6/1000 live full-term births (Shankaran, 2009). Of those affected, 15-20% will die in the postnatal period, and 25% of survivors will be left with long-term neurological disabilities (Gunn, 2000; Vannucci, 1997; Fatemi, 2009). It has become increasingly clear that peripheral immune cells infiltrate the brain parenchyma as part of the physiological response to tissue damage after HI injury. The interplay between infiltrating immune cells and brain resident cells during the inflammatory response is however dynamic and complex; in that neuro-immune crosstalk, by way of specific molecular mediators, is responsible for both neurodestructive as well as neuroprotective outcomes. Herein, we tested the hypothesis that COX-2 mediates mechanisms of brain injury and that G-CSF exerts structural and functional protection after neonatal HI.

To mimic the clinical features of HI brain injury, neonatal rat pups were subjected to unilateral carotid artery ligation followed by 2 hours of hypoxia (8% O_2 at 37°C). We used a gain and loss of function approach (pharmacological activation or inhibition, respectively) for COX-2, a neutralizing antibody for IL-15, and a gene silencer for natural killer cells in both splenectomized and non-splenectomized rats to verify the role of COX-2 in splenic immune cell responses following HI. We found that elevations in

COX-2 expression by immune cells promoted IL-15 expression in astrocytes and infiltration of inflammatory cells; additionally, down-regulated the pro-survival protein, PI3K, resulting in caspase-3 mediated neuronal death. Additionally, we investigated the efficacy of G-CSF on long-term HI-induced morphological and functional outcomes using two different dosing regimens; and found the neurotrophic factor to significantly improve behavioral and neuropathological recovery.

These results provide insight into the mechanistic basis of inflammation and indentify key components of the neuroinflammatory response after HI. Thus, we propose that COX-2 inhibition or G-CSF administration during the acute phase of injury are novel therapeutic modalities that target detrimental and beneficial mechanisms of neuroinflammation, respectively, and may offer a safe and effective option with long-term benefits for the HI-injured infant.

CHAPTER ONE

THE EVOLVING LANDSCAPE OF NEUROINFLAMMATION AFTER NEONATAL HYPOXIA-ISCHEMIA

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Abstract

Hypoxic-ischemic brain injury remains a leading cause of mortality and morbidity in neonates. The inflammatory response, which is characterized, in part, by activation of local immune cells, has been implicated as a core component for the progression of damage to the immature brain following hypoxia-ischemia (HI). However, mounting evidence implicates circulating immune cells recruited to the site of damage, as orchestrators of neuron-glial interactions and perpetuators of secondary brain injury. This suggests that re-directing our attention from the local inflammatory response toward the molecular mediators believed to link brain-immune cell interactions may be a more effective approach to mitigating the inflammatory sequelae of perinatal HI. In this review, we focus our attention on cyclooxygenase-2 (COX-2), a mediator by which peripheral immune cells may modulate signaling pathways in the brain that lead to a worsened outcome. Additionally, we present an overview of emerging therapeutic modalities that target mechanisms of neuroinflammation in the hypoxic-ischemic neonate.

Introduction

Hypoxia-ischemia (HI) occurs in one to six per 1000 live full-term births (63). Of those affected, 15-20% will die in the postnatal period, and 25% of survivors will be left with long-term neurological disabilities (24,32,70). Intrauterine asphyxia is the underlying mechanism of hypoxic injury and is a consequence of circulatory problems including clotting of placental arteries and placental abruption (50). HI in the neonate is a manifestation of systemic hypoxia combined with reduced cardiac output (48).

Studies have shown that the pathophysiology of brain injury secondary to HI consists of a biphasic profile (Figure 1). The initial phase of HI is characterized by brain acidosis and the depletion of high-energy phosphorylated compounds, such as adenosine triphosphate and phosphocreatine (12,33,63). This primary energy failure leads to the loss of membrane ionic homeostasis, depolarization of the cell, osmotic dysregulation, and inhibition of protein synthesis, further leading to necrosis (39,44). The secondary processes evolve over days after the brain insult, and are also characterized by a depletion of high-energy phosphorylated compounds however without tissue acidosis. Although the pathogenesis of secondary brain injury involves multiple pathophysiologic processes, accumulating evidence implicates the inflammatory response as a core component of damage (3,25,26).

Neuroinflammation includes initial release of pro-inflammatory mediators by injured or dying cells, activation of microglia and astrocytes, and leukocyte infiltration. It is the synergistic actions of these events that potentiate brain damage and lead to neurological dysfunction (56). However, experimental studies thus far have focused mainly on selectively targeting these mechanisms, which may explain why there are no

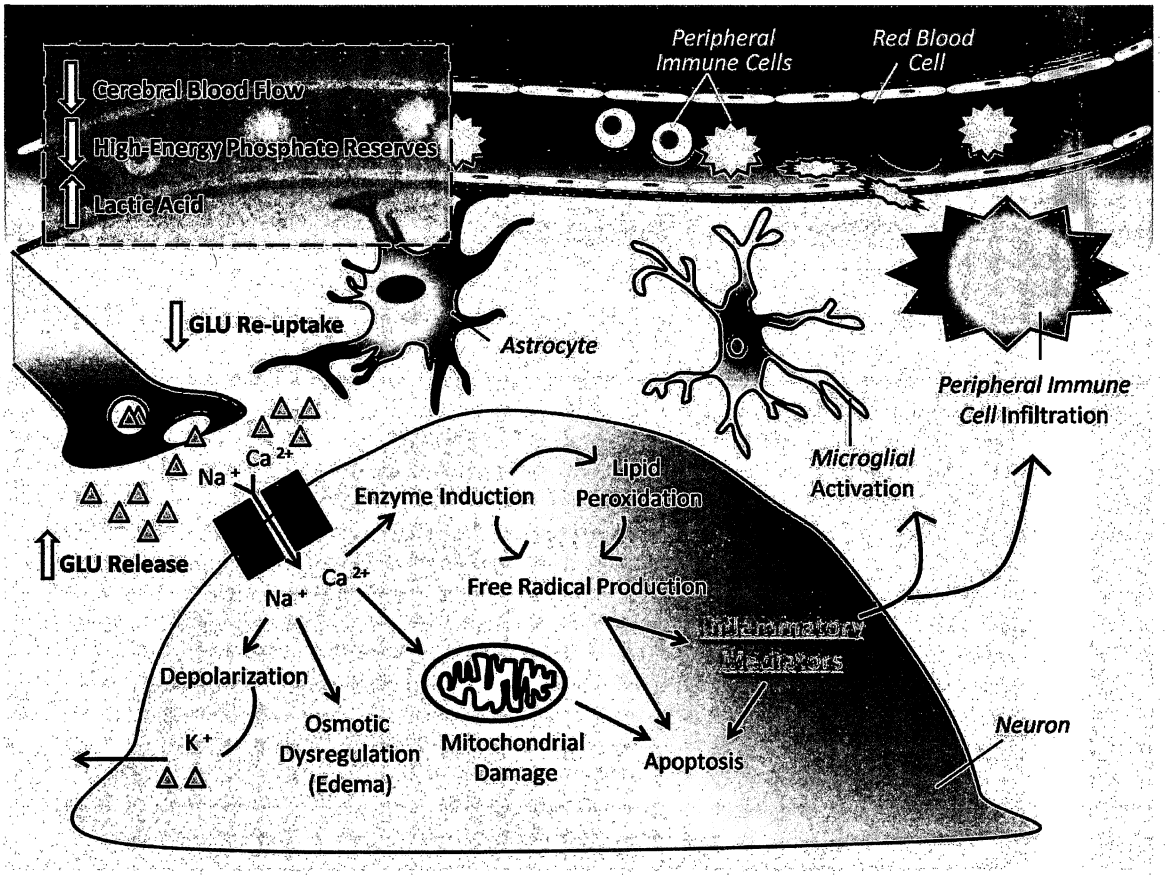
Peripheral Immune Cells
Red Blood Cell
Cerebral Blood Flow
High-Energy Phosphate Reserves
Lactic Acid
GLU Re-uptake
Astrocyte
Peripheral Immune Cell Infiltration
Microglial Activation
Ca 2+
Na +
Enzyme Induction
Lipid Peroxidation
GLU Release
Ca 2+
Free Radical Production
Na +
Inflammatory Mediators
Depolarization
K +
Osmotic Dysregulation (Edema)
Mitochondrial Damage
Apoptosis
Neuron

Figure 1. Pathophysiology of a Hypoxic-Ischemic Event. Decrease in cerebral blood flow results in a decrease of high-energy phosphate reserves (i.e., adenosine triphosphate; phosphocreatine) and a build-up of lactic acid. Loss of membrane ionic homeostasis leads to intracellular accumulation of sodium (Na^{+}), calcium (Ca^{2+}), and water (edema); thereby depolarizing the cell and releasing glutamate (triangle) and potassium (K^{+}) into the extracellular space. Intracellular calcium ion accumulation leads to enzyme induction (i.e., lipases; proteases; endonucleases) and free fatty acid elevation, which undergo peroxidation. The result is the accumulation and/or release of inflammatory mediators (i.e., cyclooxygenase-2) which can lead to apoptosis, glial activation, and peripheral immune cell infiltration.

pharmacotherapies proven clinically viable for the treatment of HI brain damage. In fact, increasing evidence suggests identifying molecular mediators responsible for orchestrating brain-immune cell interactions as a more promising approach (18,20,49).

This review will provide a brief overview of the current understanding of the local and peripheral inflammatory response involved in neonatal HI, and the role of cyclooxygenase-2 (COX-2) in brain-immune cell interactions and the progression of neuroinflammation.

Changing Landscape of Neuroinflammation

The immune response in the brain is highly complex and involves the participation of several different resident cells (Figure 2). Microglia, astrocytes, and neurons directly react and contribute to neuroinflammation in the HI-injured neonate. The role of each of these cell types in propagating the local inflammatory response is important in understanding the dynamic microenvironment.

Microglia

Microglia cells serve as specialized sensors for brain tissue damage. In response to ischemia, microglia morphologically change from a resting ramified phenotype to a motile activated amoeboid cell able to migrate to necrotic areas to remove cellular debris (43,44). However, in the process, these activated cells contribute to secondary brain injury by releasing a variety of pro-inflammatory mediators including cytokines, reactive oxygen species, complement factors, free radical species and nitric oxide, which contribute to cell death, ultimately creating a vicious perpetuating cycle (24).

Mounting evidence suggests that infiltrating peripheral immune cells may be necessary for the activation of microglia, thereby exacerbating neurodegeneration after ischemia. In an *in vitro* study, microglia when co-cultured with T-cells, become activated thereby releasing an inflammatory cytokine (14). In an *in vivo* study, removal of a population of infiltrating macrophages, neutrophils, B cells, and T cells by splenectomy appeared to reduce microglia activation and dramatically reduce brain damage (2). Systemic inhibition of monocyte/macrophage or neutrophil populations has also shown to reduce cerebral infarct volume after ischemic injury (16,17,78). However, the exact mechanism by which peripheral immune cells activate and/or propagate the local inflammatory response and enhance neuronal death remains to be determined.

Astrocytes

Astrocytes which are the predominant glial cell type in the central nervous system (CNS), have been shown to produce inflammatory mediators in a variety of brain injures including HI (61,62). Inflammatory cytokines have been associated with neonatal HI brain damage, and later development of cerebral palsy (19,24). Specifically, elevated levels of interleukin (IL)-6 in cerebrospinal fluid of asphyxiated newborns have been correlated with an increased degree of brain damage and poor neurological outcome (58). Additionally, more recent evidence has implicated IL-15 as playing a leading role in neuroinflammation in the injured immature rat brain (51). Importantly, astrocytes are the main source of both IL-6 and IL-15 in CNS injury and inflammation (24,29).

Astrocytes may also influence the local inflammatory response through their communicative partnership with neighboring cells (31). Under pathological conditions, astrocytes play a critical role in the activation of microglia (55,67), and by-products of

reactive astrocytes such as tumor necrosis factor-alpha (TNF-α) and IL-6 are associated with neuronal demise after HI (72). On the other hand, astrocytes are also a source of trophic factors, such as granulocyte-colony stimulating factor (G-CSF) (22), and are responsible for regulating neurotransmitter and ion concentrations, removing debris, and maintaining an optimal environment for neuronal function (5). Impairment of astrocyte function during HI is thought to influence neuron viability (72). Therefore, it is important to identify key molecular mediators responsible for initiating astrocyte signaling pathways involved in worsening brain injury, without eliminating the protective function of these cells.

Neurons

Once thought to be passive bystanders in neuroinflammation, neurons are now known to be playing more of an active role. As such, neurons can be a source of inflammatory mediators including complement, COX-2 and cytokines after HI (25,68). Neurons can express COX-2 at low levels under normal conditions; however, under pathological conditions, COX-2 is upregulated in response to mitogens, inflammatory mediators, and hormones (38). Induction of COX-2 expression in neurons is also driven by physiological synaptic activity (73) and acute paradigms of excitoxicity (1), whereby promoting local inflammatory reactions and injury to themselves (23,53,54). Moreover, neurons contribute to the production of pro-inflammatory mediators that can alter vascular permeability and regional blood flow, and enhance chemotactic activity and thereby promote leukocyte migration (3).

Once peripheral leukocytes and monocytes enter the brain parenchyma their actions appear to be multifaceted (77). Certain immune cell subpopulations may directly

elicit neuronal death via contact-dependent mechanisms (28) or release molecular mediators that activate resident cells, thus promoting further brain injury (4,11). In line with this concept, recent studies have shown that T-lymphocyte-deficient mice demonstrate attenuated brain injury and neurological deficits after experimental stroke (37,76). To make matters more complex, regulatory T-lymphocytes have been shown to have a protective role in the brain after stroke (45). Immune cells have been also implicated in the generation of new neurons and improvement of spatial learning and memory performance in neurodegenerative disease (7,79,80).

Figure 2. Neuroinflammatory Cascade After Brain Injury. Downstream cyclooxygenase-2 (COX-2) effectors from infiltrating peripheral immune cells activate astrocytes and microglia, which in turn, release cytokines, chemokines, reactive oxygen and nitrogen species (ROS and RNS, respectively) and complement factors. These inflammatory mediators can further activate resident brain cells, thereby amplifying neuroinflammatory signals and neuronal cell death. Excessive exposure to inflammatory mediators compromises astrocyte functions leading to downregulation of glutamate transporters, impaired glutamate re-uptake, elevated glutamate release, and decreased neurotrophic factor (i.e., granulocyte-colony stimulating factor [G-CSF]) release, all of which can lead to neuronal cell death. Neuronal release of cytokines, complement factors, and COX-2 can lead to autocrine or paracrine-mediated neuronal death.

Neuro-Glial Interactions

Astrocytes are viewed as an active participant in synaptic transmission and processing of information - a departure from the old dogma in which astrocytes were identified as merely physical supporters for neighboring neurons (5). Moreover, opening of gap-junctional communication channels links dying astrocytes in the ischemic core with penumbral cells (46). Therefore, astrocytes might compromise juxtaposed cells found in salvageable tissue that otherwise may have survived.

Studies have suggested that inflammatory mediators might be the driving force for altering astrocyte function and thereby impacting neuron-glial signaling. For example, astrocytes undergo IL-1b-induced elevations in intracellular calcium which may enhance glia-to-neuron signaling, leading to a reduction in neuron survival (9). Pro-inflammatory cytokines may also be responsible for impairing astrocyte energy metabolism thereby jeopardizing neuronal vulnerability (5). Thus it is reasonable to conceptualize that targeting cytokines may lead to a profound modulation of astrocyte function and improve neuronal survivability in this mechanism.

Central Role of COX-2

Cyclooxygenase is a rate limiting enzyme responsible for catalyzing the synthesis of prostaglandins from arachidonic acid (66). Cyclooxygenase possesses two catalytic sites: a COX active site responsible for the conversion of arachidonic acid into the endoperoxide, PGG_2; and a peroxidase active site responsible for the rapid conversion of PGG_2 into another endoperoxide, PGH_2 (10). PGH_2 is further processed to form prostaglandins, prostacyclin, and thromboxane A_2. To date, two COX isoforms have been identified. COX-1 is constitutively expressed in the brain and its by-products are thought

to contribute to normal physiological function (74). COX-2 is also constitutively expressed in the brain (neurons, astrocytes, microglia, and endothelia), but can be inducible under pathological conditions (74). In the brain, COX-2 acts as a key mediator of inflammation, orchestrating a wide spectrum of brain injuries including excitotoxic brain injury, cerebral ischemia, traumatic brain injury, and neurodegenerative disorders (52). COX-2 can propagate the neuroinflammatory response and contribute to tissue damage through the production of toxic prostanoids and reactive oxygen species (38). COX-3 has also been reported in brain tissue (15), but is a splice variant of COX-1 with unknown function (41).

COX-2 Orchestrates Immunologic Responses After HI

COX-2, a well-established mediator of adult brain injury (38), is emerging as a key player in neuroinflammation after hypoxic-ischemic brain damage in the neonate (25). Peripheral immune cells such as T-lymphocytes, B-cells, and Natural Killer cells have the capability to up-regulate COX-2 expression when activated (13). However, immune cell infiltration into the brain parenchyma is thought to also play a beneficial role through the production of neurotrophic factors (47). Activated monocytes, macrophages, and neutrophils are the peripheral cell source for the neurotrophic factor, G-CSF. Peripherally produced G-CSF can also enter the brain by crossing the intact blood-brain barrier and binding to its receptor on neurons and glial cells (57,59). In the brain, G-CSF has been shown to protect neurons and trigger neurogenesis (21). However, excessive and/or prolonged activation of inflammatory mediators can decrease neurotrophic support and neurogenesis in brain areas responsible for behavior and cognition (6,8,71). Studies suggest COX-2 may mediate suppression of G-CSF, since inhibition of COX-2 was able

to increase G-CSF production (35,36). This down-regulation of neurotrophic factors contributes to secondary brain injury and cell death, after a hypoxic-ischemic insult (69).

Emerging Therapeutic Modalities Targeting Inflammation

There are various therapeutic modalities that have attempted to modulate the neuroinflammation that results from HI brain injury in neonates. These treatment options have targeted various stages in the inflammatory cascade including COX-2 inhibition as well as investigated the use of growth factors such as G-CSF. In the following paragraphs, we will review these emerging therapeutic modalities and explore the various research that have been conducted between 1970 and 2010 using relevant literature from the National Library of Medicine and National Institute of Health Database (www.pubmed.gov).

COX-2 Inhibition

To date, many studies have investigated the anti-inflammatory properties of COX-2 inhibition and the benefits with improving neurologic outcomes after various adult brain injuries. Yet surprisingly, only one study to date has investigated the use of COX-2 inhibition on neonatal HI brain injury. The study led by Fathali et al. used postnatal day ten rat pups to assess the effects of NS398, a known selective COX-2 inhibitor, on various neurologic outcomes after right common carotid artery occlusion followed by 2 hours of hypoxia (25). The authors first described that COX-2 inhibition limited morphologic damage, improved long-term functional deficits, reversed somatic growth retardation, and lowered mortality rates after a hypoxic-ischemic injury in neonatal rats. Noteworthy, COX-2 inhibition significantly reduced the expression of IL-6,

a pro-inflammatory cytokine, and in turn, reduced the infiltration of inflammatory cells such as macrophages and neutrophils, which suggests that the increased survivability and neuroprotection provided by COX-2 may be mediated by a reduction in neuroinflammation (25).

G-CSF Administration

G-CSF is a 20 kDa protein belonging to the cytokine family of growth factors. It is responsible for stimulating the proliferation, survival, and maturation of cells committed to the neutrophilic granulocyte lineage by binding to specific G-CSF receptors (65). In addition to its role in neutropenia, G-CSF has shown to be neuroprotective in various brain injury models through direct apoptotic inhibition, inflammatory cell modulation, and/or trophic effects on neuronal cells.

One of the first studies to investigate the anti-inflammatory properties of G-CSF was conducted in 1992 by Gorgen et al. who looked at the role of G-CSF in gram negative septic rodents (30). The study showed that G-CSF could interfere with TNF-α production through a negative feedback signal. Later in 2005, both Gibson and Komie-Kobayashi would also demonstrate the anti-inflammatory role of G-CSF by showing that treatment could modulate the inflammatory response after injury (27,42). Specifically, Komie-Kobayashi demonstrated G-CSF's ability to suppress inducible nitric oxide synthase (iNOS) production and decrease activation of microglial cells expressing iNOS – according to Western blot and immunohistochemistry analysis. Gibson on the other hand, found that G-CSF treatment only suppressed the up-regulation of IL-1ß mRNA and had no effect on TNF-α and iNOS mRNA expression. Additionally, in models of

peripheral infection, G-CSF–induced JAK-STAT signaling was found to reduce TNF-α, interleukin (IL)-1ß, IL-2, IL-6, and IL-8 and elevate IL-1ß receptor antagonists (34).

In terms of its neurotrophic capabilities, various studies have confirmed G-CSF as an essential neurotrophic factor, noting its ability to stimulate the release of stem cells from the bone marrow, promoting both neural repair and neural plasticity (65). A study led by Shyu et al. in Circulation (2004) found that ischemic stroke rats treated with G-CSF, could mobilize autologous hematopoietic stem cells into circulation, enhance their translocation into ischemic brain, and significantly improve lesion repair (64). Additionally, in rat ischemic models, peripherally administered G-CSF was found to enhance structural repair and function by increasing the number of newly generated neurons in both healthy and ischemic subjects (60).

In neonatal HI, only two studies can be found on PubMed using the search criteria "G-CSF and neonatal hypoxia ischemia". Unfortunately, none of the studies looked at the role of G-CSF as an anti-inflammatory agent. Instead, the focus of attention was on the role of G-CSF in apoptosis. The first study led by Yata et al., (2007) found that five 50ug/kg G-CSF post-treatment injections over four days could reduce apoptotic neuron loss while increasing the expression of pro-survival signals (75). Specifically, the investigators found that the anti-apoptotic protein Bcl-2 declined with injury and reversed after treatment while the pro-apoptotic protein, Bax, increased following HI injury and again, was reversed following G-CSF treatment. This is in line with another study conducted by Kim et al., (2008) which found similar neuroprotective outcomes following a single injection of 50ug/kg G-CSF after injury (40).

Specific Aims

The interplay between infiltrating immune cells and brain resident cells during the inflammatory response is dynamic and complex; in that neuro-immune crosstalk, by way of specific molecular mediators, is responsible for both neurodestructive as well as neuroprotective outcomes. Therefore, the neuroinflammatory response to neonatal CNS insult may not comprise a uni-dimensional progression from ischemia to impairment, but rather multiple processes of endogenous repair mechanisms are also initiated after injury. We, therefore, propose identifying and suppressing the actions of molecular mediators believed to be responsible for causation of signaling pathways that lead to a worsened outcome and enhancing neurotrophic mediators responsible for recovery after a hypoxic-ischemic insult. Herein, we tested the hypothesis that COX-2 mediates mechanisms of brain injury and that G-CSF exerts structural and functional protection after neonatal HI. The following specific aims are proposed to address our hypothesis (Figure 2).

Specific Aim 1 is to determine the optimal dosage and broad efficacy of COX-2 inhibition after HI brain injury. Our specific hypothesis is that multiple treatments of low-dose or high-dose COX-2 inhibitor, over the first few days following HI brain insult, will reduce brain damage, and improve long-term functional deficits, and somatic and systemic organ growth.

Specific Aim 2 is to characterize the COX-2-dependent mechanisms involved in brain-immune interactions after HI brain injury. Our specific hypothesis is that COX-2, from spleen-derived immune cells, enhances neuroinflammation via IL-15 expression by astrocytes. Elevated IL-15 inhibits the pro-survival protein PI3K in neurons, thereby leading to caspase-3 activation and cell death. Interventions such as splenectomy,

pharmacological inhibition of COX-2, a neutralizing antibody for IL-15, or a gene silencer for natural killer cells are expected to provide neuroprotection against HI injury.

Specific Aim 3 is to determine the optimal regimen and broad efficacy of G-CSF administration after HI brain injury. Our specific hypothesis is that multiple treatments with the neurotrophic factor G-CSF, will improve long-term behavioral and neuropathological recovery after HI.

The proposed work will: 1) introduce novel therapeutic modalities for attenuating the morphological and neurological consequences of neonatal HI; 2) establish on a molecular level the mechanistic basis by which peripheral immune cells interact with resident brain cells and thereby propagate the neuroinflammatory response and worsen brain injury; 3) provide a basis for the clinical implication for targeting key inflammatory pathways as an effective therapeutic option with long-term benefits after a hypoxic-ischemic brain injury.

Figure 3. Schematic of Specific Aims. Aim 1 (purple arrow) will examine the short- and long-term effects of cyclooxygenase-2 inhibition after HI. Aim 2 (black arrow) will determine the COX-2 mediated mechanisms and the cell types involved in neuro-immune crosstalk. Aim 3 (blue arrow) will examine the long-term behavioral and morphological effects of G-CSF treatment.

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CHAPTER TWO

CYCLOOXYGENASE-2 INHIBITION PROVIDES LASTING PROTECTION AGAINST NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

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Abstract

The development of brain inflammation largely contributes to neonatal brain injury that may lead to a lifetime of neurologic deficits. The present study was designed to investigate whether inhibition of cyclooxygenase-2 (COX-2), a critical component of the inflammatory pathway, is neuroprotective in a neonatal rat model of cerebral hypoxia-ischemia (HI). DESIGN: Laboratory investigation. SETTING: University research laboratory. Neonatal HI was induced in postnatal day-10 Sprague-Dawley rats by ligation of the right common carotid artery followed by two hours of hypoxia (8% O_2). The pups in treatment groups were administered 10mg/kg (low dose) or 30mg/kg (high dose) of a known selective COX-2 inhibitor (NS398). Animals were euthanized at three time points: 72hrs, 2wks, or 6wks. Inflammation outcomes were assessed at 72hrs; brain damage was assessed at 2- and 6wks along with other organs (heart, spleen). Detailed neurobehavioral examination was performed at 6wks. Pharmacological inhibition of COX-2 markedly increased survivability within the first 72hrs compared to untreated rats (100% vs. 72%). Low- and high-dose NS398 significantly attenuated the loss of brain and body weights observed after HI. Neurobehavioral outcomes were significantly improved in some parameters with low dose treatment; while, high dose treatment consistently improved all neurological deficits. Immunohistochemical results showed a marked decrease in macrophage, microglial, and neutrophil abundance in ipsilateral brain of NS398 treated group along with a reduction in interleukin-6 expression. Selective COX-2 inhibition protected neonatal rats against death, progression of brain injury, growth retardation, and neurobehavioral deficits after a hypoxic-ischemic insult.

Introduction

A hypoxic-ischemic insult to neonates results not only in brain damage, but is also associated with increased mortality and somatic growth retardation (1, 2). There is no effective treatment for neonatal hypoxia-ischemia (HI). Long-term effects of HI for the survivors may include motor disability, cognitive dysfunction, and problems in learning and behavior (3). The inflammatory response is particularly detrimental in the immature brain, serving a key component in the progression of neonatal encephalopathy (4).

Cyclooxygenase-2 (COX-2), the inducible form of the enzyme and a key mediator of inflammation, is critical in different forms of brain injury such as excitotoxic brain injury, cerebral ischemia, traumatic brain injury, and neurodegenerative disorders (5). Cerebrospinal fluid concentrations of prostaglandins (PG) such as PGE_2 and PGI_2, which are downstream effectors of COX-2 enzyme, have been reported to be significantly higher in children with perinatal hypoxia (6). Moreover, pharmacological inhibition of COX-2 has been shown to be beneficial in brain and spinal cord-related injuries in adults (7, 8). To date, no study has examined the effects of COX-2 inhibition against neonatal HI-induced brain injury.

Accordingly, we hypothesized that inhibition of COX-2-induced-inflammation will reduce brain injury; improve neurological outcomes, and somatic and systemic organ growth following HI in neonates. We examined the lasting effects of COX-2 inhibition using two different doses of a selective COX-2 inhibitor, NS398, in a well established neonatal HI model in rats. We also confirmed the presence of COX-2 in neuronal cells and used the pro-inflammatory cytokine, interleukin-6 (IL-6), as an endpoint for inflammation.

Materials and Methods

Animal Groups and Operative Procedure

This study was in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Timed pregnant Sprague-Dawley rats were housed with food and water available *ad libitum.* Postnatal day-10 pups were randomly assigned to the following groups: sham, HI [Vehicle], HI+10mg/kg NS398 [NS-10], or HI+30mg/kg NS398 [NS-30]. Each litter consisted of all groups. Pups were placed on a surgical table maintained at 37^0C and anesthetized by inhalation with isoflurane (3% in mixed air and oxygen). HI-groups had right common carotid artery permanently ligated. After 1.5hours (hrs) of recovery, pups were placed in a glass jar (submerged in a water bath maintained at 37^0C) perfused with 8% oxygen for 2hrs. Rats were euthanized under general anesthesia [ketamine (80mg/kg)/xylazine (10mg/kg)] by decapitation at 72hrs, 2- and 6weeks (wks) post-HI.

Treatment Method

Some pups were treated intraperitoneally with a COX-2 inhibitor (NS398) at either 10mg/kg or 30mg/kg dosage (Cayman Chemical, Ann Arbor, MI) diluted in 10% dimethylsulfoxide (DMSO) and saline. Treatment consisted of six injections (1, 6, 24, 36, 48, and 60hrs) after hypoxia. Vehicle pups followed same injection regimen and methodology, but administered 10% DMSO in saline.

Evaluation of Brain Damage

Hemispheric weight loss has been used as the primary variable to estimate brain damage in this animal model (9). At 2- and 6wks, the brain was removed, without prior perfusion, and the hemispheres were separated by a midline incision and weighed on a high-precision balance (sensitivity ±0.001g).

Measurement of Organ Weight

After removal of brain, the spleen and heart were isolated and detached from surrounding tissue and vessels and weighed at the 6wk interval.

Assessment of Neurobehavioral Deficits

Rats were tested at 6wks and scored accordingly: 0 for immediate and correct placement; 1 for delayed and/or incomplete placement; 2 for no placement. Scores corresponded to raw values: 0 = raw value of 100; 1 = raw value of 50; 2 = raw value of 0. Methodology was as previously described (10) for the first six tests:

1. *Postural Reflex:* Assessed upper body posture and symmetry in forelimb extension (11). Rat was held by tail and lowered to 10cm above table top.
2. *Proprioceptive Limb Placing:* Rat's head was tilted 45° upwards to avoid visual and tactile contact with table. Dorsum of paw was pushed against table edge to stimulate limb muscles and joints for forelimb placement onto table top.
3. *Back Pressure Towards Edge:* Rat was moved from behind toward table edge for assessment of forward limb placement.

