PRECONDITIONING REPROGRAMS TLR SIGNALING FOLLOWING CEREBRAL ISCHEMIA: THE EMERGENCE OF A NEUROPROTECTIVE PROGRAM

By

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CERTIFICATE OF APPROVAL

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Table of Contents

List of Tables

Chapter 1

Chapter 4

List of Figures

List of Abbreviations

AP-1	Activator protein-1
BBB	Blood-brain barrier
CNS	Central nervous system
CpG ODN	Cytosine-guanine oligodeoxynucleotide- nonmethylated
DC	Dendritic cell
HSP	Heat shock protein
HMGB1	High mobility group box 1
ICAM	Intracellular adhesion molecule 1
IFN	Interferon
IκB	Inhibitor of I-kappa B
IKK	IkappaB kinase
IL	Interleukin
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCAO	Middle cerebral artery occlusion
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa-B
NK	Natural killer cell
NOS	Nitric oxide sythase

OGD	Oxygen-glucose-deprivation
pDC	Plasmacytoid dendritic cell
RIP	Receptor interacting protein
ROS	Reactive oxygen species
s-TNFR1	soluble TNFR1
SHIP1	SH2-containing inositol-5'-phosphatase 1
TAK	Transforming growth factor-beta-activated kinase-1
TBK1	TRAF-family member associated NFκB activator-binding kinase 1
TGFβ	Tumor growth factor-beta
TIA	Transient ischemic attack
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll receptor-associated activator of interferon-beta

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Forward

I have prepared my dissertation in accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health and Science University. My thesis is comprised of a general introduction, four chapters of original data, and a final section consisting of a summary, conclusions and prospectives. The references throughout the entire thesis are listed together at the end of the manuscript and conform to the style of *Stroke.*

Chapter 2 is a manuscript as it appears in the original paper published in *The Journal of Cerebral Blood Flow and Metabolism* [1]. Chapter 3 is a manuscript as it has been prepared for publication and submission to *Stroke*. Chapter 4 is a manuscript as it has been prepared for publication and submission to *The Journal of Neuroscience*. Chapter 5 is a manuscript as it has been prepared for publication and submission to *The Journal of Cerebral Blood Flow and Metabolism*. The appendix contains additional data regarding the neuroprotective effects of LPS, CpG and poly(I:C).

Abstract

Treatment with a small dose of lipopolysaccharide (LPS) prior to stroke, called LPS preconditioning, provides robust neuroprotection against the damage caused by a subsequent stroke. The molecular processes involved in the neuroprotective effects of LPS preconditioning are poorly understood. These processes likely involve signaling downstream of the cellular receptor for LPS, Toll-like receptor 4 (TLR4), as the TLR4-induced cytokine TNFα appears to play a critical role in protection. Recent findings have demonstrated that TLR family members also play a role in the *endogenous* response to stroke. Stroke causes cell injury and tissue damage, leading to the release of endogenous TLR ligands. In response to stroke, stimulation of some TLRs has been shown to exacerbate tissue damage and worsen neurological outcome. Conversely, stimulation of other TLRs appears to protect brain tissue from injury. This dual nature of TLR signaling in the context of stroke led me to hypothesize that preconditioning with TLR ligands redirects damaging TLR signaling following cerebral ischemia toward a neuroprotective pathway.

To investigate this hypothesis, other TLR ligands were assessed for their neuroprotective potential. Systemic administration of non-methylated guanine-cytosine oligodeoxynucleotides (CpG), synthetic ligands for TLR9, in advance of brain ischemia (middle cerebral artery occlusion; MCAO) significantly reduced ischemic damage in a dose and time dependent manner. CpG ODN preconditioning also provided marked neuroprotection in modeled ischemia *in vitro*. Finally, CpG pretreatment significantly

increased serum TNFα levels prior to MCAO and required TNFα for its protective effects, as mice lacking TNFα were not protected against ischemic injury by preconditioning. These findings demonstrate that the systemic administration of multiple TLR ligands can protect the brain against subsequent ischemic injury. The shared requirement for TNFα indicates that TLR ligands may employ common mechanisms that protect the brain from stroke.

Neuroprotection afforded by LPS or CpG preconditioning occurs following their systemic administration, suggesting that peripheral and central components may be involved in protection. Examination of these responses on a genomic level has the potential to reveal molecular mechanisms of neuroprotection. Assessment of the genomic response of leukocytes and brain cells 24 hours following ischemia revealed subsets of genes uniquely up-regulated in CpG pretreated mice. CpG preconditioning induced a novel response to MCAO within circulating leukocytes that was dominated by NK cell- associated genes. Further, the NK cell-associated transcriptional regulatory element (TRE) GATA-3 was over-represented in the up-regulated gene set. Preconditioning also caused a novel brain response to stroke that was dominated by Type I interferon (IFN)- associated genes and TREs. These studies indicate that CpG preconditioning invokes novel genomic responses to stroke in both blood leukocytes and brain cells. Each of these responses has the potential to profoundly protect the brain against injury, and there is evidence to suggest they may be generated downstream of TLR signaling. These findings support the hypothesis that TLR preconditioning redirects stroke-induced TLR signaling towards neuroprotective pathways.

Further genomic analysis of brains collected 24 hours following stroke in LPS pretreated animals revealed a similar set of up-regulated genes. Literature review determined that a majority of these transcripts were associated with Type I IFNs. Promoter analysis confirmed this observation, as 5 of the 14 over-represented TREs in this group were involved in Type I IFN signaling. This finding suggested the presence of Type I IFNs or interferon regulatory factors (IRFs), which up-regulate interferon-stimulated genes. Up-regulation of IFNβ was confirmed by real-time RT-PCR. Direct administration of IFNβ i.c.v at the time of stroke was sufficient for neuroprotection. However, mice lacking IFNβ were protected by LPS pretreatment, indicating that IFNβ is not necessary for LPS-induced neuroprotection. The IRF3 transcription factor, activated downstream of TLR4, induces both IFNβ and interferon-stimulated genes. Mice lacking IRF3 were not protected by LPS pretreatment. These studies constitute the first demonstration of the neuroprotective capacity of IRF3 and suggest that interferon stimulated genes, whether induced by IFNβ or by enhanced TLR signaling to IRF3, are a potent means of protecting the brain against ischemic damage. The requirement for IRF3 supports the hypothesis that TLR signaling following stroke is redirected towards a neuroprotective pathway in the context of TLR preconditioning

TLR activation by endogenous ligands following ischemia worsens stroke damage. Paradoxically, prior stimulation of TLR4 with bacterial lipopolysaccharide (LPS) provides neuroprotection against subsequent cerebral ischemic injury. The pathway by which initial TLR4 signaling occurs following stimulation with either endogenous

ligands after stroke or by LPS prior to stroke may be very similar—indicating that the timing of TLR4 stimulation is critical in determining its destructive or protective effects. Examination of mice lacking components of TLR signaling revealed that TLR4 contributed to brain injury during short durations of ischemia, but MyD88 did not. Conversely, TLR3 alleviated injury, but TRIF did not, indicating that TRIF may be responsible for the dichotomous effects of both TLR3 and TLR4 during stroke. NFκB activity was significantly increased in the brain 24 hours following stroke. At this time, IRAK-M was increased and MyD88 was decreased, suggesting an endogenous effort to suppress NFκB. LPS preconditioning causes an increase in NFκB activity within the brain prior to stroke followed by the up-regulation of the NFκB pathway inhibitors Ship-1, Tollip, and p105 either at the time of stroke or shortly thereafter, and the suppression of stroke-initiated NFκB activity. The neuroprotective effects of LPS preconditioning were independent of MyD88 but required TLR4 and TRIF. These results indicate that LPS preconditioning mirrors the endogenous response to stroke by signaling through TLR4 and TRIF to up-regulate inhibitors of the TLR-to-NFκB signaling axis. The early regulation of these inhibitors may result in the suppression of stroke-initiated NFκB activity and contribute to the protective effect of LPS preconditioning.

These studies reveal multiple functions for TLRs in ischemia and in neuroprotection. In the endogenous response to stroke, TLR signaling to NFκB, either via the MyD88 adaptor or via the Trif adaptor, is associated with worsened outcome. On the other had, TLR signaling to IRF3 is associated with neuroprotection. Preconditioning with TLR ligands tips the balance between these signaling axis following stroke, suppressing signaling to

NFκB by up-regulating inhibitors of NFκB-inducing pathways, and increasing signaling to IRF3, as evidenced by the Type I IFN genomic "fingerprint" within the brain. These findings advance the hypothesis that TLR preconditioning confers neuroprotection by redirecting stroke-initiated TLR activity, suppressing signaling to the damage-inducing transcription factor NFκB and increasing signaling to the pro-survival transcription factor IRF3.

Chapter 1

Introduction

Preface

Toll-like receptors (TLRs) are a class of evolutionarily conserved innate immune receptors that are expressed among species as diverse as sea urchins, fruit flies, and humans. In recent years, explosive progress in deciphering the physiological role of TLRs has revealed that their immunological functions have context-dependent effects. TLRs play an essential role in initiating and coordinating an effective immune response to pathogen invasion. As mediators of protection, TLRs are being explored therapeutically as vaccine and chemotherapeutic adjuvants. Conversely, in the context of aseptic tissue injury, TLRs exacerbate tissue damage—although they do so by initiating the same inflammatory response. As mediators of disease, TLRs are being examined as therapeutic targets. Until now, it has been suggested that the pharmacologic goal in such diseases should be inhibition of TLR signaling, thereby preventing the inflammatory response and minimizing subsequent damage. In my thesis work, I explored a new approach to modifying TLR signaling in aseptic disease that takes advantage of the ability of TLRs to coordinate immune responses. With this approach, the goal is not to block TLR signaling, but to transform it. The new response redirects TLR signaling following injury from one that causes cell death to one that actively protects cells from further damage. This approach represents a novel therapeutic paradigm that has the potential to inform new medical strategies for combating stroke and related diseases.

1. Significance and Rationale

Ischemia occurs when blood flow to an organ is blocked, resulting in a lack of oxygen and nutrient delivery to that organ and a decrease in the clearance of toxic metabolic byproducts. The brain is particularly sensitive to ischemia as its energy demands rely on glucose delivered from the blood rather than from stored glycogen. While early restoration of blood flow to ischemic tissues is a primary goal following stroke, it has the potential to worsen injury by generating oxygen-derived free radicals, which often cause more cell death than ischemia itself. Since neurons produce relatively low levels of anti-oxidants, they are especially susceptible to free-radical damage. Hence therapeutics that target both ischemia and reperfusion injury are required to protect the brain from injury.

Brain ischemia and reperfusion is one of the leading causes of morbidity and mortality in the United States. Each year, more than 780,000 individuals suffer from a stroke [2], 87% of which are ischemic in nature (Incidence and Prevalence: 2006 Chart Book on Cardiovascular and Lung Diseases. Bethesda, Md: National Heart, Lung, and Blood Institute; 2006). Approximately 15% of these strokes will be heralded by a transient ischemic attack (TIA), a brain ischemic event that causes minor, short-lived effects [3]. It has been found that 3-17% of all patients with a TIA will develop a frank stroke within 90 days [4-7]. Though reliable methods exist to predict which TIA patients will suffer from a subsequent stroke [8], these high-risk patients are only prescribed anticoagulants or lipid-lowering drugs to reduce their lifetime risk[9].

A large number of brain ischemic events also occur secondary to surgical interventions. As many as 0.7% of *all* surgery patients will suffer from surgery-associated brain ischemia [10]. At highest risk are patients undergoing cardiac or vascular surgeries, 10% of whom will suffer from a frank stroke during or after surgery [10-15]. Surgery-associated stroke can be insidious; recent advances in neuroimaging techniques have uncovered a previously underappreciated incidence of surgery related ischemic events. These clinically silent strokes do not cause obvious neurological deficits, but may initiate an extended period of neuropsychological decline [16-18]. Because no prophylactic therapies exist, patients are administered only acute thrombolytic therapy after the fact to reduce the extent of damage. Prophylactic neuroprotection of each of these high-risk populations has the potential to protect thousands of patients each year from adverse neurological complications and even death.

Current research into the development of stroke prophylaxis is focused on generating therapies and pharmaceuticals that work in multiple ways to achieve synergistic goals. The goal of some, such as heparin and warfarin, is the prevention of ischemic stroke. While their anticoagulant property make these drugs attractive candidates for stroke prevention in some high-risk patient populations, it makes them a poor choice for prophylaxis of surgical candidates. The goal of other antecedent strategies is to reprogram the brain such that, should a stroke occur, it employs its own endogenous mechanisms of protection. This latter strategy is known as "preconditioning". Preconditioning is defined as a small exposure to an otherwise harmful stimulus that protects the brain against subsequent injurious ischemic challenge. It is thought that mild

preconditioning exposures herald impending danger and, as such, induce endogenous protective strategies in anticipation of injury. Preconditioning allows the brain to tolerate an ischemic event, and to thus incur less damage than a brain not so preconditioned. Tolerance to ischemia in the brain can be induced by various preconditioning stimuli including brief ischemia, brief episodes of seizure, excitotoxic glutamate, exposure to inhaled anesthetics and stimulation of TLR4 [19-22].

I have undertaken my thesis work with the goal of clarifying the molecular mechanisms by which antecedent stimulation of TLRs induces endogenous strategies that protect the brain from subsequent ischemic injury. The ultimate aim of this work is to produce safe, reliable, and effective prophylactic therapies with which to combat the devastating effects of cerebral ischemia.

TLRs are a family of pattern recognition receptors that detect host-endogenous molecules associated with dead or damaged cells and tissues and initiate inflammatory processes in response. TLRs also detect pathogen-associated molecular motifs such as those found in bacterial cell walls or viral DNA. I hypothesized that it is through this dual detection ability that TLRs offer promise as targets of stroke prophylactic strategies.

The endogenous activation of two TLRs in particular, TLR2 and TLR4, has been shown to exacerbate cerebral ischemic injury. It is thought that these two TLRs are activated by damage-associated molecules generated from ischemic brain tissue and that the resultant inflammatory response increases cell death within the brain. Other TLRs, such as TLR3

and TLR7, can be activated by damage associated-molecules and thus may also have a role in ischemic damage. Interestingly, stimulation of TLR4 *prior* to ischemia, through the systemic administration of low-dose lipopolysaccharide (LPS—a TLR4 ligand of bacterial origin) protects the brain against injury. I hypothesized that stimulation of TLRs *prior* to ischemia changes TLR activity *following* ischemia. I considered a similar model of protection in which a low dose of LPS renders animals tolerant to a subsequent toxic dose of LPS. Unlike naive cells, tolerant cells exposed to LPS (or other TLR4 ligands) generate very little of the proinflammatory cytokine Tumor Necrosis Factor α (TNFα) and instead release the immunomodulatory cytokine Interferon β (IFNβ). I thus reasoned that LPS preconditioning protects the brain from ischemic injury by establishing a state of tolerance to ischemia-induced TLR signaling. In this tolerant state, the balance of all TLR activation in response to stroke tips toward the generation of IFNβ, a molecule known to be neuroprotective, rather than the damaging proinflammatory cytokine TNFα. Notably, tolerance to LPS can also be induced by prior exposure to other TLR ligands, such as the TLR9 ligand non-methylated cytosine-guanine oligodeoxynucleotides (CpG ODNs). I thus hypothesized that preconditioning with other TLR ligands will also induce a state of protective tolerance to stroke-induced TLR signaling. In this manner, preconditioning with multiple TLR ligands may not simply suppress TLR signaling in response to ischemia, but instead shift it towards a neuroprotective pathway. Such an effect would demonstrate a completely novel therapeutic approach to stroke.

1.1 *Guiding hypothesis*

The hypothesis that underlies this thesis is that preconditioning with TLR ligands redirects TLR signaling following cerebral ischemia towards a neuroprotective pathway.

Based on this hypothesis, I postulated that multiple TLR ligands redirect TLR signaling after stroke and that this redirected response is required for neuroprotection. Specifically, I postulated that:

1. **Multiple TLR ligands can precondition the brain and protect it from ischemic damage.** Activation of multiple TLRs results in tolerance to subsequent TLR stimulation. Cells pretreated with flagellin (TLR5), loxoribine (TLR7), or CpG (TLR9) do not produce TNFα upon subsequent LPS or poly(I:C) exposure (TLR4 and TLR3, respectively), but instead produce large amounts of IFNβ. If my hypothesis is correct, then preconditioning with one such molecule, CpG, will result in tolerance to subsequent cerebral ischemic injury.

2. **TLR preconditioning reprograms the endogenous response to stroke.** Upon primary exposure to TLR ligands, cells produce minimal amounts of IFNβ. However, upon secondary exposure, cells produce up to ten times more. If my hypothesis is correct and preconditioning similarly redirects subsequent TLR signaling, then stroke-induced TLR signaling will cause an increase in IFNβ and in a unique set of Type I IFN-associated gene transcripts.

3. TRIF-dependent signaling is required for TLR preconditioning. IFNβ is increasingly appreciated as a neuroprotective cytokine. The protective effects of IFNβ are likely to be generated by the Type I IFN-associated genes that it regulates. IFNβ has been shown to help maintain the integrity of the blood brain barrier (BBB) and to reduce cellular infiltration into damaged brain regions [23]. Systemic administration of IFNβ protects animals from ischemic damage in several models of cerebral ischemia [24, 25]. IRF3, a transcription factor activated downstream of TLR3 and TLR4 via the TIR-domain containing adaptor Inducing IFNβ (TRIF), can also generate Type I IFN-associated genes. IRF3 is also required for TLR-induced IFNβ production. If my hypothesis is correct and redirected TLR signaling is neuroprotective, then TRIF, IRF3, and IFNβ will be required for the protective effects of TLR preconditioning.

4. The MyD88-dependent signaling axis is suppressed after stroke following TLR preconditioning. Upon primary exposure to TLR4 ligands, cells activate the transcription factor Nuclear Factor κB (NFκB) and produce copious amounts of TNFα. However, upon secondary exposure, cells fail to activate NFκB and hence fail to generate TNFα. Inhibition of NFκB occurs through the up-regulation of inhibitors of the TLR-to-NFκB signaling axis. If my hypothesis is correct and preconditioning similarly redirects subsequent TLR signaling, then pretreatment with TLR ligands will up-regulate inhibitors of the TLR4-to-NFκB signaling axis and result in suppressed NFκB activity following stroke.

2. Toll-like Receptors

The Toll-like receptors, so-called because of their homology to the *Drosophila* Toll receptor, were first characterized in mammals by their ability to recognize pathogen-associated molecular patterns such as those found in the bacterial cell wall components peptidoglycan (TLR2) and LPS (TLR4), as well as viral dsRNA (TLR3), ssRNA (TLR7), and CpG (TLR9). Recently it has been found that in addition to their role in pathogen detection and defense, TLRs act as sentinels of tissue damage and mediate inflammatory responses to aseptic tissue injury. Host-endogenous molecules associated with damaged cells and tissues activate various TLRs (Table 1.1). Surfactant, HSP60, components of the extracellular matrix, and fibrinogen have all been shown to activate TLR4, while host HMGB1, as well as host mRNA and DNA are endogenous ligands of TLR2 (and TLR4), TLR3 and TLR9, respectively. TLRs, upon activation by either pathogen- or host-derived ligands, induce downstream signals that lead to cytokine and chemokine production and thereby initiate inflammatory responses. TLRs are primarily located on antigen presenting cells such as B cells, dendritic cells, monocytes, macrophages and microglia. In the brain, these receptors are also expressed by the cerebral endothelium and by parenchymal cells such as astrocytes, oligodendrocytes, and neurons [26-29].

2.1. *Toll-like receptor signaling*

TLR family members signal through common intracellular pathways that lead to transcription factor activation and the production of cytokines such as TNFα, IL-1β and

IL-6, and chemokines such as Rantes and Mip-1α (Figure 1) [30, 31]. Each TLR family member, with the exception of TLR3, initiates intracellular signaling via recruitment of the intracellular Toll-interleukin 1 receptor (TIR)-domain-containing adaptor Myeloid Differentiation factor 88 (MyD88). When recruited to plasma membrane-associated TLRs, either directly (TLRs 5 and 11) or via the TIR domain-containing Adaptor Protein (TIRAP) (TLRs 1, 2, 4, 6), MyD88 enlists members of the IL-1 Receptor Associated Kinase (IRAK) family, including IRAK1, IRAK2, and IRAK4, to begin a process of auto- and cross-phosphorylation. Once phosphorylated, IRAKs dissociate from MyD88 and bind TNF Receptor Associated Factor 6 (TRAF6), an E3 ligase. TRAF6 in turn activates TGFβ-Activated Kinase (TAK1) which itself activates the Inhibitor of NFκB Kinase (IKK) complex and Mitogen Activated Protein Kinase Kinase (MAPKK). The IKK complex, composed of IKKα, IKKβ and the regulatory subunit IKKγ, phosphorylates Inhibitor of Nuclear Factor κB (IκB). This leads to the ubiquitination and proteosomal degradation of IκB and the resultant release and nuclear translocation of the transcription factor NFκB. Members of the MAPK family phosphorylate and activate components of the transcription factor AP-1. Together, these transcription factors induce inflammatory cytokine production (e.g. TNFα, IL1).

MyD88 is also recruited to the endosomal receptors TLR7 and TLR9 again enlisting members of the IRAK family. Due to the endosomal location of the complex, the phosphorylated IRAKs are able to bind TRAF3 and IRF7 in addition to TRAF6 [32, 33]. This leads to the activation and nuclear localization of IRF7 with resultant Type I IFN

production. Hence these endosomal TLRs are capable of signaling to NFκB, AP-1 and IRFs, resulting in a diverse genomic response.

Endosomal TLR3 is unique among the TLRs because it does not signal through MyD88 but signals instead via recruitment of TRIF. TRIF enlists the non-canonical IKKs, TANK Binding Kinase (TBK1) and IKKε, which activate IRF3. Further, TRIF recruits TRAF6 and Receptor (TNFRSF)-Interacting serine-threonine Protein kinase 1 (RIP-1), which results in activation of MAPK and IKKα/β. Hence TLR3, like the other endosomal receptors, is capable of activating NFκB, AP-1 and IRFs.

Of all the TLRs, only TLR4 can recruit either MyD88 at the plasma membrane (via TIRAP) or TRIF at the endosomal membrane after endocytosis (via the TRIF-related adaptor molecule TRAM) [34]. TLR4 can thus induce either the pro-inflammatory cytokine TNFα via NFκB or the anti-viral cytokine IFNβ via IRF3. It is because of this dual nature that I choose to explore TLR4 as a novel therapeutic target in disease.

2.2 *Toll-like receptor expression within the brain*

The array of TLR family members expressed by a cell depends upon the cell's identity and activation status. Constitutive expression of TLRs within the brain occurs in microglia and astrocytes and is largely restricted to the circumventricular organs and meninges—areas with direct access to the circulation [35-37]. Human and murine microglia express TLRs 1-9 and generate cytokine profiles specifically tailored by the TLR

stimulated [26, 28, 38]. Similarly, human and murine astrocytes express multiple TLRs, with particularly prominent TLR3 expression [26-28, 39, 40]. Microglia and astrocytes respond differently to specific TLR engagement, reflecting their distinct roles in the brain. Microglia initiate robust cytokine and chemokine responses to stimulation of TLR2 (TNFα, IL-6, IL-10), TLR3 (TNFα, IL-6, IL-10, IL-12, CXCL-10, IFNβ), and TLR4 (TNFα, IL-6, IL-10, CXCL-10, IFNβ), yet astrocytes initiate only minor IL-6 responses to all but TLR3 stimulation [26]. These differences may be explained by the finding that microglia express TLR3 and TLR4 at the cell surface while astrocytes express these receptors intracellularly [28], and by studies demonstrating that the cellular location of TLRs influences their downstream signaling cascades [41]. The inflammatory milieu also plays a critical role in regulating TLR expression. Microglia stimulated with CpG specifically up-regulate TLR9, whereas those stimulated with a synthetic TLR3 ligand suppress all TLRs except TLR3 [38]. Similarly, astrocytes stimulated with LPS up-regulate TLRs 2 and 3 but suppress TLR4, while astrocytes exposed to RNA viruses up-regulate TLR3 and TLR9 [39]. Thus microglia and astrocytes initiate a layered and multifaceted response to TLR engagement.

Oligodendrocytes and endothelial cells express a relatively limited repertoire of TLRs. Oligodendrocytes express TLRs 2 and 3 [28], while cerebral endothelial cells constitutively express TLRs 2, 4, and 9 [42] and increase the expression of these TLRs in response to stressful stimuli, including systemic LPS and cerebral ischemia [29, 43, 44]. In response to LPS, endothelial cells up-regulate E-selectin, an NFκB-dependent molecule, and IFNβ,

an IRF3-dependent molecule, indicating that these cells utilize the TLR4-NFκB and the TLR4-IRF3 signaling axes [45].

Neurons express TLR3 and generate inflammatory cytokines (TNFα, IL-6), chemokines (CCL5, CXCL10) and antiviral molecules (IFNβ) in response to dsRNA [46]. Neurons also employ TLRs in their development and differentiation. TLRs 3 and 8 are expressed on murine neurons early in development and inhibit neurite outgrowth in an NFκB-independent manner [47]. TLR2 and TLR4 have been found on adult neural progenitor cells where they appear to elicit opposing effects. While TLR2 activation stimulates neuronal differentiation of these cells, TLR4 activation decreases proliferation and neuronal differentiation, driving these cells toward an astrocytic fate [48]. Curiously, both TLRs exert their endogenous effects in a MyD88-dependent manner, suggesting that these receptors utilize MyD88 in distinct ways. Hence even minor alterations of these fine-tuned endogenous pathways can have profound effects on cellular responses to TLR engagement.

2.3 *Toll-like receptors in brain disease*

The inflammatory responses initiated by TLRs in the brain have both beneficial and detrimental effects. Activated TLRs help clear pathogenic organisms from the brain, thereby aiding in tissue recovery and damage resolution. TLR2 has been shown to play a critical role in protection against *Streptococcus* pneumoniae meningitis [49], *Toxoplasma* gondii [50], and tuberculosis meningitis [51]. TLR2 and TLR4 are required for effective

murine immune responses to *Staphylococcus* aureaus-induced brain lesions [52]. These studies demonstrate that animals lacking TLRs suffer from decreased pathogen clearance despite increased recruitment and activation of macrophages, granulocytes and T cells. Without TLR signaling to shape the inflammatory process, these animals display dysregulated cellular and cytokine responses that are ineffective at ridding the body of invasive pathogens. This results in persistent infection and increased mortality. TLR2 and TLR4 may also help prevent the progression of Alzheimer's disease by aiding in microglial clearance of amyloid beta protein deposits [53, 54, 55]. Hence, the ability of TLRs to initiate and coordinate inflammatory responses is critical for health maintenance. In other contexts, however, TLR- initiated inflammatory responses contribute to tissue damage and increase morbidity and mortality. TLR2 contributes to neuroinflammation and mortality in HSV-1 infection [56] and TLR3 contributes to mortality in West Nile virus-induced encephalitis [57] in mouse models of these diseases. In these examples, it is not the pathogens *per se* that cause mortality, but rather the inflammatory process initiated in response to pathogen invasion that results in death. TLR2, TLR3, and TLR4, have all been implicated in either the initiation or progression of experimental autoimmune encephalitis (a mouse model of multiple sclerosis) and other demyelinating diseases [58-60], by activating microglia and other antigen-presenting cells to present self-derived antigen. TLR2 and TLR4 have further been shown to play a role in ethanol- and ganglioside- mediated neuroinflammation [61-63]. Hence, within the brain, the consequence of TLR-induced inflammation depends upon the circumstance in which it is initiated.