4. *Lateral Pressure Towards Edge:* Rat was moved from ipsilateral or contralateral side toward table edge for assessment of lateral and forward limb placement.
5. *Forelimb Placement:* Rat was held facing table edge with visual and tactile (whisker) contact with table, and assessed for forward limb placement onto table top.
6. *Lateral Limb Placement:* Rat was held parallel to table edge with tactile contact, and assessed for lateral limb abduction onto table top.
7. *T-Maze:* Assessed short-term or working memory (12). Rat was placed in the stem (40x10cm) of maze and allowed to explore until an arm (46x10cm) of maze was chosen. The sequence (10 trials) of left and right arm choices was expressed as rate of spontaneous alternation (0%=no alternation, 100%=alternation at each trial).
8. *Foot-fault:* Assessed placement dysfunction of forepaws and motor coordination, and is reliable in differentiating between ischemic and normal rats (11, 13). Rat was placed onto an elevated wire grid floor (20x40cm) for 2 minutes. Foot-faults were when a complete paw fell through openings.

Immunohistochemistry with DAB Staining

Animals (n=5/group) were perfused with 0.1M phosphate buffered saline (PBS) and fixed with 10% paraformaldehyde (formalin) diluted in PBS, via trans-cardiac approach 72hrs post-insult. The sectioned brain volume (5mm) encompassed dorsal hippocampus. Every fifth section of the tissue block was collected, and from this set, 6 random, non-adjacent sections were stained and then observed. Special care was taken to

analyze sections from the same levels of sectioning in different animals. Antigen retrieval was done on slices (10μm) by microwave irradiation in 0.1M sodium citrate (pH=6) for 10 minutes. Diaminobenzidine (DAB) staining method (ABC Staining Kit, Santa Cruz Biotech, Santa Cruz, CA) was implemented as previously described (14) for detection of COX-2 expression in the ipsilateral cortex and CA1 region of hippocampus. Antibodies included goat anti-COX-2 (1:100) and donkey anti-goat secondary antibody (1:200). All antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA), unless otherwise stated. Controls for non-specific immunohistochemical staining were done with omission of the primary antibodies.

Western Blotting of COX-2

Animals (n=5/group) were perfused (0.1M PBS) at 72hrs post-HI. Ipsilateral hemisphere was isolated then snap-frozen and kept at -80°C until analysis. Samples in (300mg/mL) extraction buffer (50mM Tris-HCl buffer [pH 7.4] with 150mM NaCl, 1% Nomide P40, 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholic acid, and 1% PMSF) and 1% protease inhibitor were homogenized with a tissue homogenizer for a total of 60 seconds (20x 3 sec pulses). The homogenate was centrifuged (15000 g for 20 min), the supernatant of the extract was collected, and the concentration of the protein samples was determined by Bradford assay (BioRad, Hercules, CA). All procedures were performed at 4°C. 40μg sample of extracted protein with 2x loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, and 5% β-mercaptoethanol) were subjected to electrophoresis on 10% polyacrylamide SDS gel (BioRad, Hercules, CA). Procedures were as previously described (15). Primary antibodies were goat anti-COX-2 and rabbit anti-β-actin. Incubation with donkey anti-

goat and donkey anti-rabbit secondary antibodies was done, respectively. Bands were detected by chemiluminescent kit (Amersham Bioscience, Piscataway, NJ) on X-ray film (Kodak, Rochester, NY). Optical density was determined using NIH Image J software and expressed relative to β-actin then to sham group.

Triple Fluorescent Labeling

Cerebral tissue was perfused (0.1M PBS), fixed (10% formalin), and sectioned (5mm volume) at 72hrs for use in triple-fluorescent labeling. Primary antibodies were goat anti-COX-2 (1:50), rabbit anti-IL-6 (1:50) with mouse anti-NeuN (1:100; Millipore Corp., Billerica, MA). Tissue slices (10μm) were blocked with 5% donkey serum in PBS at room temperature for 2hrs. Slices were incubated overnight at 4^0C, followed with respective donkey secondary antibodies conjugated with fluorescent dyes for 2hrs at room temperature in the dark, as previously described (16). Between incubations, three washes of 5min were performed at room temperature with 0.01 M PBS (pH 7.4). Controls for non-specific immunofluorescence staining were done with omission of the primary antibodies. To test for tissue autofluorescence, some sections were processed without the secondary antibodies. The ipsilateral cortex and CA1 region of hippocampus (n=5/group) were analyzed using a fluorescent microscope with digital camera (OLYMPUS BX51, Melville, NY).

Inflammatory Cell Infiltration

Animals (n=5/group) were perfused (0.1M PBS) at 72hrs post-HI. Ipsilateral hemisphere was isolated then snap-frozen and kept at -80°C until analysis of interleukin-6 (IL-6) concentration by ELISA technique (Invitrogen Corp., Carlsbad, CA) and

expressed as picogram per milligram protein. Samples in (300mg/mL) extraction buffer (50mM Tris-HCl buffer [pH 7.4] with 0.6M NaCl, 0.2% Triton X-100, 10μl aprotinin, 1μg/mL leupeptin, and 1mM PMSF) and 1% protease inhibitor were homogenized, the supernatant of the extract was collected, and the protein samples were assayed in duplicate. Cerebral tissue was perfused (0.1M PBS), fixed (10% formalin), and sectioned (5mm volume) for detection of inflammatory cell infiltration into ipsilateral cortex (n=5/group). Rabbit anti-Iba1 (1:100; Wako Chemicals USA Inc., Richmond, VA), mouse anti-CD68 (1:100; Millipore Corp., Billerica, MA), or goat anti-MPO (1:100) primary antibodies (Abcam Inc., Cambridge, MA) were added to sections (10μm). Incubation methodology was same as in triple-fluorescent labeling. An estimation of the amount of positive cells were defined as being low (<10 postive cells/per high power visual field) or high (>10 positive cells/per high power visual field). As with all staining, differences in levels of infiltrating cell markers were noted between groups by an experimenter blinded to the treatment group of each section/slide observed.

Data Analysis

Data was expressed as mean ± SEM. One-way ANOVA and Tukey test were used to determine significance in differences between means. Neurological scores were analyzed using Means of Dunn Method (except T-Maze and Foot-fault tests); mortality rates using chi square test; and assays using Dunnett's post hoc test with vehicle designated as control. Significance was accepted at $p < .05$.

Results

NS398 Protects Against HI-Related Lethality

Treatment with NS398 completely abolished the mortality evidenced in the untreated group (0% vs. 28.12%). While nine vehicle pups died within the first 72hrs following hypoxia; none of the NS398-treated pups died at any time during the experiment, indicating that survival was specifically related to COX-2 inhibition. No sham-operated pups died.

Cyclooxygenase-2 Blockade Maintains Body Weight After HI

Growth retardation is evident in both patients and experimental studies as a result of hypoxia-ischemia (2, 17). Accordingly, somatic growth retardation was apparent in vehicle rats at 2-, 3-, and 6wks (Figure 4) compared to sham. NS-30 significantly improved body weight at the 6wk time-point. Drastic differences in fur texture and appearance were detected as early as 2wks between treated and untreated rats (Figure 5).

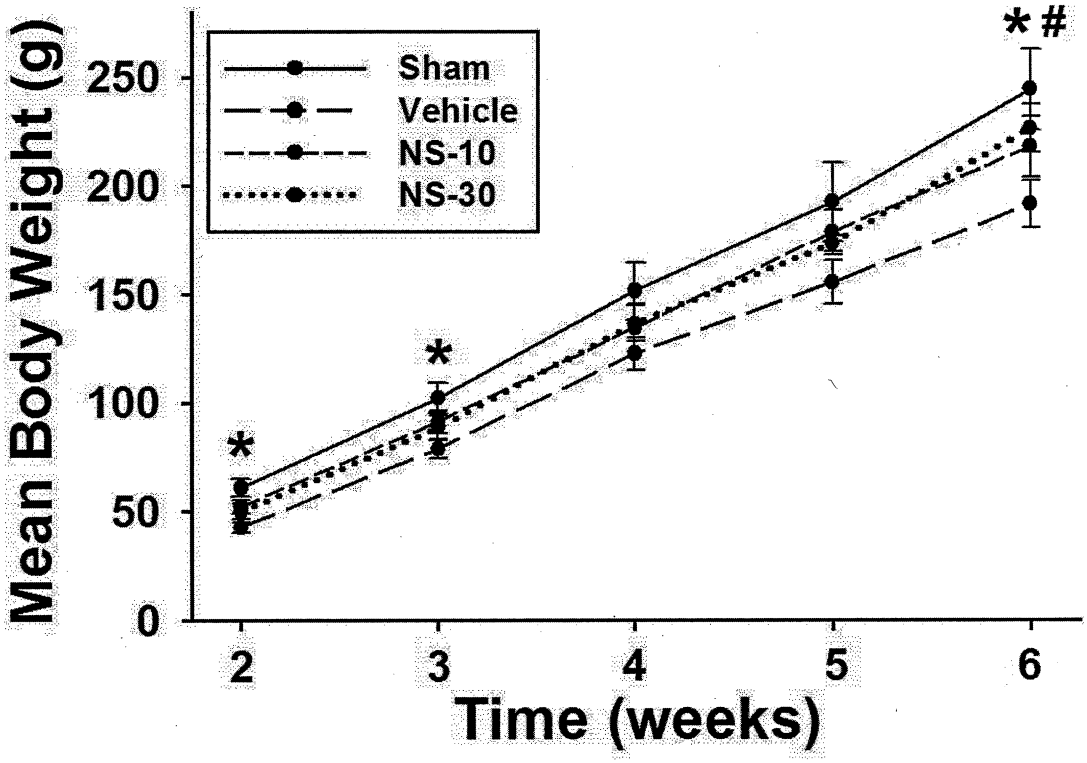
NS398 Provides Neuroprotection by Maintaining Brain Weight at Two Time Intervals

Hemispheric weight loss is an established estimate of brain damage in this animal model (9). Severe brain atrophy, marked by a reduction in right to left hemispheric weight ratio, was seen in vehicle rats at 2- and 6wks post-HI (Figure 6). Blockade of COX-2 protected rats at both time-points.

NS398 Prevents HI-Related Systemic Organ Atrophy

A reduction in spleen size following middle cerebral artery occlusion (MCAO) model of stroke (18) is correlated with the extent of brain damage (19). Our experiments supported this observation in the HI neonatal model; with vehicle having a reduced

spleen to body weight ratio (Figure 7). Treatment groups demonstrated a trend towards maintaining spleen weight; however, no statistical significance was reached. Heart to body weight ratio decreased in vehicle pups and was attenuated by NS-10 (Figure 7). Although NS-30 demonstrated a trend towards maintaining heart weight, no significance was reached.


Mean Body Weight (g)
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4
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Sham
Vehicle
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NS-30
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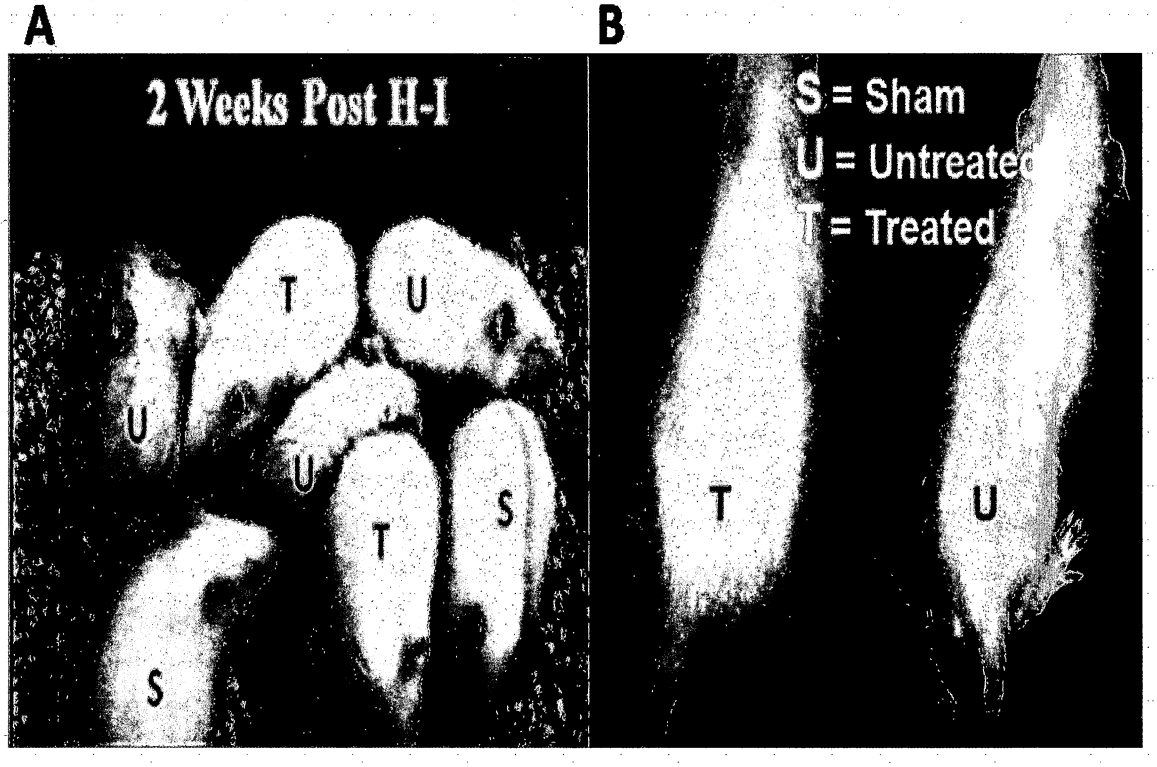
Figure 4. Long-Term Effect of COX-2 Inhibition on Body Weight. Postnatal day-10 rats were induced with a hypoxic-ischemic (HI) event: ligation of the right common carotid artery and 2-hours of hypoxia (8% O2) [Vehicle], then treated with 6 intraperitoneal injections (1, 6, 24, 36, 48, and 60-hours post-hypoxia) of a selective cyclooxygenase-2 (COX-2) inhibitor at either 10mg/kg [NS-10] or 30mg/kg [NS-30] dosage. Sham animals had same anesthesia and surgical procedure, except that the common carotid artery was not ligated. Vehicle animals had a significantly lower mean body weight at 2-, 3- and 6-weeks, compared to sham (*$p < .05$). NS-30 had long-term lasting effects as it maintained body weight compared to vehicle 6-weeks after HI (#$p < .05$). Only mean body weights of animals kept through the 6 week time-point are represented ($n = 9$/group).


A
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S = Sham
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Figure 5. Photograph of Physical Appearance of Pups at 2-Weeks After HI Insult. **A,** Differences in fur texture and appearance were detected between treated (T) and untreated rats (U). **B,** Is a close-up picture demonstrating the somatic differences between treated and untreated rats.

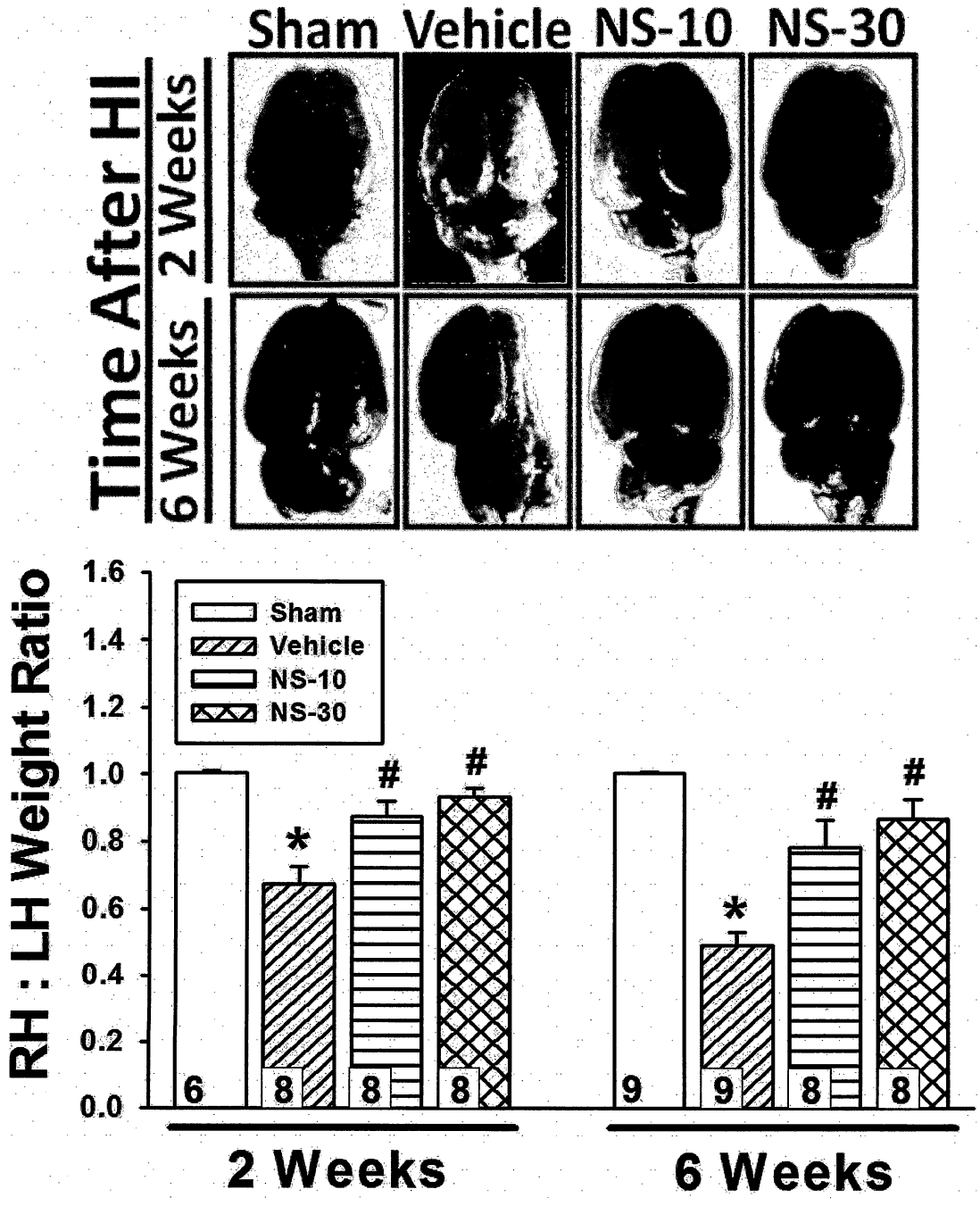
Sham
Vehicle
NS-10
NS-30
Time After HI
2 Weeks
6 Weeks
RH : LH Weight Ratio
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
Vehicle
NS-10
NS-30
*
#
6
8
9
2 Weeks
6 Weeks

Figure 6. Brain Atrophy 2- and 6-Weeks After HI Insult. NS-10 and NS-30 maintained the gross morphology of the rat brains at 2- and 6-weeks post-HI. Right to left hemispheric (RH:LH) weight ratio is representative of brain atrophy. At 2-weeks post-insult, vehicle rats had a significantly reduced RH:LH ratio compared to sham (0.67 ± .05 vs. 1.00 ± .01). This was attenuated by NS-10 (0.88 ± .04) and NS-30 (0.93 ± .03). At 6-weeks post-insult, vehicle rats had a significantly reduced RH:LH ratio compared to sham (0.49 ± .04 vs. 1.00). This was attenuated by treatment (NS-10:0.78 ± .08; NS-30:0.87 ± .06).

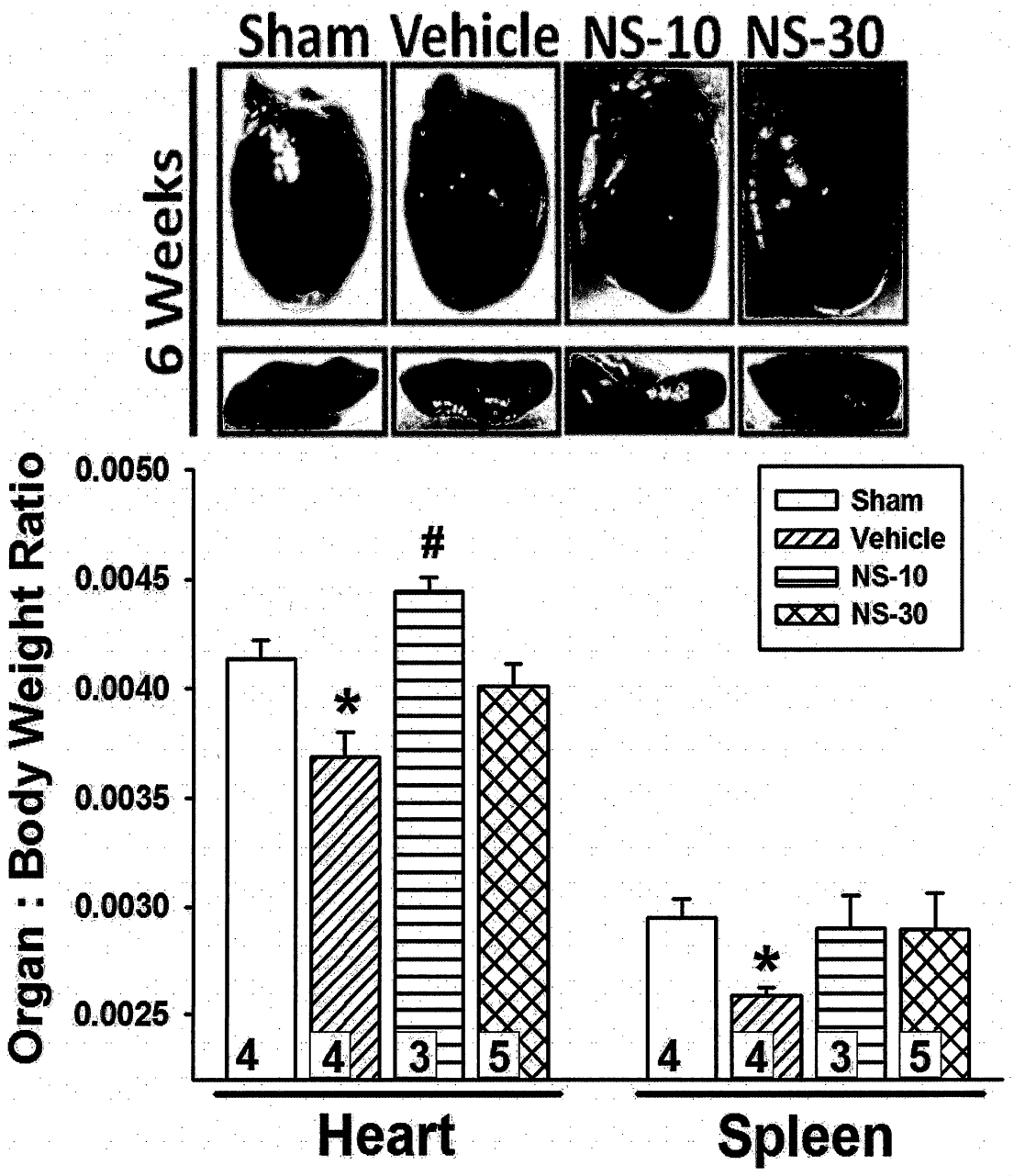
Sham Vehicle NS-10 NS-30
6 Weeks
Organ : Body Weight Ratio
0.0050
0.0045
0.0040
0.0035
0.0030
0.0025
Sham
Vehicle
NS-10
NS-30
#
*
*
4
4
3
5
4
4
3
5
Heart
Spleen

Figure 7. Heart and Spleen Weight 6-Weeks After HI Insult. Vehicle rats had a significantly reduced heart to body weight ratio (0.0037 ± .0001 vs. 0.0041 ± .0001) and spleen to body weight ratio (0.0026 vs. 0.0030 ± .0001) as compared to sham. Treatment increased the heart to body weight ratio (NS-10:0.0045 ± .0001; NS-30:0.0040 ± .0001) and the spleen to body weight ratio (NS-10: 0.0029 ± .0002; NS-30: 0.0029 ± .0002). Data represent mean ± SEM; *$p < .05$ versus sham, #$p < .05$ versus vehicle. Numbers in bars indicate animals/group.

NS398 Prevents Neurobehavior Deficits

Motor, cognitive, and behavioral deficits can be a consequence of perinatal stroke and may last a lifetime (20). Numbers in parenthesis denote mean raw score (Figure 8). Sham demonstrated a successful performance (91.67) in postural reflex test; treatment (78.57) significantly improved the gross asymmetry in posture and extension of forelimbs seen in vehicle rats (8.33). In proprioceptive limb placing test, vehicle was delayed or unable to place forelimbs onto table top (25.0). NS-30 entirely ameliorated the deficit (100.0). In back pressure towards edge test, vehicle was delayed or unable to place limbs forward (25.0); in contrast, NS-30 rats showed immediate placement (92.86). In lateral pressure towards edge test, sham (91.67) had immediate lateral and forward limb placement. This response was delayed or not present in vehicle (25.0), but markedly improved by NS-30 (85.71). In forelimb placement test, vehicle was delayed or unable to place limbs forward when allowed visual and tactile contact (33.33); NS-30 corrected performance (92.86). In lateral limb placement test, vehicle rats were unable to abduct limbs (8.33), a deficit improved by NS-30 (71.3). In T-maze, untreated animals showed a significant decline in memory (reduced %alternations) that was improved by NS-10 and more so by NS-30. Treatment also significantly attenuated the increased number of foot-faults in brain injured rats.

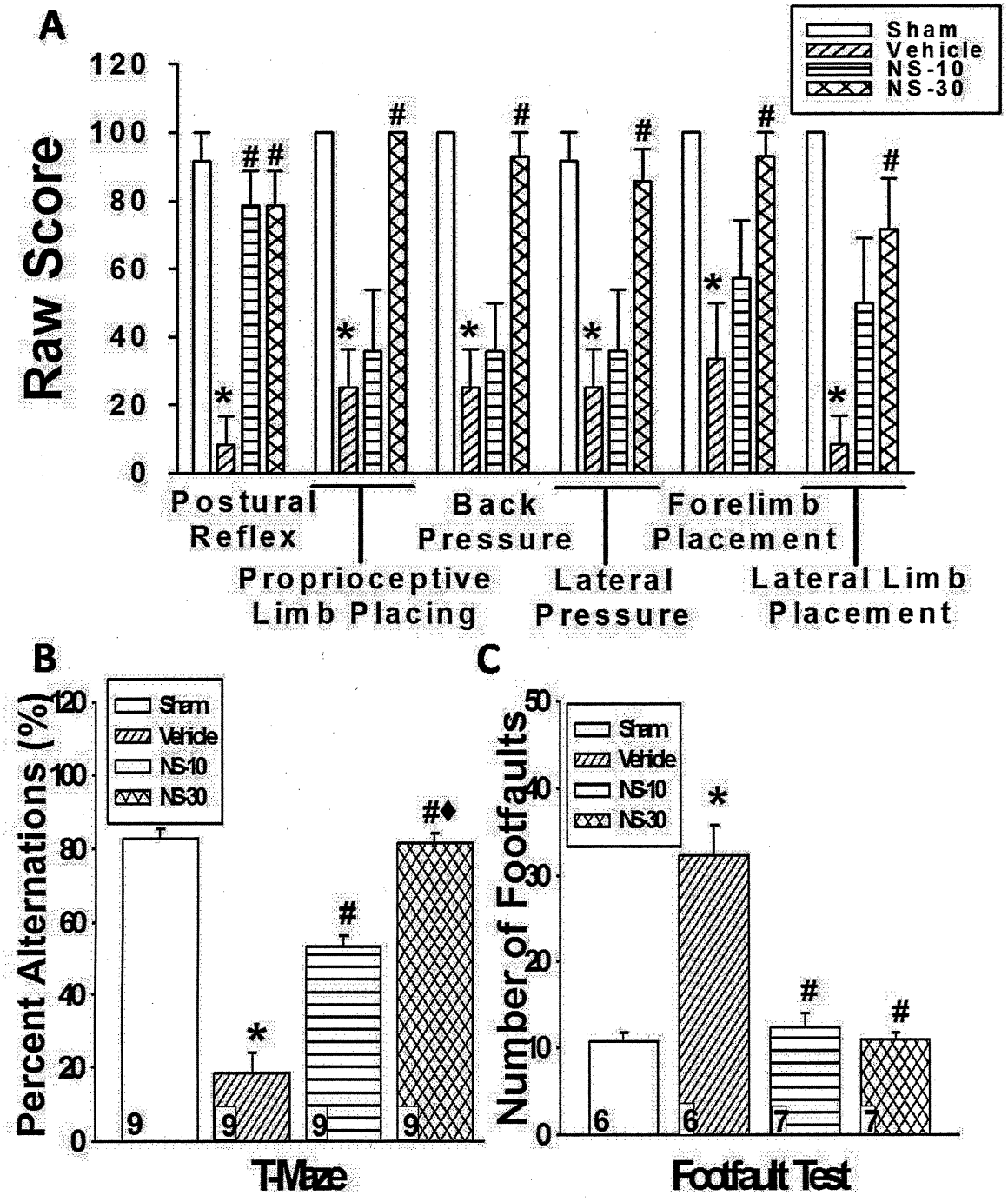
A
Raw Score
120
100
80
60
40
20
0
Sham
Vehicle
NS-10
NS-30
Postural Reflex
Proprioceptive Limb Placing
Back Pressure
Lateral Pressure
Forelimb Placement
Lateral Limb Placement
B
Percent Alternations (%)
T-Maze
C
Number of Footfaults
Footfault Test

Figure 8. Dose-Dependent Effect of COX-2 Inhibition on Neurobehavior. **A,** Rats received a raw score of 100 for immediate and correct placement; 50 for delayed and/or incomplete placement; 0 for no placement (n = 9/group). Administration of NS-30 significantly improved all assessed behavior deficits; while, NS-10 significantly improved deficits associated with the postural reflex test. Data represent *$p < .05$ versus sham, #$p < .05$ versus vehicle. **B,** Vehicle rats alternated significantly less between the two arms of the maze as compared to sham (18.52% ± 5.56 vs. 82.72% ± 2.69). Percent alternations significantly rose with administration of NS-10 (53.09% ± 3.09) and more so by NS-30 (81.48% ± 2.62). Data represent mean ± SEM; *$p < .05$ versus sham, #$p < .05$ versus vehicle, ♦$p < 0.05$ versus NS-10. **C,** Vehicle rats averaged the greatest number of foot-faults (32.33 ± 3.49), while treatment significantly reduced the deficit (NS-10:12.29 ± 1.70; NS-30:10.86 ± .86). Sham animals had an average of 10.67 ± 1.05 foot-faults. Data represent mean ± SEM; *$p < .05$ versus sham, #$p < .05$ versus vehicle. Numbers in bars indicate animals/group.

NS398 Effectively Reduces Cyclooxygenase-2 Expression

Positive staining for COX-2 was detected by DAB stain (Figure 9). The negative control showed no positive staining indicating that the primary antibody, and not non-specific immunohistochemical staining, was responsible for the positive signal. NS-30 qualitatively reduced post-HI COX-2 expression in both examined regions of the brain. Higher magnification confirmed COX-2 localization in the cytoplasm of the cell. Western blotting of COX-2 quantitatively supported significant differences between treated and vehicle groups (Figure 10).