The overwhelming and generally damaging inflammatory response of TLRs to aseptic tissue injury within the brain may be a consequence of TLR evolution in response to pathogens. In the setting of pathogen invasion, an inflammatory deluge may be the most effective means to clear microorganisms. The activation and influx of leukocytes, with the concomitant release of free radicals and tissue-destroying enzymes, assails not only the invading pathogen but also any host cells that harbor the pathogen. However, when this same powerful response is co-opted by the host to clear and resolve tissue damage, it can destroy the very cells it is meant to save. This damage-promoting characteristic is prominently observed following brain ischemia, where inflammation plays a critical role in both injury progression and resolution.

3. Cerebral ischemia

Cerebral ischemia occurs when blood flow to the brain is inhibited. Inhibition can occur locally, resulting from a thrombotic or embolic clot, or globally, resulting from systemic hypoperfusion following cardiac arrest or septic shock. In the absence of normal blood flow, oxygen and glucose delivery to the ischemic region is decreased, often to a level at which cellular energy needs cannot be met. Hence energy-dependent processes are compromised, initiating a cascade of events that culminate in cell death. Many plasma membrane ion channels depend on energy, in the form of ATP, for proper functioning. In the absence of adequate ATP levels, these channels cannot maintain ionic gradients across the plasma membrane and membrane depolarization ensues. This results in a prolonged increase in intracellular calcium that initiates several cytotoxic events. The

accumulation of intracellular calcium triggers calcium-activated proteases, lipases, and DNAses that cause cell death by simple catabolism. Additionally, calcium overload depolarizes the mitochondrial membrane, resulting in the release of reactive oxygen species, which further damage proteins and DNA, and apoptotic proteins such as cytochrome C, which activates caspase cascades that mediate cell death. Finally, intracellular calcium causes the release of the neurotransmitter glutamate. Excessive amounts of extracellular glutamate can depolarize neighboring cells for prolonged periods of time, increasing the concentration of intracellular calcium within these cells to toxic levels. In this manner, the damage cascade can be passed from cell to cell into brain regions surrounding the infarcted tissue.

Cell death within the ischemic core occurs within hours of vessel occlusion. Outside the core, where collateral blood flow attenuates cellular stress, death occurs over several days. This delayed cell death results from endogenous processes triggered in response to the ischemic injury, such as inflammation and apoptosis. Thus, therapeutically targeting the inflammatory response has the potential to salvage viable tissue and to protect neurological function.

Ischemia activates resident inflammatory cells within the brain, namely microglia and astrocytes. Once activated, these cells produce TNFα and FasL, which cause apoptotic cell death by the caspase-activating death domain in their receptors (TNFR1 and TNFR2, Fas). Microglia additionally produce reactive oxygen and nitrogen species that are toxic to neurons. Activated microglia and astrocytes release cytokines and chemokines that

activate the cerebral endothelium and aid in leukocyte accumulation and transmigration into ischemic brain tissue. One such cytokine, IL-1β, up-regulates the adhesion molecules E-selectin, intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2) and vascular adhesion molecule 1 (VCAM-1) on cerebral endothelial cells, thereby aiding neutrophil infiltration [64, 65]. The chemokines MCP-1 and MIP-1α aid in macrophage and monocyte accumulation in the ischemic region. Consistent with a deleterious role for these cytokines and chemokines, inhibition of any one of them reduces cerebral ischemic injury [66, 67].

Neutrophils are generally the first leukocyte subtype to penetrate into the ischemic brain, followed by macrophages and lymphocytes [68]. Neutrophils potentiate tissue damage and cell death by releasing oxygen free-radicals and proteolytic enzymes. Lymphocytes, too, have strong proinflammatory and tissue-damaging properties. Together, infiltrating inflammatory cells cause secondary damage to potentially salvageable tissue surrounding the ischemic core.

It is still unclear how resident inflammatory cells discern the presence of ischemia-damaged tissue. However, TLRs have been implicated in this process. TLRs detect dangerous extra-cellular conditions, such as those induced by local cell stress, and initiate inflammatory processes in response. Hence TLRs may play a critical role in initiating damaging inflammatory responses to stroke.

4. Toll-like receptors and ischemic damage

In the mouse, microglia and astrocytes express TLRs [38, 69] and it may be through TLR activation that these cells initiate the inflammatory response to stroke. Ischemia causes an early increase of TLR2 and TLR4 on neurons (0.5-6 hrs) and a delayed increase on microglia, astrocytes, and endothelial cells (24-72 hrs) [43, 70-72]. Studies using knockout mice indicate that these receptors have detrimental effects in the context of stroke (Table 1.2). Mice lacking functional TLR2 incur significantly smaller infarcts and demonstrate better functional outcomes than wild-type controls [43, 71, 72]. Similarly, mice lacking functional TLR4 are less susceptible to transient and permanent MCAO and to global cerebral ischemia (inhibition of the common carotid, and left and right subclavian arteries) and demonstrate better functional outcomes than wild-type controls [70, 71, 73-75]. Unlike wild-type mice, TLR4 deficient mice do not up-regulate Cox-2, MMP9, iNOS, or IRF1 within the brain and do not increase circulating levels of TNFα or IL-6, indicating suppressed inflammatory responses following stroke [74, 75]. TLR4 deficient mice increase the phosphorylation of Akt and GSK3B, indicating an increase in pro-survival pathways following stroke [70]. Neurons appear to be particularly sensitive to TLR stimulation. In primary cortical cultures, neurons from mice lacking TLR2 or TLR4 are resistant to cell death from glucose deprivation (a cell culture model of energy deprivation), potentially by suppressing JNK-AP-1 signaling and caspase 3 activation[71].

The endogenous TLR4 ligands HSP60, HSP70 and HMGB1 (which also binds TLR2) have been detected in the brain following injury [76-78]. Activation of either receptor by

these ligands results in the production of known mediators of ischemic injury such as TNFα and IL1β. Thus, cell death within the ischemic core leads to the release of damage-associated molecules, which, by activation of TLRs on resident brain cells, may initiate the deleterious inflammatory response to stroke (Figure 2).

5. Toll-like receptors and neuroprotection

In contrast to the detrimental role of TLRs *in response* to ischemia, stimulation of these receptors *prior* to ischemia provides robust neuroprotection. TLR4-induced tolerance to cerebral ischemia was first demonstrated with low-dose systemic administration of LPS, which protected spontaneously hypertensive rats from subsequent ischemic brain injury caused by transient MCAO [79]. Since then, LPS-induced tolerance to brain ischemia has been demonstrated in a murine model of stroke and in a porcine model of deep hypothermic circulatory arrest [80, 81] (Table 1.3).

The neuroprotective program elicited by LPS preconditioning occurs in three distinct phases. The phase between LPS administration and MCAO is termed the "initiation" stage. The phase following MCAO is termed the "effector" stage. The bridge between these two phases is termed the "reprogramming" stage.

The initiation phase of LPS preconditioning is time and dose dependent. Tolerance is achieved if LPS is administered between 1 and 7 days prior to MCAO, but no neuroprotection is observed if LPS treatment occurs 14 days before MCAO [82]. Protective

doses of LPS appear to depend on the animal model and the route of systemic administration. Protective doses range from 0.02 to 1mg/kg and have been administered via intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) routes [79, 81-86]. Tolerance induction has been shown to require *de novo* protein synthesis and a modest inflammatory response, as it can be blocked when LPS is administered simultaneous with cyclohexamide, an inhibitor of protein translation, or with the corticosteroid dexamethasone [85]. TNFα has been specifically implicated as a critical mediator in the establishment of LPS-induced ischemic tolerance. Preconditioning doses of LPS significantly increase circulating levels of TNFα and inhibition of this pro-inflammatory cytokine, either systemically or within the brain, at the time of LPS administration blocks neuroprotection [79,82]. Further, mice lacking TNFα fail to be protected by LPS preconditioning [82]. LPS-induced reactive oxygen and nitrogen species also play a role in the establishment of tolerance. Reactive oxygen species and peroxynitrite, formed by cerebral vessels in response to LPS, are both required for ischemic tolerance [83]. Hence, LPS-induced inflammatory reactions are integral components in the initiation of ischemic tolerance.

LPS-induced ischemic protection requires an inflammatory response in the initiation phase *prior* to the ischemic event, yet protection occurs in the effector phase through modulation of the inflammatory response *following* ischemia. One hallmark of LPS preconditioning is the suppression of cytotoxic TNFα signaling following stroke. Mice that have been preconditioned with LPS prior to ischemia display a pronounced suppression of the TNFα pathway following stroke, as evidenced by reduced TNFα in

the serum, and decreased levels of cellular TNFR1, but increased levels of neutralizing soluble-TNFR1, in the brain. Further, preconditioned mice express significantly lower levels of TNFR-associated death domain (TRADD), an intracellular molecule involved in TNFα-induced signaling. Preconditioned mice are thus protected from the cytotoxic effects of TNFα after cerebral ischemia [82]. Collectively, these molecular mechanisms result in dampened TNFα responses to ischemia and increased cell survival.

Modulation of the inflammatory response to stroke in preconditioned mice is also observed on the cellular level. LPS preconditioning attenuates microglial activation after stroke and reduces neutrophil infiltration into the ischemic hemisphere [80]. In addition, preconditioning changes the response of circulating leukocytes, attenuating stroke-induced neutrophilia, lymphopenia, and monocyte activation [80, 86].

Perhaps most prominently, LPS preconditioning modifies endothelial cell function following stroke. Preconditioning has been shown to prevent post-ischemic endothelial cell dysfunction and to thereby decrease BBB permeability following MCAO [86]. Maintenance of cerebrovascular function preserves cerebral blood flow in the peri-infarct region for at least 24 hours after MCAO [84, 87] and may increase collateral blood flow to compromised brain regions during the ischemic event [83]. LPS pretreatment has been shown to prevent the impairment of endothelial and smooth muscle relaxation that is normally induced by ischemia/reperfusion injury. The vasoprotective effect of LPS may be due to increases in nitric oxide. Mice lacking Inducible Nitric Oxide Synthase (iNOS) expression fail to be protected by LPS pretreatment [83], and Endothelial Nitric Oxide

Synthase (eNOS) expression within the brain is directly correlated to the time window of LPS-induced neuroprotection [84]. LPS-induced preservation of microvascular function following MCAO may be due to suppressed lymphocyte adhesion to activated endothelium. It has been suggested that this may occur by TNFα-induced suppression of endothelial activation and adhesion molecule expression [86, 88] or by prevention of cellular inflammatory responses to ischemia [80].

The mechanisms that convert the small inflammatory response in the initiation phase into the suppressed inflammatory response in the neuroprotective effector phase are largely unknown. The goal of my thesis work is to clarify the molecular underpinnings of the reprogramming phase in which this conversion occurs. Understanding the transition between initiation and effect will provide insight into the brain's endogenous protective processes and may uncover a rich source of therapeutic targets.

6. Mechanisms of neuroprotection—redirecting TLR signaling

The dual nature of TLR4 signaling in ischemic injury and neuroprotection provides insight into the mechanisms of LPS-induced neuroprotection. Studies with knockout animals have shown that TLR4 stimulation *in response* to ischemia exacerbates stroke injury. However, studies on LPS preconditioning have indicated that TLR4 stimulation *prior* to ischemia protects against cerebral ischemic injury. Together, these findings have led me to hypothesize that LPS-induced tolerance to subsequent ischemia occurs by the same molecular mechanisms that govern the similar phenomenon of LPS-induced

tolerance to subsequent LPS. Known as "endotoxin tolerance", this phenomenon occurs when pretreatment of cells or animals with a low dose of LPS renders them tolerant to the normally toxic effects of a second, higher dose of LPS. It is believed that endotoxin tolerance represents an evolutionarily conserved protective mechanism against the deleterious effects of sepsis. Cells that are tolerant to LPS are defined by their inability to generate TNFα in response to TLR4 activation. LPS tolerant cells, unlike naive cells, do not recruit MyD88 to TLR4, and fail to activate IRAK- 1 and NFκB upon TLR4 ligation [89]. The TLR4-NFκB signaling axis becomes decommissioned following a primary exposure to LPS via an elaborate negative feedback loop that involves known inhibitors of TLR signaling. Among those inhibitors are Ship-1, which prevents TLR4-MyD88 interaction, IRAK-M, a non-functional IRAK decoy, and TRIM30α, which destabilizes the TAK1 complex [90-92], each of which is up-regulated following initial exposure to LPS. Because of these inhibitors, subsequent signaling of TLR4 to NFκB is blocked and inflammatory cytokine production is suppressed. Conversely, LPS-tolerant cells produce higher levels of IFNβ upon TLR4 ligation, suggesting *increased* signaling via the TLR4-IRF3 axis [93]. Thus, pretreatment with LPS causes cells to switch their transcriptional response to TLR4 stimulation by up-regulating the IRF3- induced cytokine IFNβ and suppressing the NFκB-induced cytokine TNFα.

Similar to endotoxin tolerance, priming TLR9 with its ligand, CpG, induces a state of hypo-responsiveness to subsequent challenge with CpGs [94]. Interestingly, cross-tolerance between the two receptors has also been reported, as ligands for TLR9 induce tolerance against a subsequent challenge with a TLR4 ligand by down regulating IRAK-1 and up-

regulating IRAK-M [95] [93, 96]. Hence, CpG-pretreated cells not only produce less TNFα when secondarily challenged with LPS, they also produce significantly higher levels of IFNβ [93].

Together, these studies led me to hypothesize that TLR stimulation prior to stroke reprograms ischemia-induced TLR activation. I postulated that administration of LPS or CpG activates TLR4 or TLR9, respectively, causing a small inflammatory response, with an initial rise in TNFα. In an effort to control that inflammatory response, cells subsequently up-regulate negative feedback inhibitors of the TLR-NFκB signaling axis (Figure 3). These inhibitors remain present when cells are subsequently exposed to endogenous TLR ligands generated from ischemia-injured brain tissue. Within this new cellular environment, TLRs are unable to activate NFκB-inducing pathways. Because of this, TLR4 signaling shifts from NFκB induction to IRF3 induction, with a resultant increase in IFNβ (Figure 4).

The IRF3 transcription factor is required for LPS-induced production of IFNβ [97]. Structural information gathered from the crystallized IRF3-bound IFNβ promoter indicates that IRF3 dimers bind to each of 4 IRF transcriptional regulator elements (TREs) within the PRDIII domain of the promoter [98]. In addition to enhancing transcription by binding IRF TREs, IRF3 also enhances transcription by binding interferon-stimulated response element (ISRE) TREs. ISREs are found in the promoter regions of many interferon-stimulated genes (ISGs), and activated IRF3 has been shown to up-regulate transcription of a number of ISGs [99]. Interestingly, IFNβ itself causes the

up-regulation of many ISGs. Upon binding to the Type I IFN receptor complex, IFNβ induces JAK/STAT signaling, which activates the ISGF3 transcription factor, composed of Stat 1, Stat 2, and IRF9. ISGF3 binds to ISRE motifs within the promoters of ISGs, increasing their expression. Hence IRF3 induction of IFNβ may act to amplify ISRE-regulated gene expression, causing a feed forward loop of ISG production.

IFNβ, best known for its anti-viral effects, also has potent anti-inflammatory activities. These anti-inflammatory activities have been particularly well characterized in the brain where microglia/macrophages, astrocytes, neurons, and endothelial cells all produce IFNβ [100, 101]. Several studies have shown that IFNβ can stabilize the blood-brain barrier, potentially by reducing matrix metalloprotease production by activated glia [23, 102, 103]. Similarly, it has been shown to inhibit monocyte migration across human brain-derived endothelial cells [104] and reduce cellular infiltration into damaged brain regions [23]. On a cellular level, IFNβ can reduce reactive oxygen species [105-107], suppress inflammatory cytokine production and increase levels of IL-1Ra [108, 109], promote nerve growth factor production by astrocytes [110] and protect neurons from toxicity induced by activated microglia [111]. In addition, systemic administration of IFNβ has been shown to reduce infarct damage in rat and rabbit models of ischemic stroke [24, 25]. The neuroprotective effects of IFNβ are likely mediated by the genes it up-regulates, that is, by ISGs. Therefore, in the setting of LPS preconditioning, up-regulation of IFNβ, or of IRF3, which also up-regulates ISGs, would be expected to contribute to neuroprotection.

The studies performed in this thesis were designed to test a molecular model of TLR-induced protection from ischemic injury wherein systemic administration of TLR ligands reprograms TLR signaling in response to brain ischemia, directing it towards a neuroprotective pathway. I propose that TLR preconditioning fundamentally changes the brain's response to stroke and suggest that such reprogramming events exemplify endogenous processes that protect the brain against further injury.

Table 1.1 Endogenous Toll-like receptor ligands

TLR	Endogenous Ligand	Ligand Source	Response	Citation
TLRs 2 and 4	Hsp60	Necrotic cells	TNF-alpha and NO in macrophages	Ohashi K, et al. 2000; Vabulas RM, et al. 2001; Lenhardt S. et. al. 2008.
	Hsp70	Necrotic cells	IL-12 and ELAM-1 in macrophages	Asea A, et al.. 2002; Vabulas RM, et al. 2002.
	gp96	Necrotic cells	IL-12 in DCs	Vabulas RM, et al. 2002.
	High mobility group box 1 (HMGB1)	Released from nucleus in inflammation	Lethality in sepsis reperfusion injury	Parks JS, et al. 2004.
	Urate crystal	Deposition in joints and bursal tissues in gout	TNFα, IL-1β, TGFβ1 in macrophages	Liu-Bryan R, et al. 2005.
	Biglycan	Released from ECM	TNFα, MIP in macrophages/DCs	Schaefer L, et al. 2005.
	Hyaluronan	Degraded from larger species in ECM	Chemokine production in macrophages, activation of DCs via TLR4	Jiang D, et al. 2005; Termeer C, et al. 2002.
TLR 3	mRNA when complexed with lipofectin	Necrotic cells	DC activation and TNFα production	Kariko K, et al. 2004.
TLR 4	Hsp22 (HspB8)	Synovial fluid in rheumatoid arthritis	IL-6, TNFα, up-regulation of co-stimulatory molecules in DCs	Roelofs MF, et al. 2006.
	Fibronectin extradomain A	Tissue damage	MMP-9 in human macrophages	Okamura Y, et al. 2001.
	Surfactant protein-A	Lung surfactant	TNFα and IL-10 in macrophages	Guillot L, et al. 2002.
	Figrinogen	Extravated from vasculature after endothelial cell retraction	Chemokine production in macrophages	Smiley ST, et al. 2001.
	Heparin sulfate	Released from ECM, cell membranes	Maturation and up-regulation of co-stimulator molecules in DCs	Johnson GB, et al. J 2002.
	Beta-defensin 2-fusion protein (rmDF-2/rfv)	Epithelial antibacterial peptides	DC maturation and up-regulation of co-stimulator molecules	Birgyn A, et al. 2002.
	Minimally modified (oxidized) low density lipoprotein	Pro-inflammatory and pro-	Actin polymerization and spreading of	Miller YI, et al. 2003.

		atherogenic protein	macrophages	
	Pancreatic elastase	Pancreatic elastase	TNFα secretion in THP-1 cells	Hietaranta A, et al. 2004.
	Alpha-A crystallin	Necrotic cells	Activation of DCs	Roelofs MF, et al. 2006.
TLR 7	RNA immune complex	Necrotic cells	IFNα production by PDCs	Barrat FJ, et al. 2005.
TLRs 7 and 8	siRNAs when encapsulated into liposomes	Necrotic cells	Induction of TNFα and IL-6 in PBMCs	Sioud M. 2005.
TLR 9	Chromatin immune complex	Necrotic cells	DC activation	Boule MW, et al. 2004.
	DNA immune complex	Necrotic cells	IFNα production by pDCs	Barrat FJ, et al. 2005.

Table 1.2 The endogenous role of Toll-like receptors in cerebral ischemia

TLR knock-out	Stroke model	Animal model	Outcome	Proposed mechanism	Citation
TLR 2	MCAO via filament, 60 min, 48h reperfusion	mouse, C57BL/6J, male, 10-12 week, TLR 2 -/-	decreased infarct in TLR2 -/-	TLR 2 signaling	Ziegler G, et al. 2007.
	MCAO via filament, 60min, 24 and 72h reperfusion	mouse, C57BL/6J, male, 13-15 week, TLR 2 -/-	decreased infarct in TLR2 -/-	TLR 2 in microglia	Lehnardt S, et al. 2007.
	MCAO via filament, 60 min, 72h reperfusion	mouse, TLR 2 -/-	decreased infarct in TLR2 -/-		Tang SC, et al.. 2007.
TLR 4	permanent MCAO via electrocoagulation, 24h and 7d reperfusion	mouse, male, adult, TLR 4 -/- (C3H/HeJ and C57BL/10ScCr)	decreased infarct in TLR4 -/-	decreased inflammatory response, I.e. MMP9	Caso JR, et al. 2007.
	MCAO, 6h, embolism, 24h reperfusion	mouse, C3H/HeJ, female, 8 week, TLR 4 -/-	decreased infarct in TLR4 -/-	decreased inflammatory cytokines I.e. TNF-alpha, IL-6	Cao CX, et al. 2007.
	MCAO via filament, 60 min, 72h reperfusion	mouse, TLR 4 -/-	decreased infarct in TLR4 -/-		Tang SC, et al. 2007.
	Global cerebral ischemia/reperfusion via occlusion of CCA, LSA and RSA, 12 min, 6h reperfusion	mouse, C57BL/10ScCr, male, 8-12 week, TLR 4-/-	decreased infarct in TLR4 -/-	decrease phospho-IkB and NFκB	Hua F, et al. 2007.
	permanent MCAO via cauterization, 24h reperfusion	mouse, male, adult, TLR 4 -/- (C3H/HeJ and C57BL/HeN)	decreased infarct in TLR4 -/-, decreased subacute stress response	decreased iNOS and COX-2 in response to immobilization stress	Caso JR, et al. 2008.

Table 1.3 LPS, a TLR4 ligand, preconditions against cerebral ischemia

Ligand (TLR)	Treatment	Stroke model	Animal model	Outcome	Proposed mechanism	Citation
LPS (TLR4)	0.9 mg/kg, IV, 1-7d prior	permanent MCAO via electrocoagulation, 24h reperfusion	Rat, adult, male, spontaneously hypertensive	Reduced infarct in LPS preconditioned	IL-1, TNFα	Tasaki K, et al.. 1997.
	0.9 mg/kg, IV, 72h prior	permanent MCAO via electrocoagulation, 24h reperfusion	Rat, adult, male, spontaneously hypertensive	Reduced infarct in LPS preconditioned	Maintenance of microvascular patency	Dawson DA, et al. 1999.
	0.05 mg/kg, SC, 24h prior	transient MCAO, 6 and 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	Protection despite increased PMN infiltration	Ahmed SH, et.al. 2000.
	0.5 mg/kg, IP, 72h prior	transient MCAO, 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	Protein synthesis and inflammation dependent	Bordet R, et.al. 2000.
	0.3 mg/kg, IP, 72h prior	transient MCAO, 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	Maintenance of endothelial and smooth muscle relaxation	Bastide M, et.al. 2003.
	0.2 mg/kg, IP, 48h prior	MCAO via filament, 60 min, 48h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	Suppression of cellular inflammation	Rosenzweig HL, et al. 2004.
	0.9 mg/kg, IV, 72h prior	focal cerebral ischemia, 6h, 24h, 7d and 14d reperfusion	Rat, spontaneously hypertensive	Reduced infarct in LPS preconditioned	Preservation of local cerebral blood flow, up-regulation of eNOS	Furuya K, et al. 2005.
	0.2 mg/kg, IP, 72h prior	MCAO via filament, 60 min, 24h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	Suppression of TNFα signaling	Rosenzweig HL, et al. 2007.
	0.02 mg/kg, IV, 72h prior	Deep hypothermic circulatory arrest	neonatal piglets	Reduced infarct in LPS preconditioned	Up-regulation of inflammation inhibitors	Hickey EJ et.al. 2007.
	0.5 mg/kg, IP, 24h prior	MCAO via filament, 25min, 72h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	iNOS-induced preservation of neurovascular function and CBF	Kunz A, et al. 2007.