NS398 Reduces Cytokine Expression in the Brain

In the hippocampus (Figure 11) and cerebral cortex (Figure 12), there was strong neuronal signal for COX-2 and IL-6 in vehicle. The opposite was seen in NS-30: marked suppression of immunoreactive COX-2 and IL-6; while fluorescence was strong for neurons.

COX-2 Inhibition Reduces Inflammatory Infiltration

IL-6 protein level was significantly increased in vehicle and substantially reduced by NS-30 (Figure 13). Single-immunofluorescent labeling demonstrated a qualitative increase in expression of microglia (Iba1), macrophages (CD68), and neutrophils (MPO) after HI (Figure 14); COX-2 blockade reduced expression of these cell-types.

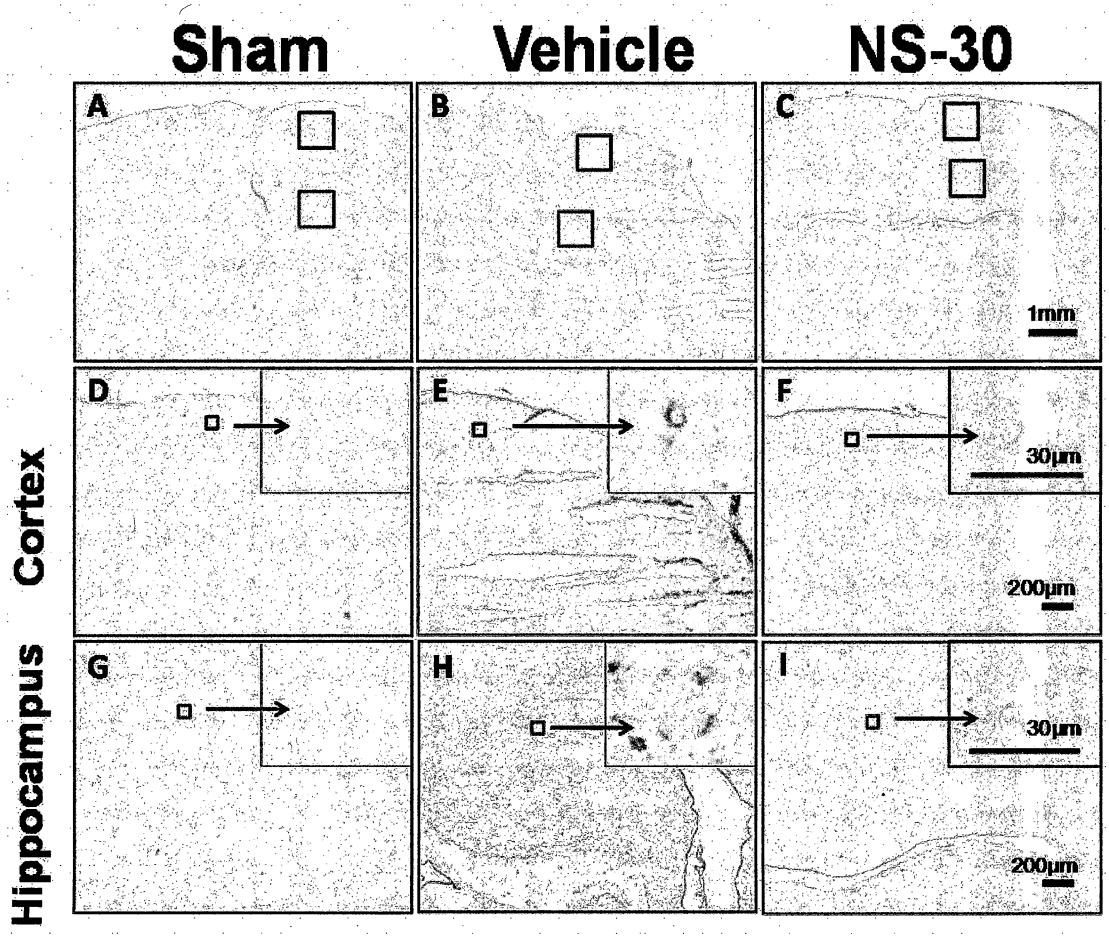
Sham
Vehicle
NS-30
A
B
C
1mm
D
E
F
30μm
200μm
Cortex
G
H
I
30μm
200μm
Hippocampus

Figure 9. Diaminobenzidin (DAB) Staining of COX-2 Expression in the Cerebral Cortex and Hippocampus. DAB stain for COX-2 in ipsilateral cerebral cortex (D-F) and CA1 region of hippocampus (G-I) qualitatively appears less in sham (A,D,G) and NS-30 (C,F,I), as compared to vehicle (B,E,H). Six non-adjacent coronal sections per brain (n = 5/group) were analyzed.

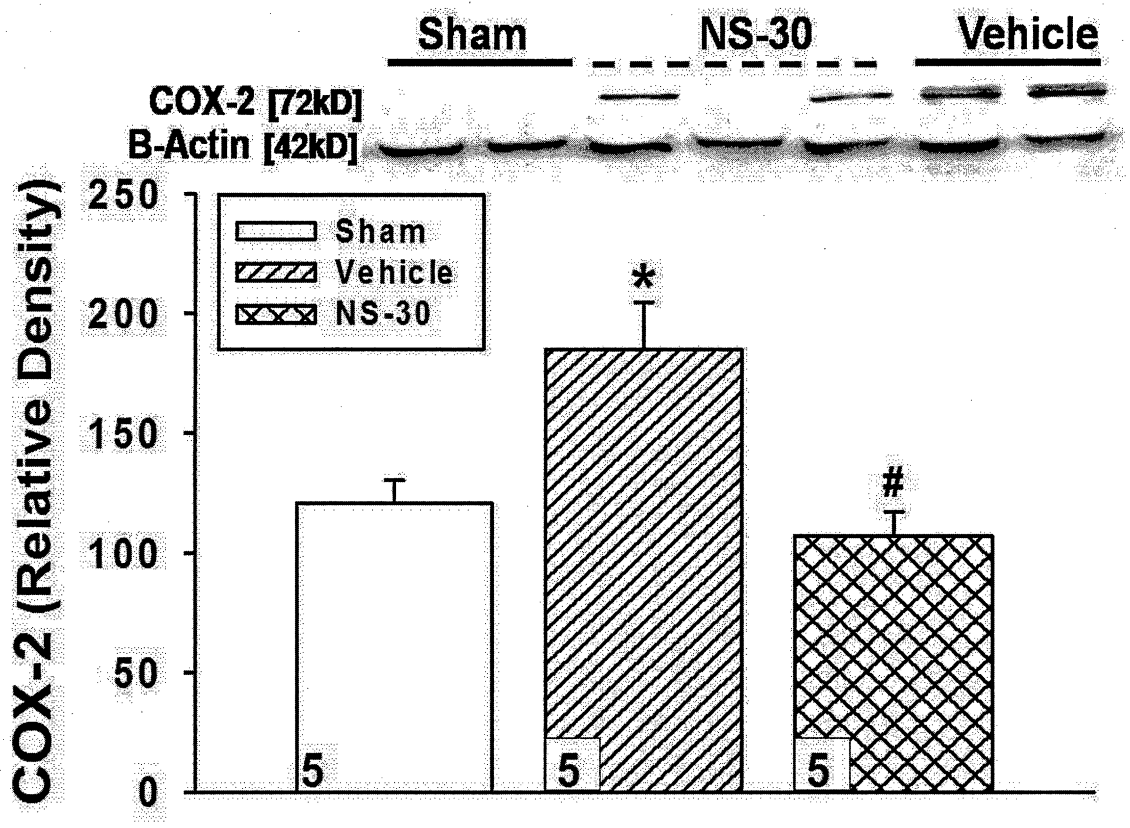
Sham
NS-30
Vehicle
COX-2 [72kD]
B-Actin [42kD]
COX-2 (Relative Density)
250
200
150
100
50
0
Sham
Vehicle
NS-30
*
#
5
5
5

Figure 10. Western Blot Analysis of COX-2 Protein. A statistically significant reduction in COX-2 expression in the ipsilateral hemisphere of NS-30 compared to vehicle (106.92 ± 10.48 vs. 185.20 ± 19.54). Sham (120.88 ± 9.30) also had significantly less COX-2 expression than the vehicle group. Data represent *$p < .05$ versus sham, #$p < .05$ versus vehicle. Numbers in bars indicate animals/group.

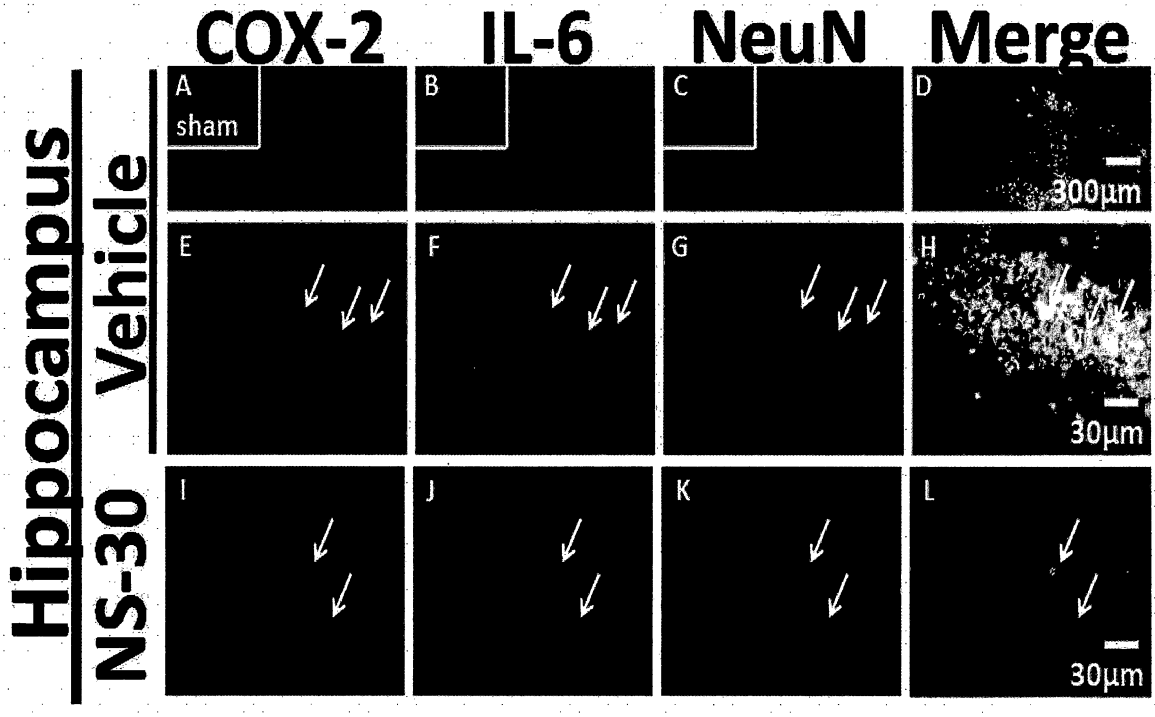
COX-2
IL-6
NeuN
Merge
Hippocampus
Vehicle
NS-30
A
sham
B
C
D
300μm
E
F
G
H
30μm
I
J
K
L
30μm

Figure 11. Interaction of COX-2 and IL-6 in Neuronal Cells of the Hippocampus. Six non-adjacent coronal sections per brain (n = 5/group) were analyzed. Immunoreactivity is shown of COX-2, IL-6 and NeuN in ipsilateral CA1 region of hippocampus. Vehicle (A-H) demonstrated strong neuronal fluorescence for COX-2 and IL-6; NS-30 (I-L) demonstrated strong fluorescence for NeuN, but weak fluorescence for COX-2 and IL-6. Sham animals are shown in subsets of (A-C).

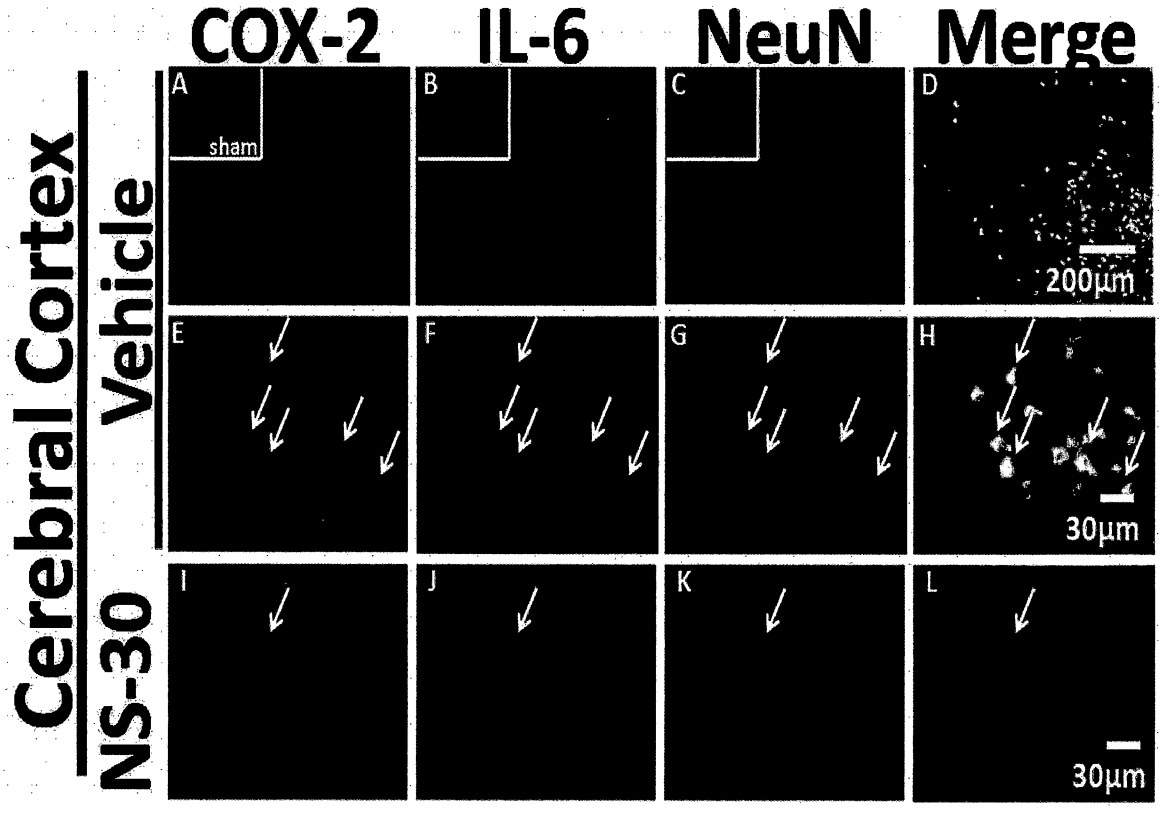
COX-2
IL-6
NeuN
Merge
Cerebral Cortex
Vehicle
NS-30
A
sham
B
C
D
200μm
E
F
G
H
30μm
I
J
K
L
30μm

Figure 12. Interaction of COX-2 and IL-6 in Neuronal Cells of the Cerebral Cortex. Six non-adjacent coronal sections per brain (n = 5/group) were analyzed. Immunoreactivity is shown of COX-2, IL-6 and NeuN in ipsilateral cerebral cortex. Vehicle (A-H) demonstrated a strong co-localization of COX-2, IL-6, and NeuN. NS-30 (I-L) treatment reduced signals of COX-2 and IL-6. Sham animals are shown in subsets of (A-C).

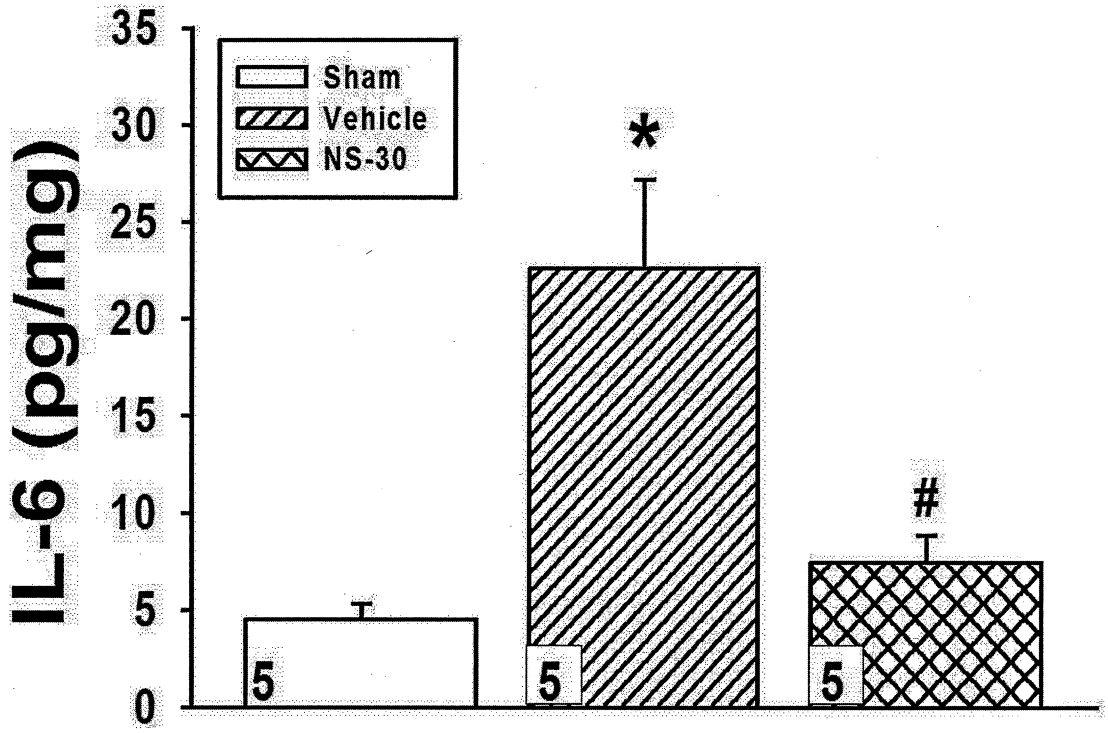
IL-6 (pg/mg)
35
30
25
20
15
10
5
0
Sham
Vehicle
NS-30
*
#
5
5
5

Figure 13. Immunosorbent Analysis of IL-6 Protein. Analysis by ELISA technique showed significant increase of IL-6 in the ipsilateral cerebral hemisphere of vehicle rats as compared to sham (22.61pg/mg ± 4.60 vs. 4.54pg/mg ± .77). Treatment with NS-30 markedly reduced IL-6 concentration (7.42pg/mg ± 1.44). Data represent *$p < .05$ versus sham, #$p < .05$ versus vehicle. Numbers in bars indicate animals/group.

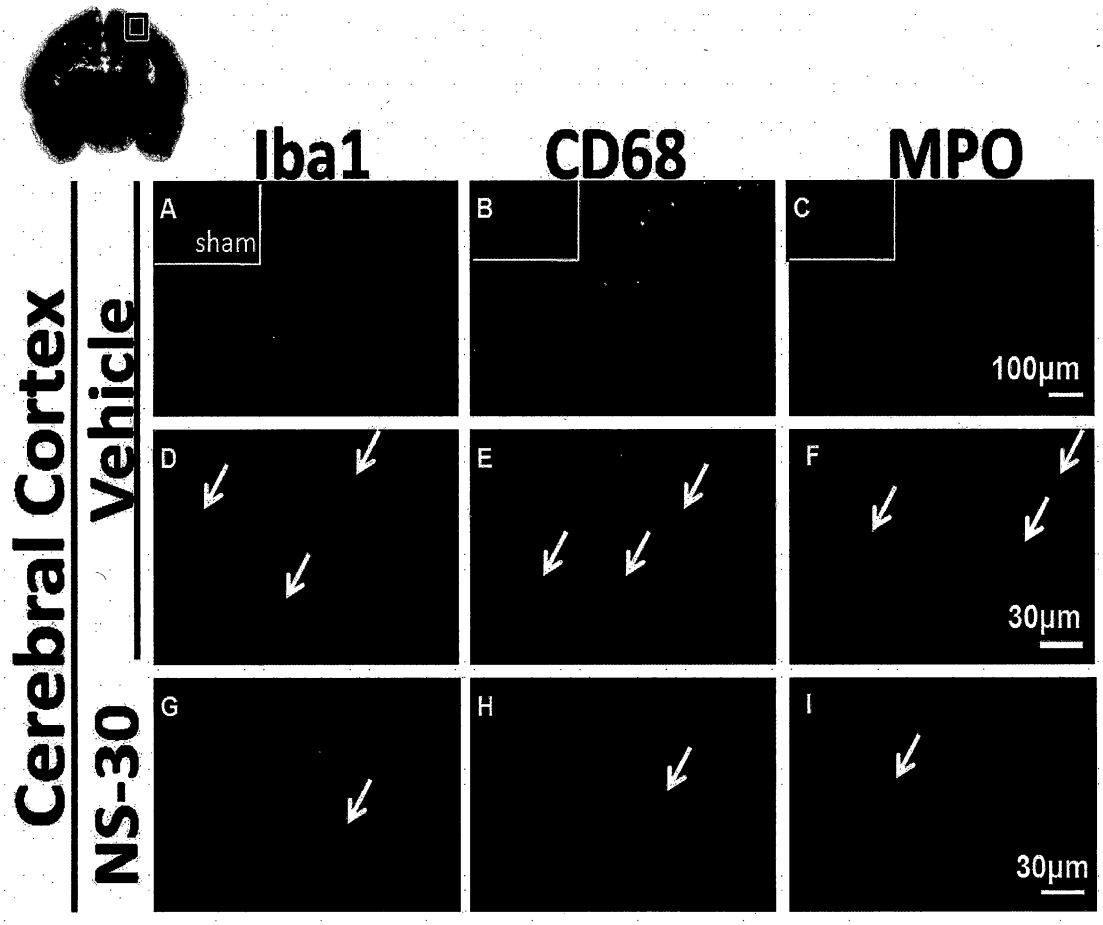
Iba1
CD68
MPO
Cerebral Cortex
Vehicle
NS-30
A
sham
B
C
100μm
D
E
F
30μm
G
H
I
30μm

Figure 14. Immunofluorescent Photomicrographs of Microglial Activation, and Macrophage and Neutrophil Expression in the Cerebral Cortex. Vehicle (A-F) pups showed marked activation of microglia (Iba1; A and D), and infiltration of macrophages (CD68; B and E) and neutrophils (MPO; C and F) in the ipsilateral cerebral cortex. NS-30 qualitatively reduced expression of all three cell markers of inflammation (G-I). Sham animals are shown in subsets of (A-C). Six non-adjacent coronal sections per brain (n = 5/group) were analyzed. Brain slice in upper left corner denotes the specific cortical area the immunofluorescent pictures represent.

Discussion

In the present study, we tested whether multiple treatments of low-dose or high-dose COX-2 inhibitor, over the first few days following brain insult, can reduce the neurodevelopmental and/or somatic consequences of the injury. We showed for the first time that COX-2 inhibition limited morphologic damage, improved long-term functional deficits, reversed somatic growth retardation and lowered mortality rates after a hypoxic-ischemic injury.

NS398 is a well known selective COX-2 inhibitor shown to have neuroprotective effects in adult rat CNS injury models (7, 8). The dosage and treatment frequency (b.i.d) of NS398 was adopted from cerebral ischemia studies in adult rats (7, 21); however, the high dose used in this study is slightly higher but comparable with that used in adult rat models (10-20mg/kg). Both, NS-10 and NS-30 decreased the brain damage, as assessed by brain weight, at both 2- and 6wks following brain injury. Trends suggested an improvement in spleen weight following NS398 treatment, and significant improvement in heart weight. Similarly, NS-30 consistently improved neurological deficits 6wks post-insult. This was an important finding, considering past therapeutic modalities in neonatology have resulted in unforeseen side effects (22, 23). The partial protective effects of NS-10 may be due to the developmental physiological status of the neonates. Some clinical studies have shown that currently used COX-2 inhibitors such as celecoxib are rapidly cleared (twice as fast) in children as compared to adults (24). Thus, the pharmacokinetics of NS398 needs to be determined in neonatal experimental models.

High dose of NS398 also showed an improvement in body weight and other somatic characteristics such as fur growth and quality. Clinical and experimental studies

have shown that neonatal HI not only causes brain damage and neurological deficits but also decreased somatic growth (2). The exact mechanism of how COX-2 inhibition affects somatic growth after neonatal HI remains to be determined.

The anti-inflammatory properties of COX-2 inhibition attenuated brain injury after neonatal HI. Over-expression of IL-6 in premature neonates is associated with severe cerebral injury (25). Studies have shown an IL-6-mediated activation of microglia around site of brain lesion; and a marked reduction of these effects in IL-6 deficient mice (26). IL-6 is also associated with increased mortality, and is an independent predictor of neurological deterioration following ischemic stroke (27, 28). Inhibition of COX-2 significantly reduced the expression of IL-6; as well as showed a marked reduction in infiltration of inflammatory cells such as macrophages and neutrophils and decreased activation of microglia in the affected brain tissue. Thus, the neuroprotective effects and increase in survivability demonstrated by COX-2 inhibition may be mediated by a reduction in IL-6 and the subsequent inflammatory response.

Recently, selective COX-2 inhibitors have come under progressively intense scrutiny due to an increased incidence of cardiovascular events among general populations treated with COX-2 inhibitors. But, the evidence-at-large remains contradictory and a host of studies both affirm and refute the putative cardiovascular harms of COX-2 inhibitors, as reviewed by Salinas G, *et al.* Furthermore, the underlying disease process and the type of cells involved may be pertinent factors in the overall effect produced by inhibition of COX-2 (29, 30). These selective inhibitors were commonly prescribed as a chronic regimen for patients with inflammatory arthritis and may thus produce entirely different effects when administered as acute regimens. Until

more definitive evidence is available, strong judgments about the harm-to-benefit ratio of COX-2 inhibitors should be withheld, as these may pre-empt valuable research into untapped benefits for the general population. As evidenced in this study, selective inhibition of COX-2 may be effective at protecting the injured neonatal brain, and be a promising therapeutic option as acute treatment after stroke with lasting beneficial effects.

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EDITORIAL

COX-2 INHIBITORS FOR ACQUIRED BRAIN INJURIES: IS THE TIME RIPE?

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Published: *Critical Care Medicine* 38(2):723-724, 2010

The timeliness of the results presented in "Cyclooxygenase-2 inhibition provides lasting protection against neonatal hypoxic-ischemic brain injury" by Fathali et al (1) pertains to our current lack of treatments for improving functional deficits after acquired brain injuries. Fathali and colleagues examine the relationships between perinatal hypoxic/ischemic brain injury, COX2 inhibition, and functional recovery, brain/body morphometrics, and the inflammatory response induced after hypoxic brain injury in neonatal rats. This work provides the basis for a better understanding of the molecular effectors of neuroinflammation, and the effects of COX2 inhibitors (coxibs) on those effectors.

The results show an increase in COX2 after brain injury that could be reversed by early posthypoxia intervention with superanalgesic doses of the COX2 inhibitor NS-398. Consistent with other neuroprotection studies, this high-dose regimen reduced subacute mortality from hypoxic/ischemic insult, stabilized chronic brain and body weight loss, and improved functional recovery in both neurologic and cognitive testing paradigms.

The evidence that prolonged elevations of COX2 expression and activity in the brain is detrimental to outcomes is overwhelming reviewed in our earlier work (2). The induction by and contribution of COX2 to inflammation in the brain has been well documented (3–5). However, there may be a case for an initial benefit of the acute COX2 response. In our brain injury studies, some data indicated (albeit indirectly) that acute COX2 activity may be beneficial (6). A few studies suggested prostaglandins may be neuroprotective, but the preponderance of data show the opposite. The effects of prostaglandin E2 in neural excitotoxicity models are limited to subacute reductions in nuclear dye uptake; no protection was seen after 48 hrs (7). McCullough et al

showed that the protective effects of prostaglandin E2 in primarily neuronal cell cultures diminished significantly in cultured brain slices, likely because of the preservation of astrocytic/neuronal interactions in organotypic cultures (8). Prostaglandin E2, likely via the EP2 receptor, mediates reduced neuroinflammatory responses in cultured neurons, but not in the presence of glia (8). Interestingly, one study has provided evidence that COX2 prostaglandins, rather than reactive oxygen species, are responsible for COX2-mediated neurotoxicity (9). Importantly, the studies in which prostanoids (or their EP2 receptors) are characterized as neuroprotective have never shown improvements in functional outcomes.

By contrast, COX2 inhibitors (e.g., 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone [DFU] and nimesulide) reduced inflammation and cell death, and improved behavioral recovery even when administered hours after brain injury (6, 10–12). These findings provide evidence that COX2 inhibitors have an extended window of opportunity to protect vulnerable brain tissue from secondary damage.

Furthermore, our studies suggest that coxibs do more than just reduce prostaglandins and free radicals. COX2 inhibition after brain injury causes arachidonic acid shunting, increasing hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids levels in the injured brain (6, 13). These cytochrome P450 epoxygenase metabolites of arachidonic acid are prime candidates for neuroprotective eicosanoids. Hydroxyeicosatetraenoic acids have been shown to block glutamatemediated excitotoxicity in cultured neurons (14). Epoxyeicosatrienoic acids block activation of

inflammatory gene induction and reduce adhesion molecule expression in endothelial cells both *in vitro* and *in vivo* (15).

It is apparent from this study by Fathali et al (1) that there are minimal age-related differences in the benefit of coxibs to the injured brain. The coxib related risks of adverse cardiovascular events (16 –21) are containable, if not insignificant, in the perinatal population. Thus, this work also represents a potential therapeutic approach after perinatal hypoxia that could improve the quality of life for children and their families.

Yet, the lack of treatments to block COX2 or its induction specifically after brain injury is due, in my opinion, to a popular collective expectation in this country of a life free from risk, rather than to any failings of researchers to provide proof-of-principle or in BioPharma's considerable efforts to develop and make available helpful tools for this indication. There are still critical questions that remain with regard to the coxibs' mechanisms of action, or whether there is any gender dependence in their utility (or risk of adverse effects).

But if the current beneficial use of thalidomide after decades of disuse (due, admittedly, to its dreadful side effects on human fetuses) is any example, it will be a long time before drugs like rofecoxib or parecoxib can be revived. Only a grassroots consciousness-raising effort by professional and patient advocates can change the repetition of a long, long wait for these potentially valuable agents in the treatment of brain injuries.

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CHAPTER THREE

IMMUNOLOGIC CELLS ORCHESTRATE NEUROINFLAMMATION VIA CYCLOOXYGENASE-2 AFTER HYPOXIA-ISCHEMIA IN NEONATAL RATS

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Abstract

Neuroimmune processes contribute to hypoxic-ischemic damage in the immature brain and may play a key role in the progression of particular variants of neonatal encephalopathy. The present study was designed to elucidate the mechanistic inter-relationship between astrocytes and neurons in response to infiltrating peripheral immune cells after experimental neonatal hypoxia-ischemia (HI). Splenectomy was performed on postnatal day-7 Sprague-Dawley rats 3 days prior to HI surgery; in which the right common carotid artery was permanently ligated followed by 2 hours of hypoxia (8% O_2). Peripheral immune cells were found to largely contribute to cerebral infarct volume at 72 hours; body weight loss, brain and systemic organ atrophy, and neurobehavioral deficits at 3 weeks. Quantitative analysis showed altered natural killer and T cell expression in spleen and brain of ischemic animals. Elevations in cyclooxygenase-2 (COX-2) expression by immune cells promoted interleukin-15 expression in astrocytes and infiltration of inflammatory cells to site of injury; additionally, down-regulated the pro-survival protein, PI3K, resulting in caspase-3 mediated neuronal death. Herein we demonstrate with the use of pharmacological inhibitors/agonists and cell-type specific siRNA that after neonatal HI, infiltrating peripheral immune cells may modulate downstream targets of cell death and neuroinflammation by COX-2 regulated signals.

Introduction

The inflammatory response, which is characterized, in part, by recruitment of circulating immune cells, has been implicated as a core component of damage to the immature brain following hypoxia-ischemia (HI) (1). Although many therapeutic interventions have been explored to prevent and/or mitigate the inflammatory sequelae of perinatal HI, few such interventions have proven clinically viable in the long run. One explanation has been that the immunoinflammatory response is multifaceted, in that activation of immune cells may have both detrimental and neuroprotective effects (2). Increasing evidence suggests that a more integrative approach to therapy may resolve this paradox (3,4). In theory, re-directing our attention from neuron-driven outcomes toward the molecular mediators believed to orchestrate brain-immune cell interactions may prove a more fruitful investigative approach in neonatal HI (5).

Cyclooxygenase-2 (COX-2), a well-established contributor to ischemic brain injury (6,7), might serve as a prime candidate for such a molecular-mediated investigation. In particular, COX-2 may mediate the mechanism by which activated immune cells induce pro-inflammatory cytokine production by astrocytes (8). Recent data suggests that enhanced interleukin-15 (IL-15) expression in astrocytes is a major propagator of inflammatory responses after central nervous system injury (9). Yet it still remains to be determined whether astrocytes respond to COX-2 effectors from infiltrating immune cells by producing IL-15, which then further orchestrates the inflammatory response and/or cell death in the immature brain.

Additionally, the degree of involvement from the innate immune system correlates with the extent of neuronal damage in the post-ischemic tissue (2). Studies

suggest dysfunction of the phosphoinositide-3-kinase (PI3K)/Akt survival pathway in triggering apoptotic cascades in the brain (10). However, it is unknown whether down-regulation of the PI3K pathway and subsequent neuronal death in HI-injured rats occurs in response to COX-2 from infiltrating immune cells.

From the ischemic stroke model comes an indication that progression of brain injury is mediated by immune cells originating in the spleen (11). We hypothesized that neurological outcomes in stroked neonatal rats can be ameliorated by targeting splenic immune cells and their modulatory functions mediated by COX-2. To elucidate the possible impact of the systemic inflammatory response on astroglia-neuron signaling, we removed the spleen, the largest pool of peripheral immune cells prior to HI and studied the short- and long-term outcomes in the neonate. We also used a gain and loss of function approach (pharmacological activation or inhibition, respectively) for COX-2, a neutralizing antibody for IL-15, and a gene silencer for natural killer (NK) cells in both splenectomized and non-splenectomized rats to verify the role of COX-2 in splenic immune cell responses following HI. Here, we identify infiltrating splenic immune cells as a major source of enhanced COX-2 expression in the ischemic brain, and implicate COX-2 for causation of signaling pathways in astrocytes and neurons that lead to a worsened outcome.

Materials and Methods

Surgical Procedures

The protocol detailing this study was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Timed pregnant Sprague-Dawley rats (Harlan Labs) were obtained and housed in individual cages under a 12 h light/dark

cycle, with food and water available *ad libitum*. Splenectomy, on postnatal day-7 pups, entailed a skin incision at the upper left quadrant of the abdomen, exteriorization of the spleen through the incision, and cauterization of the blood vessels. Un-splenectomized groups had the abdominal cavity opened, the spleen isolated, and then re-closed. Postnatal day-10 pups were placed on a surgical table maintained at 37^0C and anesthetized by inhalation with isoflurane. Briefly, HI surgery (7) entailed permanent ligation of the right common carotid artery using 5-0 surgical silk, followed by 1.5 h of recovery, then placement in a glass jar perfused for 2 h with 8% oxygen. Rats were sacrificed under general anesthesia by decapitation at 3 h, 3 d or 3 w post-HI.

Pharmacological Manipulation

Some postnatal day-9 pups were treated intraperitoneally with 30mg/kg COX-2 inhibitor (NS398; Cayman Chemical), or intraventricularly with 0.01mg/kg COX-2 agonist (4-HNE; Cayman Chemical) or with 1μl NK cell (CD161; Invitrogen) siRNA. Others were treated intraventricularly with 1.5μl IL-15 neutralizing antibody (Santa Cruz Biotech) 1 h pre-HI.

Measurement of Infarct Size

Brain tissue was collected after trans-cardial perfusion with 0.1M phosphate buffered saline; and cut at 2-mm intervals into 5 coronal sections and incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) for infarct volume measurement (35).

Evaluation of Organ Damage

Cerebral hemispheres (separated by a midline incision) of animals and spleen of un-splenectomized animals was isolated then weighed on a high-precision balance (sensitivity ± 0.001 g).

Neurological Assessment

Rats were placed onto an elevated wire grid floor (36 x 13 in) for 2 min; foot-faults were noted when a complete paw fell through the bars for assessment of motor coordination (36). Rat were placed at the base (40 x 10cm) of the maze for each trial and allowed to explore until an arm (46 x 10 cm) of the maze was chosen for assessment of short-term or working memory (37). Sequence of choices over 10 trials was expressed as the rate of spontaneous alternation. Methodology was as previously described for: Postural Reflex, Proprioceptive Limb Placing, Lateral Pressure Towards Edge, and Lateral Limb Placing (7). Animals scored 100 for immediate and correct paw placement; 50 for delayed and/or incomplete placement; 0 for no placement.

Immunofluorescence Analysis

Every fifth section of brain and spleen tissue was collected. From this set, six non-adjacent coronal sections (10μm) from the same levels of sectioning in different animals were used for fluorescent labeling (7). Antibodies used: CD161 (1:100; Serotec Co.), CD3 (1:100; Serotec Co.), COX-2 (1:100; Santa Cruz Biotech), IL-15 (1:100; Santa Cruz Biotech.), GFAP (1:100; Millipore), cleaved caspase-3 (1:100; Cell Signaling), NeuN (1:100; Millipore), Iba1 (1:100; Wako Chemicals), CD68 (1:100; Millipore), or MPO (1:100; Dako). The absolute number of positive cells per square millimeter was counted

in three peri-infarct regions of the ipsilateral cerebral cortex (7) or in three marginal zone areas immediately adjacent to the white pulp of the spleen.

Western Blot Analysis

Homogenates of ipsilateral cerebral hemispheres were processed (7) for analysis using antibodies against: COX-2 (1:300), CD161 (1:200), CD3 (1:200), PI3K (1:300; Santa Cruz Biotech), pro caspase-3 (1:1000; Cell Signaling), cleaved caspase-3 (1:1000), or beta actin (1:1000, Santa Cruz Biotech). Incubation with respective secondary antibodies was done. Optical density was determined using NIH Image J software and expressed relative to beta actin.

RT-PCR Analysis of NK Cells

Total RNA was isolated from the ipsilateral hemisphere with TRIZOL reagent and cDNA prepared from 1μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) (38). The thermal cycle profile of 35 cycles for PCR amplification (GAPDH: 22 cycles) by means of thermocycler (iCycler; BIO-RAD) was: 1) denaturing (1 min, 94°C); 2) annealing primers (1 min, 55°C); and 3) extending primers (1.5 min, 72°C). A portion of 10 μl of PCR products was electrophoresed in 2% agarose gel in Tris-borate-EDTA buffer. Densities of bands were determined by Bio-Rad Quantity One graphic software, and were expressed relative to GAPDH bands.

Klrb1b (CD161) (39):

Forward: 5'-GTTCTAGACTCGGCTGTGCTTGCCT-3'

Reverse: 5'-CTGAATTCTGGTAAAGTAATCGAGGTACG-3'

GAPDH (38):

Forward: 5'-ACCACAGTCCATGCCATCAC-3'

Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'

Assessment of Cell Death

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) method was used according to manufacturer's instructions (Roche Diagnostics-Applied Science). Sections were mounted with anti-fade mounting medium (Molecular Probes) under glass cover-slips, and digital microphotographs were taken separately for each stain and merged using Magnafire software (Optronics).

Data Analysis

Observers were blind to the actual animal groupings. Results were expressed as mean ± SEM. One-way ANOVA Holm-Sidak correction were used to determine significance in differences between means. Kruskal-Wallis ANOVA followed by Dunn's test was used for neurobehavioral analysis. When only two groups were available for comparison, the unpaired Student's *t*-test was implemented. $P < 0.05$ was taken as significant.

Results

Splenectomy Attenuates HI-Induced Cerebral Infarct

The spleen contains the largest reservoir of immune cells (11). To detect whether the spleen is the major source of peripheral immune cells that infiltrate the ischemic brain and contribute to cerebral infarction after HI, we quantified primary (3 h post-HI) and secondary (3 d post-HI) infarct volume of splenectomized and un-splenectomized rats

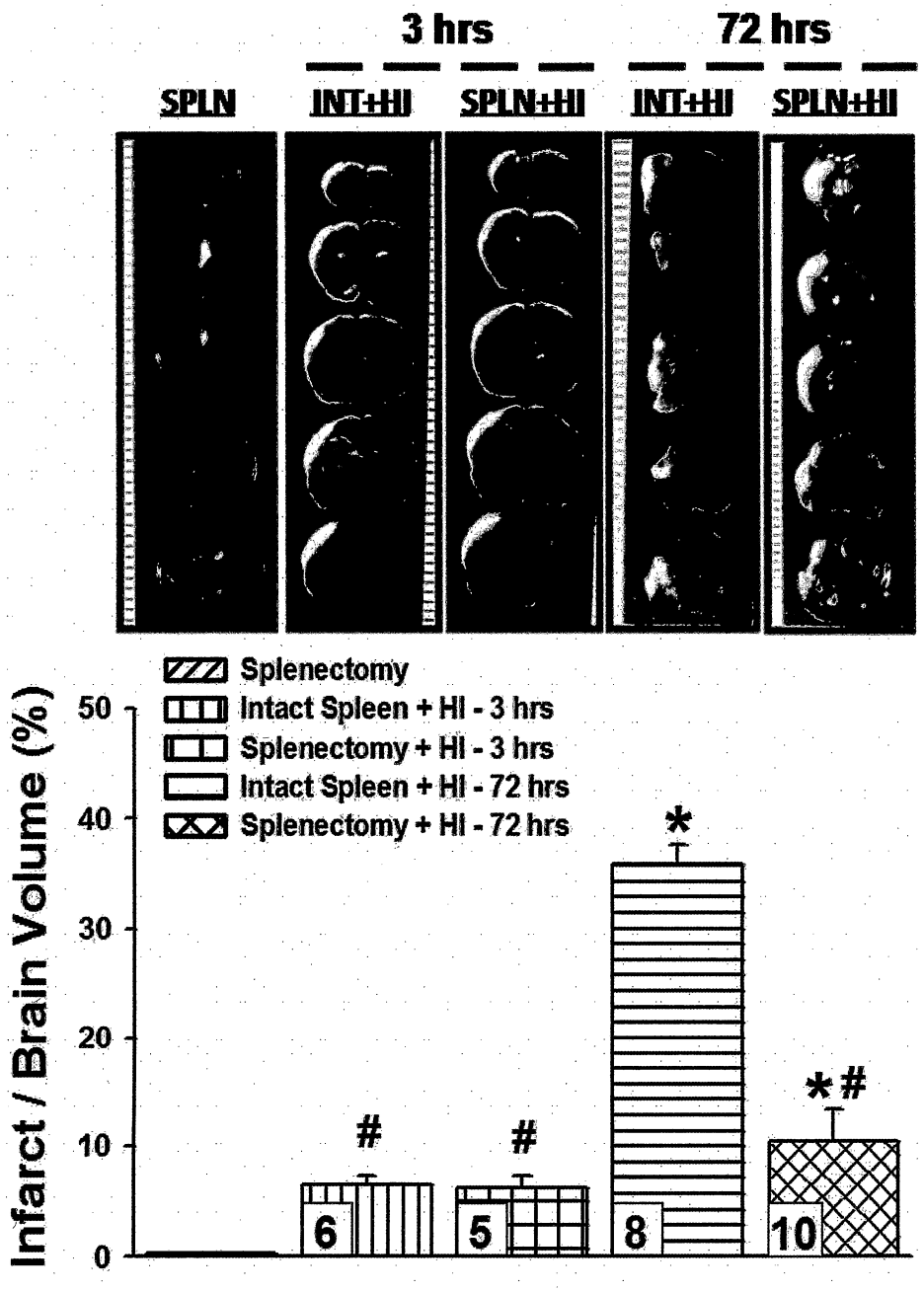

3 hrs
72 hrs
SPLN
INT+HI
SPLN+HI
INT+HI
SPLN+HI
Splenectomy
Intact Spleen + HI - 3 hrs
Splenectomy + HI - 3 hrs
Intact Spleen + HI - 72 hrs
Splenectomy + HI - 72 hrs
Infarct / Brain Volume (%)
50
40
30
20
10
0
#
#
*
*#
6
5
8
10

Figure 15. Primary and Secondary Brain Injury Expressed as Percent Infarct Volume. Infarct volumes at 3 hours and 72 hours in splenectomy (SPLN), intact spleen with hypoxia-ischemia (INT+HI), and splenectomy with HI (SPLN+HI) rats. $^{*}P < 0.001$ versus splenectomy, $^{\#}P < 0.001$ versus intact spleen + HI – 72hrs. Data represent mean ± SEM. One-way ANOVA with Holm-Sidak correction. Numbers in bars indicate animals/group.

(Figure 15). Removal of the spleen prior to HI had no effect on primary infarct (6.19 ± 1.11 vs. 6.50 ± 0.77), but significantly reduced secondary infarct (10.45 ± 2.94 vs. 35.93 ± 1.69). Splenectomized animals with no brain injury are shown as a control.

Splenectomy Attenuates HI-Induced Extended Brain Damage

In order to determine the role of the spleen in long-term HI-related brain damage, we used reductions in spleen size (12) and hemispheric weight (13) as parameters for brain damage. HI-injured rats had significant spleen and brain atrophy compared to sham animals 3 w post-HI (Figure 16). Comparisons of right to left hemispheric weight showed significant attenuation of brain loss in HI rats with prior splenectomy (0.85 ± .03 vs. 0.72 ± .04). Brain weight ratios of splenectomized animals without brain injury were not significantly different than sham (0.99 ± .01 vs. 0.98 ± .01).

Splenectomy Improves Short- and Long-Term Body Weight After HI

Body weight gain post-HI is an indicator of general well-being (14). Accordingly, we measured body weight gain over the first 3 d after HI insult and found HI-injured rats with an intact spleen gained less weight during the acute phase as compared to their splenectomized counterparts (Figure 17). Next, weekly body weights were taken to determine if these somatic differences had lasting effects (Figure 18). The HI rats with an intact spleen were unable to catch up to the weight of sham rats for the entirety of the study; while splenic removal prior to HI insult maintained the weight of the animals at all weekly time-intervals.

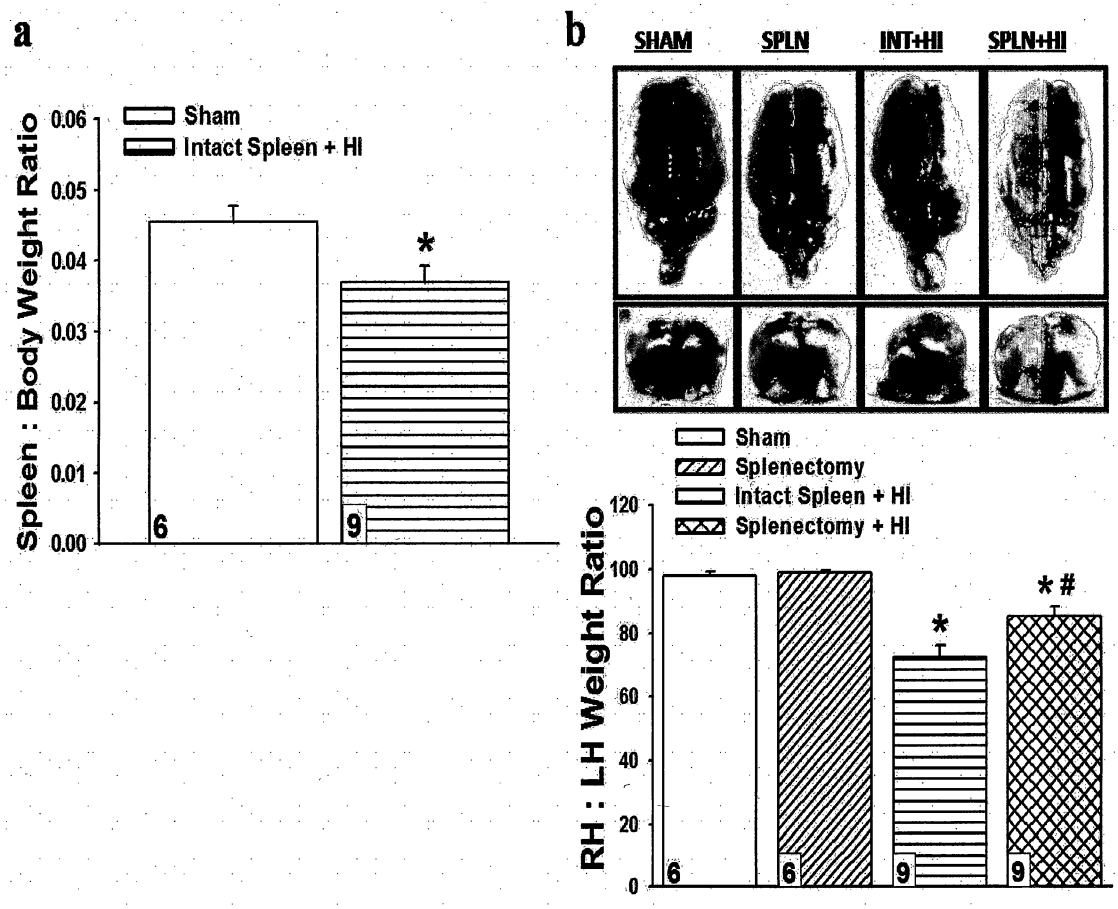

a
Spleen : Body Weight Ratio
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Sham
Intact Spleen + HI
*
6
9
b
SHAM
SPLN
INT+HI
SPLN+HI
Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
RH : LH Weight Ratio
120
100
80
60
40
20
0
*
*#
6
6
9
9

Figure 16. Long-Term HI-Related Brain Damage Assessed by Spleen and Brain Weights. (**a**) Spleen to body weight ratio of sham and intact spleen with HI rats at 3 weeks. $^{*}P < 0.05$ versus sham (**b**) Right to left hemispheric (RH:LH) weight ratio representing brain atrophy at 3 weeks. $^{*}P < 0.001$ versus sham, $^{\#}P < 0.001$ versus intact spleen + HI. Data represent mean ± SEM. Unpaired Student's *t*-test (**a**); one-way ANOVA with Holm-Sidak correction (**b**). Numbers in bars indicate animals/group.

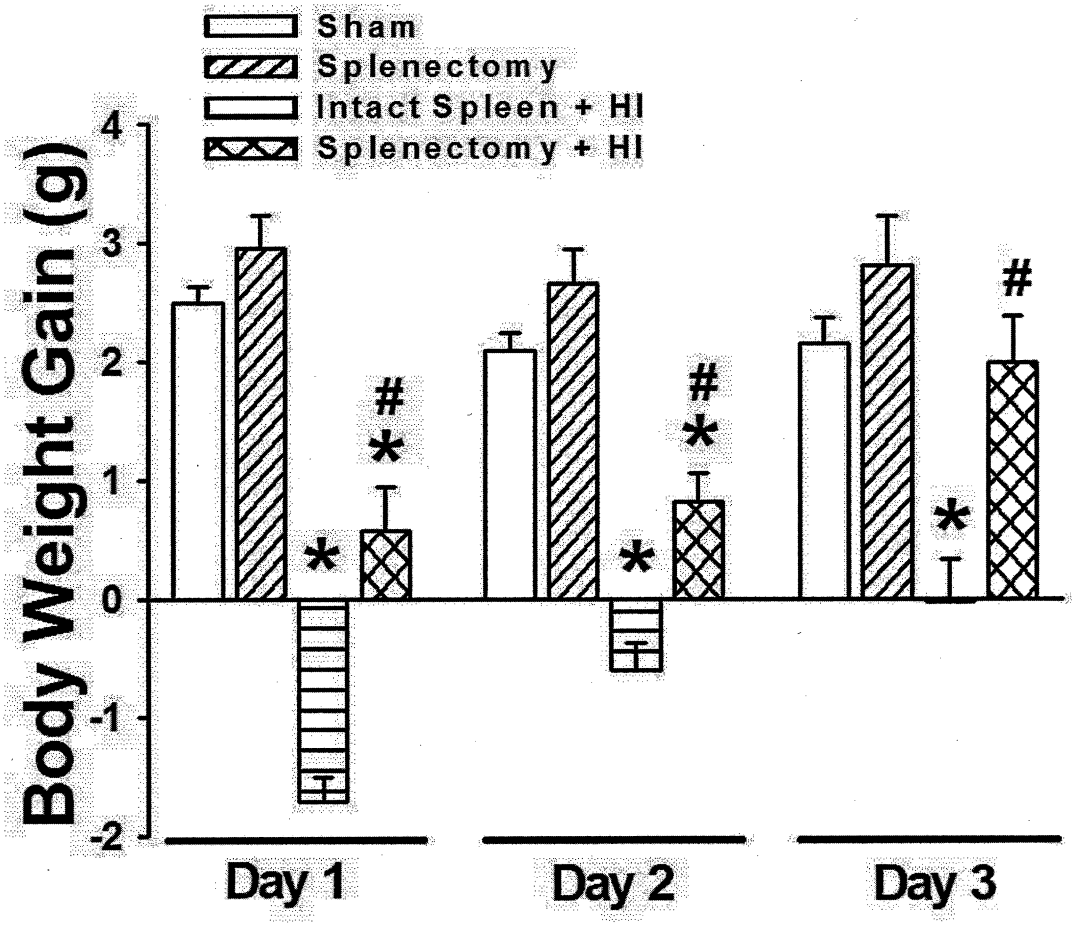

Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Body Weight Gain (g)
4
3
2
1
0
-1
-2
*
#
Day 1
Day 2
Day 3

Figure 17. Acute Body Weight Gain. Daily body weight gain of animals during the first 3 days after HI insult. $^{*}P < 0.001$ versus sham, $^{\#}P < 0.001$ versus intact spleen + HI. Data represent mean ± SEM. One-way ANOVA with Holm-Sidak correction.

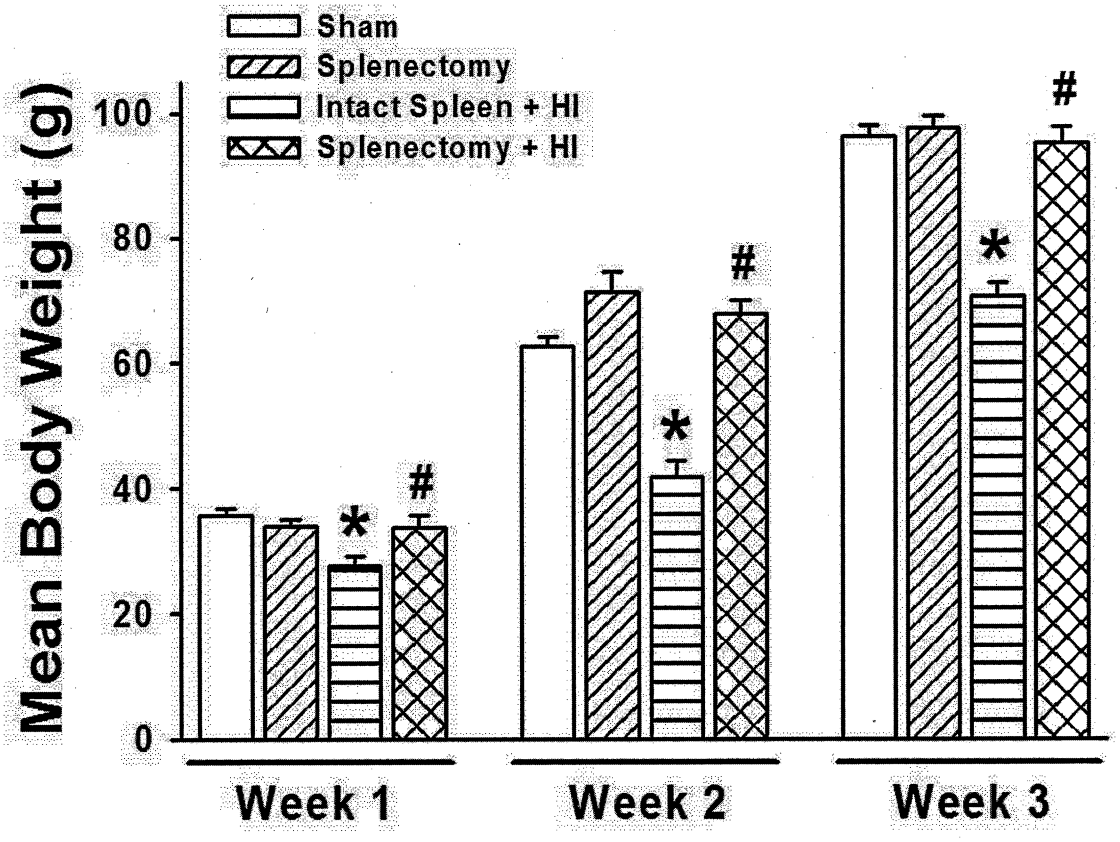

Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Mean Body Weight (g)
100
80
60
40
20
0
*
#
Week 1
Week 2
Week 3

Figure 18. Weekly Mean Body Weight. Weekly mean body weight of animals over 3 weeks. Week 1: $^{*}P < 0.01$ versus sham, $^{\#}P < 0.01$ versus intact spleen + HI; Week 2 and 3: $^{*}P < 0.001$ versus sham, $^{\#}P < 0.001$ versus intact spleen + HI. Data represent mean ± SEM. One-way ANOVA with Holm-Sidak correction.

Splenectomy Improves HI-Induced Neurological Behavior Deficits

Lifetime consequences of perinatal HI may include motor, cognitive, and behavioral deficits (15). Several functional tests were used to evaluate the spleen's role on long-term neurological behavior (Figure 19). When the spleen was intact, an ischemic event resulted in a significant decline in motor coordination (Foot-fault: 33.33 ± 2.39), memory (T-maze: 34.57% ± 4.70), and sensory function (Proprioceptive Limb Placing: 22.22 ± 8.78); while, prior removal of the spleen improved these outcomes (19.99 ± 2.77, 70.37% ± 3.21, and 83.33 ± 11.79, respectively). Additionally, HI rats with an intact spleen demonstrated significant asymmetry in posture and extension of forelimbs (Postural Reflex: 50.00), and reduced ability for correct placement of limbs (Lateral Pressure: 22.78 ± 8.78; Lateral Limb Placing: 38.89 ± 13.89). Although splenectomy prior to HI appeared to improve these deficits (77.78 ± 8.78, 66.67 ± 11.79, 77.78 ± 8.78, respectively), significance was not reached.

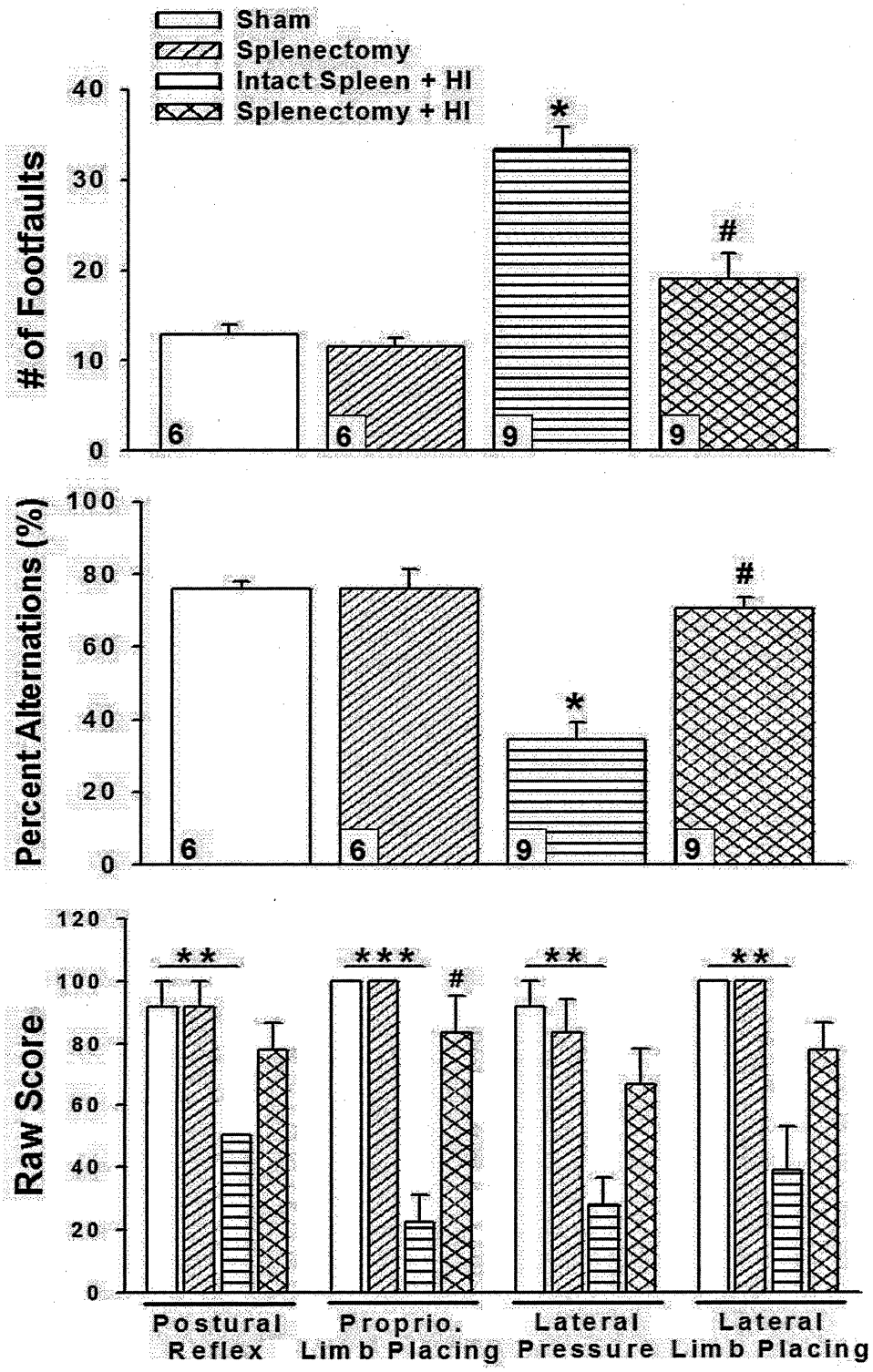

Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
# of Footfaults
40
30
20
10
0
6
6
9
9
*
#
Percent Alternations (%)
100
80
60
40
20
0
6
6
9
9
*
#
Raw Score
120
100
80
60
40
20
0
**
***
#
**
**
Postural Reflex
Proprio. Limb Placing
Lateral Pressure
Lateral Limb Placing

Figure 19. Neurological Behavior 3 Weeks After HI Insult. $^{*}P < 0.05$ versus sham, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$ versus intact spleen + HI. Data represent mean ± SEM. Kruskal-Wallis ANOVA with Dunn's correction.