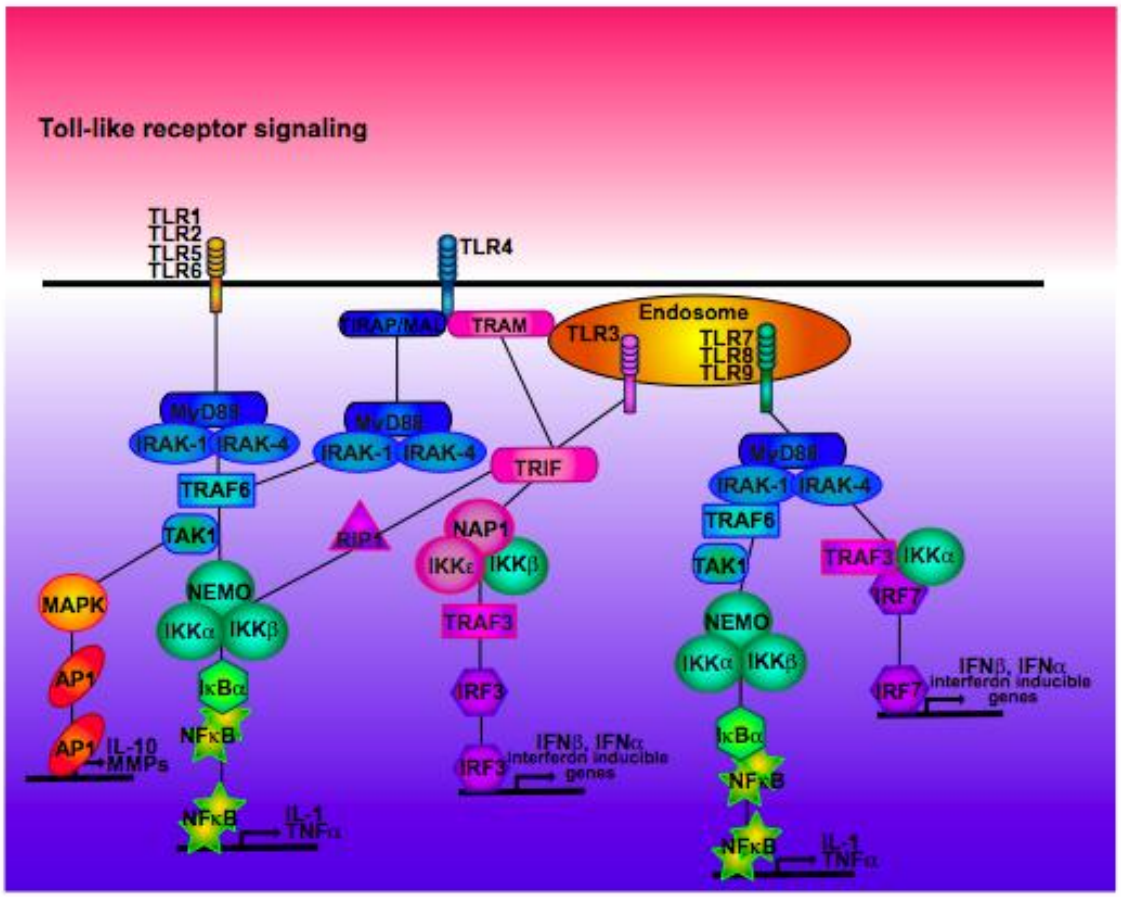

Figure 1.1. Schematic of Toll-like receptor signaling

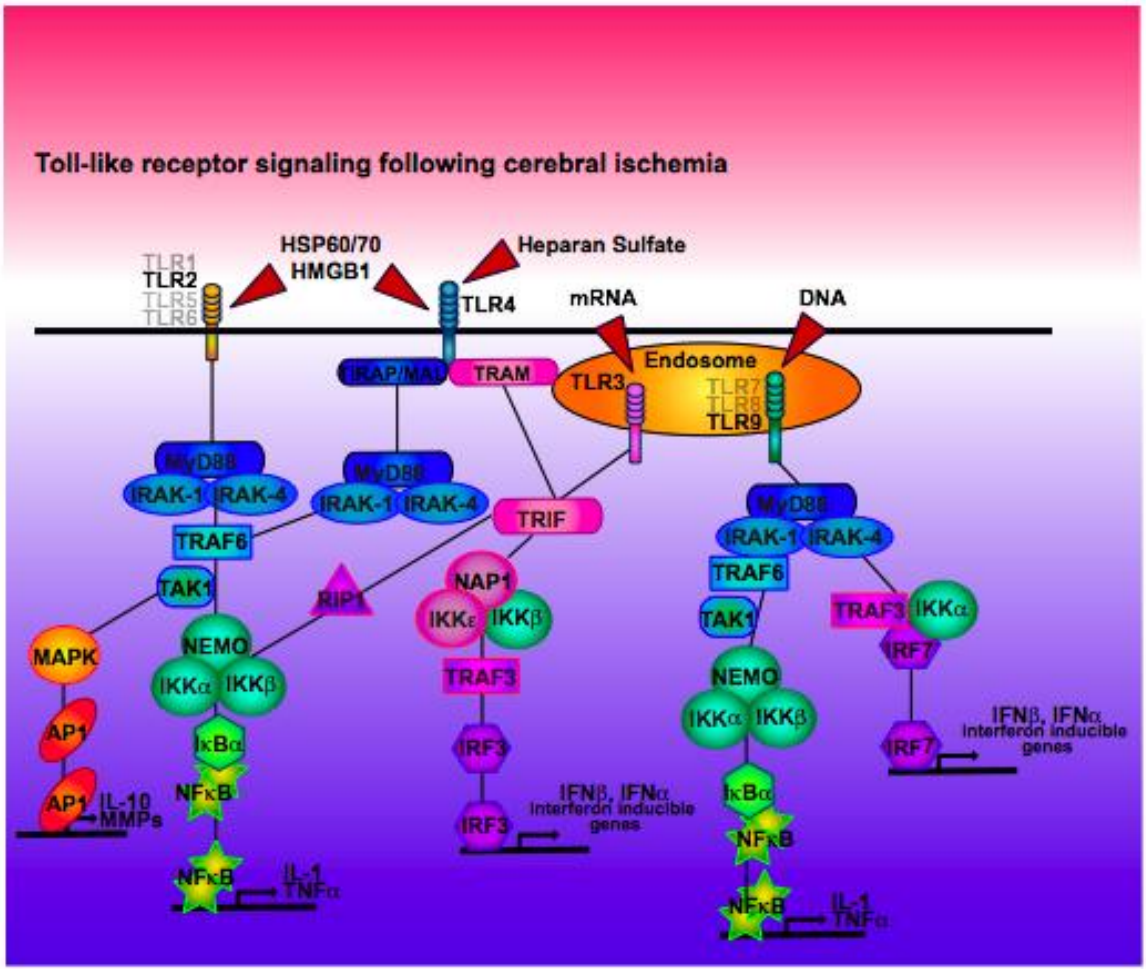

Figure 1.2. Schematic of Toll-like receptor signaling following cerebral ischemia

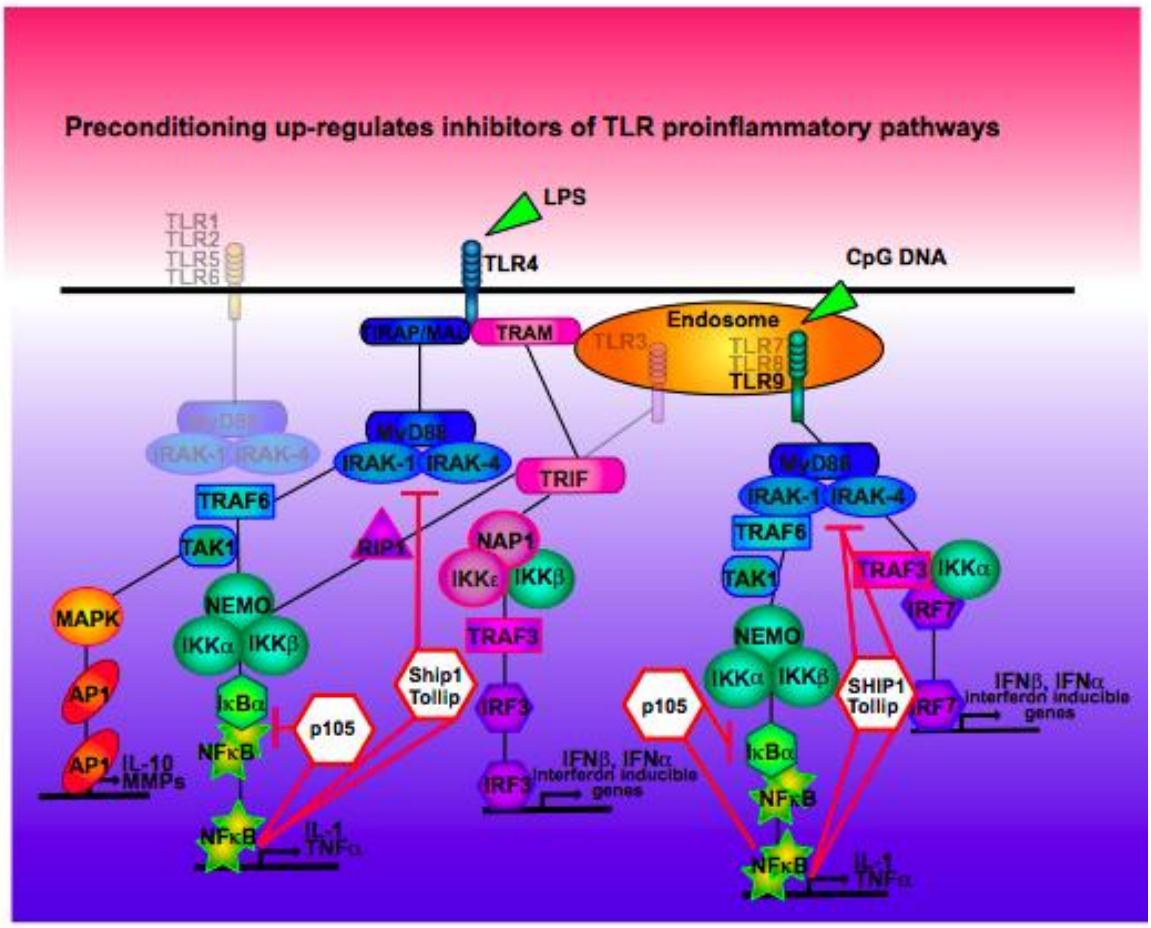

Figure 1.3. Schematic of Toll-like receptor signaling in response to preconditioning stimulation with TLR ligands

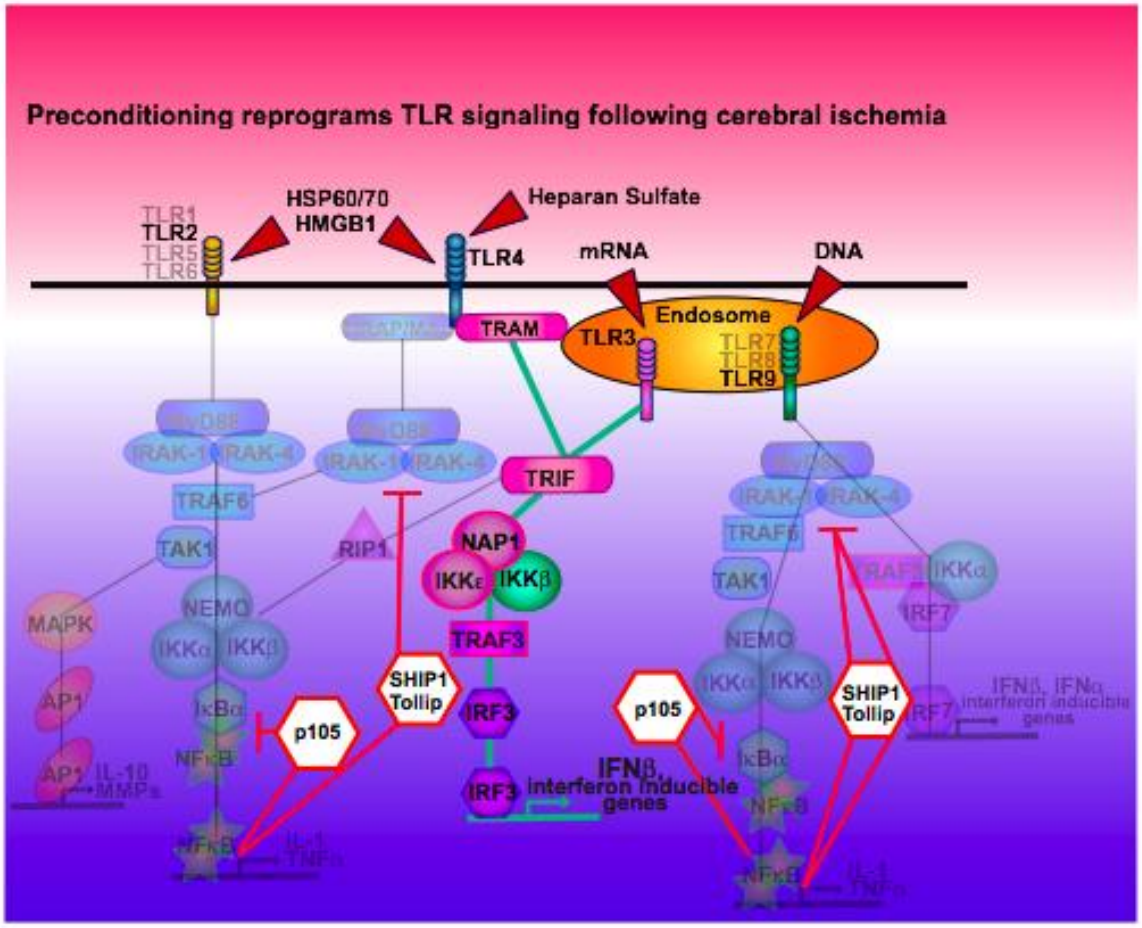

Figure 1.4. Schematic of Toll-like receptor signaling following cerebral ischemia in the context of LPS preconditioning

Materials and Methods

Mice: C57Bl/6 mice (male, 8 to 10 weeks approximately 25 grams) were obtained from Jackson Laboratories (West Sacramento, California, USA) and from the National Cancer Institute (Frederick, MD). IFNβ knockout mice were kindly provided by Dr. Leanderson of Lund University. IRF3 knockout mice were procured from RIKEN BioResource Center, Tsukuba, Japan. Both strains were backcrossed onto the C57Bl/6 background for at least 8 generations. TLR4 knockout mice (C57Bl/10ScSn) and their wild-type counterparts (C57Bl/10ScN), TLR3 knockout mice (B6;129S1-Tlr3^{tm1Flv}/J) and their wild type counterparts (B6;129SF1/J) and TRIF knockout mice (C57Bl/6J-Ticam1^{LPS2}/J) were obtained from Jackson Laboratories. TNFα knock-out mice (B6.129S-Tnf^{tm1Gkl}/J), were also obtained from Jackson Laboratories. This strain is backcrossed at least 5 generations to C57Bl/6 at Jackson Laboratories. MyD88 knockout mice (C57Bl/6 background) were a kind gift of Dr. Shizuo Akira (Osaka University, Osaka Japan) and were bred in our facility. All mice are housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

Drug Treatment: Mice were given a 200 ul intraperitoneal injection of saline, LPS (0.2 - 1.0 mg/kg; *Escherichia coli* serotype *055:B5*; Sigma) or CpG ODN (0.5 - 1.6mg/kg; tcc atg acg ttc ctg acg tt; Invivogen). For all studies except those that determined the time

window of protection, mice were injected 72 hr prior to MCAO. For the time window of protection, mice were treated from 1-14 days prior to MCAO.

Reagents: ODN2088 (tcc tgg cgg gga agt), a mouse-specific TLR9 signaling inhibitor [112, 113], were obtained from Invivogen. In addition, endotoxin levels were determined to be negligible (<0.125EU/mg). A control ODN (Invivogen; tcc atg agc ttc ctg agc tt) was used which contained the same sequence as 1826 but the CpG dinucleotides have been replaced by GpC dinucleotides. Western blots were performed using antibodies against IRAK-M (ProSci, 2355), MyD88 (R&D, AF3109), Ship-1 (Santa Cruz, sc8425), Tollip (AbCam, Ab37155), and Actin beta (Santa Cruz, sc1616R).

Oxygen glucose deprivation in vitro: Primary mouse mixed cortical cultures were prepared from E15-E17 mouse fetuses. Cortices were dissected and dissociated with Trypsin-EDTA (Gibco) and plated at a density of 1×10^6 cells/ml onto coverslips coated with poly-L-Ornithine (15mg/L). Cells were cultured in Neurobasal media (containing 4.5g/L glucose; supplemented with Glutamax and B27-AO; Gibco) for 5 days prior to each experiment. Cultures consisted of ~60% neurons (range 53-66%) as determined by staining for NeuN (Chemicon), with less than 5% astrocytes ($GFAP^+$; Sigma) and less than 5% microglia (tomato $lectin^+$; Vector Labs). Oxygen-glucose-deprivation (OGD) was performed by removal of the culture medium and replacement with D-PBS (Gibco) followed by incubation in an anaerobic atmosphere of 85% N_2, 10% CO_2, 5% H_2 at 37°C for 3 h. The anaerobic conditions within the chamber were monitored using an electronic oxygen/hydrogen analyzer (Coy Laboratories). OGD was terminated by replacement of the exposure medium with Neurobasal medium (containing 4.5g/L glucose;

supplemented with Glutamax and B27-AO) and return of the cells to a normoxic incubator. Control plates were kept in the normoxic incubator during the OGD interval.

Cell Death Evaluation in vitro: Cell death *in vitro* was examined 24 hr following OGD by means of fluorescent, cell-permeable, DNA-binding dyes: propidium iodide (PI), as an indicator of cell death, and 4',6-diamidino-2-phenylindole (DAPI), as an indicator of the total number of cells. Coverslips were incubated with PI (1.5ug/ml, Sigma) for 2 min, washed with PBS and fixed for 30 min in 10% formalin. Coverslips are mounted on slides with Vectashield mounting medium containing DAPI (Vector labs). Stained cells were visualized with a fluorescent microscope (Leica GMBH) and analyzed using Metmorph7 software (Molecular Devices Corp., Downington, PA). The number of PI and DAPI stained cells were counted in two random fields of view on each coverslip, and percent death was calculated as mean (PI)/(DAPI) x 100 per field of view. Each treatment was performed with triplicate coverslips within an experiment and the entire experiment was repeated three or more times.

Surgery: Cerebral focal ischemia was induced by MCAO as published previously [114]. Mice were briefly induced with 3% isoflurane and maintained with 1.5-2% throughout the surgery. The middle cerebral artery (MCA) was blocked by threading silicone-coated 8-0 monofilament nylon surgical suture through the external carotid to the internal carotid, and finally blocking its bifurcation into the MCA and anterior cerebral artery. The filament was maintained for 60 min (unless otherwise noted) while the mice were maintained under anesthesia. The filament was removed, and blood flow restored. Cerebral blood flow was monitored with Laser Doppler Flowmetry (Transonic System

Inc.). Temperature was maintained at 37°C±0.5°C with a rectal thermometer-controlled heating pad and lamp (Harvard Apparatus). All surgical procedures were performed under an operating stereomicroscope. After surgery mice were kept alive for 24 hr on a heating pad with access to soft food and water and were then sacrificed. We consistently have a survival rate for the MCAO procedure that exceeds 85%.

Infarct Measurement: Mice were deeply anesthetized with isoflurane, then perfused with ice-cold saline containing 2U/mL heparin. Brains were removed rapidly, placed on a tissue slicer and covered with agarose (1.5%). The olfactory bulbs were removed and the remainder of the brain was sectioned into 1-mm slices beginning from the rostral end, for a total of 7 slices. The area of infarction was visualized by incubating the sections in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich) in PBS for 15 min at 37^{o}C. The sections were then transferred to 10% formalin (Sigma Aldrich). Images of the sections were scanned, and the hemispheres and areas of infarct were measured using ImageJ software [115]. The measurements were multiplied by the section thickness and summed over the entire brain to yield volume measurements. Ischemic damage data was calculated using the indirect method to minimize error introduced from edema. % Infarct = (contralateral hemisphere volume – volume of non-infarcted tissue of the ipsilateral hemisphere)/(contralateral hemisphere volume) x 100 [116].

Quantification of Serum TNFα: Blood was taken from mice (cardiac puncture) and allowed to clot for 2 hr at room temperature. The blood was centrifuged (2000 rpm, 20 min) and the clear serum was removed and stored at –80^{o}C until analyzed. Serum TNFα

was measured using an ELISA available commercially from R&D Systems (Minneapolis, MN, USA). The assay sensitivity is ~5.1 pg/ml. All samples were run in duplicate.

RNA isolation: Mice were anesthetized and blood was obtained via retro-orbital puncture. Animals were perfused with saline and, under RNase-free conditions, a 1 mm section was removed for infarct area analysis. The ipsilateral cortex region from the frontal 4 mm was snap frozen. Total RNA was isolated from the blood using the Qiagen PAXgene Blood RNA Kit and from the brain using the Qiagen RNeasy Lipid Mini Kit (Qiagen Inc.). RNA from individual animals was hybridized to single arrays.

Experimental Design for Gene Expression Studies: C57/BL6 mice were divided into 6 groups with 4 animals per group: Groups 1 and 2 received a saline injection followed 72 hr later with a 45 min MCAO. Group 3, and 4 received an LPS injection followed 72 hr later with a 45 min MCAO. Groups 5 and 6 received CpG injection followed 72 hr later with a 45 min MCAO. Groups 1, 3, and 5 were sacrificed 3 hr following start of occlusion. Groups 2, 4, and 6 were sacrificed 24 hr following start of occlusion. At time of sacrifice mice were anesthetized, then perfused with heparinized saline. A group of 6 mice were included as unhandled controls. Under RNase-free conditions, a 1 mm section was removed (4 mm from rostral end) for infarct area analysis by TTC staining. The ipsilateral cortex region from the frontal 4 mm was isolated and snap frozen in liquid nitrogen.

GeneChip Expression Analyses: Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health & Sciences University Gene Microarray Shared

Resource. RNA samples were labeled using the NuGEN Ovation Biotin RNA Amplification and Labeling System_V1. Hybridization was performed as described in the Affymetrix technical manual (Affymetrix, Santa Clara, CA). Labeled RNA was hybridized to test arrays containing control probe sets and samples that did not meet empirically defined cutoffs within the core facility were remade. Quality-tested samples were hybridized to the MOE430 2.0 array. The array image was processed with Affymetrix GeneChip Operating Software (GCOS). Data was normalized using the Robust Multichip Average method [117]. The normalized data was then analyzed using a two-way ANOVA model for each gene, using conditions and time as groups. Post hoc comparisons were made using the unhandled mice as a control group. P-values were adjusted for multiple comparisons using the method of Hochberg and Benjamini [118]. Genes were considered significantly regulated if the adjusted p value was less than 0.05 and the fold change in regulation was greater than or equal to 2.

Transcriptional regulatory network analysis. Using the web based program: Promoter Analysis and Interaction Network Toolset (PAINT) version 3.5 [119], we examined the predicted regulatory elements associated with the unique gene regulation identified by microarray. In brief, using PAINT we obtained the 5000 bp upstream sequence for the transcripts represented on the MOE430 Affymetrix gene chip (33,635 transcripts were identified with 5000 bp of upstream sequence). PAINT identified putative transcription factor binding sequences (TREs) in these upstream sequences using the TRANSFAC PRO database version 10.4. This pool of genes and identified TREs was used as our reference comparison group. The statistical component of PAINT (FDR adjusted p value

set at $\leq$0.2) was used to determine the over represented TREs in individual gene clusters compared to the reference comparison group (i.e. uniquely expressed genes in LPS preconditioned mice compared to 33,635 member reference group).

Intracerebral Ventricular Injection of IFNβ during MCAO. rmIFNβ (Cell Sciences, Canton, MA) or vehicle (saline) was injected into the left lateral ventricle as previously described [120]. Injections (1ul) of either rmIFNβ (200U) or saline were administered immediately before and after surgery (60 min MCAO). Infarct volume was measured 24 hr following stroke.

Quantitative Real-time PCR for IFN-b. RNA was treated with DNase and transcribed into cDNA using the Omniscript RT Kit (Qiagen). Real-time PCR (RT-PCR) reactions were performed in a volume of 25 ul using TaqMan PCR Master Mix (Applied Biosystems). For IFNβ TaqMan Gene Expression Assay Mix for mouse IFNβ was used (ABI # Mm00439546_S1). Primers and probe for β-Actin were obtained from Integrated DNA Technologies: forward: 5'-AGAGGGAAATCGTGCGTGAC-3'; reverse: 5'-CAATAGTGATGACCTGGCCGT-3'; probe: CACTGCCGCATCCTCTTCCTCCC. Samples were run on an ABI-prism 7700 (Applied Biosystems, Foster City, CA). Results were analyzed using ABI sequence detection software. The relative quantification of IFNβ was determined using the comparative CT method (2^{-DDC_T}) described in ABI User Bulletin #2. Results were normalized to b-actin and presented relative to unhandled mice. All reactions were performed in triplicate.

Electrophoretic Mobility Shift Assay: Nuclear protein extracts were prepared from tissue dissected from the ipsilateral cortex. Homogenized tissue was incubated in Buffer A (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF) for 5 minutes on ice, centrifuged at 3000 rpm for 5 minutes at 4°C, and the supernatant saved as cytoplasmic extract. Pellets were washed once in Buffer B (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 0.5%NP-40, 1mM Dtt, 1mM PMSF), then resuspended in Buffer C(250mM Tris pH7.8, 60mM KCl, 1mM DTT, 1mM PMSF), and freeze-thawed 3 times in liquid nitrogen. All buffers contained a protease inhibitor cocktail (Roche). After cenTRIFuging at 10,000 rpm for 10 min at 4°C, supernatant was saved as nuclear extract. Nuclear protein concentrations were determined using the BCA method (Pierce-Endogen). Electrophoretic mobility shift assays were performed using the Promega Gel Shift Assay System according to the manufacturer's instructions. Briefly, 15ug of nuclear protein was incubated with ^{32}P-labeled NFκB consensus oligonucleotide (Promega), either with or without unlabeled competitor oligonucleotide, unlabeled non-competitor oligonucleotide, or anti-p65 antibody (Santa Cruz). Samples were electrophoresed on a 4% acrylamide gel, dried and exposed to phosphorimager overnight. The densitometry of the gel bands was analyzed using scanning integrated optical density software (ImageJ).

Western Blotting: Protein extraction was performed as described previously [120] with some modifications. Briefly, tissue samples were dissected from the ipsilateral cortex and lysed in a buffer containing a protease inhibitor cocktail (Roche). Protein concentration were determined using the BCA method (Pierce-Endogen). Protein samples (50ug) were denatured in a gel-loading buffer (Bio-Rad Laboratories) at 100°C for 5 min and then

loaded onto 12% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories). Following electrophoresis, proteins were transferred to polyvinylodene difluoride membranes (Bio-Rad Laboratories) and incubated with primary antibodies at 4°C overnight. Membranes were then incubated with anti-rabbit, anti-goat, or anti-mouse IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and detected by chemiluminescence (NEN Life Science Products) and exposure to Kodak film (Biomax). Images were captured using an Epson scanner and the densitometry of the gel bands, including actin-beta as a loading control, was analyzed using ImageJ.

Statistical Analysis: All statistical analyses were performed using Prism (Graphpad). Mean differences were analyzed using Students T test, or one-way or two-way ANOVA with Bonferroni's post hoc test. Data are represented as mean $\pm$ standard error of the mean (SEM) and differences were considered statistically significant when $p<0.05$.

Chapter 2- Manuscript #1

Toll-like Receptor 9: A New Target of Ischemic Preconditioning in the Brain

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Chapter 2 is a manuscript as it appears in the original paper published in the Journal of Cerebral Blood Flow and Metabolism [1].

Abstract

Preconditioning with the toll-like receptor 4 (TLR4) ligand, lipopolysaccharide, provides neuroprotection against subsequent cerebral ischemic brain injury, through a TNFα dependent process. Here we report the first evidence that another TLR, TLR9, can induce neuroprotection. We show that the TLR9 ligand (CpG ODN) can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury. Our studies show that systemic administration of CpG ODN 1826 in advance of brain ischemia (middle cerebral artery occlusion; MCAO) reduces ischemic damage up to 60% in a dose and time dependent manner. We also offer evidence that CpG ODN preconditioning can provide direct protection to CNS cells as we have found marked neuroprotection in modeled ischemia *in vitro*. Finally, we show that CpG preconditioning significantly increases serum TNFα levels prior to MCAO and show that TNFα is required for subsequent reduction in damage, as mice lacking TNFα are not protected against ischemic injury by CpG preconditioning. Our studies demonstrate that preconditioning with a TLR9 ligand, induces neuroprotection against ischemic injury through a mechanism that shares common elements with LPS preconditioning via TLR4.

Introduction

Toll-like receptors (TLRs) are a family of pattern recognition receptors involved in the identification of, and response to foreign pathogens. To date at least 11 TLRs have been identified in mammals, and each recognize different pathogen-associated molecular patterns (PAMPs). Although the stimuli are different, the resultant signaling cascades are all mediated through toll/interleukin-1 receptor (TIR) domain containing adapters, the most prominent being MyD88, with subsequent activation of NFκB. TLRs are broadly distributed on immune cells and thus play an important role in initiation of innate and adaptive immune responses.

TLR4 was the first TLR identified in mammals and is the most widely studied of the TLRs. Endotoxin (lipopolysaccharide; LPS), a cell surface component of gram-negative bacteria, binds to TLR4 and at high levels can cause death through septic shock. An interesting counter-point is that low concentrations of LPS actually induce a protective state against a subsequent lethal dose of LPS reviewed in [121]. This phenomenon, referred to as endotoxin tolerance, has been studied for over 50 years, yet the molecular mechanisms are incompletely understood. It is known, however, that many pro-inflammatory cytokines and cytotoxic mediators that are normally elicited by LPS, fail to be induced by a second exposure to LPS. Instead, new signaling proteins and anti-inflammatory pathways are increased in the setting of endotoxin tolerance reviewed in [121, 122].