Cerebral Ischemia Alters Expression of Immune Cells in Spleen and Brain

Leukocytes are major effectors of damage after cerebral ischemia (16,17). However, the contributions of peripheral immune cells to the ischemic neonatal brain are unclear (18); and due to the vast differences in immune response between neonates and adults, much of the insight obtained from adult studies cannot be inferred to be the same in the immature brain (19). Accordingly, we sought to determine the leukocyte subsets possibly released by the spleen (Figure 20) and found there to be a significant reduction in immunoreactive-NK (1923 ± 175 cells/mm^2 vs. 5381 ± 244 cells/mm^2) and T (3010 ± 202 cells/mm^2 vs. 4326 ± 233 cells/mm^2) cells after HI compared to sham. The same trends between groups were also observed after quantitative analysis (NK: 65.02 ± 12.78 vs. 101.50 ± 2.10; T: 65.43 ± 5.24 vs. 100.00 ± 5.31). Next, we verified whether the spleen is a major source of invading leukocytes in the post-ischemic brain (Figure 21). Results indicated rats with an intact spleen had increased NK (186.67 ± 29.52 cells/mm^2 vs. 28.00 ± 12.52 cells/mm^2) and T (93.33 ± 11.81 cells/mm^2 vs. 23.33 ± 8.61 cells/mm^2) cells after HI compared to sham; while prior splenectomy significantly reduced these cell populations (NK cells: 14.00 ± 6.26 cells/mm^2; T cell: 42.00 ± 6.26 cells/mm^2).

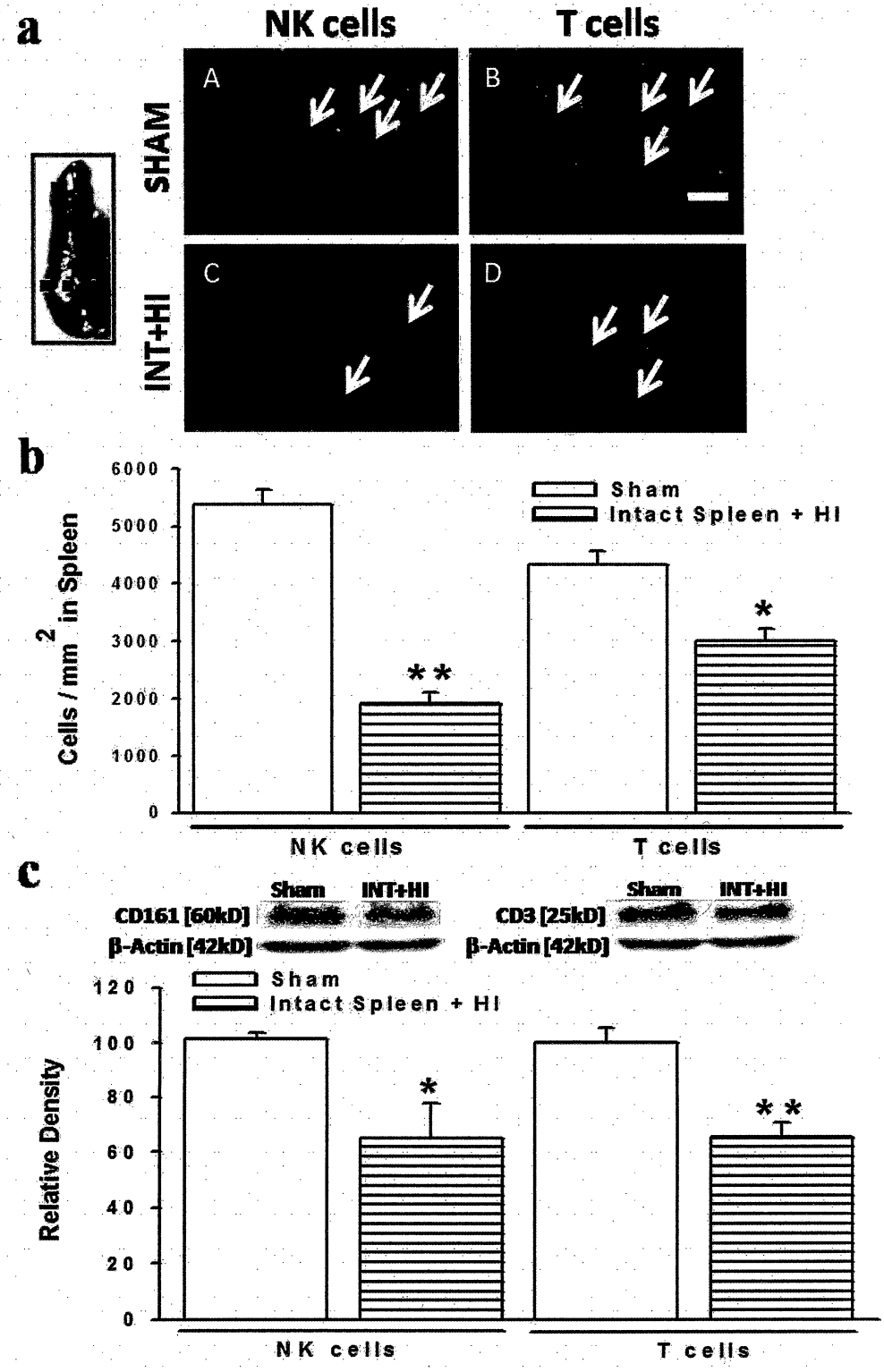
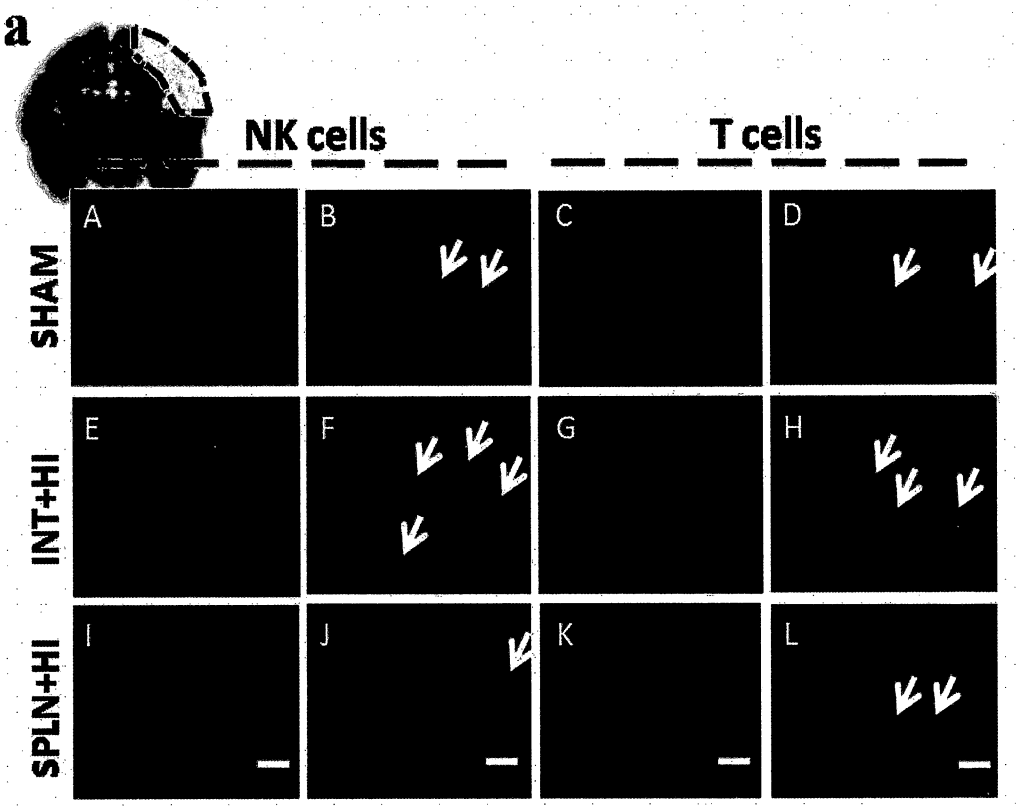

a
NK cells
T cells
A
B
C
D
SHAM
INT+HI
b
Cells / mm 2 in Spleen
6000
5000
4000
3000
2000
1000
0
Sham
Intact Spleen + HI
**
*
NK cells
T cells
c
Sham
INT+HI
CD161 [60kD]
β-Actin [42kD]
CD3 [25kD]
β-Actin [42kD]
Relative Density
120
100
80
60
40
20
0
Sham
Intact Spleen + HI
*
**
NK cells
T cells

Figure 20. Natural Killer (NK) and T Cell Populations in Spleen Tissue. (**a**) NK and T immunoreactive cells from sectioned spleen tissue between dotted lines of sham and intact spleen with HI (INT+HI) rats. Scale bar, 60 μm. (**b**) Absolute number of NK and T cells per square millimeter region in three marginal zone areas of the spleen immediately adjacent to the white pulp (n = 6/group). NK: $^{**}P < 0.001$ versus sham, T: $^{*}P < 0.01$ versus sham. (**c**) Splenic tissue probed for NK (CD161) and T (CD3) cell expression, relative to beta actin, on western blots. NK (n = 4/group): $^{*}P < 0.05$ versus sham, T (n = 5/group): $^{**}P < 0.01$ versus sham. Data represent mean ± SEM; Unpaired Student's *t*-test.


a
NK cells
T cells
SHAM
INT+HI
SPLN+HI
A
B
C
D
E
F
G
H
I
J
K
L

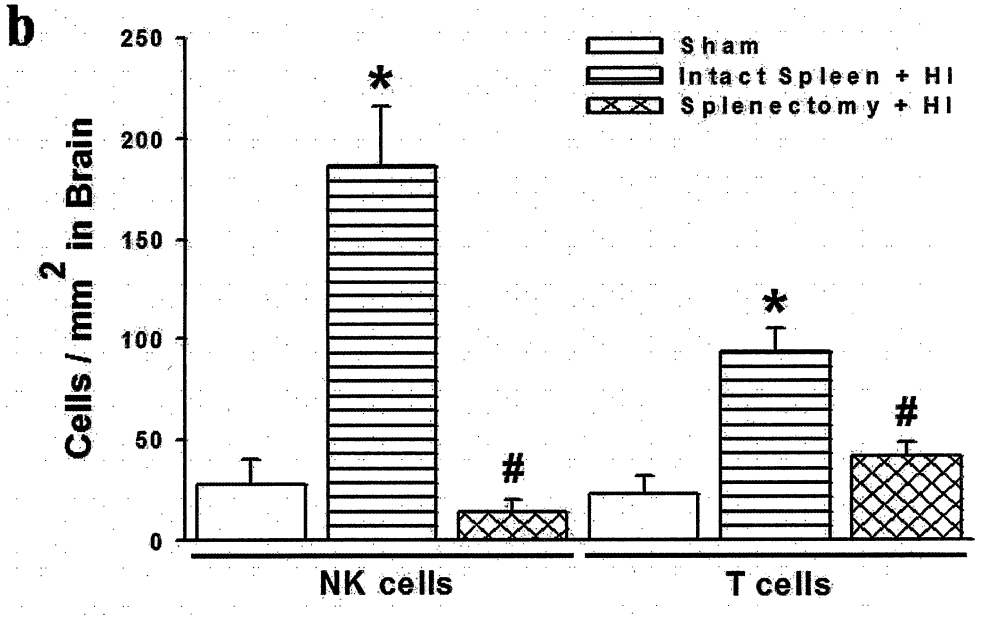

b
Cells / mm 2 in Brain
250
200
150
100
50
0
Sham
Intact Spleen + HI
Splenectomy + HI
*
#
*
#
NK cells
T cells

Figure 21. Natural Killer (NK) and T Cell Populations in Brain Tissue. **(a)** Immunofluorescent evaluation of NK and T cell populations in ipsilateral cerebral cortex (within dotted region) of sham, INT+HI, and splenectomy with HI (SPLN+HI) rats. Scale bar, 300 μm (panels I,K) or 30 μm (panels J,L). **(b)** Absolute number of NK and T cells per square millimeter (n = 6/group). $^{*}P < 0.001$ versus sham, $^{\#}P < 0.001$ versus intact spleen + HI. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

Splenic Immune Cells May be a Major Source of COX-2 in HI Brain

COX-2 is a major effecter of ischemic cerebral injury (6,7). Therefore, we sought to determine the contribution of the spleen on COX-2 expression in the HI-injured brain (Figure 22). Results showed that COX-2 expression was elevated in HI rats with an intact spleen, but significantly suppressed by prior splenectomy. To verify the efficacy of the inhibitor (NS398) and agonist (4-HNE) used in our experimental studies for suppressing and upregulating COX-2, respectively; the pharmacological agents were administered to HI-induced rats with or without a spleen. NS398 to HI rats with an intact spleen significantly reduced COX-2 density compared to their untreated counterpart; but, there was no significance between NS398-treated or –untreated HI rats with prior splenectomy. 4-HNE significantly enhanced COX-2 expression in HI-injured groups, although less so in splenectomized rats.

COX-2 May Contribute to HI-Induced Body Weight Reduction

To elucidate whether the low body weights of HI rats with an intact spleen were COX-2-mediated, rats were administered NS398 or 4-HNE on postnatal day-9 followed one day later by sham- or HI-surgery. We found NS398 maintained body weights after HI (Figure 23, left-side); while 4-HNE groups showed a trend for lower body weights (Figure 23, right-side).

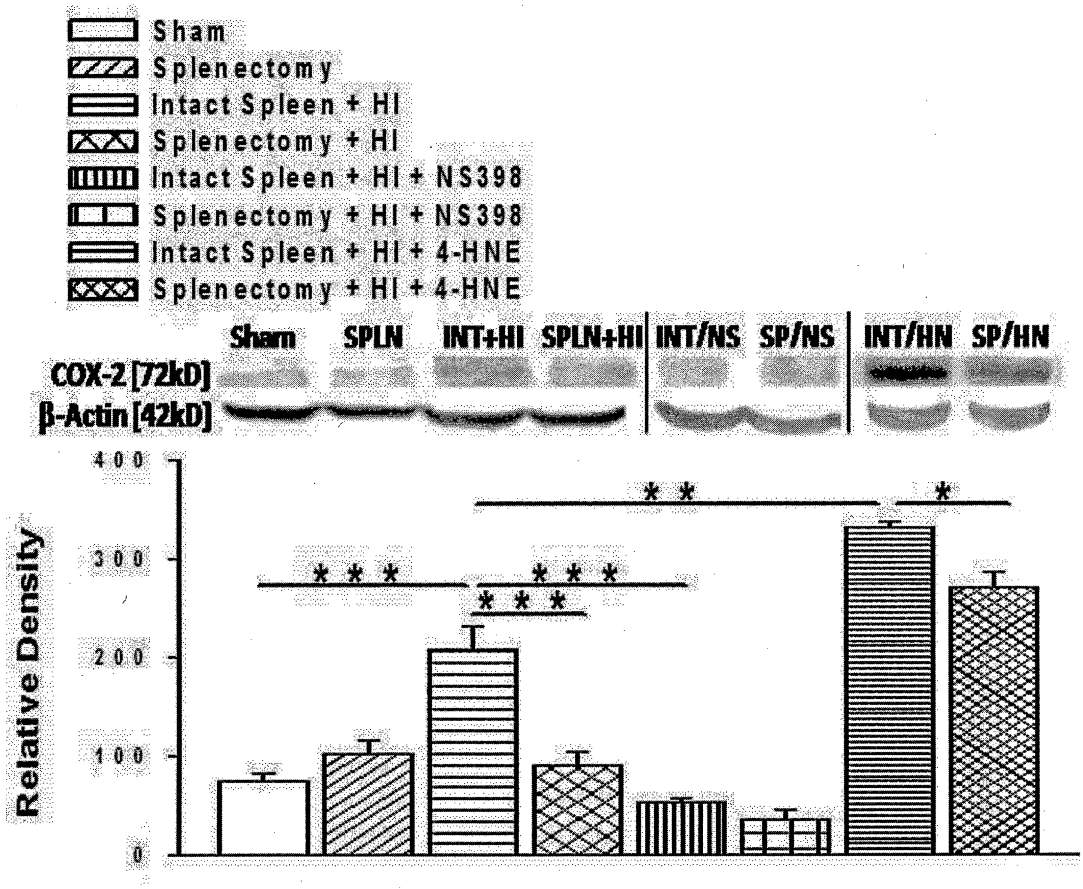

Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Intact Spleen + HI + NS398
Splenectomy + HI + NS398
Intact Spleen + HI + 4-HNE
Splenectomy + HI + 4-HNE
Sham
SPLN
INT+HI
SPLN+HI
INT/NS
SP/NS
INT/HN
SP/HN
COX-2 [72kD]
β-Actin [42kD]
Relative Density
400
300
200
100
0
* * *
* * *
* * *
* *
*

Figure 22. Western Blot Analysis of COX-2 Protein. COX-2 protein levels relative to beta actin in the ipsilateral hemisphere of rats. Comparisons made after administration of COX-2 inhibitor (NS398) or agonist (4-HNE) (n = 5/group; interventions: n = 4/group). $^{*}P = 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

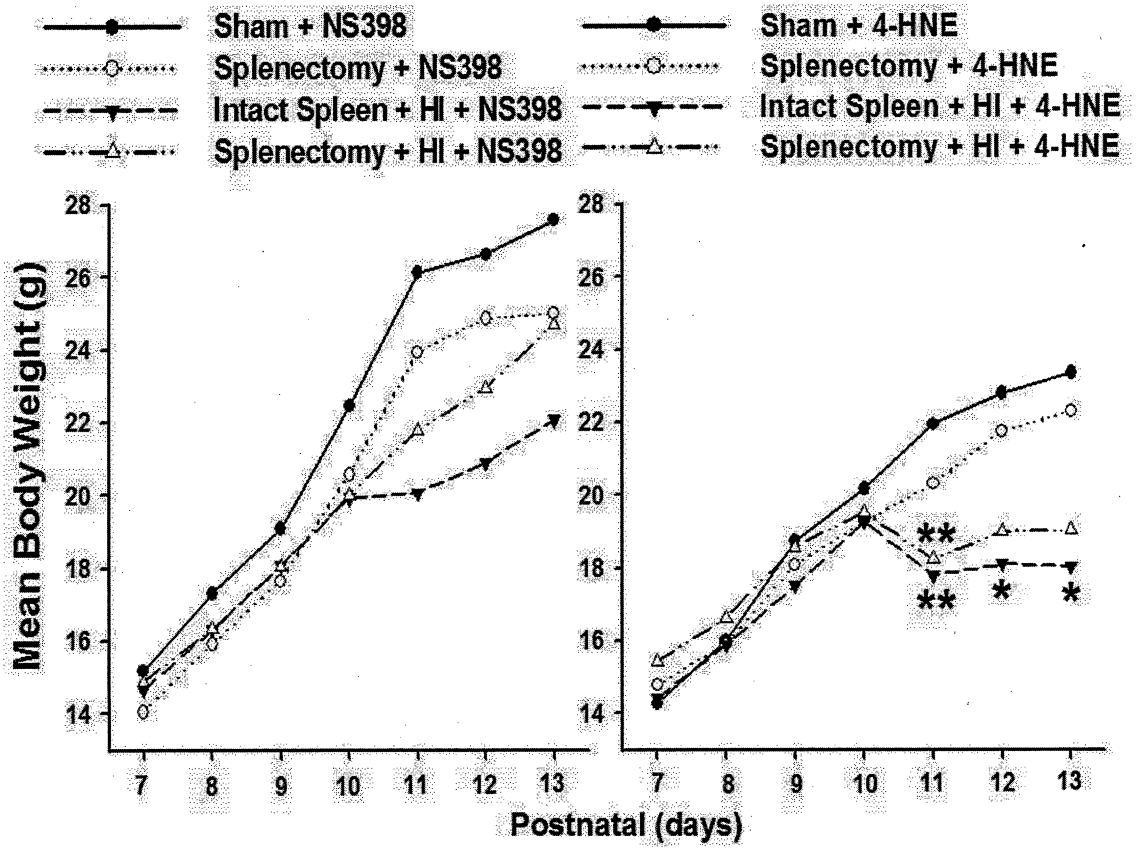

Sham + NS398
Splenectomy + NS398
Intact Spleen + HI + NS398
Splenectomy + HI + NS398
Sham + 4-HNE
Splenectomy + 4-HNE
Intact Spleen + HI + 4-HNE
Splenectomy + HI + 4-HNE
Mean Body Weight (g)
Postnatal (days)
**
**
*
*

Figure 23. Mean Body Weight of Pups Administered COX-2 Inhibitor (NS398) or Agonist (4-HNE). Daily mean body weight of rats administered NS398 (left-side of figure) or 4-HNE (right-side of figure) on postnatal day 9 followed by HI insult on day 10. Individual body weights were compared for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

COX-2 Co-Localizes With Immune Cell Subsets After HI

To determine whether the immune cell subsets infiltrating the ischemic brain were involved in expressing COX-2, double-immunofluorescence was performed on brain sections for comparison of splenectomized and un-splenectomized HI-injured rats (Figure 24). Results showed elevated COX-2-expressing NK and T cells in brains of HI rats with an intact spleen (79.33 ± 13.36 and 56.00 ± 7.23 cells/mm^2, respectively) compared to sham (32.67 ± 13.36 and 14.00 ± 6.26 cells/mm^2, respectively). Splenectomy prior to HI significantly reduced NK/COX-2 (14.00 ± 6.26 cells/mm^2) and T/COX-2 (28.00 ± 7.23 cells/mm^2) populations. Since NK cells appeared to be the majority in the ischemic tissue, NK siRNA was administered to verify its role on COX-2 expression. The efficacy of the siRNA was provided by RT-PCR (Figure 25) and further analysis demonstrated equal COX-2 levels between ischemic and non-ischemic groups (Figure 26).

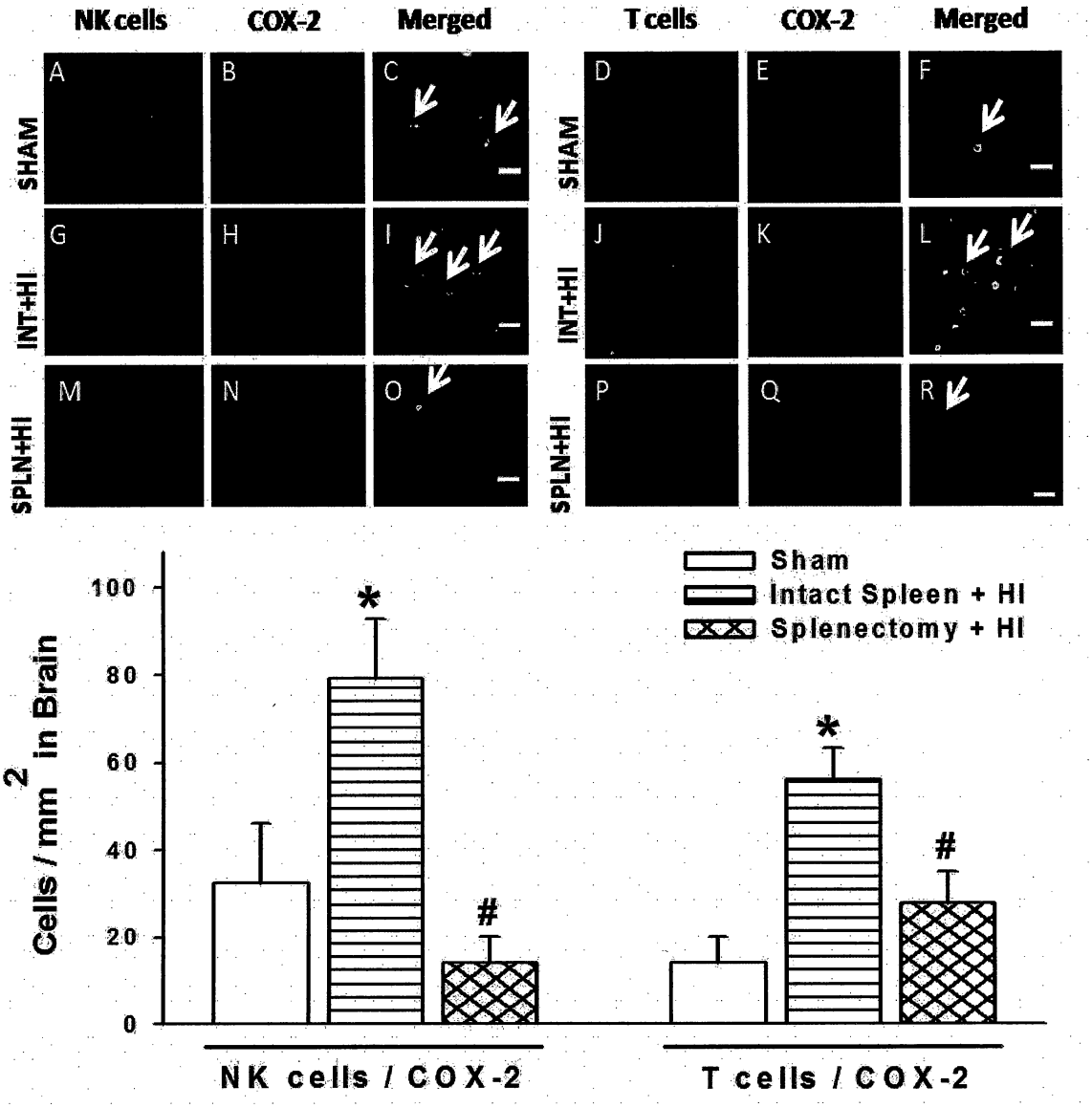

NK cells
COX-2
Merged
T cells
COX-2
Merged
SHAM
INT+HI
SPLN+HI
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Sham
Intact Spleen + HI
Splenectomy + HI
Cells / mm 2 in Brain
100
80
60
40
20
0
*
#
*
#
NK cells / COX-2
T cells / COX-2

Figure 24. Quantification of COX-2 Immunoreactive Natural Killer (NK) or T Cells. Immunohistochemical analysis of ipsilateral cerebral cortex double-stained with antibodies against NK cells and COX-2 or T cells and COX-2. Scale bar, 30 μm. Absolute number of NK and T cells co-expressing COX-2 per square millimeter (n = 6/group). $^{*}P < 0.01$ versus sham, $^{\#}P < 0.01$ versus intact spleen + HI. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

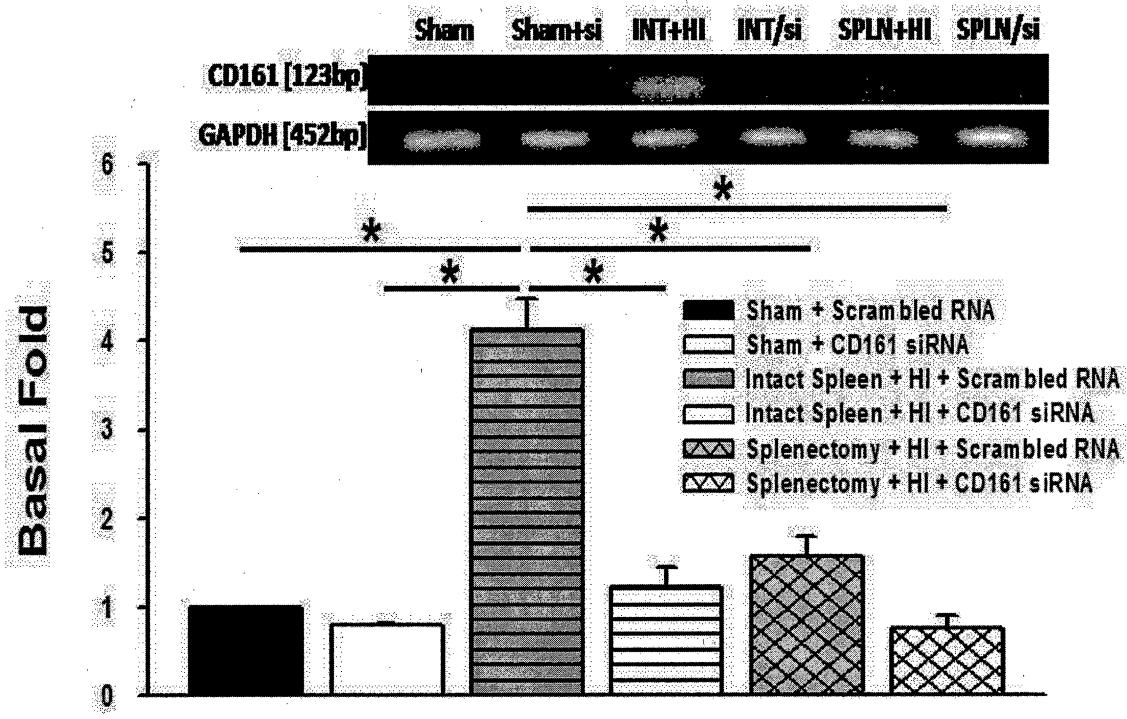

Sham
Sham+si
INT+HI
INT/si
SPLN+HI
SPLN/si
CD161 [123bp]
GAPDH [452bp]
Basal Fold
0
1
2
3
4
5
6
*
*
*
*
*
Sham + Scrambled RNA
Sham + CD161 siRNA
Intact Spleen + HI + Scrambled RNA
Intact Spleen + HI + CD161 siRNA
Splenectomy + HI + Scrambled RNA
Splenectomy + HI + CD161 siRNA

Figure 25. RT-PCR Analysis of Natural Killer Cell (CD161) mRNA. NK cell mRNA expression levels in rats pre-treated with scrambled RNA or NK (CD161) small interfering (si) RNA (n = 6/group). $^{*}P < 0.001$. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

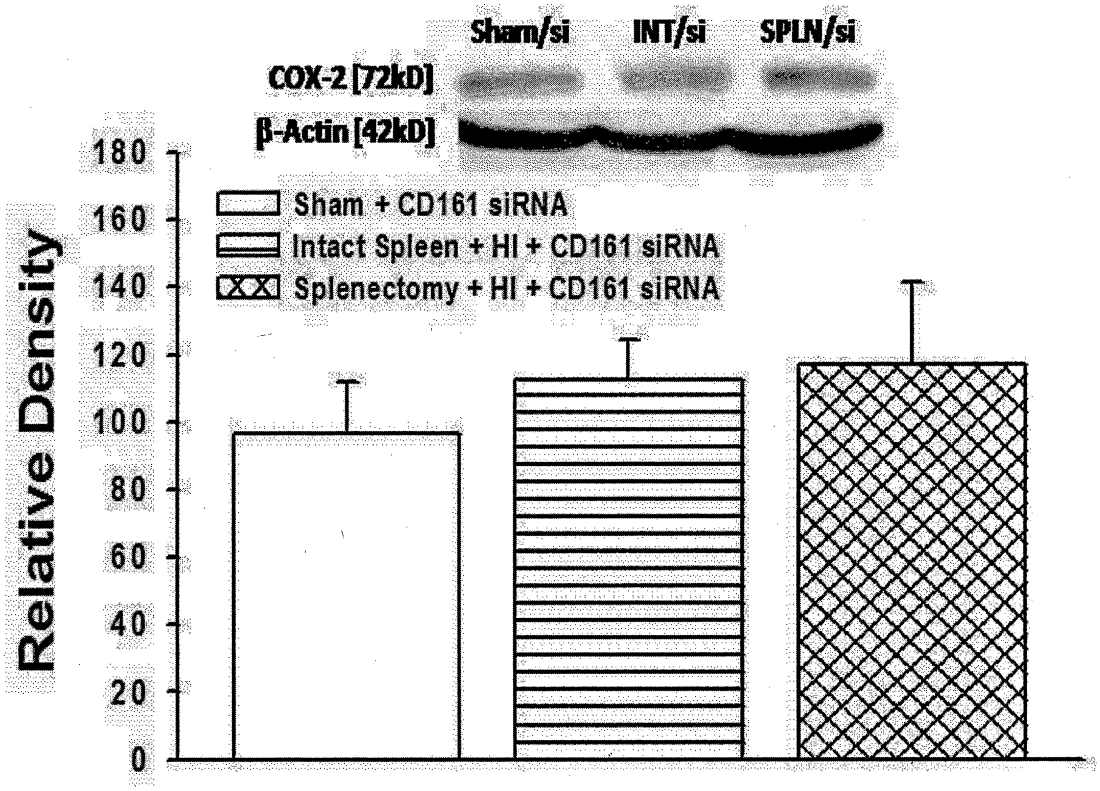

Sham/si
INT/si
SPLN/si
COX-2 [72kD]
β-Actin [42kD]
Relative Density
180
160
140
120
100
80
60
40
20
0
Sham + CD161 siRNA
Intact Spleen + HI + CD161 siRNA
Splenectomy + HI + CD161 siRNA

Figure 26. Western Blot Analysis of COX-2 Protein in Pups Administered Natural Killer Cell (CD161) siRNA. COX-2 protein levels relative to beta actin in the ipsilateral hemisphere of rats pre-treated with CD161 siRNA (n = 4/group). Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.

COX-2 May Promote IL-15 Expression by Astrocytes

By-products of reactive astrocytes play a key role in regulating the extent of the immune response (20). Therefore, we sought to elucidate whether peripheral immune cells propagate the neuroinflammatory response by inducing expression of the pro-inflammatory cytokine IL-15 in astrocytes (Figure 27). Ischemic cortical areas of rats with an intact spleen revealed elevated IL-15-immunoreactive astrocytes, but a marked reduction was evident in splenectomized animals. Next we verified whether these differences were COX-2-mediated, and found suppressed IL-15 immunoreactivity in NS398-treated HI animals. While 4-HNE enhanced IL-15-expressing astrocytes, co-localization was substantially less in HI rats with prior splenectomy. To determine the role of IL-15 in HI-induced damage, we evaluated infarct volume (Figure 28) and body weights (Figure 29) of rats after administration of IL-15 neutralizing antibody and found significant improvements in both parameters.

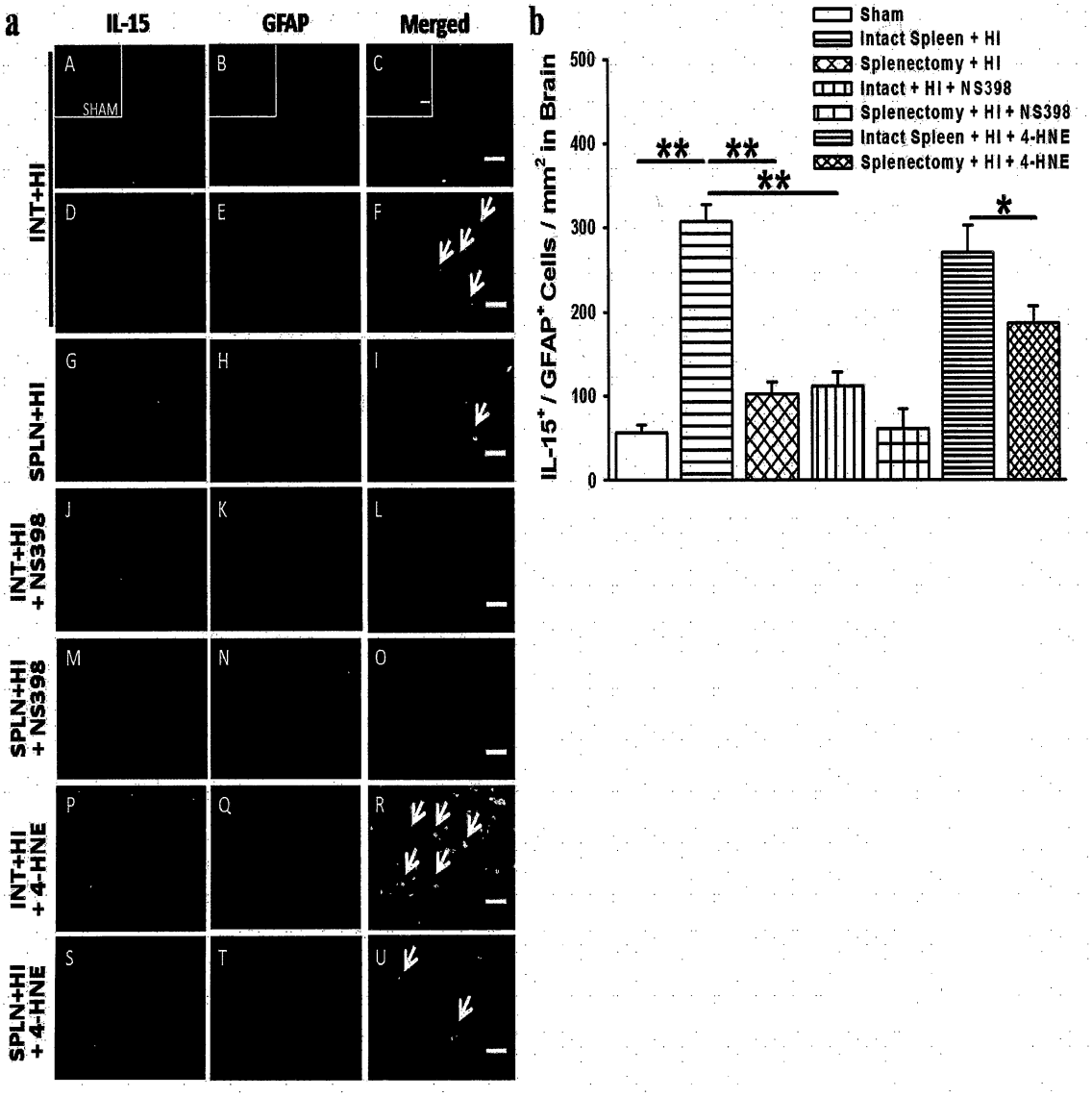
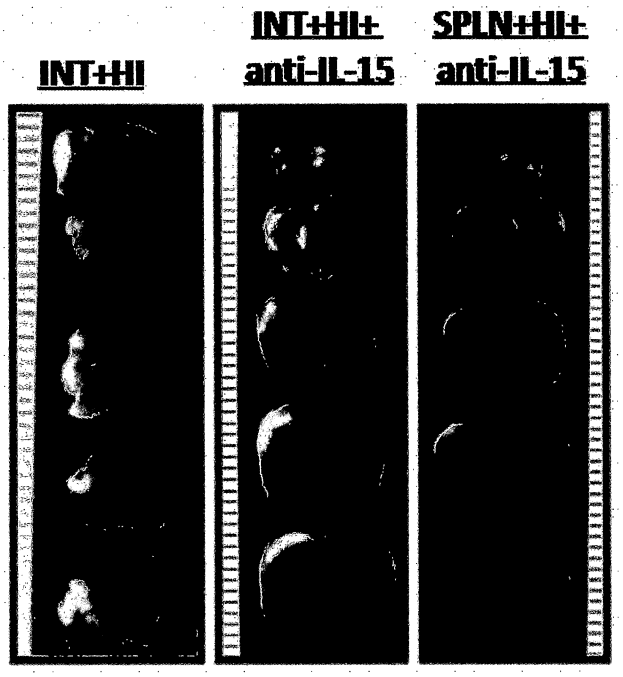
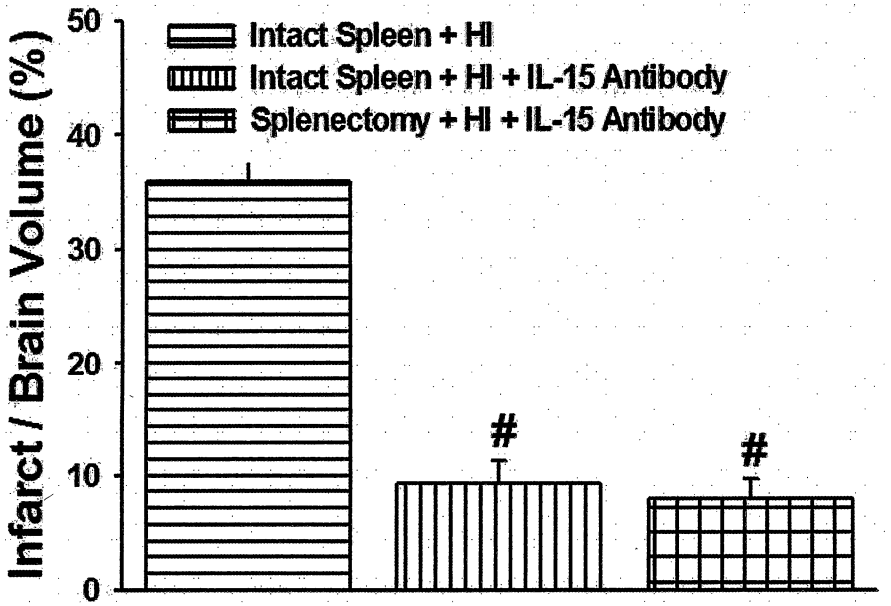

a
IL-15
GFAP
Merged
A
SHAM
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
INT+HI
SPLN+HI
INT+HI + NS398
SPLN+HI + NS398
INT+HI + 4-HNE
SPLN+HI + 4-HNE
b
Sham
Intact Spleen + HI
Splenectomy + HI
Intact + HI + NS398
Splenectomy + HI + NS398
Intact Spleen + HI + 4-HNE
Splenectomy + HI + 4-HNE
IL-15+ / GFAP+ Cells / mm2 in Brain
500
400
300
200
100
0
**
**
**
*

Figure 27. Quantification of Interluekin-15 (IL-15) Immunoreactive Astrocytes. (**a**) Immunofluorescent evaluation of IL-15 expression by astrocytes (GFAP). Comparisons made after administration of COX-2 inhibitor (NS398) or agonist (4-HNE). Scale bar, 300 μm (panel C) or 30 μm (subset panel C and others). (**b**) Absolute number of astrocytes co-expressing IL-15 per square millimeter (n = 6/group). $^{*}P < 0.05$, $^{**}P < 0.001$. Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction.


INT+HI
INT+HI+ anti-IL-15
SPLN+HI+ anti-IL-15


Intact Spleen + HI
Intact Spleen + HI + IL-15 Antibody
Splenectomy + HI + IL-15 Antibody
Infarct / Brain Volume (%)
50
40
30
20
10
0
#
#

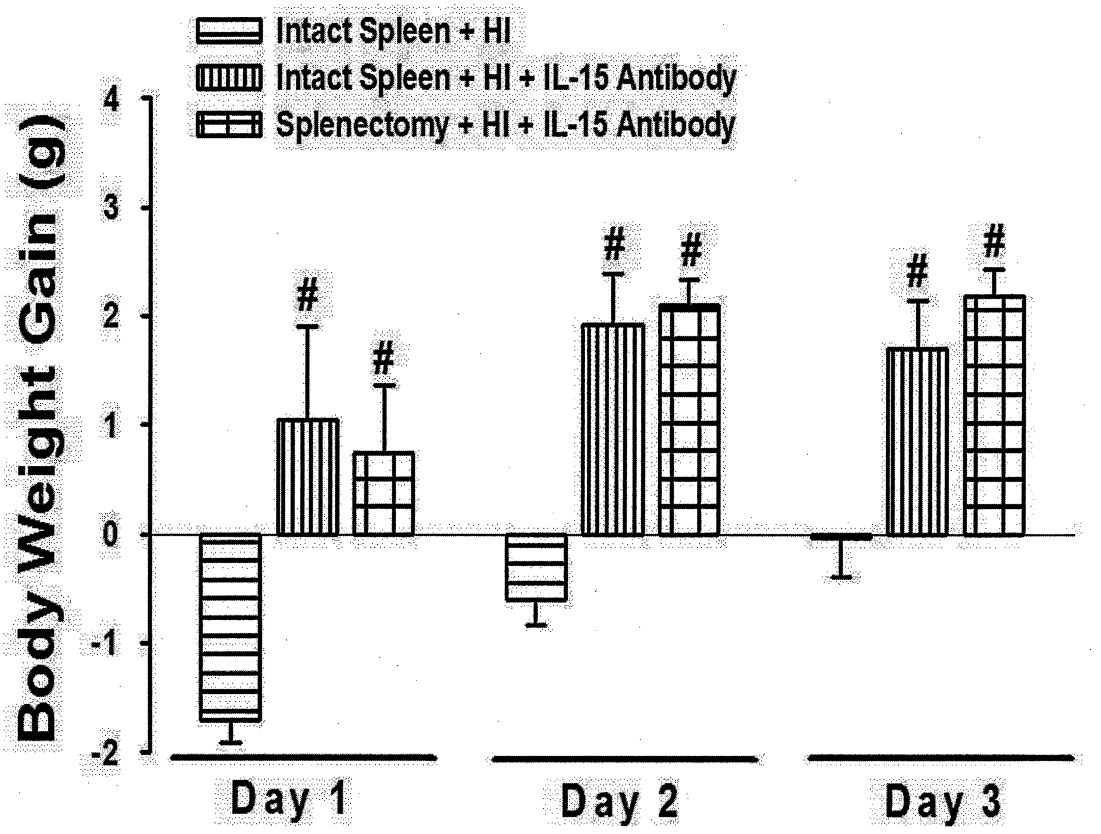

Intact Spleen + HI
Intact Spleen + HI + IL-15 Antibody
Splenectomy + HI + IL-15 Antibody
Body Weight Gain (g)
4
3
2
1
0
-1
-2
#
Day 1
Day 2
Day 3

Figure 29. Daily Weight Gain of Pups Administered Interleukin-15 (IL-15) Neutralizing Antibody Prior to HI. Daily body weight gain, during first 3 days post-HI, of rats administered IL-15 neutralizing antibody. Data represent mean ± SEM; One-way ANOVA with Holm-Sidak correction. $^{\#}P < 0.001$ versus intact spleen + HI.

COX-2 May Mediate the Local Inflammatory Response

Microglia, macrophage, and neutrophil numbers in the ischemic hemisphere are greatest 3 d post-stroke (2). To determine the role of peripheral immune cells in propagating inflammatory cell infiltration and microglia activation at the site of injury; single-immunofluorescent staining in the ipsilateral cerebral cortex were done for markers of microglia, macrophages, and neutrophils. We found HI-induced rats with an intact spleen showed elevated expression of these inflammatory cells, while prior splenectomy suppressed this outcome (Figure 30). NS398 to HI-injured rats with an intact spleen also reduced inflammatory cell abundance. On the other hand, 4-HNE significantly increased neuroinflammation although in splenectomized rats there were still less activated microglia and macrophages.

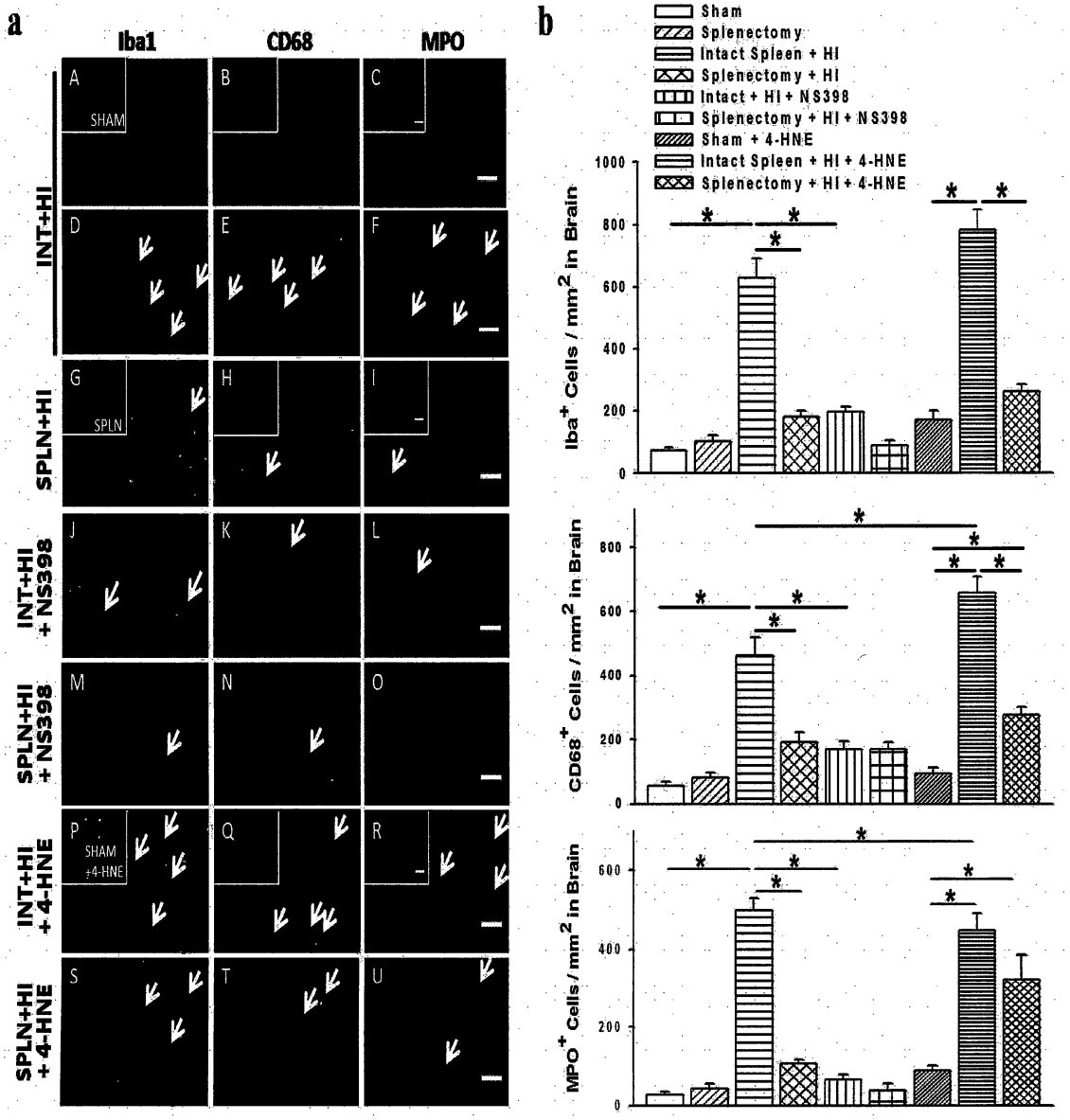

a
Iba1
CD68
MPO
INT+HI
SPLN+HI
INT+HI + NS398
SPLN+HI + NS398
INT+HI + 4-HNE
SPLN+HI + 4-HNE
SHAM
SPLN
SHAM +4-HNE
b
Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Intact + HI + NS398
Splenectomy + HI + NS398
Sham + 4-HNE
Intact Spleen + HI + 4-HNE
Splenectomy + HI + 4-HNE
Iba+ Cells / mm2 in Brain
CD68+ Cells / mm2 in Brain
MPO+ Cells / mm2 in Brain

Figure 30. COX-2 Mediated Inflammatory Cell Abundance in the Cerebral Cortex. (**a**) Immunofluorescent evaluation of microglia (Iba1) activation, and expression of macrophages (CD68) and neutrophils (MPO) in cerebral cortex of rats. Comparisons made after administration of COX-2 inhibitor (NS398) or agonist (4-HNE). Scale bar, 300 μm (panel C) or 30 μm (subset panel C and others). (**b**) Absolute number of microglia, macrophage, and neutrophil abundance per square millimeter (n = 6/group). Data represent mean ± SEM; One-way ANOVA with Holm-Sidak correction. $^{*}P < 0.001$.

COX-2 May Promote Cell Death Pathway

To determine whether infiltrating immune cells in the injured brain exert detrimental effects by down-regulating specific pro-survival cellular signals, the expression of PI3K was measured and found to be significantly reduced in HI-injured animals with an intact spleen compared to those with prior splenectomy (Figure 31). NK cells may be responsible for these differences as PI3K levels were equal between groups after NK cell siRNA administration. Next, we determined whether IL-15 regulates PI3K expression and found that IL-15 inhibition normalized PI3K levels for HI-injured rats with an intact spleen. To then investigate the molecular mechanism by which decreased PI3K may have an apoptotic effect, we quantified pro- and cleaved-caspase 3 levels. We found that HI-injured rats with splenectomy had higher levels of pro-caspase-3 and lower levels of the activated form of the protein compared to those with intact spleen (Figure 32). Moreover, we found that NS398 to HI rats with an intact spleen decreased the expression of activated caspase-3 while the reverse was shown with 4-HNE.

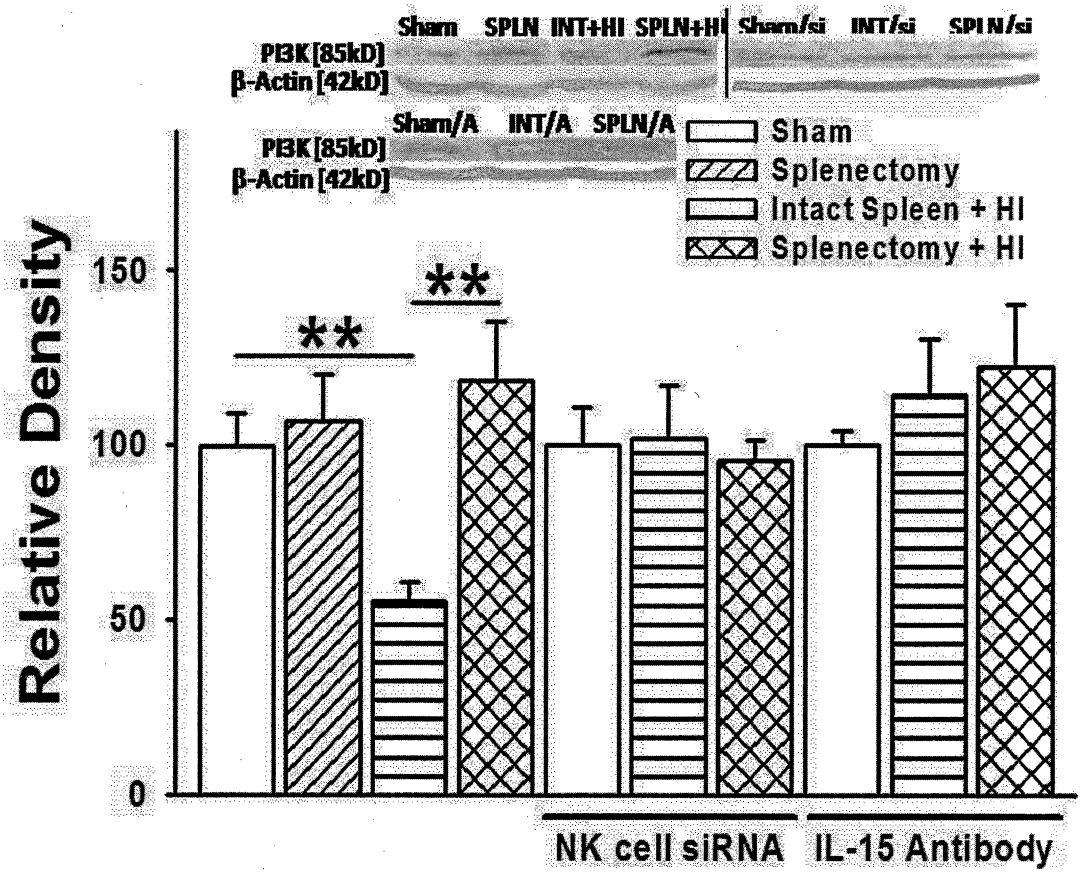

Sham
SPLN
INT+HI
SPLN+HI
Sham/si
INT/si
SPLN/si
PI3K [85kD]
β-Actin [42kD]
Sham/A
INT/A
SPLN/A
PI3K [85kD]
β-Actin [42kD]
Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Relative Density
150
100
50
0
**
**
NK cell siRNA
IL-15 Antibody

Figure 31. Western Blot Analysis of PI3K Protein. Phosphoinositide-3-kinase (PI3K) protein levels relative to beta actin in the ipsilateral hemisphere of rats. Comparisons made after administration of NK cell siRNA (si) or IL-15 neutralizing antibody (A) (n = 5/group; interventions: n = 4/group). Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction. $^{**}P < 0.01$

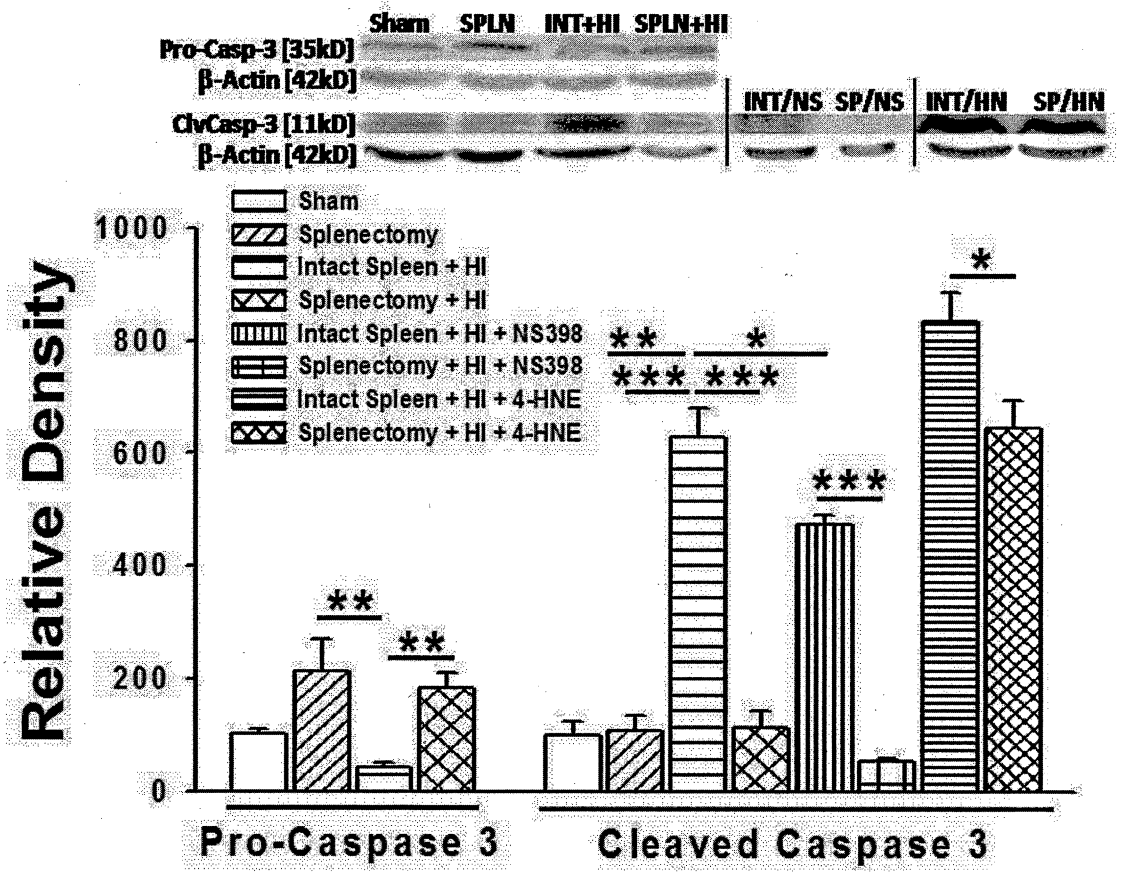
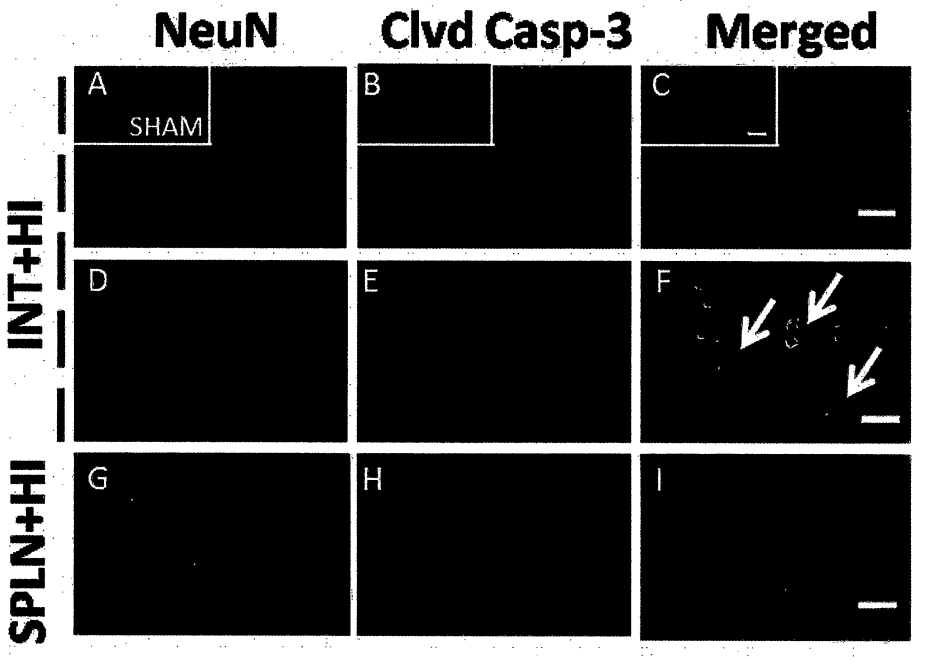

Sham
SPLN
INT+HI
SPLN+HI
Pro-Casp-3 [35kD]
β-Actin [42kD]
INT/NS
SP/NS
INT/HN
SP/HN
ClvCasp-3 [11kD]
β-Actin [42kD]
Sham
Splenectomy
Intact Spleen + HI
Splenectomy + HI
Intact Spleen + HI + NS398
Splenectomy + HI + NS398
Intact Spleen + HI + 4-HNE
Splenectomy + HI + 4-HNE
Relative Density
1000
800
600
400
200
0
**
**
**
***
***
***
*
*
Pro-Caspase 3
Cleaved Caspase 3

Figure 32. Western Blot Analysis of Pro- and Cleaved-Caspase 3. Pro-caspase 3 and cleaved caspase 3 protein levels relative to beta actin in the ipsilateral hemisphere of rats. Comparisons made after administration of COX-2 inhibitor (NS398; NS) or agonist (4-HNE; HN) (n = 5/group; interventions: n = 4/group). Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Splenectomy Attenuates Caspase-Mediated Neuronal Death

An immunofluorescent study was done to examine whether neuronal cells were involved in the caspase-mediated pathways induced by infiltrating immune cells. We found elevated cleaved caspase-3-expressing neurons in HI rats with an intact spleen as compared to splenectomized rats (Figure 33). TUNEL analysis was done to determine whether the effects on cell death were limited to neurons (Figure 34). Results showed that HI rats with an intact spleen abundantly expressed TUNEL-positive cells localized in the nucleus of neuronal cells and not astrocytes, thereby further supporting our proposed mechanism for astrocyte-neuron dynamics in response to immune cells after HI (Figure 35).