More recently other TLRs have been shown to induce protection against a subsequent challenge with the same ligand, a state commonly referred to as self-tolerance. Priming of TLR2, TLR5 or TLR9 with their respective ligands induces a state of hypo-responsiveness to a subsequent challenge with their corresponding ligands [94, 123-125]. This shared phenomenon is expected given the similarities in signaling pathways of the TLR family members. Interestingly, cross-tolerance (or hetero-tolerance) between two differing receptors has also been reported, as ligands for TLR2 and TLR9 induce tolerance against a subsequent challenge with LPS [94, 124-126].

However, hetero-tolerance is not induced with all combinations of TLRs and differences in ability to induce hetero-tolerance have been reported depending on the model [94, 127]. For example, Dalpke and colleagues, although able to show hetero-tolerance between TLR2, TLR4 and TLR9 in a macrophage cell line, and a similar hetero-tolerance with TLR2 and TLR4 in an *in vivo* paradigm, failed to induce hetero-tolerance with TLR9 *in vivo*. In fact, pretreatment with unmethylated CpG oligodeoxyneucleotides (ODNs), the ligand for TLR9, actually enhanced TNFα production *in vivo* in response to LPS and LTA, making the system hyper-responsive [94]. These inconsistencies suggest a complicated interplay between the varying TLR signaling pathways that is more complex than a simple feedback suppression of inflammatory signals within a cell.

In addition to the phenomenon of cross-tolerance that exists among the different TLRs, tolerance against ischemic injury can be induced by LPS in various organs such as heart, brain and kidney [79, 128, 129]. Although the mechanism of protection in these models is even

less well understood, the paradigm appears similar in that a small inflammatory response is initiated that mitigates the subsequent damaging inflammatory response associated with the secondary stimuli. In the case of brain ischemia, a systemic low dose of LPS delivered, at least 1day but not longer than 7 days, prior to stroke reduces the ischemic injury [79, 80, 85, 130]. A critical role for TNFα has been shown by us [82] and others [79] wherein LPS-induced tolerance to ischemia fails to occur in the absence of TNFα.

Similarities among the known TLR signaling pathways and their shared ability to induce hetero-tolerance between certain members of the TLR family has lead us to hypothesize that other TLR ligands may also provide neuroprotection against ischemic brain injury. Further, we postulated that TNFα may play a central role in conferring protection. To test our hypothesis we examined the protective potential of CpG ODN 1826, a mouse specific TLR9 ligand. We chose to examine TLR9 because, similar to TLR4, it is coupled to the signaling adapter, MyD88. In addition, activation of TLR9 by CpG ODNs increases serum TNFα levels in mice within 6 hrs of administration [131, 132]. TLR4 and TLR9 display a similar cell type distribution as both are expressed by multiple systemic immune cell types [69, 133], and on cells of the central nervous system [38, 39, 71]. CpG ODNs are currently approved for human trials as vaccines and cancer therapies reviewed in [134], which makes them particularly well-suited for therapeutic development for use in stroke neuroprotection. Here we report that ligand activation of TLR9 induces neuronal protection against brain ischemia. We show that neuroprotection is time and dose dependent. In addition, we report that TNFα plays an essential role in CpG ODN-induced ischemic tolerance, just as it does in LPS-induced tolerance to ischemic brain injury.

These data are the first to indicate that TLR9 is a target for the induction of tolerance against ischemic injury in the brain.

Materials and Methods

Mice: C57Bl/6 mice (male, 8 to 10 weeks) were obtained from Jackson Laboratories (West Sacramento, California, USA). TNFα knock-out mice (B6.129S-Tnf^{tm1Gkl}/J), were also obtained from Jackson Laboratories. This strain is backcrossed at least 5 generations to C57Bl/6 at Jackson Laboratories. All mice are housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

Reagents: ODN1826 (tcc atg acg ttc ctg acg tt), a mouse-specific phosphothioate CpG-ODN ligand for TLR9, and ODN2088 (tcc tgg cgg gga agt), a mouse-specific TLR9 signaling inhibitor [112, 113], were obtained from Invivogen. Invivogen has confirmed the specificity of ODN1826 for mouse TLR9 by testing against cells transfected with the other TLR family members (personal communication). In addition, endotoxin levels were determined to be negligible (<0.125EU/mg). A control ODN (Invivogen; tcc atg agc ttc ctg agc tt) was used which contained the same sequence as 1826 but the CpG dinucleotides have been replaced by GpC dinucleotides (shown in bold). Therefore, it does not stimulate TLR9.

Oxygen glucose deprivation in vitro: Primary mouse mixed cortical cultures were prepared from E15-E17 mouse fetuses. Cortices were dissected and dissociated with Trypsin-EDTA (Gibco) and plated at a density of 1 x 10^6 cells/ml onto coverslips coated with poly-L-Ornithine (15mg/L). Cells were cultured in Neurobasal media (containing 4.5g/L glucose; supplemented with Glutamax and B27-AO; Gibco) for 5 days prior to each experiment. Cultures consisted of ~60% neurons (range 53-66%) as determined by staining for NeuN (Chemicon), with less than 5% astrocytes ($GFAP^+$; Sigma) and less than 5% microglia (tomato $lectin^+$; Vector Labs). Oxygen-glucose-deprivation (OGD) was performed by removal of the culture medium and replacement with D-PBS (Gibco) followed by incubation in an anaerobic atmosphere of 85% N_2, 10% CO_2, 5% H_2 at 37°C for 3 h. The anaerobic conditions within the chamber were monitored using an electronic oxygen/hydrogen analyzer (Coy Laboratories). OGD was terminated by replacement of the exposure medium with Neurobasal medium (containing 4.5g/L glucose; supplemented with Glutamax and B27-AO) and return of the cells to a normoxic incubator. Control plates were kept in the normoxic incubator during the OGD interval.

Cell Death Evaluation in vitro: Cell death *in vitro* was examined 24 hr following OGD by means of fluorescent, cell-permeable, DNA-binding dyes: propidium iodide (PI), as an indicator of cell death, and 4',6-diamidino-2-phenylindole (DAPI), as an indicator of the total number of cells. Coverslips were incubated with PI (1.5ug/ml, Sigma) for 2 min, washed with PBS and fixed for 30 min in 10% formalin. Coverslips are mounted on slides with Vectashield mounting medium containing DAPI (Vector labs). Stained cells

were visualized with a fluorescent microscope (Leica GMBH) and analyzed using Metmorph7 software (Molecular Devices Corp., Downington, PA). The number of PI and DAPI stained cells were counted in two random fields of view on each coverslip, and percent death was calculated as mean (PI)/(DAPI) x 100 per field of view. Each treatment was performed with triplicate coverslips within an experiment and the entire experiment was repeated three or more times.

Drug treatments: CpG ODN 1826 and the saline vehicle were administered by intraperitoneal (i.p.) injection in a volume of 200ul. For the dose response studies mice were injected 72 hr prior middle cerebral artery occlusion (MCAO). For the time window of protection mice were treated from 1-14 days prior to MCAO.

Surgery: Cerebral focal ischemia was induced by MCAO as published previously [114]. Mice were briefly induced with 3% isoflurane and maintained with 1.5-2% throughout the surgery. The middle cerebral artery (MCA) was blocked by threading silicone-coated 8-0 monofilament nylon surgical suture through the external carotid to the internal carotid, and finally blocking its bifurcation into the MCA and anterior cerebral artery. The filament was maintained for 60 min (unless otherwise noted) while the mice were maintained under anesthesia. The filament was removed, and blood flow restored. Cerebral blood flow was monitored with Laser Doppler Flowmetry (Transonic System Inc.). Temperature was maintained at 37°C±0.5°C with a rectal thermometer-controlled heating pad and lamp (Harvard Apparatus). All surgical procedures were performed under an operating stereomicroscope. After surgery mice were kept alive for 24 hr on a

heating pad with access to soft food and water and were then sacrificed. We consistently have a survival rate for the MCAO procedure that exceeds 85%.

Infarct Measurement: Mice were deeply anesthetized with isoflurane, then perfused with ice-cold saline containing 2U/mL heparin. Brains were removed rapidly, placed on a tissue slicer and covered with agarose (1.5%). The olfactory bulbs were removed and the remainder of the brain was sectioned into 1-mm slices beginning from the rostral end, for a total of 7 slices. The area of infarction was visualized by incubating the sections in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich) in PBS for 15 min at 37^{o}C. The sections were then transferred to 10% formalin (Sigma Aldrich). Images of the sections were scanned, and the hemispheres and areas of infarct were measured using ImageJ software [115]. The measurements were multiplied by the section thickness and summed over the entire brain to yield volume measurements. Ischemic damage data was calculated using the indirect method to minimize error introduced from edema. % Infarct = (contralateral hemisphere volume – volume of non-infarcted tissue of the ipsilateral hemisphere)/(contralateral hemisphere volume) x 100 [116].

Quantification of Serum TNFα: Blood was taken from mice (cardiac puncture) and allowed to clot for 2 hr at room temperature. The blood was centrifuged (2000 rpm, 20 min) and the clear serum was removed and stored at –80^{o}C until analyzed. Serum TNFα was measured using an ELISA available commercially from R&D Systems (Minneapolis, MN, USA). The assay sensitivity is ~5.1 pg/ml. All samples were run in duplicate.

Statistical Analysis: All statistical analyses were performed using Prism (Graphpad). Mean differences were analyzed using one-way ANOVA with Bonferroni's post hoc test. Data are represented as mean ± standard error of the mean (SEM) and differences were considered statistically significant when $p<0.05$.

Results

CpG ODN preconditions against ischemia-induced neuronal cell death in vitro.

We tested whether the TLR9 ligand, CpG ODN, would induce tolerance to ischemic cell death in an *in vitro* model of ischemia, oxygen-glucose-deprivation (OGD). Mouse mixed cortical cultures subjected to 3 hr of OGD showed 56% cell death compared to untreated control cultures. Cultures preconditioned by exposure to CpG ODN 1826 (0.5–5.0 ug/ml) for 24 hr prior to OGD showed a dose-dependent reduction in cell death (Figure 2.1A). A dose of 1ug/ml CpG ODN produced maximal protection, which resulted in a 60% reduction in cell death following exposure to OGD. No significant cell death was detected in cultures treated with CpG ODN alone (Figure 2.1A). Our findings show that pretreatment with CpG ODN provides significant protection from cell death induced by exposure to modeled ischemia (OGD) and suggests that TLR9 is a new target for preconditioning against ischemic neuronal injury.

We used a TLR9 specific antagonist (ODN 2088), to confirm that protection was induced specifically via TLR9 signaling [112, 113]. Mixed cortical cultures were preconditioned with CpG ODN 1826 (1ug/ml) in the presence or absence of the TLR9 antagonist, ODN 2088

(2 or 5 ug/ml) for 24 hr prior to OGD (3 hr). ODN 2088 abolished the protective effect of CpG 1826 at both doses, but failed to inhibit the protective effect of LPS (1ug/ml; Sigma L-2880), a TLR4 agonist that we have shown induces tolerance to ischemia in vitro [82] (Figure 2.1B). These data, suggest that CpG 1826 signals through TLR9 to induce protection against OGD.

Preconditioning with CpG reduces ischemic damage in an *in vivo* model of stroke.

We next examined the protective potential of CpG ODN treatment in a mouse model of stroke. Mice were preconditioned with varying doses of CpG ODN 1826 (5-40 ug; i.p.) 72 hr prior to MCAO. Twenty-four hours following MCAO mice were sacrificed and infarct damage was determined. Pretreatment with CpG ODN reduced the infarct size significantly at doses of 20 and 40ug (56.5 and 57.5% reduction respectively; Figure 2.2). Thus, CpG ODN delivered systemically to mice preconditions the brain in a dose-dependent manner leading to marked tolerance to ischemic brain injury. To confirm that the protection was specific to the CpG ODN, we tested a control ODN that contained the same sequence as 1826 but the CpG dinucleotides were replaced by GpC dinucleotides. No significant protection was observed in mice preconditioned with 20 ug of the control ODN (data not shown). The reduction in infarct size reported here at 24 hr post MCAO remains evident in mice sacrificed 72 hr post MCAO (unpublished observation).

Preconditioning time window of CpG induced neuroprotection.

LPS preconditioning induces ischemic neuroprotection in the brain by 1 day—an effect that last for at least 7 days but is lost by 14 days following treatment [82]. To determine the time window of

neuroprotection induced by CpG ODN 1826, we administered CpG ODNs (20 ug; i.p.) 1, 3, 7 and 14 days prior to MCAO. We found pretreatment with CpG ODNs induced significant neuroprotection within 1 day (46% reduction in infarct volume)— a neuroprotective effect that remains evident at 3 days (61% reduction) but diminished by 7 days. By 14 days post administration, the protective effect is completely abolished in the CpG treated mice (Figure 2.3). Thus, CpG preconditioning provides a time window of neuroprotection that lasts ~ 1 week and is lost by 2 weeks following administration. This time window of protection mirrors that seen with systemic administration of LPS.

TNFα is required for CpG induced neuroprotection.

Previous work in the model of LPS preconditioning against brain ischemia has demonstrated that the presence of TNFα plays an essential role in conferring neuroprotection [79, 82]. We postulated the CpG ODN preconditioning via TLR9 may have a similar requirement for the presence of TNFα. We first tested whether CpG ODN administration increased serum levels of TNFα. We found significant increases in TNFα levels in the serum as early as 1 hr post-injection (400 pg/ml compared to vehicle treated mice which were below the level of detection; Figure 2.4). The timing of the increase in TNFα was similar to what we have previously reported with LPS preconditioning [82], however the magnitude is significantly less than that observed for preconditioning levels of LPS (3092 pg/ml). As with LPS, the response diminishes quickly and returns to baseline by 72 hr.

We investigated whether TNFα is required for CpG preconditioning by testing the neuroprotective effect of CpG ODN treatment in TNFα knock-out mice (B6.129S-Tnf^{tm1Gkl}/J; $TNF\alpha^{-/-}$). We administered CpG ODN 1826 to $TNF\alpha^{-/-}$ and control mice (C57Bl/6; $TNF\alpha^{+/+}$) 72 hr prior to 40 min MCAO. Control mice pretreated with CpG showed a significant reduction in ischemic injury (46% reduction) as expected. In contrast, $TNF\alpha^{-/-}$ mice pretreated with CpG did not demonstrate any reduction in infarct size (saline treated = 31.9±7.7% vs. CpG treated = 29.3±6.8%; Figure 2.5). These data suggest that TNFα plays an essential role in mediating CpG-induced neuroprotection against ischemic injury.

Discussion

We report the first evidence that a TLR9 ligand (CpG ODN) can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury. This finding indicates that TLR9, in addition to TLR4, can induce preconditioning in the brain. Our studies show that systemic administration of CpG ODN 1826 in advance of brain ischemia reduces ischemic damage in a dose and time dependent manner. We offer evidence that CpG ODN preconditioning can provide direct protection to CNS cells as we have found marked neuroprotection in modeled ischemia *in vitro*. In addition, using our *in vitro* model we show that CpG ODN specifically acts through TLR9 to induce neuroprotection. Finally, our studies support a critical role for TNFα in CpG-induced neuroprotection. This latter observation suggests that the mechanism of neuroprotection between LPS and CpG preconditioning share common elements.

Previous studies have demonstrated that LPS, acting through TLR4 can induce cross-tolerance and provide neuroprotection against ischemic injury in the brain. We have posited that heterologous tolerance induction against brain ischemia extends beyond TLR4 to other TLRs. We demonstrate here that cross-tolerance by TLR4 is not unique to this particular TLR as we show that a TLR9 agonist, CpG ODN, also induces cross-tolerance against an ischemic insult.

It is known that TLR4 couples to both MyD88 and TRIF dependent pathways and that the MyD88 cascade culminates in NFκB-mediated induction of pro-inflammatory cytokines (i.e. TNFα, IL6, IL1b). TNFα, which is required for TLR4 induced tolerance against ischemic injury [79, 82], may be induced via the MyD88 cascade. We hypothesize that signaling through TLR9 would also induce tolerance via a MyD88 dependent mechanism as TLR9 signals exclusively through MyD88 with no evidence for TRIF dependent signaling [135, 136]. Studies to explore this possibility are in progress in our laboratory and should provide important information broadly regarding the molecular mechanisms underlying tolerance to injury.

Thus far, the known ligands for TLR9 include bacterial DNA containing unmethylated CpG motifs, certain double-stranded DNA viruses and synthetic CpG ODNs such as CpG-ODN 1826 used in the studies described here [137-139]. Synthetic CpG ODNs have been shown to confer protection to mice against subsequent challenge from a variety of bacteria, viruses, parasites and prions [140]. Protection against pathogen challenge typically occurred within 48 hr and lasted for several weeks [141, 142]—such a time window is similar

to our findings with CpG-ODN induced ischemic tolerance shown here and our recent report of LPS-induced ischemic tolerance to stroke injury [82].

The data presented here provides the first evidence that in addition to tolerance (protection) against foreign pathogens, CpG ODN administration protects against a stimulus that is unrelated to a foreign pathogen, namely ischemic injury. CpG ODN-induced neuroprotection occurs following systemic administration in a mouse model of stroke. TNFα appears to be critical to the induction of ischemic tolerance by CpG preconditioning as we show here that CpG ODN administration fails to protect TNFα deficient mice against ischemic brain injury. In wild type mice, systemic administration of CpG increased TNFα in the plasma, which suggests that the actions of TNF may occur in the periphery. Further support for this lies in the observation by Nawashiro et al that systemic administration of TNFα itself preconditions against stroke injury [143]. In addition, LPS preconditioning against ischemic brain injury is abolished by systemic blockade of TNFα using TNF-binding protein [79]. Thus, CpG ODN preconditioning *in vivo* very likely provides neuroprotection through a TNFα dependent mechanism similar to that seen with LPS preconditioning.

We also report that CpG induces neuroprotection when directly applied to mixed cortical cultures *in vitro* which are subsequently subjected to oxygen-glucose deprivation. The mechanism of this more direct route of CpG interaction in the CNS is still unclear. These cultures contain neurons, astrocytes and microglia. Until very recently, astrocytes and microglia, but not neurons were known to express TLRs, including TLR9 [38, 144]. Neurons

have also been reported to express TLRs, although the extent to which they are targets of cell signaling is not yet clear. [71]. Thus, CpG treatment may modulate the cytokine response to injury in glial cells which in turn may have a protective effect on neurons indirectly or through more direct modulation by activation of TLR9 on neurons leading to altered neuronal signaling in the setting of injury. Whether TNFα has a critical role in CpG-induced tolerance *in vitro* is of interest and as such, the subject of future studies in our laboratory.

We have recently reported genomic evidence that preconditioning via LPS activation of TLR4 produces a tolerant state in the brain via inflammatory mediators that are neuroprotective (e.g. Type I interferons) [145]. In addition, in the setting of LPS-induced tolerance, there is a marked absence of deleterious inflammatory mediators (IL-6, MIP1a, TRAF6) generally found in ischemic brain injury. It is possible that these particular features of neuroprotection (suppressed proinflammatory mediators/increased neuroprotective cytokines) are common to preconditioning stimuli that act through TLRs.

The demonstration that ischemic tolerance in the brain occurs through TLR9, in addition to TLR4, raises the possibility that this is a conserved feature of all TLRs. Recognition that TLR9 is a new target for preconditioning broadens the range of potential antecedent therapies for brain ischemia, such as in the setting of coronary artery bypass grafting (~300,000 procedures annually) where patients are at risk of cerebral morbidity reviewed in [146]. Phase II clinical trials are already in progress with CpG ODNs for use in adjuvant and anti-cancer therapies reviewed in [134]. Thus, CpG ODNs may offer great translational promise as a prophylactic treatment against cerebral morbidity for ‘at risk’ patients.

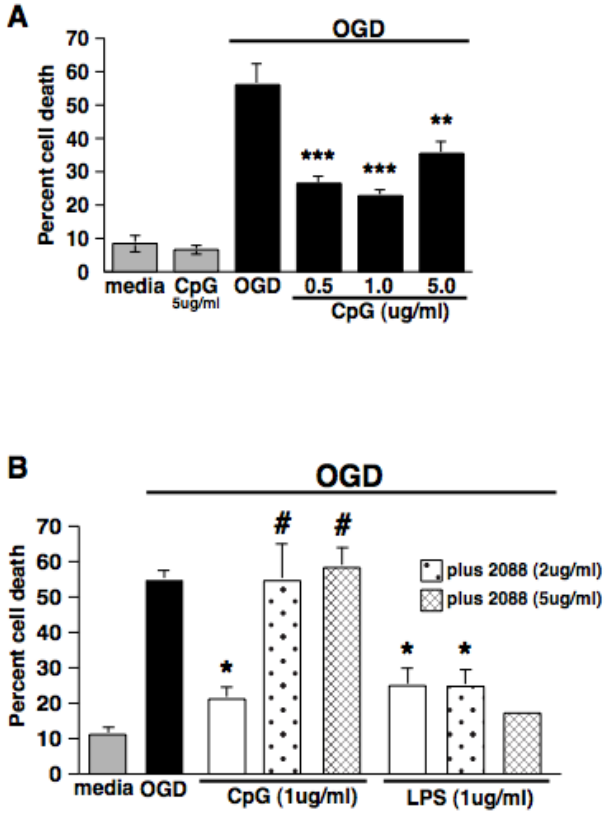

Figure 2.1. CpG protects primary mixed cortical cultures from OGD-induced cell death through TLR9. A) Mixed cortical cultures were stimulated with increasing doses of CpG 1826 (0.5-5 ug/ml) 24 hr prior to 3 hr OGD. Cell death was assessed 24 hr following OGD by propidium iodide (PI) staining. For all experiments, values are mean $\pm$ SEM, **p<0.01, ***p<0.001 versus media-treated OGD controls, n=5 individually repeated experiments. CpG 1826 treatment alone at the highest dose (5ug/ml) did not result in increased cell death over media alone (grey bar). B) Mixed cortical cultures were stimulated with either CpG or the TLR4 agonist LPS (1ug/ml) in the presence or absence of the TLR9 antagonist ODN 2088 (2 or 5 ug/ml) 24 hr prior to 3 hr OGD. Cell death was assessed 24 hr following OGD by PI staining. Values are mean + SEM, *p<0.05 vs. media-treated OGD controls, #p<0.05 vs. CpG-treated OGD; n=2-4 individually repeated experiments, except for LPS +5ug/ml ODN 2088 which represents a single experiment.

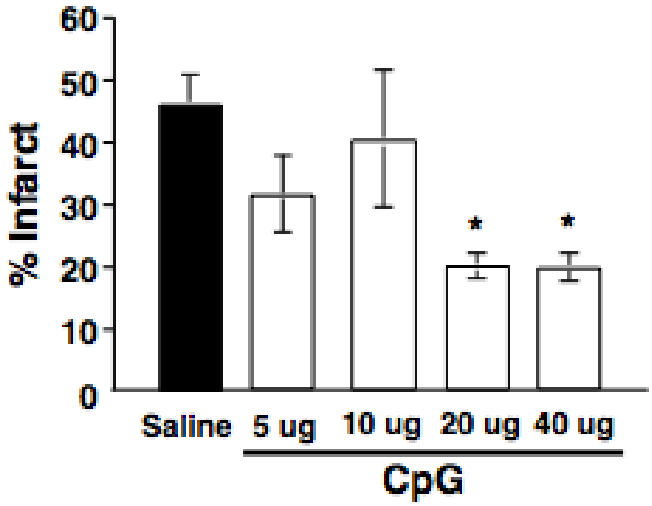

Figure 2.2. Preconditioning with CpG reduces infarct size in a mouse model of focal ischemia. C57BL/6 (males, 6-10/dose) received various doses of CpG 1826 (5-40ug; i.p.) 72 hr prior to ischemic challenge (60 min MCAO). Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM; *$p<0.05$ comparison to saline controls by one way ANOVA followed by Bonferroni's multiple comparison test.

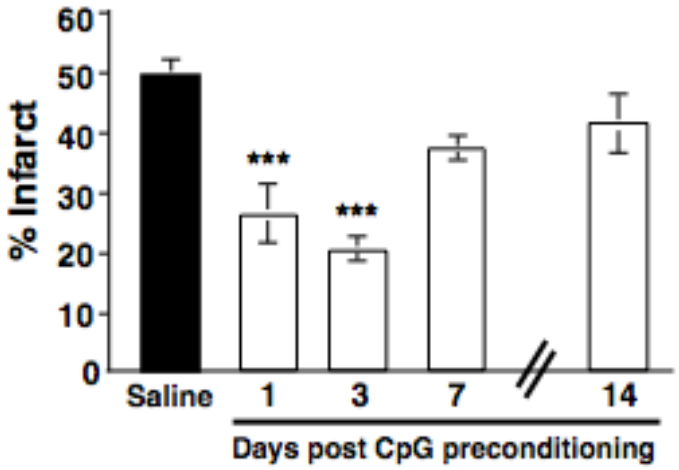

Figure 2.3. Time window of CpG preconditioning. C57Bl/6 mice received an injection of saline (4 mice/time point) or CpG (20ug; i.p.) 1, 3, 7 or 14 days (6 mice/time point) prior to 60 min MCAO. Infarct volume was determined 24 hr following MCAO by TTC staining. No statistical difference was observed between the saline groups, thus they were combined for analysis. Values are group means ± SEM; ***$p<0.001$ comparison to saline controls by one way ANOVA followed by Bonferroni's multiple comparison test.

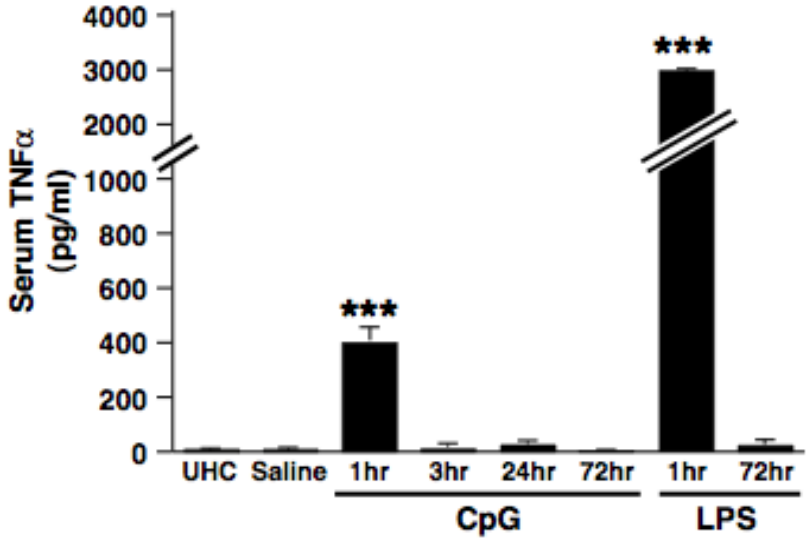

Figure 2.4. Serum TNF-α levels significantly increased 1-hour post CpG treatment. Mice (n=4/time point) were administered CpG 1826 (20 ug; i.p.) and blood was collected at 1, 3, 24 or 72 hrs post injection. Blood was allowed to clot for 2 hr at room temperature and the serum collected. TNFα levels (pg/ml of blood) were measured with a TNFα ELISA (R&D Systems). Values are group means ± SEM; ***$p < 0.001$ by two way ANOVA followed by Bonferroni's multiple comparison test. LPS (5 ug; i.p.) treated mice were included in the same experiment for comparison.