NeuN
Clvd Casp-3
Merged
A
SHAM
B
C
D
E
F
G
H
I
INT+HI
SPLN+HI

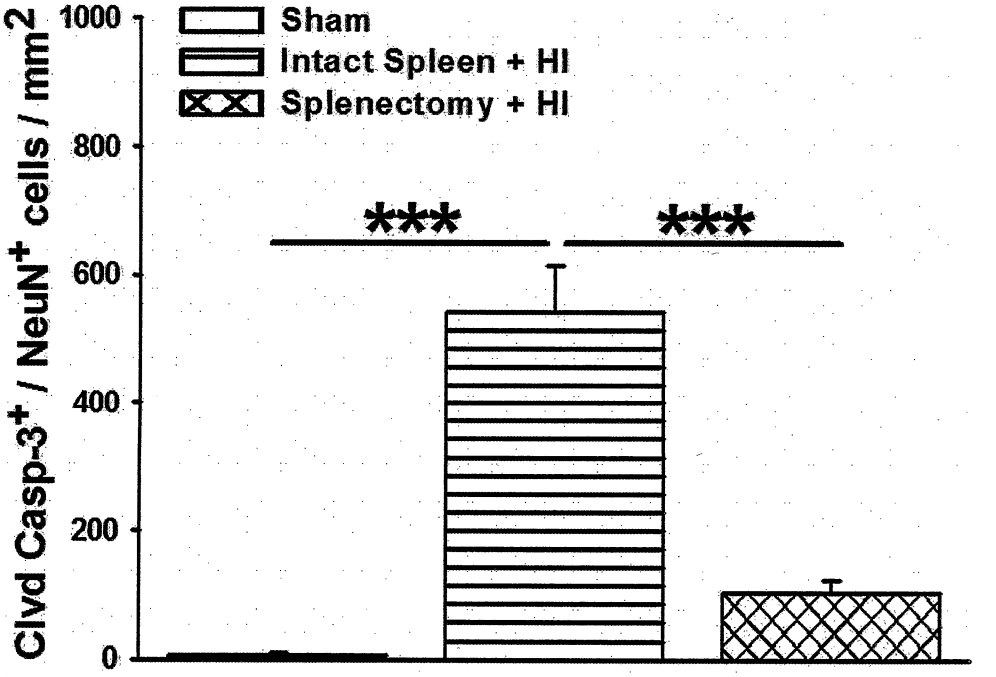

Sham
Intact Spleen + HI
Splenectomy + HI
Clvd Casp-3+ / NeuN+ cells / mm2
1000
800
600
400
200
0
***
***

Figure 33. Quantification of Cleaved-Caspase 3 Immunoreactive Neuronal Cells. Immunofluorescence stain for cleaved caspase-3 (clvd casp-3) in neurons (NeuN) of the ipsilateral cerebral cortex. Scale bar, 300 μm (panel C) or 30 μm (subset panel C and others). Absolute number of neurons co-expressing cleaved caspase-3 per square millimeter (n = 6/group). Data represent mean ± SEM; one-way ANOVA with Holm-Sidak correction. $^{***}P < 0.001$.

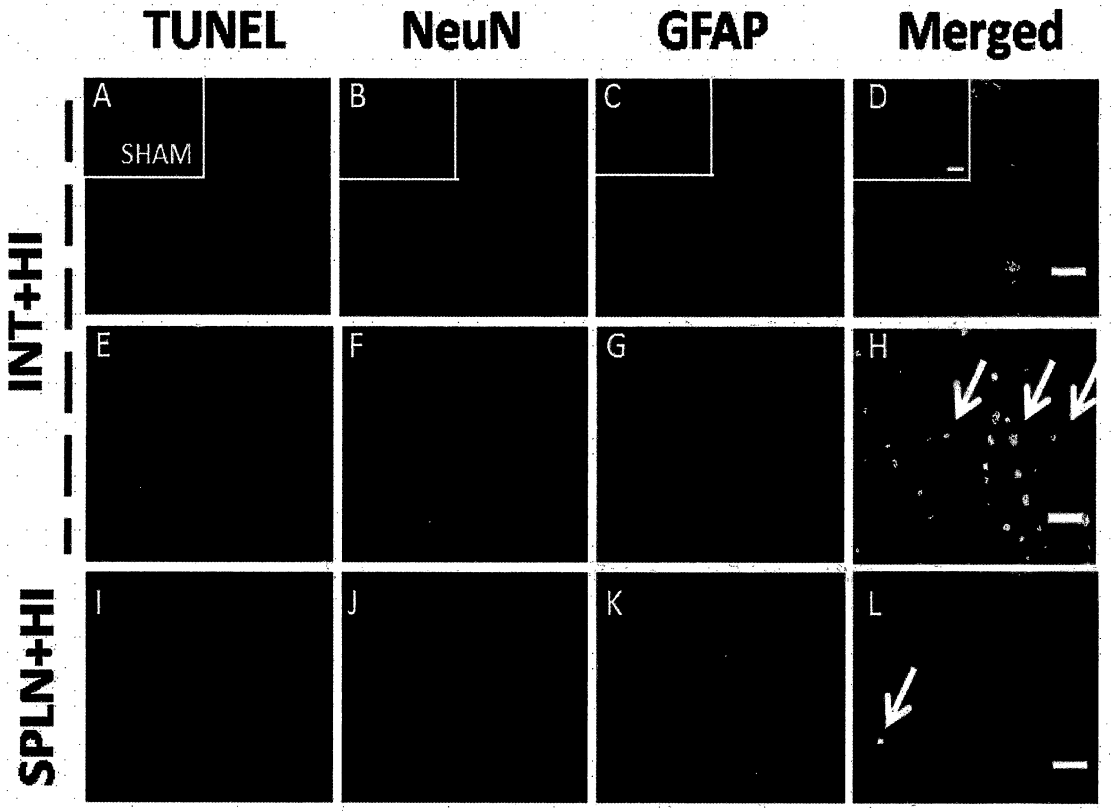

TUNEL
NeuN
GFAP
Merged
A
SHAM
B
C
D
INT+HI
E
F
G
H
SPLN+HI
I
J
K
L

Figure 34. TUNEL-Positive Cells in the Cerebral Cortex After HI Insult. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) with markers for neurons (NeuN) and astrocytes (GFAP) (n = 6/group). Scale bar, 300 μm (panel D) or 30 μm (subset panel D and others).

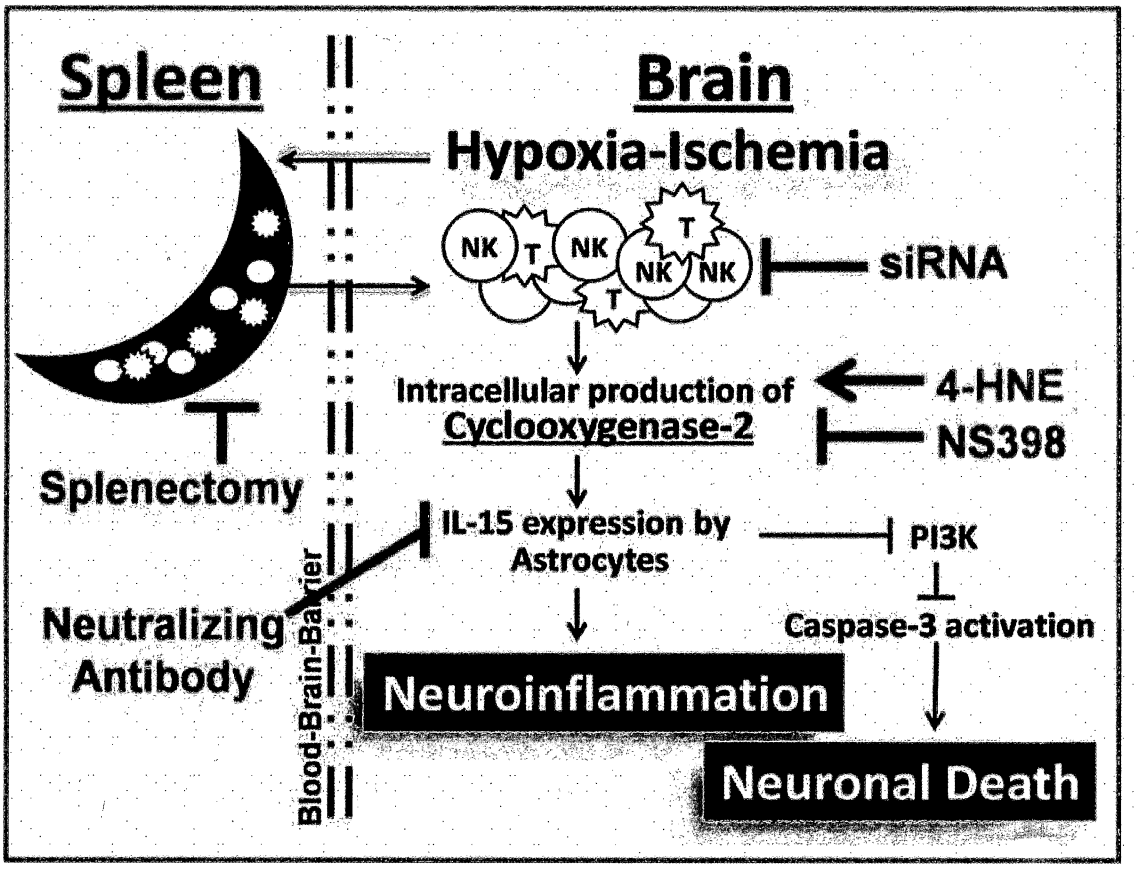

Spleen
Brain
Hypoxia-Ischemia
NK
T
NK
T
NK
NK
T
siRNA
Intracellular production of
Cyclooxygenase-2
4-HNE
NS398
Splenectomy
IL-15 expression by
Astrocytes
PI3K
Neutralizing
Antibody
Blood-Brain-Barrier
Caspase-3 activation
Neuroinflammation
Neuronal Death

Figure 35. Proposed Mechanism with Sites of Intervention.

Discussion

In this study we characterize the mechanistic interaction between the components of the glia-neuron unit in response to immigrating peripheral immune cells after a hypoxic-ischemic insult in the neonatal rat. Our major new finding is that spleen-derived immune cells serve as principal mediators of infarct growth, long-term brain atrophy, somatic restriction, and functional deterioration following neonatal HI; and propose an underlying mechanism by which infiltrating immune cells may cause these neuropathological outcomes.

We evidenced marked reductions in infarct volume in HI-injured animals with prior splenectomy as compared to animals with an intact spleen. Since the operative variable was splenectomy, our data in a compelling way links HI inflammatory responses to splenic immune cell population. Previous reports indicated that inflammatory responses are a core component in the sequelae of neonatal HI, comprising a significant portion of secondary brain injury (21). In this study we demonstrate that the magnitude of inflammatory response in the ischemic immature brain may be proportional to the extent of peripheral immune cell invasion and suggest that these cells employ COX-2 to modulate astrocyte-neuron signaling and the extent of injury.

More specifically, we have found that the influx of COX-2 expressing immune cells enhances astrocytic IL-15 production and caspase-3-mediated neuronal death, which in sum may propagate brain damage. While various brain cells can produce COX-2, splenic cells appear to be a crucial source as splenectomy dramatically reduced COX-2 expression in the ischemic brain. Of the many spleen-derived immune cells that may be involved in COX-2 production, we focused on NK cells which are a component of the

innate immune system, and T cells which are typically part of the adaptive immune system. Indeed our findings suggest a substantial involvement of NK cells in the production of COX-2 after HI; however to verify this, a gene silencer against NK cells was administered. Results demonstrated that knocking down CD161 of NK cells restored baseline levels of COX-2 in brain injured animals with an intact spleen, further suggesting NK cells may be a key spleen-derived cell type involved in COX-2 production and the progression of brain damage.

Inflammation in the central nervous system, a major component of brain damage, is maintained by cytokine cascades triggered by reactive astrocytes and microglia (22,23). Recent evidence implicates IL-15 as a significant activator of resting microglia after LPS stimulation, with astrocytes being a major source of IL-15 (9). In line with these findings, we observed an increase in IL-15 expression by astrocytes, as well as increases in neutrophil and macrophage abundance and microglia activation in brain injured animals. On the other hand, splenic removal prior to HI-insult markedly attenuated these associated changes. This led us to believe that down-stream COX-2 effectors from splenic immune cells may be potent stimulators of IL-15 production by binding to E-prostanoid receptors, which are present on astrocytes (24). In fact, pharmacological blockade of COX-2 afforded the same reductions in IL-15 mediated inflammatory propagation, as did splenectomy in the HI-injured rats. These COX-2 regulated signaling patterns were further supported when opposite effects (enhanced IL-15 and inflammatory propagation) were seen in HI-injured rats with administration of 4-HNE, a COX-2 agonist. It is important to note that 4-HNE can induce COX-2 mRNA and protein expression in many different cell types (25,26). This may explain the 4-HNE

induced elevation in COX-2 expression, increases in astrocytic IL-15 expression and inflammatory cell infiltration in the ischemic hemisphere, irrespective of splenectomy.

Although, other lymphocyte-exclusive by-products, such as interleukin-2 (IL-2), have also been shown to stimulate IL-15 production (27), experimental studies using IL-2-deficient mice have reported no changes in immune function (28). Previous studies showed that IL-15 plays a major role in inflammatory infiltration and activation of NK cells (29,30). Concordantly, our study shows that COX-2 blockade, which results in reduced IL-15 brain expression, also decreases infiltration of inflammatory cells. Splenectomy, by eliminating a large source of COX-2 formed by invading NK immune cells, may reduce IL-15 expression and the subsequent inflammatory response thereby reducing brain injury and improving functional performance.

Modulation of COX-2 is important not only for regulation of neuroinflammation, but chronically elevated levels of IL-15 may also lead to neuronal death (9). Consequently, we found that inhibition of IL-15 in the ischemic brain lead to increased PI3K levels. Moreover, co-culture studies incubated with COX inhibitors suppress glial-mediated paracrine damage to neurons (24). In line with these studies, we found that a reduction in COX-2-expressing NK cells correlated to increased PI3K levels and decreased cleaved caspase-3 expression in neurons. Furthermore, the HI-induced elevations in cell death appear to be neuron-limited and not include astrocytes. This supports our notion that astrocytes are actively participating in production of down-stream effectors that induce neurodegeneration. Future studies are needed to determine the exact mechanism of COX-2-mediated increases in cell death proteins after HI, including IL-15 binding to the IL-15/PI3K neuronal complex (27).

In the present study we confirm that the secondary brain injury in the HI-induced neonate includes apoptosis (31) and activation of local inflammation (32) both of which may be triggered by spleen-derived NK cells. Depletion of splenic immune cells through splenectomy led to a lasting and robust improvement across most neurobehavioral tasks. However, it cannot be ruled out that the splenectomy-induced neuroprotection afforded 3 w post-HI may, in fact, not be a direct effect of there being less COX-2 producing immune cells to infiltrate the ischemic brain in the acute stage of HI. At later stages, studies have shown that adaptive immune responses promote production of neurotrophic factors involved in remodeling the post-ischemic brain (33). Therefore, a possible explanation for the long-term benefits in our study could be that immunologic cells involved in enhancing brain plasticity within weeks after HI may have migrated from other lymphoid organs in the absence of the spleen (34). Nevertheless, this amelioration of long-term outcomes provides the basis for the clinical relevance of this study.

In summary, we have demonstrated that the spleen appears to be a major source of peripheral immunologic cells that infiltrate and exacerbate brain damage through their interactions with astrocytes and neurons. Consequently, a depletion of splenic cells offers lasting protection against HI-induced neonatal brain injury. We also identified a previously unrecognized COX-2-dependent link between splenic immune cells and signaling pathways controlling cellular inflammatory targets and neuronal death after neonatal HI. We believe that the findings of this study provide mechanistic basis for potential novel modalities ameliorating long-term outcomes of neonatal hypoxia-ischemia.

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CHAPTER FOUR

LONG-TERM EVALUATION OF GRANULOCYTE-COLONY STIMULATING FACTOR ON HYPOXIC-ISCHEMIC BRAIN DAMAGE IN INFANT RATS

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Abstract

Hypoxia-ischemia (HI), as a major cause of fetal brain damage, has long-lasting neurological implications. Therefore, therapeutic interventions that attenuate the neuropathological outcome of HI while also improving the neurofunctional outcome are of paramount clinical importance. The aim of this study was to investigate the long-term functional and protective actions of granulocyte-colony stimulating factor (G-CSF) treatment in an experimental model of cerebral HI. Postnatal day-7 Sprague-Dawley rats were subjected to HI surgery which entailed ligation of the right common carotid artery followed by 2 hours of hypoxia (8% O_2). Treatment consisted of subcutaneous injection of G-CSF at 1 hour after hypoxia followed by an additional one injection per day for 5 days (6 total injections) or for 10 days (11 total injections). Animals were euthanized 5 weeks post-insult for extensive evaluation of neurological deficits and assessment of brain, spleen, heart, and liver damage. G-CSF treatment promoted somatic growth, and prevented brain atrophy and under-development of the heart. Moreover, reflexes, limb placing, muscle strength, motor coordination, short-term memory, and exploratory behavior were all significantly improved by both G-CSF dosing regimens. Long-term neuroprotection afforded by G-CSF in both morphological and functional parameters after a hypoxic-ischemic event in the neonate provides a rationale for exploring clinical translation.

Introduction

Neonatal hypoxia-ischemia (HI) is a major cause of long-term neurological disturbances, such as behavioral alterations and motor deficits including cerebral palsy, mental retardation, and epilepsy [1, 2]. In spite of advancements in obstetric and neonatal intensive care, HI brain damage with severe neurological sequelae remains an important clinical problem [3]. Therefore, the efficacy of potential neuroprotective treatments on long-term functional brain recovery is of significant translational importance.

Granulocyte-colony stimulating factor (G-CSF), a neurotropic factor involved in proliferation, differentiation, and functional integration of neural cells [4], is a neuroprotective agent in a wide spectrum of experimental models of neurological disease [5-11]. Protection ranges from reductions in infarct size during the acute phase to attenuation of long-term functional neurological deficits [12, 13]. Promotion of neurogenesis and angiogenesis are processes by which G-CSF exerts beneficial effects on adult post-stroke recovery [14-16]; however, due to the vast differences in the pathophysiology of immature and adult brains, data gathered from adult studies do not necessarily infer synonymous outcomes in neonatal medicine. Therefore, the effects of G-CSF treatment on cerebral infarct volume at 2 weeks (w) [17] and cerebral atrophy at 3 w [6] after HI have previously been explored. Although no study as of date has examined whether these G-CSF-induced morphological benefits translate into improvements to sensorimotor deficits and memory 5w after neonatal HI; and whether there is an additive benefit against long-term brain and systemic organ atrophy provided with additional administration of G-CSF.

Accordingly, we hypothesized that G-CSF has lasting neuroprotective actions by improving both morphological and behavioral endpoints after HI-injury in neonatal rats. We used two different dosing regimens to implicate the neurotrophic capabilities of G-CSF to be responsible for the structural preservation and improved functional outcome.

Materials and Methods

Animal Groups and Surgical Procedure

This study was in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Postnatal day-7 Sprague-Dawley rats were randomly assigned to the following groups: sham, HI [Vehicle], HI + G-CSF daily for 5 days [G-CSF(5d)], or HI + G-CSF daily for 10 days [G-CSF(10d)]. HI-groups were anesthetized with 3% isoflurane and had the right common carotid artery permanently ligated followed by 1.5 hours (h) of recovery. Afterwards, pups were placed in a glass jar (submerged in a water bath maintained at 37^0C) perfused with 8% O_2/92% N_2 for 2 h. Sham rats had the common carotid artery exposed, but not ligated. All rats were sacrificed 5 w after HI surgery under general anesthesia [ketamine (80mg/kg)/xylazine (10mg/kg)] by decapitation.

Treatment Method

Some pups were treated subcutaneously with G-CSF (Amgen Inc., Thousand Oaks, CA) at 50μg/kg dosage diluted in saline. Treatment was injected at 1 h after hypoxia followed by an additional one injection per day for 5 days (6 total injections) or

for 10 days (11 total injections). Vehicle pups received subcutaneous injections of saline following the same regimen.

Evaluation of Brain Damage and Systemic Organ Weight

The HI animal model results in brain damage exclusive to the ipsilateral side [18, 19]; commonly assessed by hemispheric brain weight loss which is highly correlated to histological loss of brain tissue [20, 21]. Brain tissue (sham = 5; vehicle = 13; G-CSF (5d) = 12; G-CSF (10d) = 13) was removed and the hemispheres were separated by a midline incision and weighed on a high-precision balance (sensitivity ± 0.001g). Data was expressed as a ratio of ipsilateral (right) to contralateral (left) hemispheric weights. The heart, spleen, and liver were also isolated and weighed. Data for systemic organs was expressed as a ratio of organ weight to body weight.

Assessment of Neurobehavioral Deficits

The behavior of the rats was blindly evaluated using 8 sensorimotor (postural reflex, back pressure, lateral pressure, proprioceptive limb placing, lateral placement, forelimb placement, foot-fault, and rotarod) tests [2, 20]; and a test (T-maze) to ascertain short-term or working memory, as well as complex cortical function [22, 23]. Methodology was as previously described for the first 6 sensorimotor tests [2] and scored accordingly: 0 for immediate and correct placement; 1 for delayed and/or incomplete placement; 2 for no placement. Scores corresponded to raw values: 0 score = 100; 1 score = 50; 2 score = 0. In the foot-fault test, the rat was placed on a horizontal grid floor (36 x 13 in, square size 3 x 3 cm, wire diameter 0.4 cm) for a duration of 2 min. A foot-fault was noted when a paw fell through an opening in the grid floor. In the rotarod test, rats

were placed on a rotating treadmill (diameter 14 cm) initially at rest (stationary) for a maximum of 1 min. In the second round of testing, the treadmill was set in motion at a constant speed of 5 rotations per minute (rpm) for a maximum of 1 min. Finally in the third round of testing, the treadmill was set in motion at an accelerated speed of 5-40 rpm for a maximum of 2 min. Each animal had 2 trials/round of testing. The time spent by the animal on the rotarod during each round was noted. In the T-maze test, rats were placed at the base of the T-maze (stem 40 x 10 cm, arm 46 x 10 cm) and allowed to explore until an arm of the maze was chosen. Each animal was given 10 trials and the sequence of right and left arm choices were expressed as the percent of spontaneous alternation.

Data Analysis

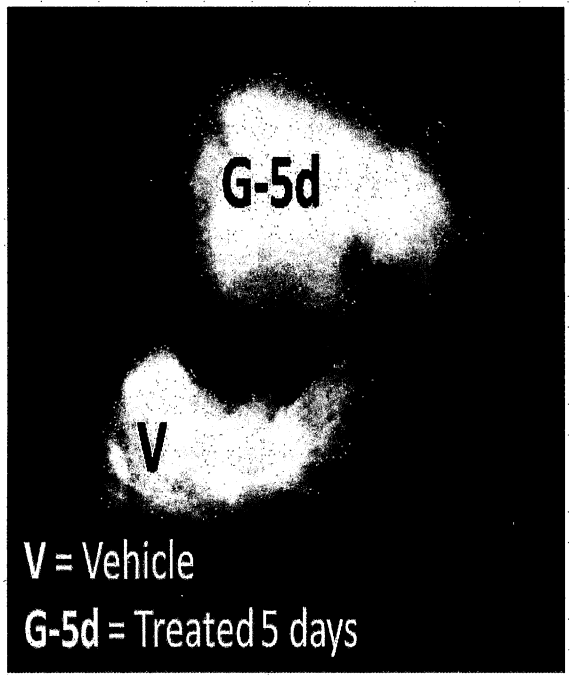
Data was expressed as mean ± SEM. Using a commercially available software (Sigma Stat 3.0.1, Aspire Software, Ashburn, VA), one-way ANOVA and Tukey test were implemented to determine significance in differences between groups. Kruskal-Wallis ANOVA followed by Dunn's test was used for neurobehavioral analysis. Significance was accepted at $p < 0.05$.

Results

G-CSF Promotes Physical Development

HI-induced somatic growth retardation starting from 1 d after insult is a common finding in animal experiments [3, 24]. Representative pictures demonstrate the significant differences in physical development between rats injected with vehicle or G-CSF, at the completion of 5 day dosing regimen or 10 day dosing regimen (Figure 36). Vehicle rats gained significantly less weight than sham after 1 w following HI (7.73 ± 0.42 vs. 13.38

± 0.42; Figure 37); an effect attenuated by both G-CSF treatment regimens (5d: 10.71 ± 0.53; 10d: 10.49 ± 0.62). After 3 w following HI, G-CSF(5d) continued to improve weight gain as compared to vehicle (66.54 ± 1.85 vs. 58.87 ± 1.89). Vehicle pups however did catch up in weight since the amount of weight gained over the entirety of the study was not significantly less than that of sham rats (125.01 ± 3.30 vs. 136.12 ± 7.40). On the other hand, rats treated with 5 days of G-CSF (143.39 ± 3.58) gained significantly more weight over 5 w as compared to vehicle rats, but did not differ from those treated for 10 days with G-CSF (136.09 ± 4.60).


G-5d
V
V = Vehicle
G-5d = Treated 5 days

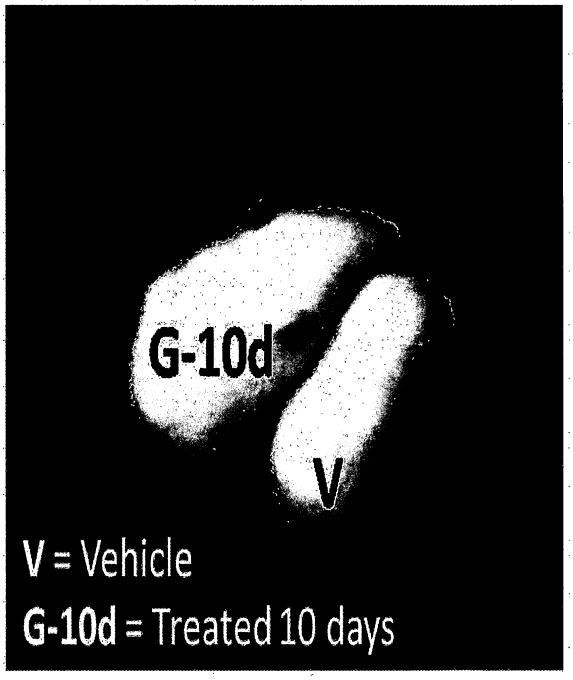

G-10d
V
V = Vehicle
G-10d = Treated 10 days

Figure 36. Photographs of Physical Appearance of Pups at the Completion of Treatment. There are marked differences in physical development at the completion of 5 d of G-CSF treatment (G-5d) or 10 d of G-CSF treatment (G-10d), as compared to vehicle.

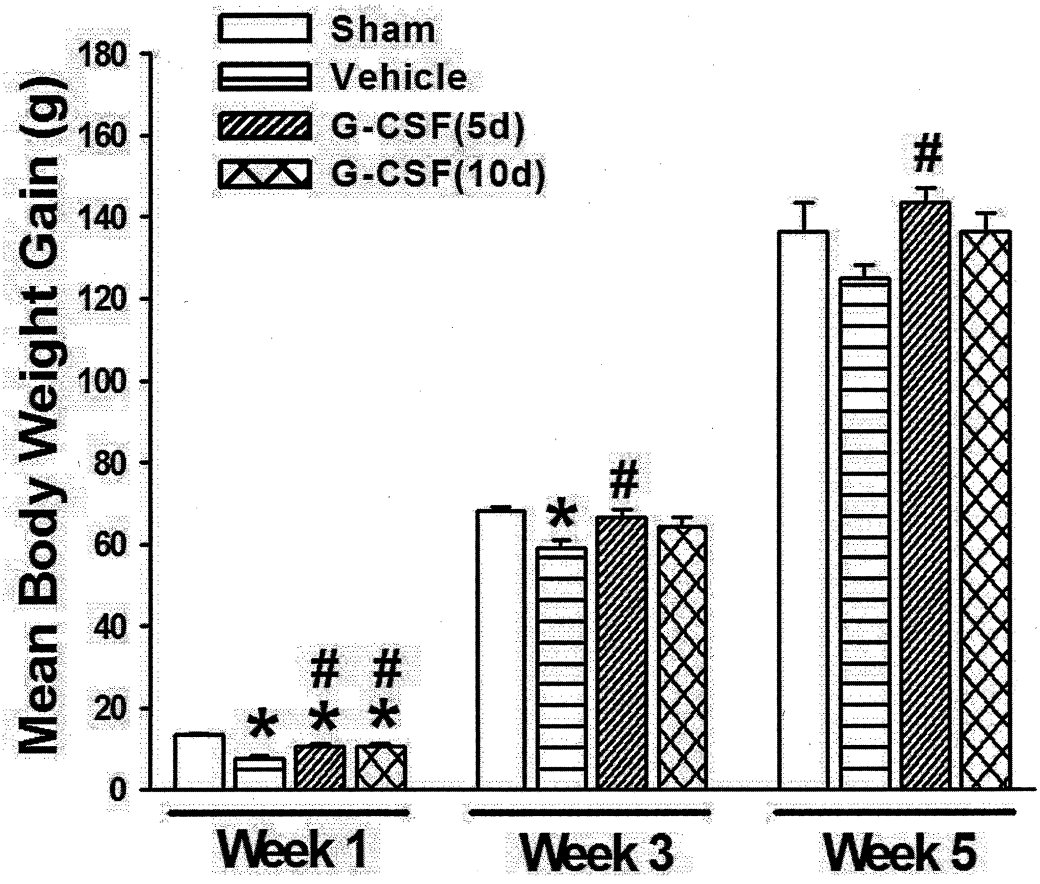
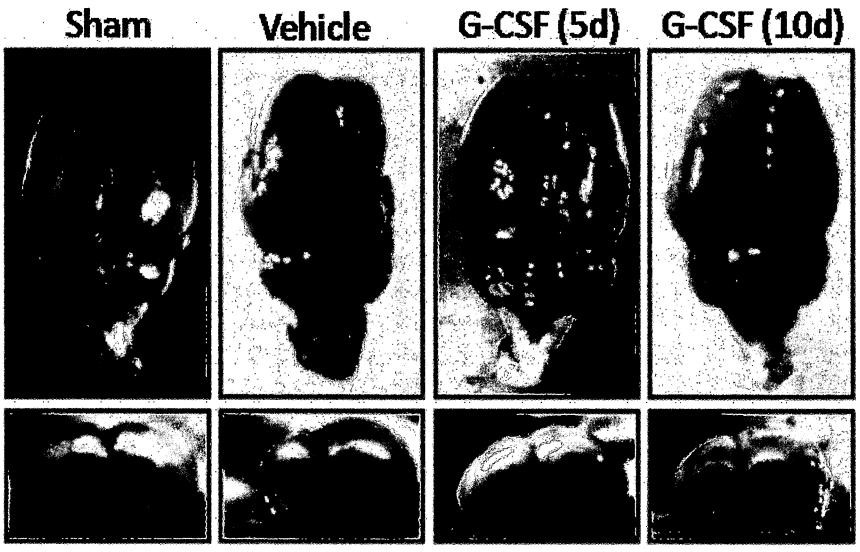

Sham
Vehicle
G-CSF(5d)
G-CSF(10d)
Mean Body Weight Gain (g)
180
160
140
120
100
80
60
40
20
0
*
*
*
#
#
*
#
#
Week 1
Week 3
Week 5

Figure 37. Effect of G-CSF Treatment on Weekly Mean Body Weight Gain. Vehicle rats gained significantly less weight at 1 week and 3 weeks post-HI, but appeared to catch up to weight of sham rats after 5 weeks. Both treatment regimens improved weight gain at the 1 week time point; however the G-CSF(5d) rats gained an average weight significantly greater than vehicle rats at all tested intervals. Data represent mean ± SEM; *$p < 0.05$ versus sham, #$p < 0.05$ versus vehicle.

G-CSF Maintains Brain and Systemic Organ Weight

Neonatal encephalopathy involves multiple organs and not just the brain [25, 26]; therefore, effects of treatment interventions should be explored across multiple organ systems [27]. HI-injury resulted in significant brain atrophy of the lesioned hemisphere (19.60 ± 3.09%); remarkably, treatment with G-CSF (5d: 8.70 ± 2.18%; 10d: 9.70 ± 2.39%) demonstrated less damage to the brain tissue 5 w post-insult (Figure 38). Although vehicle rats appeared to have a smaller heart compared to sham, statistical significance was not reached (Figure 39). When compared to G-CSF(5d)-treated rats, vehicle rats had a 16.11% reduction in heart to body ratio. Although G-CSF(5d) treatment appeared to additionally improve spleen and liver weights, no statistical differences were detected between groups.


Sham
Vehicle
G-CSF (5d)
G-CSF (10d)

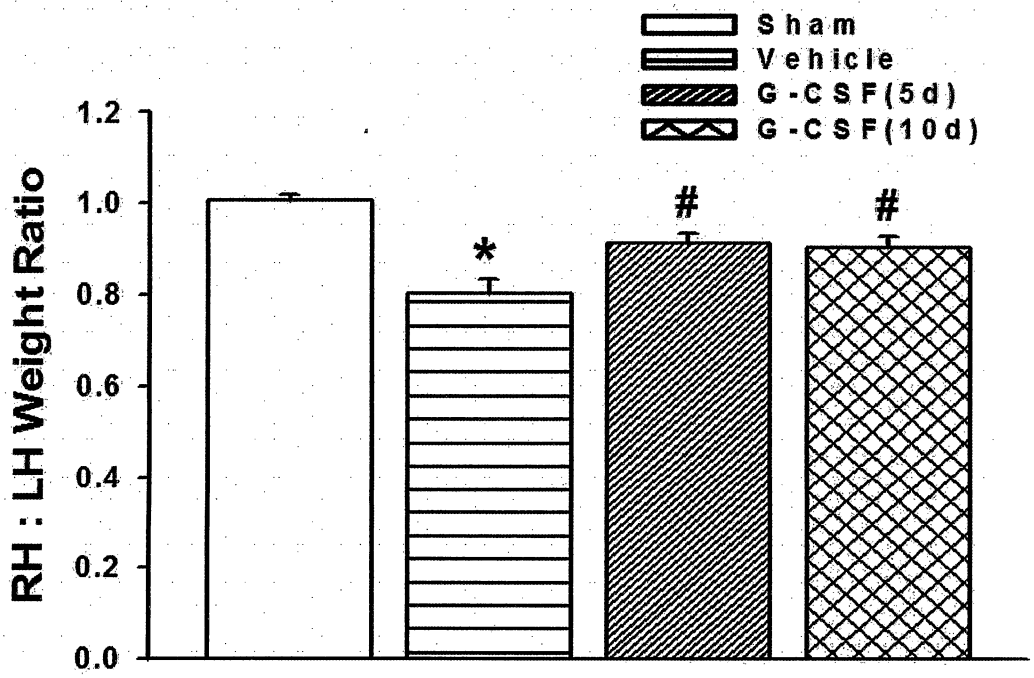

Sham
Vehicle
G-CSF(5d)
G-CSF(10d)
RH : LH Weight Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
#

Figure 38. Brain Atrophy 5-Weeks After HI Insult. Significant loss of right-to-left hemispheric (RH:LH) weight ratio is evident in vehicle rats and improved by G-CSF(5d) and G-CSF(10d). Data represent mean ± SEM; *$p < 0.05$ versus sham, #$p < 0.05$ versus vehicle.

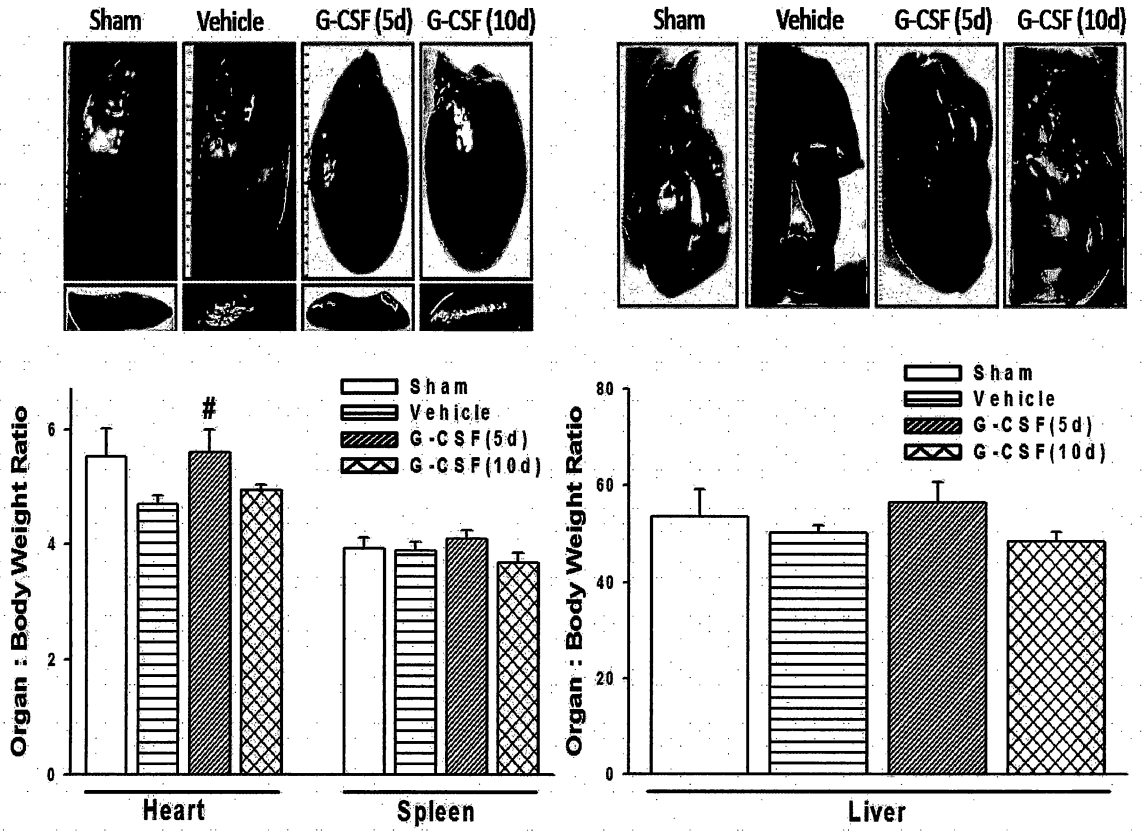

Sham
Vehicle
G-CSF (5d)
G-CSF (10d)
Sham
Vehicle
G-CSF (5d)
G-CSF (10d)
Organ : Body Weight Ratio
#
Sham
Vehicle
G-CSF(5d)
G-CSF(10d)
0
2
4
6
Heart
Spleen
Organ : Body Weight Ratio
Sham
Vehicle
G-CSF(5d)
G-CSF(10d)
0
20
40
60
80
Liver

Figure 39. Heart, Spleen, and Liver Weights 5-Weeks After HI Insult. G-CSF(5d) significantly improved heart-to-body weight ratio as compared to vehicle. Representative pictures of organs. Data represent mean ± SEM; #$p < 0.05$ versus vehicle.

G-CSF Ameliorates HI-Induced Functional Deficits

In all behavior tests, vehicle rats performed significantly worse than sham. This showed consistency in inducing severe damage across all tested brain regions. Numbers in parenthesis represent mean raw score. In the postural reflex test, the contralateral forelimb of the vehicle rats was completely flexed (16.67 ± 6.29); while treatment with G-CSF(5d) and G-CSF(10d) significantly improved this deficit (5d: 72.22 ± 8.78; 10d: 66.67 ± 8.33; Figure 40). In the remaining 5 placement tests, the vehicle rats averaged a raw score ranging from 20.00 ± 6.55 to 43.33 ± 4.54; this meant vehicle rats were consistently unresponsive in these tasks. Although the G-CSF(10d) significantly improved these deficits across tasks, G-CSF(5d) did so in all but the proprioceptive limb placing test. Rats subjected to HI displayed significantly reduced sensorimotor coordination as assessed using the foot-fault test compared to sham (34.20 ± 2.27 vs. 12.4 ± 1.57). Both G-CSF treatment for 5 d (21.11 ± 1.76) and 10 d (17.56 ± 0.99) significantly attenuated the HI-induced deficits. Vehicle rats also displayed a significant reduction in muscle strength and motor coordination compared to sham (32.57 ± 3.45 vs. 50.24 ± 2.54), as assessed by the rotarod test. Treatment with G-CSF (5d: 50.49 ± 2.94; 10d: 58.02 ± 1.04) improved the latency to fall period when the treadmill was stationary. When set at constant velocity, G-CSF (5d: 54.39 ± 2.38; 10d: 59.49 ± 0.51) significantly improved the HI-induced (40.57 ± 4.14) deficits. During acceleration of the treadmill, animals from both G-CSF regimens (5d: 56.81 ± 4.10; 10d: 65.83 ± 4.11) again performed significantly better than vehicle (32.44 ± 2.46). In the T-maze, vehicle rats demonstrated a significant reduction in exploratory behavior and short-term memory as compared to sham (33.33% ± 3.07 vs. 73.33% ± 2.72). These deficits were significantly

improved by both G-CSF regimens (5d: 56.79% ± 4.32; 10d: 61.73% ± 1.95); however, there was a difference in performance between G-CSF(5d) rats and sham.

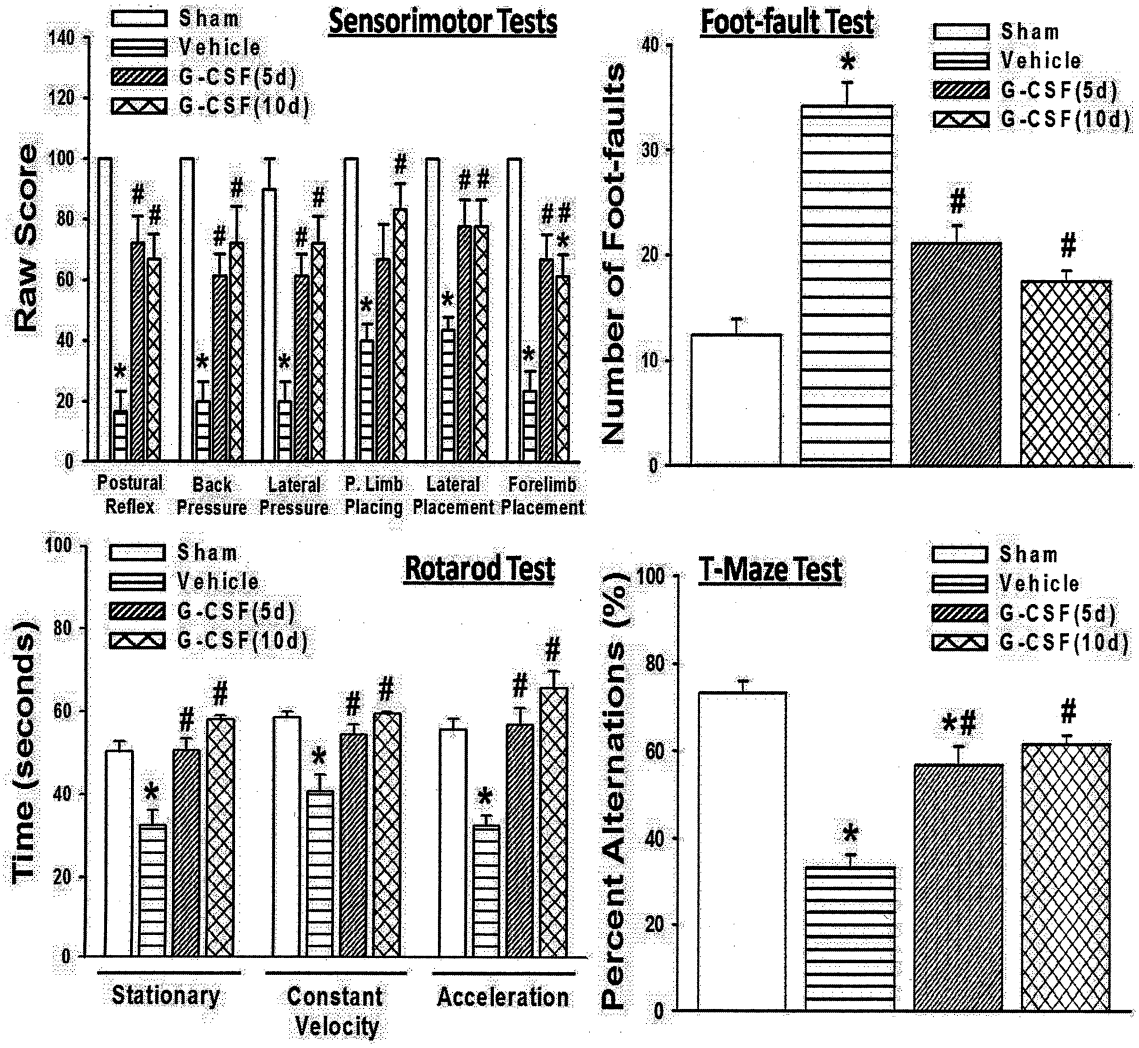

Sensorimotor Tests
Sham
Vehicle
G-CSF(5d)
G-CSF(10d)
Raw Score
Postural Reflex
Back Pressure
Lateral Pressure
P. Limb Placing
Lateral Placement
Forelimb Placement
Foot-fault Test
Number of Foot-faults
Rotarod Test
Time (seconds)
Stationary
Constant Velocity
Acceleration
T-Maze Test
Percent Alternations (%)

Figure 40. Functional Outcome at 5-Weeks After HI Insult. Sensorimotor tests: 100 for immediate and correct placement; 50 for delayed and/or incomplete placement; 0 for no placement. Vehicle rats did significantly worse than sham in all tests: postural reflex, back pressure, lateral pressure, proprioceptive limb placing (P. limb placing), lateral placement, and forelimb placement. G-CSF(5d) improved deficits in all but those associated with the P. limb placing test; while G-CSF(10d) corrected all tested deficits. Vehicle rats had a greater number of foot-faults during the 2 min testing interval; while, both treatment regimens attenuated these deficits. In the Rotarod test, vehicle rats performed significantly worse under all testing conditions: stationary, constant velocity (5 rpm) and accelerating (5-40 rpm). In the T-Maze test, vehicle and G-CSF(5d) rats alternated significantly less between the two arms of the maze as compared to sham; however, G-CSF(5d) and G-CSF (10d) performed significantly better at this task than vehicle. Data represent mean ± SEM; *$p < .05$ versus sham, #$p < .05$ versus vehicle.

Discussion

This study demonstrates the long-term efficacy of G-CSF administration on behavioral and neuropathological recovery in an established rat model of neonatal HI injury. To our knowledge, our data is the first to demonstrate the effect of multiple treatments of G-CSF on HI-induced sensorimotor and memory impairment. The importance of the present findings may be highlighted by: 1) the elucidation of a beneficial treatment regimen with long-term, brain and system organ protection; 2) the significant improvement in neurological function across a battery of tests, which renders these results important for the treatment of neonatal encephalopathy in the clinical setting.

Experimental stroke studies have found treatment with G-CSF to be well tolerated without major side effects [28]; of which, may include, mild to moderate bone and/or musculoskeletal pain, anemia, thrombocytopenia, and injection site reactions [29]. Nevertheless, the safety profile of G-CSF appears to be fairly innocuous, even after years of administration to patients with severe neutropenia [29]. In experimental neonatal HI, a single dose of G-CSF immediately after hypoxia is neuroprotective 2 w following insult [17]; while multiple doses confer morphological benefits up to 3 w [6]. Based on these findings, evidence that neuronal damage likely develops over a period of time [30], and that neuroreparative processes may need stimulation after 24 h when the inflammatory responses have declined [31], we chose to administer multiple doses of G-CSF over 5 days or 10 days following HI. The efficacy of the treatment regimen was determined by its ability to improve physical development, protect brain tissue, and improve long-term functional outcome.

Fetal growth retardation is a HI-related outcome, and can be used as an indicator of general well-being [17]. From the body weight data, it is evident that G-CSF treatment significantly improves physical development during the critical period following brain injury. Whilst both treatment protocols initially promoted weight gain after HI, the rats treated with G-CSF(5d) gained significantly more weight at the end of 5 w compared to their vehicle-administered counterpart. Moreover, previous studies have shown that low body weight is accompanied by a decreased heart weight [32] – an effect we found to be counteracted by G-CSF(5d). An explanation has been that slow growing pups have a significantly lower number of cardiomyocytes, as well as qualitative changes of the subcellular structures [32]. Other systemic organs affected by fetal growth retardation are the spleen and liver; possibly due to oxygen deprivation and inadequate macro- and micronutrients during fetal life resulting from the preferential blood flow to vital organs such as the brain and heart [33]. However, our results show that these acute affects do not have long-term implications, and that the neonatal rat compensates as there were no differences found between groups when comparing spleen and liver weights.

Although no significant differences were found between groups, the G-CSF(5d) treated animals appeared to have a larger body, heart, spleen, and liver weights compared to those in the sham group. This finding may spark concern about unintentional organ hypertrophy upon prolonged treatment with growth factors. In fact, spleen enlargements have been reported during repetitive G-CSF administration – a likely result of G-CSF-induced extramedullary hematopoiesis [34]. However, the observed trend toward organ hypertrophy in the 5 day treated group may be entirely incidental since more prolonged treatment (i.e, 10 day) did not demonstrate the same trend. Aside from promoting weight

gain, G-CSF treatment also protected brain integrity in the neonate. Both G-CSF(5d) and G-CSF(10d) prevented the long-term loss of brain tissue. The anti-apoptotic [6], anti-inflammatory [35], and excitoprotective [36] properties of G-CSF may be responsible for the long-term attenuation in brain damage; however the exact mechanism is yet unclear.

Preservation of structural integrity by G-CSF treatment resulted in improved motor performance. Brain regions, such as the sensorimotor cortex and hippocampus, are critical for the maintenance of sensorimotor function and are adversely affected by HI insult [37]. Accordingly, damage to these vulnerable brain regions severely affected functional performance of vehicle rats. Conversely, G-CSF-induced morphological protection manifested into muscle strength, motor coordination, reflexes, limb placing, short-term memory, and exploratory behavior similar to that of control animals. The recovery processes that are activated and/or amplified by G-CSF, and thereby exert beneficial effects on post-HI recovery are yet to be elucidated.

G-CSF is an attractive candidate as a therapeutic modality for the human neonate, and may be expanded as treatment to other clinical fields that share common pathophysiological features, such as neonatal stroke, global cerebral ischemia, and neonatal hemorrhagic brain injury. In the clinical setting, G-CSF has been administered to neutropenic neonates with sepsis once daily for 3-5 days. Based on this, and the positive results we obtained from 5 day treatment with G-CSF, the most relevant time period of treatment in the human might be a 5 day dosing regimen. Our experimental study (i.e, use of a clinically-relevant animal model, multiple treatment regimens, and neurobehavioral assessment) provides a foundation for exploring clinical translation. However, we anticipate the need for a dose response study, as well as a more

comprehensive evaluation of safety [38] before moving G-CSF to the clinical setting as treatment against neonatal hypoxic-ischemic brain damage.

Overall, the findings from the present study provide new insight to the therapeutic repertoire of G-CSF. Specifically, that treatment with G-CSF attenuates long-term brain damage and spares the functional integrity responsible for behavior after neonatal hypoxia-ischemia.

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CHAPTER FIVE

FINAL REMARKS

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Discussion

The data presented here suggests targeting of key components of the neuroinflammatory response confers neuroprotection after a hypoxic-ischemic insult. Specifically, we found: 1) Inhibition of COX-2 attenuates the HI-induced consequences by reducing brain damage, and improving long-term functional deficits, and somatic and systemic organ growth; 2) Splenic immune cells to be the major source of COX-2 in the ischemic brain; and that COX-2 modulates downstream targets of neuroinflammation and cell death in astrocytes and neurons, respectively; 3) Short-term administration of the neurotrophic factor, G-CSF, improves long-term behavioral and neuropathological recovery after a hypoxic-ischemic insult.

Neuroprotective Effects of COX-2 Inhibition

To replicate the clinical features seen in human infants after asphyxia, we used a well-established rodent model that combines unilateral carotid artery ligation with exposure to 2 hours of hypoxia (1). This model has been very informative for understanding the underlying mechanisms of brain injury from peri-natal hypoxia, and for the testing of potential neuroprotective agents (2). A hypoxic-ischemic insult is associated with increased mortality and somatic growth retardation in both patients and experimental studies (3,4,5). In accordance to these findings, we found a higher incidence of mortality and lower body weight in HI injured rats. In contrast, 100% of animals that were treated with a COX-2 inhibitor survived the injury and were able to maintain their body weights to that of control animals. Aside from these HI-induced consequences, interruption of placental blood flow can affect cerebral and systemic function (6,7); therefore, potential treatment modalities should address not only cerebral protection but

rather involve multiple organ systems. As such, we found that animals that had experienced a HI insult had significant brain and systemic organ tissue loss compared to that of control. This effect was counteracted when animals were treated with a COX-inhibitor. Moreover, attenuation of brain atrophy by blockade of COX-2 appeared to also result in improved motor, cognitive, and behavioral function 6 weeks after HI injury. This long-term assessment of treated animals was a particularly important finding considering past therapeutic modalities in neonatology have resulted in unforeseen side-effects (8,9). These results suggest that inhibition of COX-2 may provide major benefits for brain and systemic organ integrity, neurobehavioral deficits, and survival after neonatal HI. Collectively, these data provide a foundation for an investigation of COX-2-dependent mechanisms in neonatal hypoxic-ischemic brain injury.

COX-2-Dependent Mechanisms in Brain-Immune Interactions

Having established that COX-2 is a mediator of brain damage after a hypoxic-ischemic event, we sought to determine whether it plays a role in brain-immune cell interactions. First, to elucidate the possible impact of the systemic inflammatory response on astroglia-neuron signaling, we removed the spleen, the largest pool of peripheral immune cells prior to HI and studied the short- and long-term outcomes in the neonate.

Next, we sought to determine whether COX-2 mediates the mechanism by which activated immune cells induce pro-inflammatory cytokine production by astrocytes (16). This is especially important since recent data suggests that enhanced interleukin-15 (IL-15) expression in astrocytes is a major propagator of inflammatory responses after central nervous system injury (17). In addition, the degree of involvement from the innate immune system has been shown to correlate with the extent of neuronal damage in the

post-ischemic tissue (18). Moreover, studies suggest dysfunction of the phosphoinositide-3-kinase (PI3K)/Akt survival pathway to be responsible for triggering apoptotic cascades in the brain (19). However, it was still unknown whether down-regulation of the PI3K pathway and subsequent neuronal death in HI-injured rats occurs in response to COX-2 from infiltrating immune cells. Therefore, we used a gain and loss of function approach (pharmacological activation or inhibition, respectively) for COX-2, a neutralizing antibody for IL-15, and a gene silencer for natural killer (NK) cells in both splenectomized and non-splenectomized rats to verify the role of COX-2 in splenic immune cell responses following HI.

Our major new finding was that spleen-derived immune cells serve as a major source of COX-2 in the ischemic brain, and are principal mediators of infarct growth, long-term brain atrophy, somatic restriction, and functional deterioration following neonatal HI. More specifically, we found that the influx of COX-2 expressing immune cells enhances astrocytic IL-15 production and caspase-3-mediated neuronal death, which in sum propagates brain damage. In line with other studies, our data confirms that the secondary brain injury in the HI-induced neonate includes apoptosis (20) and activation of local inflammation (21) both of which may be triggered by spleen-derived immune cells. We believe that the findings of this study provide mechanistic basis for potential novel modalities ameliorating long-term outcomes of neonatal hypoxia-ischemia.

Long-Term Behavioral and Morphological Effects of G-CSF Treatment

Excessive and/or prolonged activation of inflammatory mediators can decrease neurotrophic support and neurogenesis in brain areas responsible for behavior and cognition (10,11). For example, LPS-induced cognitive impairment and elevated levels of

TNFa and IL-1 are associated with decreased hippocampal expression of brain-derived neurotrophic factor and reduced neurogenesis (12). Blockade of IL-1 receptor, the use of IL-1 receptor knock-out mice, or transplantation of IL-1 receptor antagonist secreting neural precursor cells can prevent the effects on behavior, cognition, neurotrophic factors, and neurogenesis (10,11,13). In line with these findings, studies have shown that selective inhibition of COX-2 can increase G-CSF production (14,15), therefore, we sought to determine the direct effect of G-CSF administration on HI-induced long-term behavioral and neuropathological outcome. We found that animals that had experienced a hypoxic-ischemic insult had severe brain atrophy. Additionally, these animals demonstrated reflexes, limb placing, muscle strength, motor coordination, short-term memory, and exploratory behavior that were significantly worse than control animals. In contrast, HI-injured animals that were treated with G-CSF showed less brain tissue loss and scored similarly to the control animals; thereby, implicating the neurotrophic capabilities of G-CSF to be responsible for the structural preservation and improved functional outcome.

Summary

In summary, the emerging landscape of neuroinflammation reveals highly complex interactions involving neurons, glia, and peripheral immune cells in the neonatal brain injured by HI. The mechanism of these multidirectional communications and their specific involvement in brain injury began to unveil only recently. It is however important to further dissect molecular orchestrators of these interaction in order to devise novel therapeutics with increased likelihood of success in clinical trials. Recent studies demonstrated a critical involvement of COX-2 in brain inflammation after HI and implied

the use of COX inhibitors in treatment for neonatal encephalopathy in the clinical setting. While COX-2 appears as a major neuroinflammatory mediator after HI, G-CSF can negatively modulate inflammatory responses in the immature brain. Interestingly, COX-2 can also mitigate G-CSF action and thereby can compromise neuronal survivability in the brain. Collectively, these finding suggest that COX-2 inhibitors and exogenous G-CSF are promising treatment modalities on which to rely.

Perspective

Based on the results obtained so far it is also reasonable to anticipate that COX-2 plays a major role in mediating neuro-glial interactions as well as in orchestrating immunological cell response in HI-induced brain injury. Further studies of this matter are warranted.

In the adult stroke model, splenectomy prior to cerebral ischemia reduced brain injury by elimination of the largest pool of immunological cells in the system. Consequently, it positively verified the involvement of peripheral immune cells in the mechanism of brain injury progression after stroke. Considering that all clinical trials with anti-flammatory agents against stroke failed, these latest findings may point towards the reassessment as to whether anti-inflammatory therapies for stroke can reduce the peripheral immune system's involvement. These new evaluation criteria would also stand true for numerous candidate treatments of neonatal HI currently tested in neuroscience labs, including G-CSF. In addition, it would be worthwhile to develop immunomodulatory therapies aimed at switching immune response after HI to Th_2 cells. A significant progress in this field should not come as a surprise quite soon considering latest studies employing "beneficial" subtypes of T cells (Th_2 /Th_3) to support

neuroprotection and/or regeneration. However, the existing studies of neuro-glial-immune interactions have been conducted almost exclusively in adult stroke models. In addition, few relevant studies of neonatal HI included unsexed animals. Thus the peripheral immune involvement in neonatal HI awaits further investigations, considering distinct characteristics of the developing brain and immunologic immaturity of the neonate. In conclusion, there is a need to decipher molecular circuitry of neuro-glial-immune communications in the hypoxic-ischemic neonatal brain with gender specific investigations. It is believed that targeting master mediators of these interactions may pave the way to the first successful clinical trial with therapeutic agents that combat neuroinflammation after neonatal HI.

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