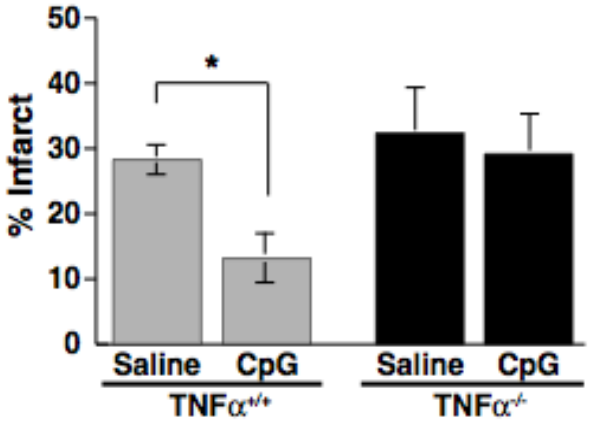

Figure 2.5. TNFα induction is required for CpG preconditioning. Control (C57Bl/6; TNFα$^{+/+}$) or TNFα knockout (B6.129S-Tnf^{tm1Gkl}/J; TNFα$^{-/-}$) mice were treated with CpG 1826 (20 ug; i.p.) at 72 hr prior to 40 min MCAO and infarcts were assessed 24 hr post MCAO. Values are mean + SEM, *$p<0.05$ versus saline treatment, n = 4-6 mice/group.

Chapter 3- Manuscript #2

Inflammation and the Emerging Role of the Toll-like Receptor System in Acute Brain Ischemia

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Chapter 3 is a manuscript as it appears in the original paper published in the journal *Stroke.*

Abstract

BACKGROUND AND PURPOSE: Systemic administration of cytosine-guanine oligodeoxynucleotides (CpG ODNs) provides neuroprotection against subsequent cerebral ischemic injury. We examined the genomic response of leukocytes and brain cells following ischemia in the context of CpG preconditioning.

METHODS: RNA was isolated from circulating leukocytes and ischemic cortex 3 and 24 hours after middle cerebral artery occlusion (MCAO) following CpG or saline pretreatment and subjected to microarray analysis. Genes uniquely up-regulated in CpG pretreated mice were examined for over-represented transcriptional regulatory elements (TREs).

RESULTS: CpG preconditioning induced a novel response to MCAO within circulating leukocytes that was dominated by NK cell- associated genes and the GATA-3 TRE. Preconditioning also caused a novel brain response to stroke that was dominated by Type I interferon- associated genes and TREs.

CONCLUSION: CpG preconditioning invokes novel leukocyte and brain responses to stroke. In this, CpG may be a unique preconditioning agent, coordinating peripheral and brain responses to protect against ischemic injury.

Introduction

Bacterial non-methylated cytosine-guanine oligodeoxynucleotide motifs (CpG ODNs) alert the body to infection through activation of Toll-like receptor 9 (TLR9). In mice, TLR9 is expressed by B cells, plasmacytoid dendritic cells (pDCs), macrophages, microglia, and astrocytes. TLR9-activated cells produce the pro-inflammatory cytokines TNFα, IFNα, and IL-12. These cytokines further activate monocytes, neutrophils, natural killer cells (NK cells), and T cells, facilitating a coordinated inflammatory response to pathogen invasion.

Pre-exposure to CpG reprograms the cellular response to subsequent TLR stimulation. Unlike naïve cells, macrophages pre-treated with CpG do not generate TNFα in response to TLR4 stimulation, instead generating IFNβ [93]. Furthermore, systemic administration of CpG increases resistance to polymicrobial sepsis [147]. Hence pre-exposure to CpG redirects both cellular and systemic responses to subsequent TLR stimulation.

Systemic administration of CpG also protects the brain from subsequent ischemic damage[1]. Such 'CpG preconditioning' is time and dose dependent and requires TNFα. The precise mechanisms responsible for CpG preconditioning are not well understood, but likely involve both direct cellular processes and coordinated systemic responses that minimize ischemic damage.

We hypothesize that CpG preconditioning reprograms the response of the brain and the peripheral immune system to subsequent stroke. Here we provide evidence for such reprogramming and consider its potential neuroprotective consequences.

Results

CpG preconditioning induces a NK cell-associated peripheral response to stroke

We evaluated RNA from blood leukocytes 24 hours following MCAO using Affymetrix oligonucleotide microarrays. We found 422 genes to be differentially regulated in CpG pretreated animals relative to saline. We next identified over-represented transcriptional regulatory elements (TREs) in the genes uniquely increased in CpG preconditioned animals. In those genes for which upstream sequence was available for analysis (234) a single TRE, GATA-3, was over-represented with an adjusted p value = 0.118. A network depiction of interactions between GATA-3 and genes in the CpG preconditioned cluster is displayed in Figure 3.1. GATA-3 is linked to 53% of the genes within this up-regulated cluster (124 of 234). GATA-3 plays a critical role in the development of NK cells. Literature review identified 24 of the up-regulated genes as NK cell-associated: Klra5, Klra7, Klra8, Klra10, Klra18, Klra22, Klrb1a, Klrb1c, Klrb1f, Klrc1, Klrc2, Klre1, Klrg1, Klrk1, Rantes, Cma1, Eomes, Fasl, Gzmb, Il2rb, Ncr1, Ndg1, Prf1, and T-bet. CpG activates NK cells indirectly via IL-12 released from activated dendritic cells (DCs). Serum IL-12 levels were significantly increased 24 hours after MCAO in preconditioned animals (data not shown). Together, our data demonstrate that CpG preconditioning induces a novel, systemic NK cell response to stroke.

CpG preconditioning induces a Type I IFN-associated brain response to stroke

We evaluated RNA from ischemic cortex 24 hours following MCAO using Affymetrix oligonucleotide microarrays. We found 223 genes to be differentially regulated in CpG pretreated animals relative to saline. We next identified over-represented TREs in the genes uniquely up-regulated in CpG preconditioned animals. In those genes for which upstream sequence was available for analysis (136) we identified 4 over-represented TREs with an adjusted p value < 0.1. Notably, each TRE was Type I interferon (IFN) -associated (IRF, IRF8, ISRE, HMG-1Y). A network depiction of interactions between the identified TREs and the genes in the CpG preconditioned cluster is displayed in Figure 3.2. The IFN-associated TREs are linked to 64% of the genes within this up-regulated cluster (88 of 136). Literature review identified 12 of the up-regulated genes as Type I IFN-associated: Oas1a, MHC class I (H2-D1, H2-K1, H2-L, H2-Q6), Ifi203, Ifi204, Ifi205, Ifi27, Isg20l1, Lmp7, and Psmb9. Thus an altered signaling cascade involving Type I IFNs exists in the brain following stroke in CpG preconditioned mice.

Discussion

We report the first evidence that CpG preconditioning alters the genomic response to stroke in circulating leukocytes and in the brain. We demonstrate a distinct pattern of NK cell activity in the blood and a clear enhancement of Type I IFN signaling in the brain following MCAO. This pattern of up-regulated gene expression underscores a *unique* response to brain ischemia that may actively protect the brain from injury.

CpG preconditioning induced a novel genomic response in blood leukocytes that was evident 24 hours after stroke. Of those genes uniquely up-regulated in preconditioned animals, a majority contained the GATA-3 TRE, which is required for NK cell development. Additionally, 24 of the up-regulated genes were NK cell-related and serum IL-12 was increased at this time, supporting the notion of increased NK cell activity.

This unique systemic response may play a role in neuroprotection as NK cells have been shown to limit damaging neuroinflammation in experimental autoimmune encephalomyelitis (EAE) [148]. Interestingly, administration of CpG ODNs prior to EAE induction also reduces disease severity [149]. Furthermore, treatment with CpG inhibits inflammatory arthritis in an IL-12- and NK cell- dependent manner [150]. Hence, CpG may also initiate a protective NK cell response to cerebral ischemia.

CpG preconditioning induced a novel genomic response in the brain that was evident 24 hours after stroke. Of those genes uniquely up-regulated in preconditioned animals, a majority contained one or more Type I IFN-associated TREs. Moreover, 12 of the up-regulated genes were associated with Type I IFN signaling, further supporting a role for IFNs following stroke in preconditioned animals.

Microglial, astrocytes, endothelial cells and neurons all produce the Type I IFN IFNβ. IFNβ can stabilize the blood-brain barrier [23], suppress inflammatory cytokines [108], and protect neurons from cytotoxic microglia [111]. Systemic administration of IFNβ reduces

infarct damage in several models of ischemic stroke [24, 25]. Hence an increase in Type I IFN signaling within the brain has the potential to be neuroprotective.

Our data supports a shift toward Type I IFN signaling following stroke in CpG pretreated animals. How might this shift occur? Mice lacking TLR4 incur significantly less damage from MCAO than wild-type controls [74], indicating a damaging role for this receptor in ischemic injury. Pretreatment with CpG shifts the cellular response to subsequent stimulation of TLR4, leading to a suppression of TNFα and an increase in IFNβ. A similar series of events might occur following CpG preconditioning wherein pretreatment with CpG shifts the response of TLR4 to subsequent stimulation with endogenous ligands, such as HSP60, released after stroke, and potentially leads to suppressed cytotoxic TNFα and enhanced neuroprotective IFNβ.

Alternatively, the systemic increase in NK cell activity may explain the Type I IFN shift in the brain. NK cells promote the release of IFNα from pDCs in a CpG- or IL-12-dependent manner [151, 152]. Hence pretreatment with CpG may activate DCs to produce IL-12, thereby activating NK cells which, in turn, induce pDCs to produce IFNα.

We have shown that CpG preconditioning reprograms the peripheral and central responses to stroke. The appearance of novel NK cell and interferon genomic "fingerprints" after ischemia indicates that CpG preconditioning fundamentally changes the body's inflammatory response to stroke. This is consistent with our previous reports of reprogramming in which ischemic and LPS preconditioning induce novel, protective sets

of gene transcripts following stroke [145, 153]. CpG appears to be a unique preconditioning agent, coordinating both systemic and central immune components to actively protect the body from ischemic injury.

Materials and Methods

Mice: C57Bl/6 mice (male, 8-10 weeks) were obtained from Jackson Laboratories (West Sacramento, CA, USA). All mice were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

Drug Treatments: CpG ODN 1826 (20-40ug; 200μl; Invivogen, San Diego, CA, USA) or saline was administered by intraperitoneal injection 72 hours before MCAO.

Surgery: Mice were anesthetized with isoflurane and ischemia was induced by MCAO as published previously[114]. Cerebral blood flow was monitored with laser Doppler flowmetry and temperature was maintained at 37°C. After surgery, mice were kept for 24 hours on a heating pad with access to soft food and water.

RNA isolation: Mice were anesthetized and blood was obtained via retro-orbital puncture. Animals were perfused with saline and, under RNase-free conditions, a 1 mm section was removed for infarct area analysis. The ipsilateral cortex region from the frontal 4 mm was

snap frozen. Total RNA was isolated from the blood using the Qiagen PAXgene Blood RNA Kit and from the brain using the Qiagen RNeasy Lipid Mini Kit (Qiagen Inc.). RNA from individual animals was hybridized to single arrays.

GeneChip Expression Analyses: Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health & Sciences University Gene Microarray Shared Resource. RNA samples were labeled using the NuGEN Ovation Biotin RNA Amplification and Labeling System_V1. Quality-tested samples were hybridized to the MOE430 2.0 array and processed with Affymetrix GeneChip Operating Software (GCOS). Data was normalized using the Robust Multichip Average method. Normalized data was analyzed by multivariate ANOVA for each gene. P-values were adjusted for multiple comparisons using the Hochberg and Benjamini method. Significance was determined by $p < 0.05$ and fold change ≥ 2 for blood analyses and ≥ 1.5 for brain analyses.

Transcriptional regulatory network analysis: For our reference comparison group, we identified putative TREs in the upstream sequence of transcripts represented on the MOE430 Affymetrix gene chip using TRANSFAC PRO database version 10.4. We then determined the over-represented TREs in the uniquely up-regulated gene cluster compared to the reference group using Promoter Analysis and Interaction Network Toolset (PAINT) version 3.5.

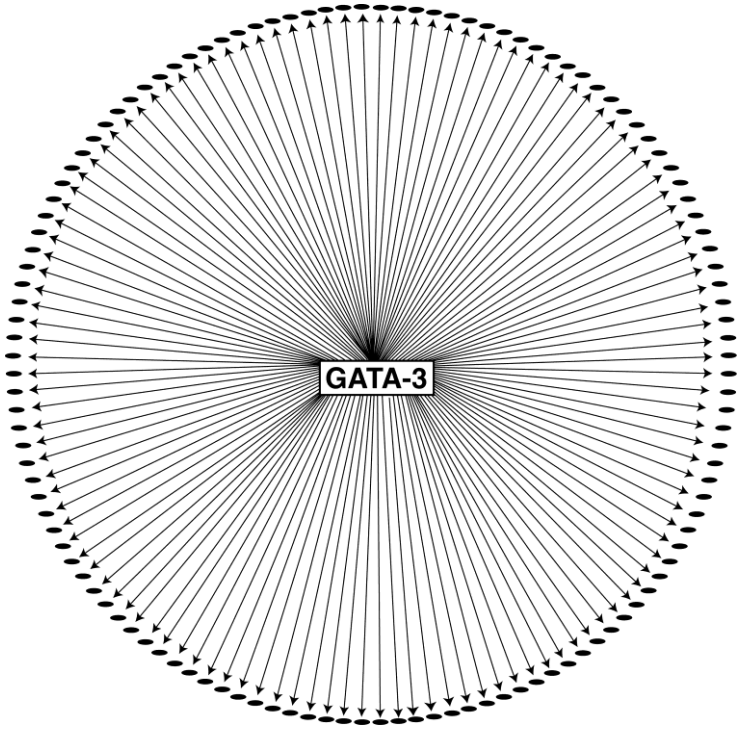

Figure 3.1. The GATA-3 TRE is over-represented in blood leukocytes 24 hours following stroke in CpG preconditioned mice. A PAINT- generated Hypothesis Gene-TRE Network depicting the genes uniquely up-regulated by CpG preconditioning 24 hours after MCAO that contain the GATA-3 TRE. Genes are depicted as ovals. P value threshold set at 0.2

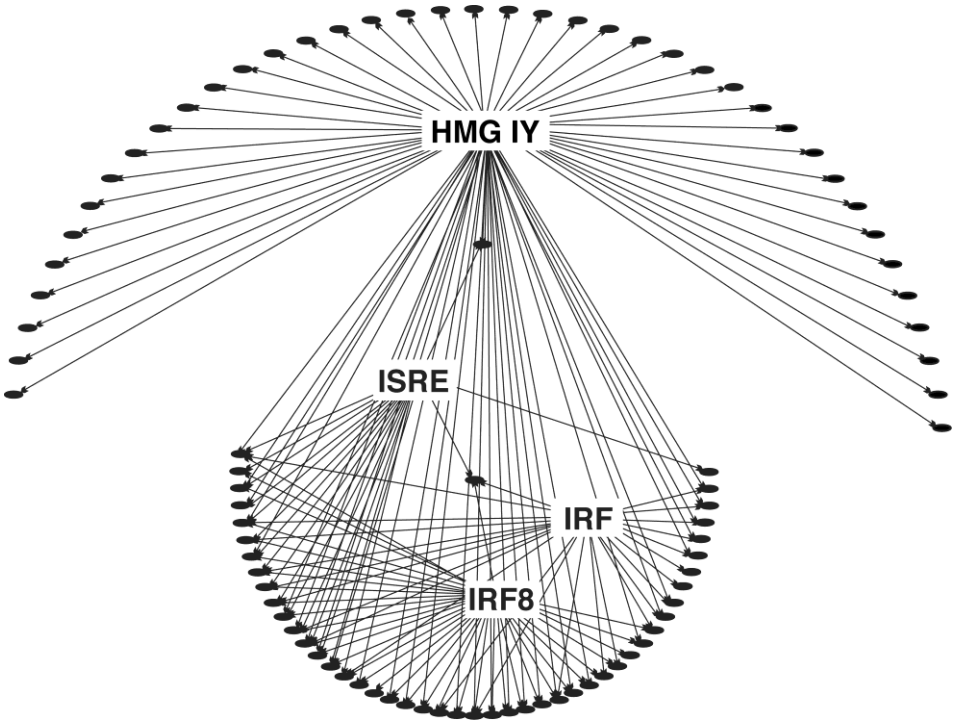

Figure 3.2. Type I interferon-associated TREs are over-represented in the brain 24 hours following stroke in CpG preconditioned mice. A PAINT- generated Hypothesis Gene-TRE Network shows the relationships between the genes uniquely up-regulated by CpG preconditioning 24 hours after MCAO and the TREs shared in common. Genes are depicted as ovals. P value threshold set at 0.1.

Chapter 4- Manuscript #3

Systemic LPS protects the brain from ischemic injury by reprogramming the brain's response to stroke: a critical role for IRF3

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Chapter 4 is a manuscript as it has been submitted to *The Journal of Neuroscience.*

Abstract

Lipopolysaccharide (LPS) preconditioning provides neuroprotection against subsequent cerebral ischemic injury through activation of its receptor, Toll-like receptor 4 (TLR4). Paradoxically, TLR activation by endogenous ligands following ischemia worsens stroke damage. Here, we define a novel, protective role for TLRs following ischemia in the context of LPS preconditioning. Microarray analysis of brains collected 24 hours following stroke revealed a unique set of up-regulated genes in LPS pretreated animals. Literature review determined that a majority of these transcripts are associated with Type I interferons (IFNs). Promoter analysis confirmed this observation, as 5 of the 14 over-represented transcriptional regulatory elements in this group were involved in Type I IFN signaling. This finding suggested the presence of Type I IFNs or interferon regulatory factors (IRFs), which up-regulate interferon-stimulated genes. Up-regulation of IFNβ was confirmed by real-time RT-PCR. Direct administration of IFNβ i.c.v at the time of stroke was sufficient for neuroprotection. However, mice lacking IFNβ were protected by LPS pretreatment, indicating that IFNβ is not necessary for LPS-induced neuroprotection. The IRF3 transcription factor, activated downstream of TLR4, induces both IFNβ and interferon-stimulated genes. Mice lacking IRF3 were not protected by LPS pretreatment. Our studies constitute the first demonstration of the neuroprotective capacity of IRF3 and suggest that interferon stimulated genes, whether induced by IFNβ or by enhanced TLR signaling to IRF3, are a potent means of protecting the brain against ischemic damage.

Introduction

It is increasingly clear that Toll-like receptor (TLR) signaling worsens stroke injury. Mice lacking TLR2 or TLR4 are less susceptible to damage in multiple models of cerebral ischemia [43, 72, 75]. TLRs are expressed by microglia, astrocytes and endothelial cells and are activated by the damage-associated molecules HSP70 (TLR4) and HMGB1 (TLRs 2 and 4), present in the brain following ischemia [77, 78, 154]. TLR activation induces production of the inflammatory molecules TNFα, IL1β, and iNOS, and other cytotoxic mediators that increase tissue damage.

Though TLR4 activation *following* stroke exacerbates injury, activation of TLR4 *prior* to stroke protects the brain from damage. Systemic administration of lipopolysaccharide (LPS), a potent TLR4 ligand of bacterial origin, renders animals tolerant to injury in several models of cerebral ischemia [79-81]

LPS-induced tolerance to ischemic injury mirrors the phenomenon of LPS-induced tolerance to LPS. Initial exposure of macrophages to LPS induces pro-inflammatory TNFα, but upon subsequent exposure to LPS, TNFα production is reduced markedly due to disrupted signaling through the TLR4 adaptor molecule MyD88 [121, 122, 155]. Conversely, macrophages produce little IFNβ upon initial exposure to LPS, but *enhance* IFNβ production upon secondary exposure [93], suggesting up-regulated TLR4 signaling through the TRIF adaptor molecule. Thus, pretreatment with LPS may cause cells to switch their dominant TLR4 signaling pathway.

We suggest that these molecular mechanisms that modulate MyD88 and TRIF may play a role in LPS-induced tolerance to stroke damage. We hypothesize that pretreatment with LPS reprograms the brain's response to stroke by redirecting stroke-induced TLR4 signaling. This redirection suppresses the inflammatory response to stroke and enhances the Type I IFN response. We have previously demonstrated suppression of the inflammatory response to stroke, specifically that of TNFα, following LPS preconditioning, [80, 82] and found such suppression to be neuroprotective. Here, we explore the enhancement of a Type I IFN response which occurs downstream of the TRIF adaptor molecule.

TLR4 signaling through TRIF activates the transcription factor IRF3, which is required for IFNβ production. IFNβ, administered systemically, reduces ischemic brain damage [24, 25], likely through activation of interferon-stimulated genes (ISGs). Thus, enhanced TLR4 signaling to TRIF-IRF3-IFNβ would be expected to contribute to neuroprotection.

IRF3 itself may have similar neuroprotective effects. IRF3 binds to interferon stimulated response elements (ISREs) within gene promoters, increasing the expression of many ISGs to the same extent that Type I IFNs do [156]. Hence activation of IRF3 may independently result in protection from ischemic stroke.

Pretreatment or preconditioning with LPS changes the cellular environment such that subsequent activation of TLR4 increases signaling to IRF3 and up-regulates the

neuroprotective cytokine IFNβ. We propose that LPS preconditioning reprograms subsequent activation of TLR4 during ischemia, which leads to an increase in neuroprotective Type I IFN signaling. Here we provide evidence for such reprogramming and its neuroprotective consequences.

Results

LPS preconditioning induces a novel genomic response to stroke.

We have reported previously that systemic administration of LPS (0.2 mg/kg) 3 days prior to MCAO reduces damage due to ischemia in the brain [80, 82]. To identify potential mechanisms of protection, we examined the transcriptional profile of neuroprotection. RNA was isolated from the cortex of LPS and saline treated mice 24 hours following MCAO. RNA isolated from unhandled mice was used as a baseline control group (see Methods). Using Affymetrix oligonucleotide microarrays we found that the majority of genes regulated 24 hours after MCAO (~60%) did not depend on the preconditioning stimulus (Figure 4.1; red region), and were regulated as a general response to stroke. However, a significant number of genes were regulated only in preconditioned animals, with 23% (176 out of 755) specific to LPS preconditioned mice (Figure 4.1; green region). Hence, following stroke, LPS preconditioning induces the regulation of a unique set of genes not evident in saline pretreated mice. These findings suggest a distinct reprogrammed response is initiated following stroke in LPS preconditioned mice.

LPS preconditioning induces a Type I interferon-associated response to stroke.

To characterize the protective response to stroke injury in LPS-preconditioned mice we determined the functional profile of the genes regulated 24 hrs following stroke. Based on the Affymetrix Netaffx website (https://www.affymetrix.com/analysis/netaffx/index.affx), the Stanford-Online Universal Resource for Clones and ESTs website (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and literature review we determined putative functions for the modulated genes. Figure 4.2 shows the functional categories of genes expressed in response to stroke common to saline and LPS treated animals (far left), and those genes unique to saline pretreatment (middle) and LPS pretreatment (far right). Of those genes uniquely regulated after stroke in LPS preconditioned animals, 38% (47 out of 123 genes of known function) are involved in defense/inflammation (Figure 4.2 far right, green wedge), and constitute a novel inflammatory signature in these animals. Further literature analysis of these defense/inflammation genes revealed that 53% (25 out of 47) are associated with type I interferon signaling (Table 4.1), all of which are up-regulated at this time point.

Interferon transcriptional regulatory elements are associated with LPS preconditioning.

We identified transcriptional regulatory elements (TREs) associated with the unique gene regulation detected in the LPS and saline preconditioned animals using the web-based program: Promoter Analysis and Interaction Network Toolset (PAINT) version 3.5. We compared the TREs identified in the cluster of genes uniquely increased in LPS

preconditioned mice 24 hrs following stroke (158 genes, Figure 4.1) to a reference cluster consisting of ~33,000 transcripts from the MOE430 gene chip to determine over-represented TREs associated with the genes in the preconditioned cluster. We performed the same comparison using the cluster of genes uniquely increased in the saline pretreated mice 24 hrs following stroke (128 genes, Figure 4.1). Analysis of the LPS preconditioned group identified 14 TREs with an adjusted p value of less than 0.2 while the saline pretreated cluster revealed only 5 over-represented TREs (Table 4.2). Five of the 14 identified TREs in the LPS preconditioned cluster are interferon-associated (IRF [V$IRF_Q6 and V$IRF_Q6_01], IRF8, ISRE, IRF7). A network depiction of interactions between the identified TREs and the genes in the LPS preconditioned cluster is displayed in Figure 4.3. The interferon-associated TREs (in red) are linked to a substantial number of the genes shown (60%; 76 of 127). This interferon-dominated response implies that an altered signaling cascade that involves an interferon-defined pathway may exist following stroke in LPS preconditioned mice.

Increased levels of IFNβ following stroke in LPS preconditioned mice.

The increase in interferon inducible genes and over-representation of interferon-associated TREs suggested that IFNβ may be present in the brain cortex following stroke in LPS preconditioned mice. Using real time PCR we examined the levels of IFNβ transcript in the brain following stroke in LPS preconditioned and saline treated mice. IFNβ levels were increased following stroke in the preconditioned and non-preconditioned mice compared to unhandled controls (Figure 4.4A). However, levels in LPS preconditioned mice were 9x higher at 3 hr (LPS treated 59.4 $\pm$ 22 vs. saline treated

6.7 ± 3; p<0.0001) and 3.5x higher at 24 hr (LPS treated 45.3 ± 23 vs. saline treated 11.7 ± 6; p<0.0001) post stroke. We examined levels of IFNβ just prior to MCAO (72hrs post injection) to confirm that the increase in IFNβ following stroke was independent of any residual increase of IFNβ resultant from the preconditioning LPS injection. Levels of IFNβ in LPS and saline treated mice were statistically equivalent to unhandled controls (1.49 ± 1.4 and 0.74 ± 0.6 respectively; data not shown). Thus, following stroke, mice preconditioned with LPS mount a more robust IFNβ response to ischemic injury.

IFNβ protects against ischemic injury.

Systemic administration of IFNβ improves stroke outcome in rodents [25] and rabbits [24]. Here we tested whether IFNβ administration in the brain provides protection against stroke. We injected C57BL/6 mice i.c.v. with recombinant mouse IFNβ immediately prior to and following MCAO and measured infarct size 24 hrs later. Animals treated with IFNβ showed a significant reduction in infarct volume versus vehicle treated mice (31.9 ± 4% vs. 49.4 ± 2%; p<0.001; Figure 4.4B). This result supports the notion that increased expression of IFNβ within the brain would confer protection from ischemic injury.

IFNβ is not required for LPS-induced protection from brain ischemia.

Given that LPS preconditioning leads to increased IFNβ within the brain, and that exogenous administration of IFNβ protects the brain against ischemic damage, we tested whether IFNβ is a critical effector of LPS-induced ischemic protection. We first determined whether IFNβ is involved in the brain's endogenous response to stroke. IFNβ

knockout mice were subjected to 40' MCAO followed by 72 hrs of reperfusion. Wild type and IFNβ knockout mice displayed infarcts of similar size (38.5 ± 2% vs. 39.5 ± 1%; p=0.7; Figure 4.5A). Thus IFNβ does not play a critical role in the brain's usual response to ischemia. We then determined whether IFNβ is a required effector of LPS-induced ischemic tolerance. IFNβ knockout mice were pretreated with LPS (1mg/kg) 72 hours prior to 40 min MCAO, and sacrificed 24 hrs later. Figure 4.5B shows that LPS preconditioning protects IFNβ knockout mice from ischemic damage (47% infarct reduction). Thus, although IFNβ is sufficient for neuroprotection, is in not required for LPS-induced ischemic tolerance.

IRF3 is required for LPS-induced protection from brain ischemia.

LPS preconditioning up-regulates interferon-associated transcripts following MCAO but does not require IFNβ. This suggests the involvement of an alternative interferon-associated pathway that relies on the transcription factor IRF3 that binds to ISRE and IRF TREs. Therefore we tested whether IRF3 is a critical effector of LPS-induced ischemic protection. First we determined whether IRF3 is involved in the brain's endogenous response to stroke. IRF3 knockout mice were subjected to 40 min MCAO followed by 72 hrs of reperfusion. IRF3 knockout mice displayed infarcts of similar size to wild type mice (42.2 ± 5% vs. 43.8 ± 4%; p=0.8; Figure 4.6A). Thus IRF3 does not play a critical role in the brain's usual response to ischemia. Next we determined whether IRF3 is a required effector of LPS-induced ischemic tolerance. IRF3 knockout mice were pretreated with LPS (1mg/kg) 72 hours prior to 40 min MCAO, and sacrificed 24 hrs later. Figure 4.6B shows that IRF3 knockout mice fail to be preconditioned with LPS

(17.3% vs. 53.2% reduction). Hence, IRF3 is required for the protective effects of LPS pretreatment.

Discussion

We propose a molecular model of LPS-induced neuroprotection from ischemic injury wherein systemic LPS preconditioning reprograms TLR4 signaling in response to stroke, directing it towards a neuroprotective pathway. Administration of LPS prior to brain ischemia alters the brain's transcriptional response to stroke, eliciting a new pattern of gene regulation 24 hours after ischemia. Of the 176 genes differentially regulated by LPS pretreatment, 90% are up-regulated, suggesting that LPS pretreatment may induce actively protective processes following cerebral ischemia. Thirty-eight percent of the genes in this group are involved in defense and inflammation, and of those, ~60% are related to Type I interferon signaling. Promoter analysis of this group identified 5 of the 14 over-represented TREs as IFN-associated. IRF3 and IFNβ, two molecules downstream of TLR4 signaling, have the capacity to elicit such a transcriptional response. We found that LPS preconditioning increases IFNβ within the brain 3 and 24 hours after stroke. We then tested whether the increase in IFNβ within the brain might confer neuroprotection. Mice lacking IFNβ incurred infarcts of similar size to wild type mice, suggesting that endogenous IFNβ does not protect the brain from ischemic injury. However, exogenous administration of IFNβ i.c.v. at the time of stroke conferred significant protection against ischemic damage, indicating that local up-regulation of this cytokine may be neuroprotective. Nevertheless, IFNβ was not required for LPS-induced protection, as

preconditioning protected IFNβ-deficient mice from stroke damage. We concluded that IFNβ is sufficient but not necessary for LPS-induced ischemic tolerance. This finding indicated that a different IFN-associated factor might be involved in effecting LPS-induced neuroprotection. Thus we postulated that IRF3 might be a critical mediator of LPS preconditioning. We tested whether IRF3 is involved in the brain's natural response to stroke. Mice lacking IRF3 incurred infarcts of similar size to wild type mice, suggesting that IRF3 is not part of the brain's endogenous response to ischemia. However, LPS preconditioning failed to protect IRF3-deficient mice from ischemic damage. Therefore, we conclude that IRF3 is necessary for the neuroprotective effects of LPS preconditioning and present the first evidence of a protective role for this transcription factor.

We have previously reported that LPS preconditioning suppresses stroke-induced inflammation, cellular infiltration and pro-inflammatory cytokine production. Here we show that LPS preconditioning enhances the production of Type I IFN-associated genes following stroke and protects the brain via IRF3. Together our data support a model of redirected TLR4 signaling that resembles endotoxin tolerance. Cells made tolerant to endotoxin (lipopolysaccharide, LPS) are known to suppress the pro-inflammatory MyD88-TNFα pathway by up-regulating pathway inhibitors, namely IRAK-M, Tollip, Ship1 and Trim30α, among others [92, 157], which results in decreased inflammatory cytokine responses upon secondary exposure to TLR4 ligands. Inhibition of these pathways shunts subsequent TLR4 signaling down the TRAM-IRF3-IFNβ pathway and results in enhanced production of IFNβ [96]. Similarly, LPS preconditioning may up-

regulate inflammatory pathway inhibitors that shunt subsequent TLR signaling down the TRAM-IRF3-IFNβ pathway. Thus, in the setting of ischemia, release of endogenous TLR ligands would be expected to lead to TLR signaling that is shunted down the TRAM-IRF3-IFNβ pathway and results in up-regulation of IFNβ.

Our data support a model of redirected TLR4 signaling following stroke in preconditioned animals. Unlike control animals, LPS preconditioned animals demonstrate a significant up-regulation of IFN-associated genes following stroke which, based our microarray data, is likely to be produced via the TLR4-TRIF-IRF3 pathway. At 24 hrs following stroke, we detected several interferon-associated genes previously shown to be induced by TLR4-to-TRIF signaling, including Ifit1, Ifit3, and Oasl2. Ifit1 and Ifit3 are up-regulated following LPS treatment of peritoneal macrophages from MyD88-deficient mice [158]. Similarly, Ifit2 and Oasl1, close family members of the genes found on our array, are also up-regulated in these cells, suggesting that these genes are all products of TRIF-dependent signaling. Together, this data suggests that the Type I IFN "fingerprint" is generated downstream of TLR4-TRIF-IRF3 and supports the concept of TLR4 reprogramming.

IRF3 is required for TLR4-induced production of IFNβ. Structural information gathered from the crystallized IRF3-bound IFNβ promoter indicates that IRF3 dimers bind to each of 4 IRF TREs within the promoter region. In addition to enhancing transcription by binding IRF TREs, IRF3 enhances transcription by binding ISRE TREs. ISREs are found in the promoter regions of many ISGs and activated IRF3 has been shown to up-regulate

transcription of a number of these genes. Our microarray data demonstrate an up-regulation of ISGs following ischemia in preconditioned animals, and an over-representation of both IRF and ISRE TREs within this group of genes. Furthermore, we have shown that IRF3 is required for LPS-induced neuroprotection. Hence LPS preconditioning may redirect stroke-induced TLR4 signaling towards activation of IRF3, resulting in up-regulation of neuroprotective ISGs.

The potential for ISGs to confer neuroprotection is evinced by the protective actions of IFNβ. Upon binding to the Type I IFN receptor complex, IFNβ induces JAK/STAT signaling, which activates the ISGF3 transcription factor, comprised of Stat1, Stat2 and IRF9. ISGF3 binds to ISRE motifs within the promoters of ISGs, increasing their expression. Presumably, it is the ISGs that render the neuroprotective effects of IFNβ̃ We have shown that direct administration of IFNβ protects the brain against ischemic injury. IFNβ may confer protection by stabilizing the blood-brain barrier (BBB) [23, 102], or by reducing cellular infiltration into damaged brain regions [25]. IFNβ has been shown to reduce reactive oxygen species [105-107], suppress inflammatory cytokine production [108], promote nerve growth factor production by astrocytes [110] and protect neurons from toxicity induced by activated microglia [111]. In addition, systemic administration of IFNβ reduces tissue damage in rat and rabbit models of ischemic stroke [24, 25]. It should be noted that Maier and colleagues were unable to attenuate ischemic brain injury following systemic administration of IFNβ in a rat model of focal ischemia (Maier CM 2006). The authors propose that their occlusion model caused less disruption of the BBB and thus IFNβ was not able to reach the affected brain. In our model, IFNβ is administered i.c.v.,

hence disruption of the BBB is likely not a factor. We speculate that the neuroprotective actions of IFNβ occur through the actions of the ISGs induced. As IRF3 can regulate transcription of the same ISGs, we suggest that the neuroprotective function of IRF3 in the context of LPS preconditioning lies in its ability to up-regulate these genes. In this context, IRF3 induction of IFNβ may act to amplify ISRE-regulated gene expression, causing a feed forward loop of ISG production.

Our data suggest that systemic administration of LPS reprograms TLR4-expressing cells within the brain. TLR4 is widely expressed in the brain [37, 38, 159] and many studies have shown that peripheral LPS induces a pro-inflammatory response within the brain [160, 161] However, it is unclear whether LPS crosses the BBB and/or whether it induces peripheral cytokines which, in turn, cross into the brain. Recent evidence suggests that systemic LPS elicits TLR4 signaling in the brain independent of peripheral cytokine responses [37, 162]. However, other researchers have failed to find LPS within the brain parenchyma following systemic administration [29]. It is clear that LPS binds to cerebral endothelial cells [29, 163]. As these cells are an interface between the systemic circulation and the brain parenchyma, they may help integrate information from both compartments. Hence, reprogramming of TLR4 may occur within the cerebral endothelium.

In summary, we have shown that LPS preconditioning reprograms the brain's response to stroke and causes a Type I IFN response, with a critical and protective role for IRF3. These reprogramming events may exemplify endogenous processes that protect the brain against further injury and suggests that that LPS preconditioning fundamentally changes

the brain's response to stroke. This is the first demonstration that a preconditioning stimulus results in an interferon "fingerprint" after the ischemic event and the first report of a neuroprotective role for IRF3. LPS appears to be a unique preconditioning agent, as it does not simply suppress ischemia-induced damaging pathways, but confers active protection to the brain in conditions of ischemia.

Materials and Methods

LPS Treatment: Mice were given a 200 ul intraperitoneal injection of saline or LPS (0.2 - 1.0 mg/kg; *Escherichia coli* serotype *055:B5*; Sigma).

Mice: C57BL/6 mice (male, 8-12 weeks, approximately 25 grams) were purchased from the National Cancer Institute (Frederick, MD). IFNβ knockout mice were kindly provided by Dr. Leanderson of Lund University. IRF3 knockout mice were procured from RIKEN BioResource Center, Tsukuba, Japan. Both strains were backcrossed onto the C57Bl/6 background for at least 8 generations. All mice were housed in an American Association for Laboratory Animal Care-approved facility. Procedures were conducted according to Oregon Health and Science University, Institutional Animal Care and Use Committee, and National Institutes of Health guidelines.

Middle Cerebral Artery Occlusion (MCAO): Mice were anesthetized with 4% halothane and subjected to MCAO using the monofilament suture method described previously [68]. Briefly, a silicone-coated 8-0 monofilament nylon surgical suture was threaded through

the external carotid artery to the internal carotid artery to block the middle cerebral artery, and maintained intraluminally for 40, 45, or 60 min. The suture was then removed to restore blood flow. Cerebral blood flow was monitored throughout surgery by laser Doppler flowmetry. Body temperature was maintained at 37°C with a thermostat-controlled heating pad.

Infarct evaluation: To visualize the region of infarction, 6 x 1 mm coronal midsections were placed in 1.5% 2,3,5 triphenyltetrazolium chloride (TTC) in 0.9% phosphate buffered saline and stained at 37°C for 15 min [164]. The infarct size was determined from computer-scanned images of the hemispheres using NIH images analyses. To account for edema within the infarct region, infarct area for each section was computed indirectly as: 100 x (contralateral hemisphere area - area of live tissue on ipsilateral hemisphere)/(contralateral hemisphere area) [116].

Experimental Design for Gene Expression Studies: C57/BL6 mice were divided into 2 groups with 4 animals per group: Group 1 received a saline injection followed 72 hr later with a 45 min MCAO. Group 2 received an LPS injection followed 72 hr later with a 45 min MCAO. Both groups were sacrificed 24 hr following start of occlusion. At time of sacrifice mice were anesthetized, then perfused with heparinized saline. A group of 6 mice were included as unhandled controls. Under RNase-free conditions, a 1 mm section was removed (4 mm from rostral end) for infarct area analysis by TTC staining. The ipsilateral cortex region from the frontal 4 mm was isolated and snap frozen in liquid nitrogen.

RNA isolation: Total RNA was isolated using the Qiagen RNeasy Lipid Mini Kit (Qiagen Inc.). RNA from individual animals was hybridized to single arrays as described below.

GeneChip Expression Analyses: Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health & Sciences University Gene Microarray Shared Resource. RNA samples were labeled using the NuGEN Ovation Biotin RNA Amplification and Labeling System_V1. Hybridization was performed as described in the Affymetrix technical manual (Affymetrix, Santa Clara, CA). Labeled RNA was hybridized to test arrays containing control probe sets and samples that did not meet empirically defined cutoffs within the core facility were remade. Quality-tested samples were hybridized to the MOE430 2.0 array. The array image was processed with Affymetrix GeneChip Operating Software (GCOS). Data was normalized using the Robust Multichip Average method [117]. The normalized data was then analyzed using a two-way ANOVA model for each gene, using conditions and time as groups. Post hoc comparisons were made using the unhandled mice as a control group. P-values were adjusted for multiple comparisons using the method of Hochberg and Benjamini [118]. Genes were considered significantly regulated if the adjusted p value was less than 0.05 and the fold change in regulation was greater than or equal to 2.

Transcriptional regulatory network analysis. Using the web based program: Promoter Analysis and Interaction Network Toolset (PAINT) version 3.5 [119], we examined the

predicted regulatory elements associated with the unique gene regulation identified by microarray. In brief, using PAINT we obtained the 5000 bp upstream sequence for the transcripts represented on the MOE430 Affymetrix gene chip (33,635 transcripts were identified with 5000 bp of upstream sequence). PAINT identified putative transcription factor binding sequences (TREs) in these upstream sequences using the TRANSFAC PRO database version 10.4. This pool of genes and identified TREs was used as our reference comparison group. The statistical component of PAINT (FDR adjusted p value set at $\leq$0.2) was used to determined the over represented TREs in individual gene clusters compared to the reference comparison group (i.e. uniquely expressed genes in LPS preconditioned mice compared to 33,635 member reference group).

Intracerebral Ventricular Injection of IFNβ during MCAO. rmIFNβ (Cell Sciences, Canton, MA) or vehicle (saline) was injected into the left lateral ventricle as previously described [120]. Injections (1ul) of either rmIFNβ (200U) or saline were administered immediately before and after surgery (60 min MCAO). Infarct volume was measured 24 hr following stroke.

Quantitative Real-time PCR for IFN-b. RNA was treated with DNase and transcribed into cDNA using the Omniscript RT Kit (Qiagen). Real-time PCR (RT-PCR) reactions were performed in a volume of 25 ul using TaqMan PCR Master Mix (Applied Biosystems). For IFNβ TaqMan Gene Expression Assay Mix for mouse IFNβ was used (ABI # Mm00439546_S1). Primers and probe for β-Actin were obtained from Integrated DNA Technologies: forward: 5'-AGAGGGAAATCGTGCGTGAC-3'; reverse: 5'-

CAATAGTGATGACCTGGCCGT-3'; probe: CACTGCCGCATCCTCTTCCTCCC. Samples were run on an ABI-prism 7700 (Applied Biosystems, Foster City, CA). Results were analyzed using ABI sequence detection software. The relative quantification of IFNβ was determined using the comparative CT method (2^{-DDC_T}) described in ABI User Bulletin #2. Results were normalized to b-actin and presented relative to unhandled mice. All reactions were performed in triplicate.

Acknowledgments

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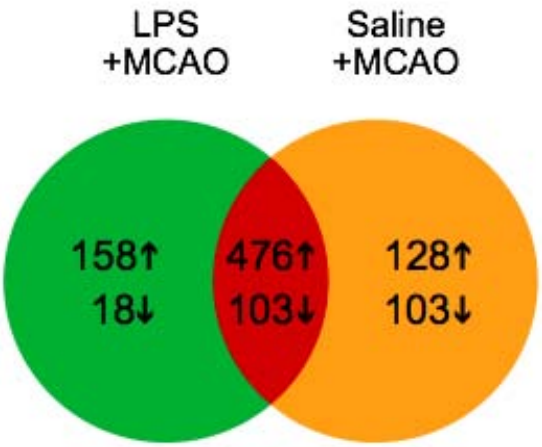

Figure 4.1. LPS preconditioning induces a unique set of genes in response to MCAO. C57Bl/6 mice were preconditioned with LPS (5ug) or saline 72 hr prior to MCAO (45 min). At 24 hr post MCAO mice (n=8/timepoint) were sacrificed and the ipsilateral cortical brain tissue was collected. RNA was isolated and hybridized to Affymetrix gene chips (MOE430). Venn diagram showing the number of genes differentially regulated in each condition compared to unhandled controls. Arrows indicate increased or decreased regulation.

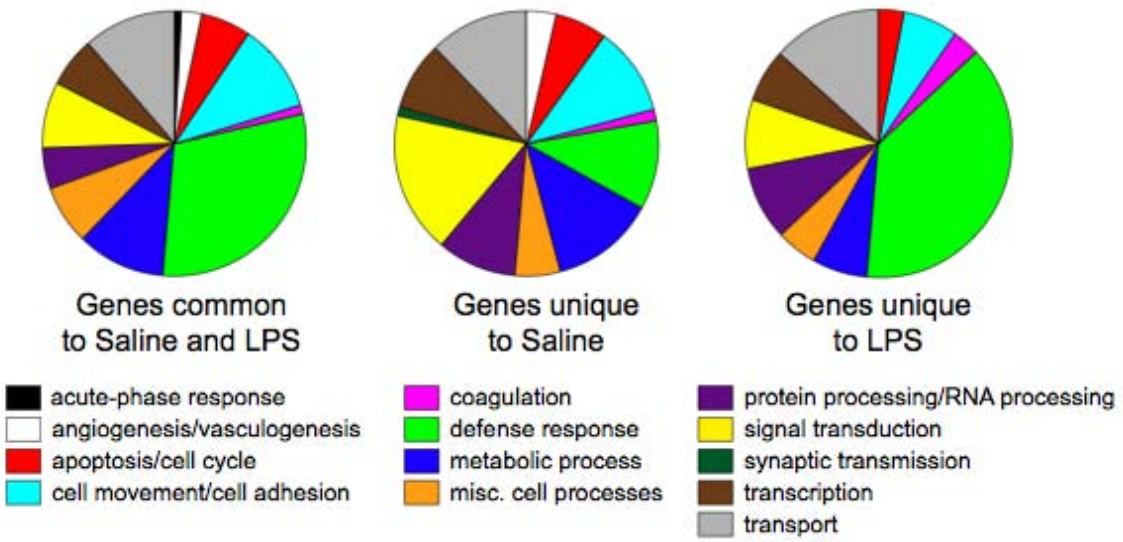

Figure 4.2. LPS preconditioning induces a unique defense/inflammatory response 24 hrs following stroke. Putative biological functions were assigned to the regulated genes using available public databases and literature review. Data is depicted as pie charts of genes regulated 24 hrs following MCAO that are shared in common between saline and LPS preconditioning (left panel), unique to saline (center panel) and unique to LPS (right panel). Only genes in which putative functions were available are graphed.

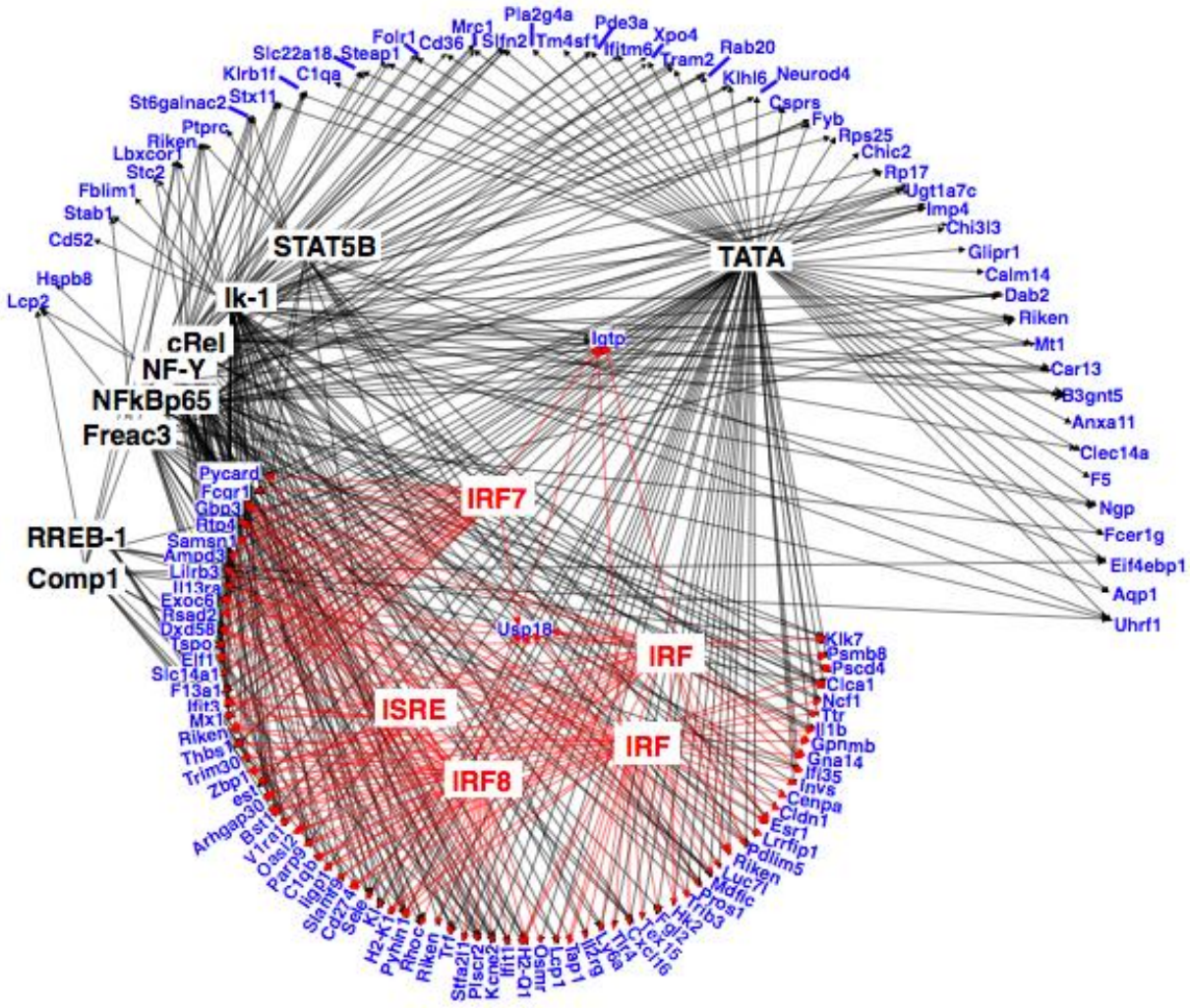

Figure 4.3. Interferon related TREs identified in the majority of genes increased following stroke in LPS preconditioned mice. Hypothesis Gene-TRE network showing the relationship of the identified TREs to the genes increased following stroke in LPS preconditioned mice. Genes are depicted in blue, interferon associated TREs represented in red and non-interferon TREs in black. P-value threshold set at 0.2.

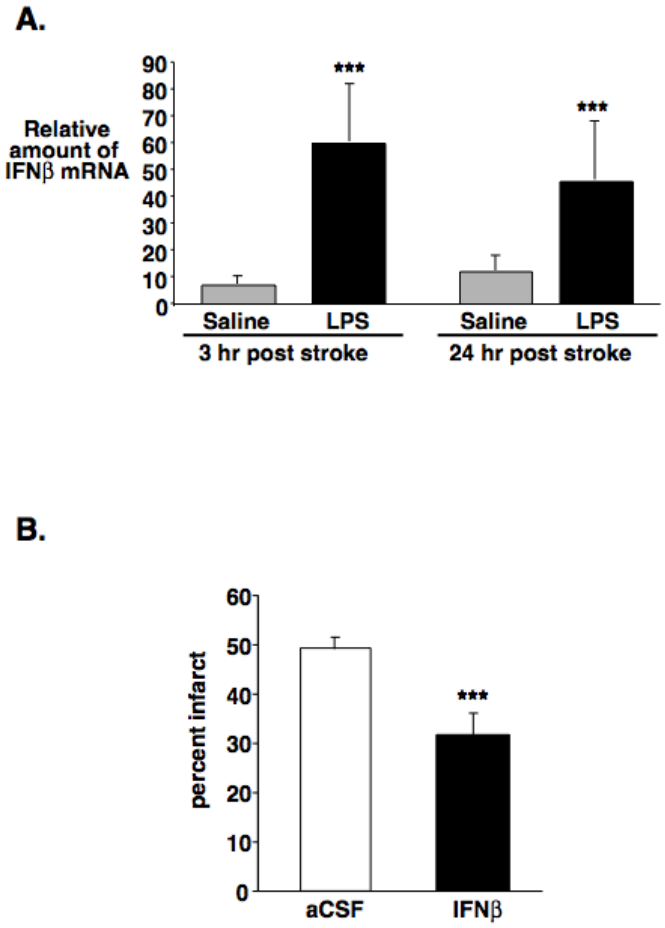

Figure 4.4. Increased levels of IFNβ following MCAO in LPS preconditioned mice may contribute to neuroprotection. A) Real-time PCR analysis was performed on RNA derived from the cortices following MCAO (3 and 24hr) of mice either preconditioned with LPS or saline. β-actin was used as a loading control. Results are presented as fold increase relative to unhandled controls. N= 3-4 mice/group; data are group means ± SEM; an overall effect of treatment was observed by two way ANOVA p=0.01. B) C57BL/6 mice were administered rmIFNβ (2x200U; i.c.v.) or artificial spinal fluid (aCSF) immediately prior to and following 60 min MCAO. Infarct volume was measured 24 hrs following surgery using TTC staining (n=9 and 10 respectively). Data shown are group means ± SEM; *** $p<0.001$ by Students T-Test.

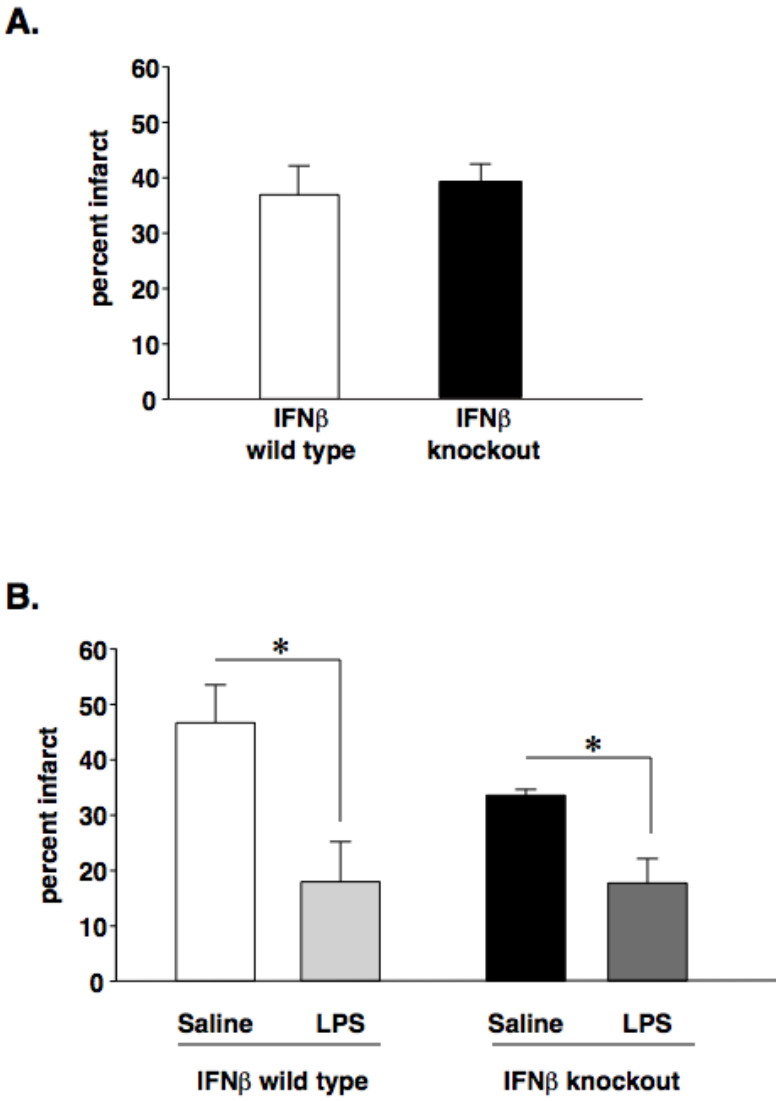

Figure 4.5. IFNβ is not required for LPS preconditioning. A) IFNβ knockout mice and their wild type counterparts were subjected to 40 min MCAO. Infarct volume was measured 72 hrs following surgery using TTC staining. Data shown are group means ± SEM; p = 0.7 by Students T-Test. B) IFNβ knockout and wild type mice were pretreated with LPS (1mg/kg) or saline 72 hours prior to 40 min MCAO. Infarct volume was measured 24 hrs following surgery using TTC staining. Data shown are group means ±

SEM; ** p<0.01, * p<0.05 by Two way ANOVA with Bonferroni post test, n = 6-11 per group.

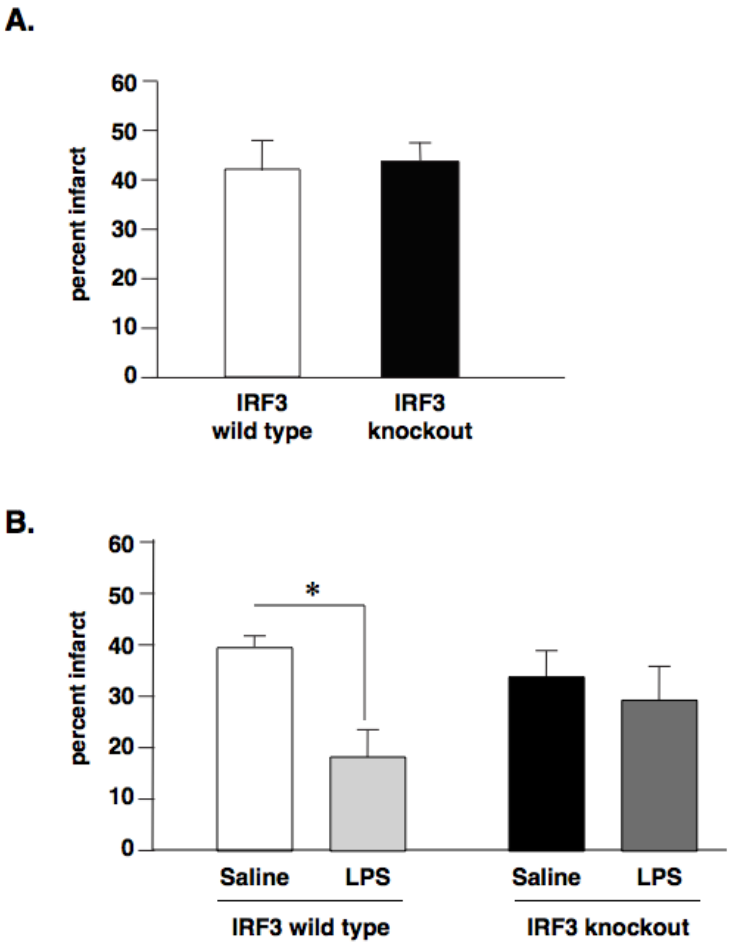

Figure 4.6 IRF3 is an essential mediator of LPS preconditioning. A) IRF3 knockout mice and their wild type counterparts were subjected to 40 min MCAO. Infarct volume was measured 72 hrs following surgery using TTC staining. Data shown are group means ± SEM; p = 0.8 by Students T-Test. B) IRF3 knockout and wild type mice were pretreated with LPS (1mg/kg) or saline 72 hours prior to 40 min MCAO. Infarct volume

was measured 24 hrs following surgery using TTC staining. Data shown are group means ± SEM; * $p<0.05$ by Two way ANOVA with Bonferroni post test, n = 7-10 per group.

Table 4.1 Defense response genes increased 24 hr following stroke exclusively in LPS preconditioned mice which are associated with type I interferon signaling [a]

Gene Name	Symbol	Reference
2'-5' oligoadenylate synthetase-like 2	Oasl2	165, 166
Complement component 1, q subcomponent, beta polypeptide	C1qb	167
Fc receptor, IgE, high affinity I, gamma polypeptide	Fcer1g	168
Fc receptor, IgG, high affinity I	Fcgr1	166
guanylate nucleotide binding protein 3	Gbp3	169
histocompatibility 2, D region	H2-L	170, 171
histocompatibility 2, D region locus 1	H2-D1	170, 171
histocompatibility 2, K1, K region	H2-K1	170
histocompatibility 2, Q region locus 1	H2-Q1	170
interferon gamma induced GTPase	Igtp	165
interferon inducible GTPase 1	Iigp1	172
interferon-induced protein 35	Ifi35	173
interferon-induced protein with tetratricopeptide repeats 1	Ifit1	173
interferon-induced protein with tetratricopeptide repeats 3	Ifit3	165
Interleukin 1 beta	Il1b	168
myxovirus (influenza virus) resistance 1	Mx1	165
protein tyrosine phosphatase, non-receptor type 6	Ptpn6, SHP1	174
proteasome (prosome, macropain) subunit, beta type 8	Psmb8, Lmp7	166
radical S-adenosyl methionine domain containing 2	Rsad2, VIPERIN	165, 166
retinoic acid-inducible protein I	RIG-1, Ddx58	175

schlafen 2	Slfn2	[176]
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Tap1	[174]
ubiquitin specific peptidase 18	Usp18	[166]
Z-DNA binding protein 1	Zbp1, DLM-1	[166]
interferon induced transmembrane protein 6	Ifitm6, fragilis5	Possibly regulated by IFN [177]
a. Identified by literature review.		

Table 4.2 TREs identified as significantly over represented in genes induced 24 hrs following stroke in LPS preconditioned mice

Transcriptional Regulatory Element	Adjusted p values for over-representation	
	LPS group	saline group
c-Rel/V$CREL_01	0.00	>1.00
IRF/V$IRF_Q6	0.00	>1.00
IRF/V$IRF_Q6_01	0.01	>1.00
NF-kappaB (p65)/V$NFKAPPAB65_01	0.01	0.86
RREB-1/V$RREB1_01	0.01	>1.00
IRF-8/V$ICSBP_Q6	0.03	0.99
NF-Y/V$NFY_Q6	0.03	>1.00
ISRE/V$ISRE_01	0.07	>1.00
STAT5B (homodimer)/V$STAT5B_01	0.07	>1.00
IRF-7/V$IRF7_01	0.15	>1.00
COMP1/V$COMP1_01	0.17	0.37
Freac-3/V$FREAC3_01	0.17	>1.00
Muscle TATA box/V$MTATA_B	0.17	>1.00
Ik1/V$IK1_01	0.199	0.64
S8/V$S8_01	>1.00	0.00
E2/V$E2_01	0.69	0.0024
C/EBPbeta/V$CEBPB_02	>1.00	0.13
HNF-1/V$HNF1_C	>1.00	0.13
Myogenin/NF-1/MYOGNF1_01	0.94	0.19

Chapter 5- Manuscript #4

LPS preconditioning signals through TLR4 and TRIF to up-regulate inhibitors of NFκB-inducing pathways and inhibit stroke-induced NFκB activity

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Chapter 5 contains data for a paper in preparation for *The Journal of Cerebral Blood Flow and Metabolism.*

Abstract

TLR activation by endogenous ligands following ischemia worsens stroke damage. Paradoxically, prior stimulation of TLR4 with bacterial lipopolysaccharide (LPS) provides neuroprotection against subsequent cerebral ischemic injury. Here, we demonstrate that LPS preconditioning, through activation of TLR4 and the TRIF adaptor molecule, leads to the inhibition of the TLR-to-NFκB signaling axis following stroke and results in the suppression of stroke-induced NFκB activity. Examination of mice lacking components of TLR signaling revealed that TLR4 contributes to brain injury during short durations of ischemia, but MyD88 does not. Conversely, TLR3 alleviates injury, but TRIF does not, indicating that TRIF may be responsible for the effects of both TLR3 and TLR4 during stroke. NFκB activity is significantly increased in the brain 24 hours following stroke. At this time, IRAK-M is increased and MyD88 is decreased, suggesting an endogenous attempt to suppress NFκB. LPS preconditioning causes an increase in NFκB activity within the brain prior to stroke followed by the up-regulation of the NFκB pathway inhibitors Ship-1, Tollip, and p105 either at the time of stroke or shortly thereafter, and the suppression of stroke-initiated NFκB activity. The neuroprotective effects of LPS preconditioning are independent of MyD88 but require TLR4 and TRIF. Our results indicate that LPS preconditioning mirrors the endogenous response to stroke by signaling through TLR4 and TRIF to up-regulate inhibitors of the TLR-to-NFκB signaling axis. The early regulation of these inhibitors may result in the suppression of

stroke-initiated NFκB activity and contribute to the protective effect of LPS preconditioning.

Introduction

The inflammatory response that is initiated after stroke can cause further tissue damage and cell death in the brain. Toll-like receptors (TLRs) are part of the inflammatory response. TLR2 and TLR4 have been shown to worsen tissue damage in several models of cerebral ischemia [43, 72, 75]. TLRs are expressed by microglia, astrocytes, endothelial cells, and neurons and are activated by damage-associated molecules such as HSP70 (TLR4) and HMGB1 (TLRs 2 and 4), found in the brain following ischemia [77, 78, 154]. Activation of these receptors causes the release of the pro-inflammatory molecules TNFα and iNOS, and other cytotoxic mediators.

TLRs are pattern recognition receptors that signal through common intracellular pathways and cause transcription factor activation and the production of cytokines and chemokines. Each TLR family member, with the exception of TLR3, initiates signaling via recruitment of the intracellular MyD88 adaptor. When recruited to plasma membrane-associated TLRs such as TLR2 and TLR4, MyD88 activates IRAK molecules that bind TRAF6. This leads to the activation of the IKK complex and MAPKKs and results in the nuclear translocation of the transcription factors NFκB and AP-1. Together, these transcription factors induce inflammatory cytokine production. Endosomal TLR3 is unique among the TLRs because it does not signal through MyD88 but signals exclusively through the TRIF adaptor. TRIF enlists the non-canonical IKKs, TBK1 and

IKKε, which activate the transcription factor IRF3 leading to transcription of the anti-viral cytokine IFNβ. TRIF also recruits RIP-1 and TRAF6, resulting in activation of MAPK and IKKα/β. Hence TLR3 is capable of activating NFκB, AP-1 and IRFs. Of all the TLRs, only TLR4 can recruit either MyD88 at the plasma membrane or, after being endocytosed, TRIF at the endosomal membrane [41]. TLR4 can thus induce TNFα via NFκB or IFNβ via IRF3. It is not known which pathway is responsible for the damaging effects of TLR4 activation following stroke.

In addition to binding damage-associated ligands, TLRs also detect pathogen-associated molecules. Primary exposure of macrophages to lipopolysaccharide (LPS), a potent TLR4 ligand of bacterial origin, activates NFκB and generates pro-inflammatory TNFα. Subsequently, macrophages suppress NFκB-inducing pathways by up-regulating pathway inhibitors, such that these cells generate markedly less TNFα upon secondary exposure to LPS [121, 122, 155]. In this manner, robust inflammatory responses to pathogen invasion can be controlled and extinguished.

A similar scenario may occur during the inflammatory response to stroke; one aspect of the brain's endogenous response to stroke may be to regulate stroke-induced TLR-to-NFκB signaling pathway. We hypothesize that TLR signaling in response to stroke initiates the production of damaging pro-inflammatory mediators, then dampens subsequent production of these mediators by blocking NFκB-inducing pathways.

Though TLR4 activation *following* stroke exacerbates injury, activation of TLR4 *prior* to stroke protects the brain from damage. LPS, administered systemically, renders animals tolerant to injury in several models of cerebral ischemia [79] [80, 81]. The molecular mechanisms by which LPS confers tolerance to subsequent ischemia may be similar to the mechanisms by which LPS confers tolerance to subsequent LPS. We hypothesize that LPS preconditioning leads to the *early* regulation of NFκB-inducing pathways, thereby suppressing stroke-induced NFκB activity. NFκB has been shown to exacerbate stroke injury[178-181], thus suppression of its activity would be expected to protect brain tissue from further damage.

We suggest that LPS preconditioning enlists endogenous strategies of neuroprotection. Without preconditioning, these strategies are belatedly induced and may provide some measure of protection from further damage. Preconditioning with LPS, we hypothesize, enlists these strategies *early*, thereby preventing much of the damaging inflammatory response to stroke and protecting brain tissue from injury.

Results

TLR4 knockout mice display reduced damage to focal ischemia in a dose-dependent manner

TLR4 is activated by a variety of host- endogenous molecules generated from damaged tissue such as HSP60 and HMGB1 [76, 182]. Stimulation of TLR4 by such molecules subsequent to ischemia might exacerbate the inflammatory response and thus contribute

to damage. Other researchers have assigned a detrimental role to TLR4 by examining infarct damage following a single duration of MCAO[70, 71, 73-75]. We asked whether this role is dependent on the duration of MCAO. To determine the effects of endogenous TLR4 signaling in response to various lengths of ischemia, TLR4 knockout mice (C57BL/10ScNJ) and wild-type controls (C57BL/10ScSnJ) were subjected to MCAO lasting 40, 50, or 60 minutes. Infarct volume was assessed via TTC staining 24 hours after reperfusion. Mice deficient in TLR4 suffered from significantly smaller infarcts than their wild-type counterparts when MCAO lasted 40 minutes (28.8% ± 5.0 versus 43.7% ± 3.8, respectively) or 50 minutes (34.8% ± 4 versus 48.3% ± 4, respectively), but incurred infarcts of similar size to wild-type mice when MCAO lasted 60 minutes (42% ± 2.8 vs. 44.7% ± 3.3, respectively) (Figure 5.1). These data indicate that endogenous TLR4 activity significantly contributes to cerebral ischemic damage following short durations of ischemia, but has negligible effects following long durations of ischemia.

TLR3 knockout mice incur significantly larger infarcts than wild-type mice

Signaling through TLR3 has been shown to sustain neuronal survival by the production of neuroprotective molecules [27]. Endogenous ligands of TLR3, such as mRNA, are released from necrotic cells and might be present in the brain following ischemia. We thus asked whether endogenous TLR3 activity protects against cerebral ischemic injury. TLR3 knockout mice and wild-type controls were subjected to 40 minutes of MCAO and infarct volume was assessed via TTC staining 24 hours after reperfusion. Mice deficient in TLR3 incurred significantly larger infarcts than wild-type mice (24.4% ± 4.3 versus

37.3% ± 4.2, respectively, Figure 5.2). These results indicate that TLR3 signaling following cerebral ischemia protects brain tissue from damage.

TRIF knockout mice incur infarcts of similar size to wild-type mice

Both TLR3 and TLR4 signal through the TRIF adaptor molecule, yet these receptors have opposite effects on stroke outcome. We postulated that removal of TRIF would reveal which of these two receptors contributes more to ischemic outcome. We therefore asked whether signaling through TRIF contributes to or protects from cerebral ischemic injury. TRIF knockout mice and wild-type controls were subjected to 40 minutes of MCAO and infarct volume was assessed via TTC staining 24 hours after reperfusion. Mice deficient in TRIF incurred infarcts of similar sizes to those of wild-type mice (32.0% ± 3 versus 29.2% ± 5.5, respectively, Figure 5.3). These data suggest that, on balance, TRIF signaling neither worsens nor protects against ischemic injury.

MyD88 knockout mice incur infarcts of similar size to wild-type mice

TLR4 also signals through the MyD88 adaptor molecule. We next asked whether MyD88 contributes to ischemic injury. MyD88 knockout mice and wild-type controls were subjected to 40 minutes of MCAO and infarct volume was assessed via TTC staining 24 hours after reperfusion. Forty minutes of MCAO was chosen because it is at this duration that TLR4 knockout mice incur significantly less damage than wild-type mice. However, mice deficient in MyD88 incurred infarcts of similar sizes to those of wild-type mice (40.4% ± 3.3 versus 36.0% ± 3, respectively, Figure 5.4). These data indicate that

MyD88 does not contribute to ischemic injury, hence the damage initiated by TLR signaling following stroke may not occur through MyD88.

Stroke increases NFκBp65 DNA binding activity in the brain

TLR4 contributes to ischemic injury. One transcription factor activated downstream of TLR4 is NFκB. We thus hypothesized that stroke would lead to an increase in the DNA binding activity of NFκB. Ipsilateral cortex was collected 1, 3, and 24 hours following stroke. Electrophoretic mobility shift assays were performed to assess the DNA binding activity of NFκB in the brain at these time points. We detected a significant increase in NFκB activity in the brain 24 hours following stroke (Figure 5.5A). Supershift assays confirmed the identity of the DNA-bound transcription factor as the p65 subunit of NFκB (Figure 5.5B). Hence stroke causes an increase the DNA binding activity of the TLR4-induced transcription factor NFκB.

Stroke causes the late regulation of NFκB-inducing pathways

We have shown an increase in NFκB activity within the brain 24 hours after cerebral ischemia. NFκB has been show to worsen ischemic damage, hence we postulated that endogenous mechanisms might be employed to suppress stroke-induced NFκB activity, thereby preventing further damage. Western blot analysis revealed the up-regulation of IRAK-M, a non-functional IRAK decoy, and the down-regulation of MyD88 24 hours

after stroke in control animals (Figure 5.6). Hence one endogenous response to stroke-induced NFκB activity appears to be the regulation of NFκB-inducing pathways.

LPS preconditioning increases NFκB activity in the brain before ischemia and suppresses ischemia-induced NFκB activity

Although endogenous stimulation of TLR4 following stroke worsens ischemic injury, stimulation of this receptor prior to stroke alleviates subsequent damage. TLR4 and stroke both activate NFκB. We hypothesized that, like LPS-induced tolerance to subsequent LPS, LPS-induced ischemic tolerance entails the suppression of insult-induced NFκB activity. Mice were injected with either LPS (1mg/kg) or saline and nuclear protein from the ipsilateral cortex was collected either following injection or following subsequent stroke. Electrophoretic mobility shift assays were performed to assess the DNA binding activity of NFκB in the brain at these time points. LPS caused a significant decrease in NFκB DNA binding activity 3 hours after administration followed by a significant increase 24 and 72 hours after administration (Figure 5.7A). Following stroke, LPS preconditioning prevented the increase in stroke-induced NFκB activity (Figure 5.7B). Supershift assays confirmed the identity of the DNA-bound transcription factor as the p65 subunit of NFκB (data not shown). These results indicate that systemic

LPS administration increases NFκB activity within the brain following administration and prevents stroke-induced NFκB activity.

LPS preconditioning causes the early regulation of NFκB-inducing pathways

Cells that are exposed to LPS signal through pro-inflammatory pathways to initiate inflammatory responses. Cells then up-regulate inhibitors of inflammatory pathways that dampen and control this response. We postulated that, in a similar manner, LPS preconditioning would cause the up-regulation of inhibitory molecules that could act to dampen and control the subsequent inflammatory response to stroke. Western blot analysis revealed that LPS preconditioning caused the up-regulation of Ship-1, a phosphatase that inhibits TLR-MyD88 interactions, at the time of stroke and 24 hours after MCAO (Figure 5.8A), Tollip, a molecule that binds IRAK-1 and suppresses its activity, 3 and 24 hours after MCAO (Figure 5.8B), and p105, a non-canonical IκB, 24 hours after MCAO (data not shown). Interestingly, unlike control animals, LPS preconditioned mice displayed no regulation of IRAK-M or MyD88 following stroke. Hence, LPS preconditioning caused the early regulation of NFκB-inducing pathways that differs from the regulation endogenously induced later following stroke in control animals.

TLR4 signaling is required for LPS-induced neuroprotection

We next examined the role of TLR4 in LPS preconditioning. TLR4 deficient mice (C57BL/10ScNJ) and their wild-type counterparts (C57BL/10ScSnJ) were subjected to 40 min of MCAO followed by 24 hrs of reperfusion. Animals were either pretreated with

LPS (i.p.; 1mg/kg) or saline 72 hrs prior to ischemia. Following reperfusion, infarct volume was assessed via TTC staining. LPS preconditioning significantly reduced infarct volume in wild-type mice (percent reduction from saline 46.2% ± 7.1, $p<0.01$, Figure 5.9). However, preconditioning had no effect on infarct volume in mice deficient for TLR4 ($p = 0.3$). Hence the neuroprotective effects of LPS preconditioning are mediated through TLR4.

MyD88 is not required for LPS-induced neuroprotection

We next asked if the TLR4 adaptor molecule MyD88 is required for LPS-induced neuroprotection. MyD88-deficient and wild-type mice were subjected to 40 min of MCAO followed by 24 hrs of reperfusion. Animals were either pretreated with LPS (i.p.; 1mg/kg) or saline 72 hrs prior to ischemia. Following reperfusion, infarct volume was assessed via TTC staining. Both wild-type and knockout mice were significantly protected by LPS pretreatment (percent reduction from saline 44.4% ± 13.6, $p<0.05$, and 44.7% ± 6.1, $p<0.01$, respectively, Figure 5.10) Hence the neuroprotective effects of LPS preconditioning are not mediated through MyD88.

TRIF is required for LPS-induced neuroprotection

We next examined the role of the TRIF adaptor in LPS preconditioning. TRIF-deficient and wild-type mice were subjected to 40 min of MCAO followed by 24 hrs of reperfusion. Animals were either pretreated with LPS (i.p.; 1mg/kg) or saline 72 hrs prior to ischemia. Following reperfusion, infarct volume was assessed via TTC staining. LPS preconditioning reduced infarct volume in wild-type mice (percent reduction from saline

27.6% ± 17.9, $p<0.05$, Figure 5.11). However, preconditioning had no effect on infarct volume in TRIF knockout mice ($p = 0.3$). Hence the neuroprotective effects of LPS preconditioning are mediated by TRIF.

Discussion

We propose a molecular model of LPS preconditioning wherein stimulation of TLR4 prior to ischemia signals through TRIF to activate NFκB in the brain. This leads to a small inflammatory response and the subsequent up-regulation of inflammatory inhibitors, including inhibitors of NFκB-inducing pathways. These inhibitors are present at the time of stroke, and prevent activated TLRs from signaling to NFκB. Hence, stroke-induced NFκB activity in the brain is suppressed. In this manner, LPS preconditioning induces endogenous mechanisms of neuroprotection early after the ischemic event, thereby preventing damaging inflammatory sequella.

The TRIF adaptor molecule is emerging as the primary adaptor through which TLR4 signals. A recent study demonstrated that TRIF-dependent signaling causes 75% of the transcriptional response to LPS in macrophage [183]. We suggest that TRIF-dependent signaling is responsible for the deleterious effects of TLR4 activation following stroke. We found that while TLR4 activity worsens ischemic damage, TLR3 activity alleviates injury. TLR3 signals exclusively through TRIF, yet TRIF itself does not appear to

alleviate injury. We suggest that TRIF is used by both TLR3 and TLR4 and that it has opposing functions downstream of these two receptors. In the brain, TLR3 is primarily expressed by astrocytes and stimulation of TLR3 on these cells causes the generation of several factors that support neuronal survival [27]. Neurons have also been shown to express TLR3 and to produce the neuroprotective cytokine IFNβ when stimulated withTLR3 lignads [184]. This suggests that TRIF signaling downstream of TLR3 activates neuroprotective pathways in these cells. On the other hand, TLR4 is primarily expressed by microglia and TLR4-induced activation of microglial leads to oligodendrocyte injury [159] and neurodegeneration [185]. This suggests that TRIF signaling downstream of TLR4 may activate damaging pathways in microglia. Thus, it appears that TLR3 and TLR4 are preferentially expressed by different cell types in the brain and may initiate different actions through TRIF-dependent signaling.

One potential outcome of TLR4 stimulation following stroke is the activation of NFκB. We found a significant increase in NFκB DNA binding activity in the brain 24 hours after stroke. NFκB is known to worsen ischemic injury, hence it may be through activation of this transcription factor that TLR4 exerts its deleterious effects. One endogenous mechanism employed by activated cells to keep inflammatory responses in check is the regulation of inflammatory signaling pathways. We found evidence for such regulation 24 hours after stroke when MyD88 expression was decreased and IRAK-M was increased. IRAK-M has not been shown to be expressed in brain parechymal tissue, indicating that infiltrating cells such as macrophage and neutrophils may be regulating these molecules. Hence, one mechanism of endogenous protection from stroke-initiated

inflammatory damage may be a belated suppression of NFκB-inducing pathways in infiltrating cells.

LPS preconditioning causes the up-regulation of inflammation inhibitors prior to, or just after, the ischemic event. In this manner, LPS pretreatment may shift stroke-induced TLR4 signaling toward a neuroprotective pathway similar to that initiated by TLR3. LPS-induced tolerance to ischemic injury mirrors the phenomenon of LPS-induced tolerance to LPS. Initial exposure of macrophages to LPS causes the production of pro-inflammatory TNFα, but upon subsequent exposure to LPS, TNFα production is reduced markedly due to suppressed NFκB-inducing pathways [121, 122, 155]. This suppression is achieved by the up-regulation of pathway inhibitors, namely IRAK-M, Tollip, Ship1 and Trim30α [92, 157] following the initial LPS exposure. Conversely, macrophages produce little IFNβ upon initial exposure to LPS, but *enhance* IFNβ production upon secondary exposure [93], suggesting up-regulated TLR4 signaling through IRF3-inducing pathways. Our data support a similar model of redirected TLR4 signaling following stroke. We have shown that LPS preconditioning up-regulates the inflammatory pathway inhibitors Ship-1, Tollip, and p105, which may block TLR4 signaling from TRIF to NFκB. Tollip and p105 have been shown to be expressed within the brain parenchyma [186-188], indicating that microglial may be regulating these molecules. LPS pretreatment led to a significant reduction in stroke-induced NFκB activity. Because this pathway is blocked, subsequent TLR4 signaling may be shunted down the IRF3-inducing pathway. We have previously shown that LPS preconditioning enhances the production of Type I IFN-associated genes following stroke and protects the brain via IRF3. Together, these data support a model of

redirected stroke-induced TLR4 signaling. This redirection is initiated by LPS preconditioning and contributes to its neuroprotective effects.

The finding that LPS preconditioning requires TRIF, and not MyD88, supports the idea that TLR4 signals primarily through the TRIF adaptor molecule. We first confirmed that TLR4 mediates the protective effects of LPS preconditioning by demonstrating that mice lacking TLR4 are not protected by LPS pretreatment. We then found that, of the two adaptor molecules utilized by TLR4, only TRIF was required for LPS-induced neuroprotection. This demonstrates for the first time that TRIF-mediated signaling prior to stroke protects against ischemic injury.

In summary, we have shown that LPS preconditioning utilizes TLR4-TRIF pathways to up-regulate inhibitors of NFκB-inducing pathways that are present in the brain early after stroke. We suggest that this mirrors the endogenous response to stroke, but differs in the timing of induction. Preconditioning activates the endogenous response earlier, redirecting TLR4 signaling at the time of stroke. Redirected TLR4 signaling results in suppressed pro-inflammatory signaling to NFκB and enhanced pro-survival signaling to IRF3. Together, these responses transform TLR4 signaling following stroke from one that increases cell death to one that actively protects the brain from further injury.

Materials and Methods

Mice: C57Bl/6 mice (male, 8 to 10 weeks) were obtained from Jackson Laboratories (West Sacramento, California, USA). TLR4 knockout mice (C57Bl/10ScSn) and their wild-type counterparts (C57Bl/10ScN), TLR3 knockout mice (B6;129S1-Tlr3^{tm1Flv}/J) and their wild type counterparts (B6;129SF1/J) and TRIF knockout mice (C57Bl/6J-Ticam1^{LPS2}/J) were also obtained from Jackson Laboratories. MyD88 knockout mice (C57Bl/6 background) were a kind gift of Dr. Shizuo Akira (Osaka University, Osaka Japan) and were bred in our facility. All mice are housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

LPS Treatment: Mice were given a 200 ul intraperitoneal injection of saline or LPS (1.0 mg/kg; *Escherichia coli* serotype *055:B5*; Sigma).

Reagents: Western blots were performed using antibodies against IRAK-M (ProSci, 2355), MyD88 (R&D, AF3109), Ship-1 (Santa Cruz, sc8425), Tollip (AbCam, Ab37155), and Actin beta (Santa Cruz, sc1616R).

Middle Cerebral Artery Occlusion (MCAO): Mice were anesthetized with 4% halothane and subjected to MCAO using the monofilament suture method described previously [68]. Briefly, a silicone-coated 8-0 monofilament nylon surgical suture was threaded through the external carotid artery to the internal carotid artery to block the middle cerebral

artery, and maintained intraluminally for between 40 and 60 min. The suture was then removed to restore blood flow. Cerebral blood flow was monitored throughout surgery by laser Doppler flowmetry. Body temperature was maintained at 37°C with a thermostat-controlled heating pad.

Infarct evaluation: To visualize the region of infarction, 6 x 1 mm coronal midsections were placed in 1.5% 2,3,5 triphenyltetrazolium chloride (TTC) in 0.9% phosphate buffered saline and stained at 37°C for 15 min [164]. The infarct size was determined from computer-scanned images of the hemispheres using NIH images analyses. To account for edema within the infarct region, infarct area for each section was computed indirectly as: 100 x (contralateral hemisphere area - area of live tissue on ipsilateral hemisphere)/(contralateral hemisphere area) [116].

Electrophoretic Mobility Shift Assay: Nuclear protein extracts were prepared from tissue dissected from the ipsilateral cortex. Homogenized tissue was incubated in Buffer A (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF) for 5 minutes on ice, centrifuged at 3000 rpm for 5 minutes at 4°C, and the supernatant saved as cytoplasmic extract. Pellets were washed once in Buffer B (10mM Hepes-KOH pH7.9, 60mM KCl, 1mM EDTA, 0.5%NP-40, 1mM Dtt, 1mM PMSF), then resuspended in Buffer C(250mM Tris pH7.8, 60mM KCl, 1mM DTT, 1mM PMSF), and freeze-thawed 3 times in liquid nitrogen. All buffers contained a protease inhibitor cocktail (Roche). After cenTRIFuging at 10,000 rpm for 10 min at 4°C, supernatant was saved as nuclear extract. Nuclear protein concentrations were determined using the BCA method (Pierce-

Endogen). Electrophoretic mobility shift assays were performed using the Promega Gel Shift Assay System according to the manufacturer's instructions. Briefly, 15ug of nuclear protein was incubated with ^{32}P-labeled NFκB consensus oligonucleotide (Promega), either with or without unlabeled competitor oligonucleotide, unlabeled non-competitor oligonucleotide, or anti-p65 antibody (Santa Cruz). Samples were electrophoresed on a 4% acrylamide gel, dried and exposed to phosphorimager overnight. The densitometry of the gel bands was analyzed using scanning integrated optical density software (ImageJ).

Western Blotting: Protein extraction was performed as described previously [120] with some modifications. Briefly, tissue samples were dissected from the ipsilateral cortex and lysed in a buffer containing a protease inhibitor cocktail (Roche). Protein concentration were determined using the BCA method (Pierce-Endogen). Protein samples (50ug) were denatured in a gel-loading buffer (Bio-Rad Laboratories) at 100°C for 5 min and then loaded onto 12% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories). Following electrophoresis, proteins were transferred to polyvinylodene difluoride membranes (Bio-Rad Laboratories) and incubated with primary antibodies at 4°C overnight. Membranes were then incubated with anti-rabbit, anti-goat, or anti-mouse IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and detected by chemiluminescence (NEN Life Science Products) and exposure to Kodak film (Biomax). Images were captured using an Epson scanner and the densitometry of the gel bands, including actin-beta as a loading control, was analyzed using ImageJ.

Acknowledgements

The authors would like to thank Rebecca Williams Karnesky for excellent technical support. This work was supported by National Institutes of Health grant R01 NS050567 (MS-P).

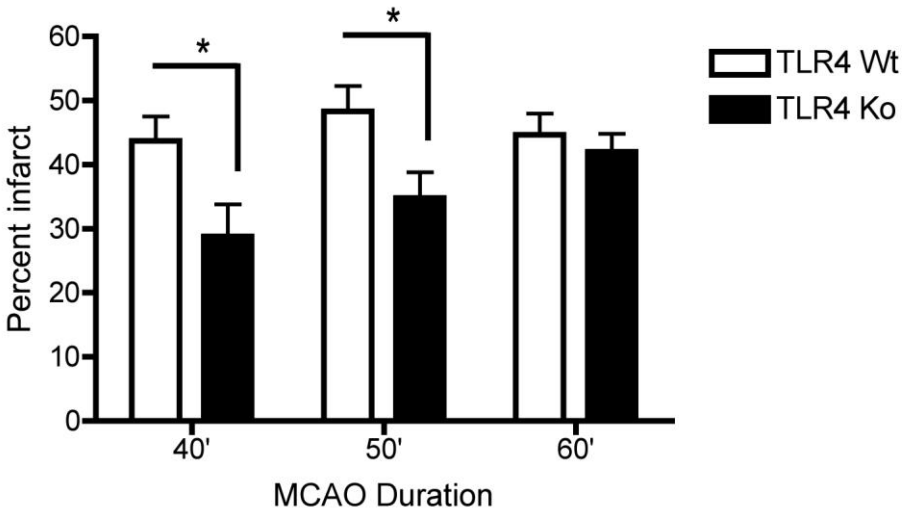

Figure 5.1. TLR4 knockout mice incur significantly smaller infarcts than wild-type mice in a dose dependent manner. TLR4 knockout mice and wild-type counterparts

were subjected to MCAO of 40, 50, or 60 min duration. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM; an overall effect of genotype was detected by two way ANOVA; *p<0.05 comparison to wild-type controls by Students T test

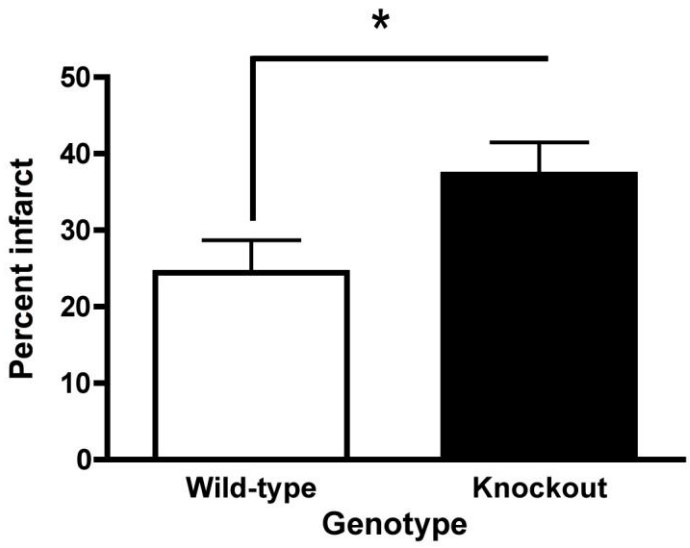

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Figure 5.2. TLR3 knockout mice incur significantly larger infarcts than wild-type mice. TLR3 knockout mice and wild-type controls were subjected to 40 min MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM; *p<0.05 comparison to wild-type controls by Students T test.

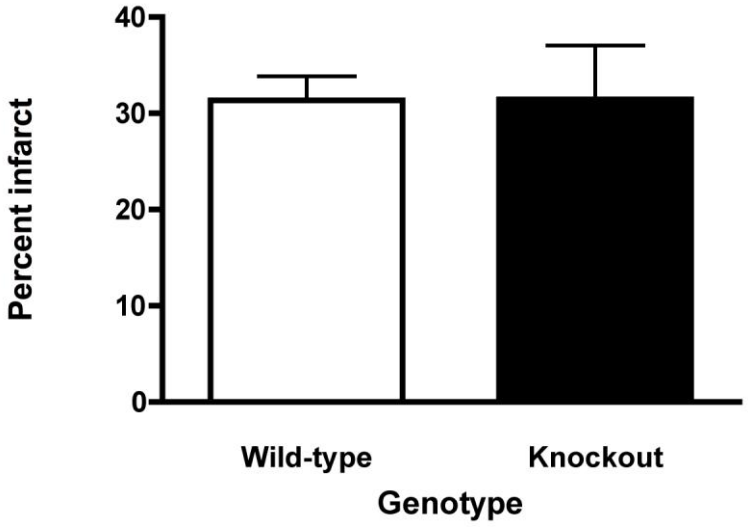


Figure 5.3. TRIF knockout mice incur infarcts of similar size to wild-type mice. MyD88 knockout mice and wild-type controls were subjected to 40 min MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM.

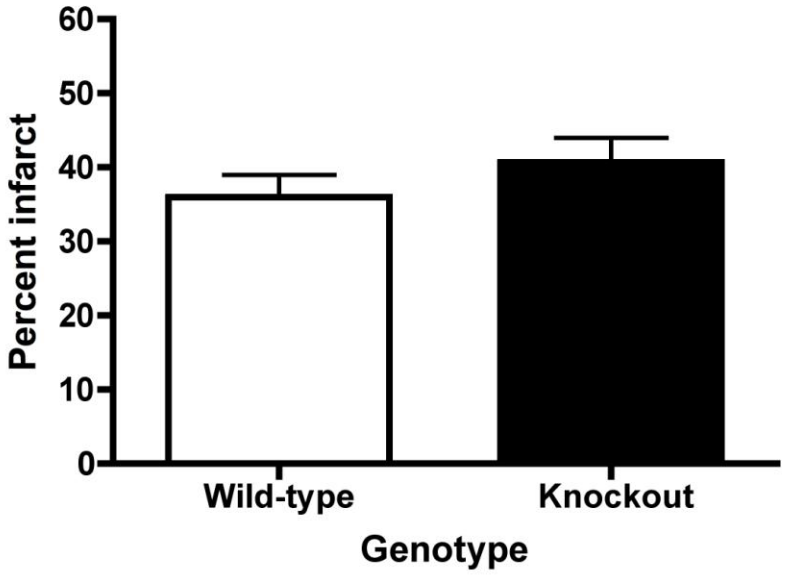


Figure 5.4. MyD88 knockout mice incur infarcts of similar size to wild-type mice. MyD88 knockout mice and wild-type controls were subjected to 40 min MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM.

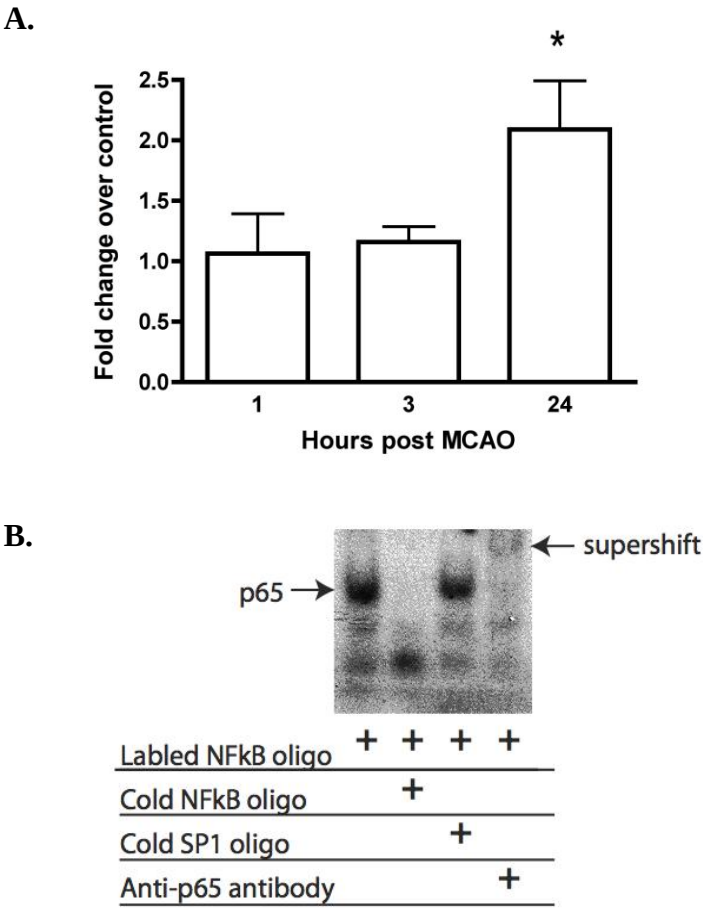
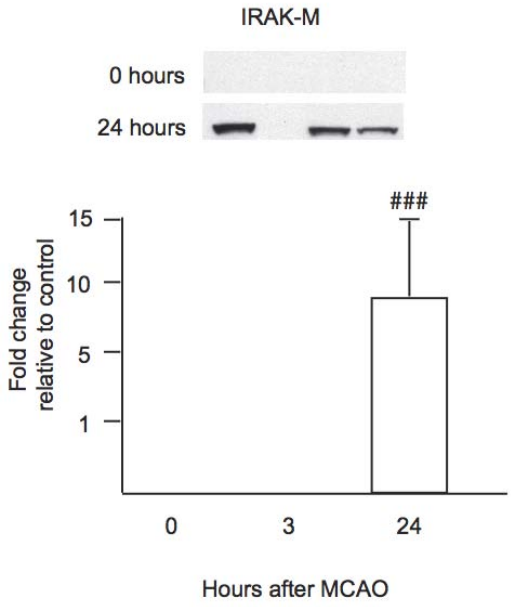


Figure 5.5. Stroke increases the DNA-binding activity of NFκBp65 in the brain. Gel shift assays were performed on nuclear protein isolated from ipsilateral cortex at various

times following stroke. Stroke significantly increased the binding activity of NFκB within the brain after 24 hours. B. Representative image of a supershift assay revealing the activity of the NFκBp65 subunit following LPS administration and following MCAO. DNA binding activities were assessed by phosphorimage intensity and are reported here as fold change over saline; n=3-4 / treatment / time; *p<0.05 by Students T test.

A.



B.

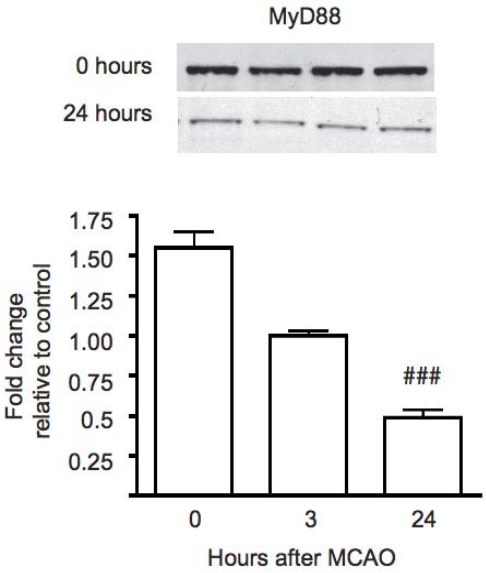


Figure 5.6. The endogenous response to stroke includes inhibition of NFκB-inducing pathway 24 hours after ischemia. A. Western blot analysis of total protein isolated from ipsilateral cortices following MCAO indicates the up-regulation of IRAK-M 24 hours after stroke in control animals. B. MyD88 protein is suppressed 24 hrs after stroke in control animals. N=3-4 /treatment /time; data shown are average band intensities relative to unhandled control; $^{\#}$ = difference from unhandled control, $^{\#\#\#}p<0.001$.

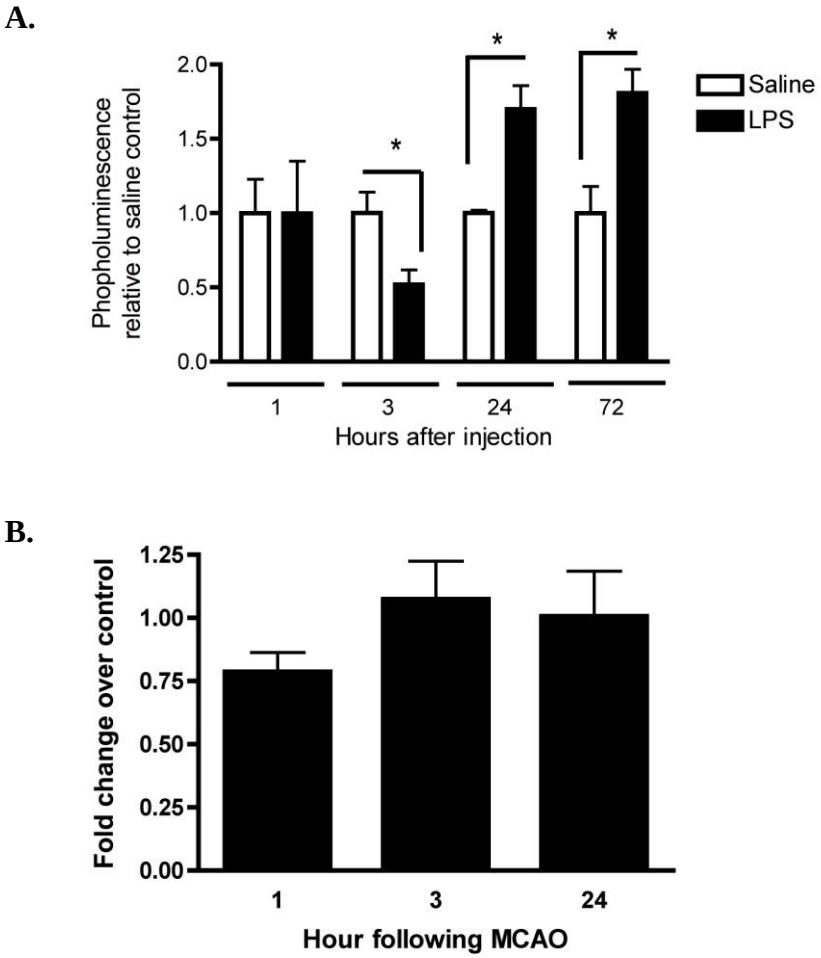


Figure 5.7. Systemic LPS administration increases NFκB activity in the brain prior to stroke, and decreases NFκB activity following stroke. A. Gel shift assays were

performed on nuclear protein isolated from ipsilateral cortex at various times following LPS or saline administration. LPS significantly increased the binding activity of NFκB 24 and 72 hours after administration. B. LPS preconditioning inhibited stroke-induced NFκB DNA binding activity 24 hours after MCAO. DNA binding activities were assessed by phosphorimage intensity and are reported here as fold change over saline; n=3-4 / treatment / time; *p<0.05 by Students T test.

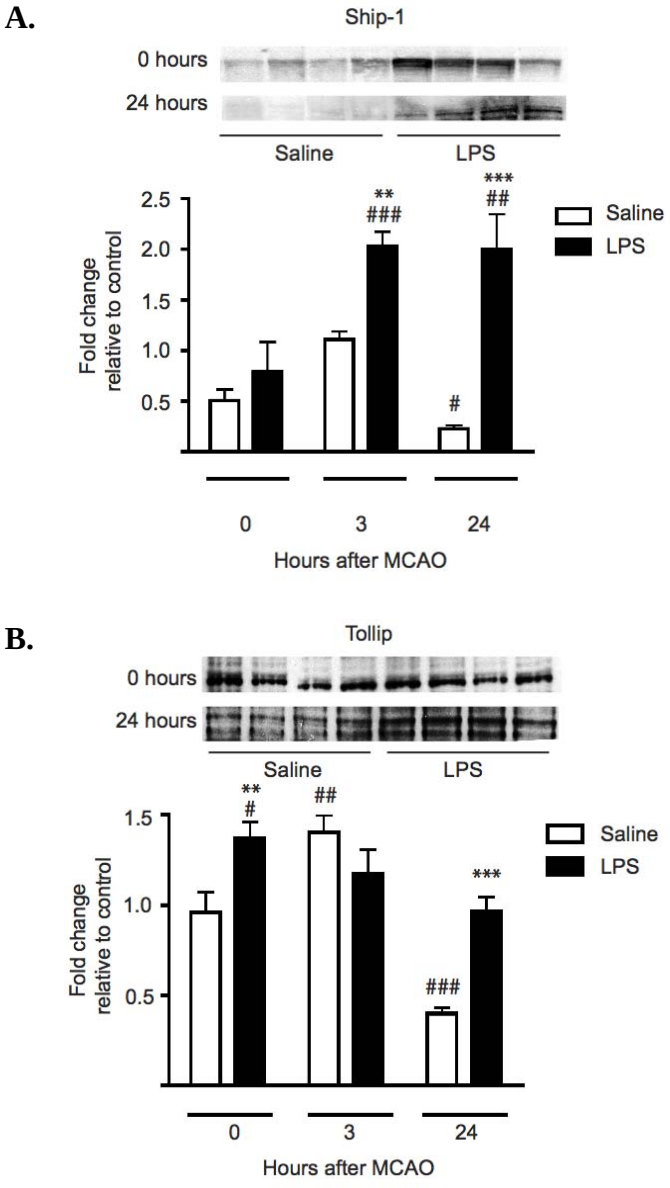


Figure 5.8. LPS preconditioning causes an early up-regulation of MyD88 pathway inhibitors. A. Western blot analysis of total protein isolated from ipsilateral cortices following MCAO indicates the up-regulation of Ship-1 3 and 24 hours after stroke in LPS pretreated animals. B. LPS preconditioning causes the early up-regulation of Tollip following MCAO. N=3-4 /treatment /time; data shown are average band intensities relative to unhandled control; * = difference from saline, # = difference from unhandled control, *,#p<0.05, **,##p<0.01, ***,###p<0.00

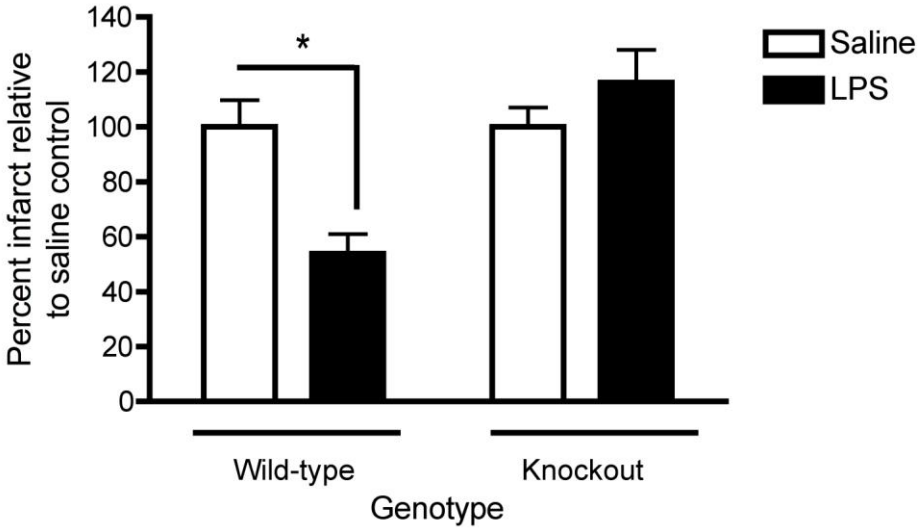


Figure 5.9. LPS preconditioning does not protect TLR4 knockout mice from cerebral ischemic injury. TLR4 knockout mice and wild-type controls were administered LPS (1mg/km) or saline, i.p., 72 hours prior to 40 minute MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM. *p<0.05 by two way ANOVA.

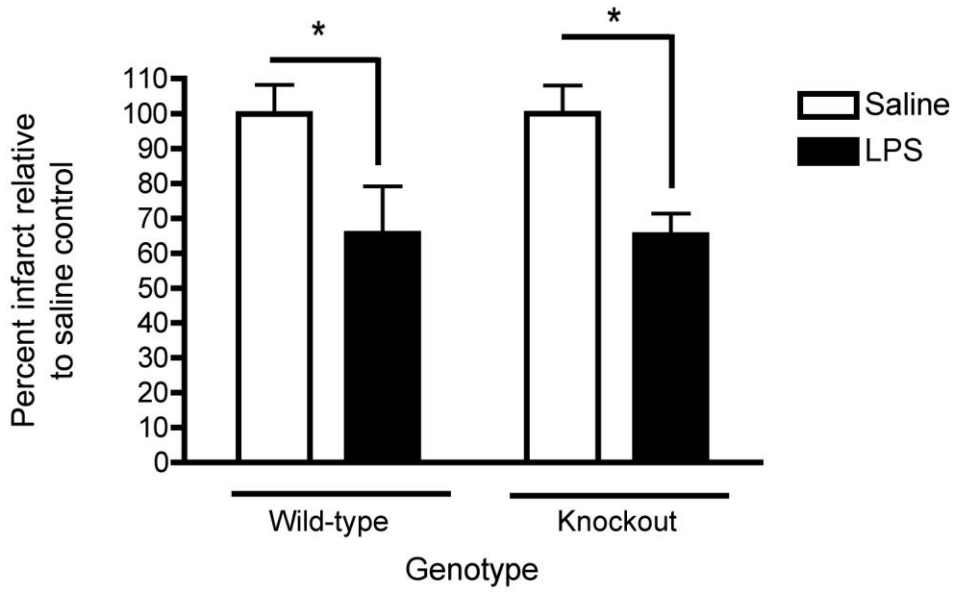


Figure 5.10. LPS preconditioning protects MyD88 knockout mice from stroke injury. MyD88 knockout mice and wild-type controls were administered LPS (1mg/km) or saline, i.p., 72 hours prior to 40 minute MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM. An overall effect of treatment was assessed by two way ANOVA, *p<0.05 by Students T test.

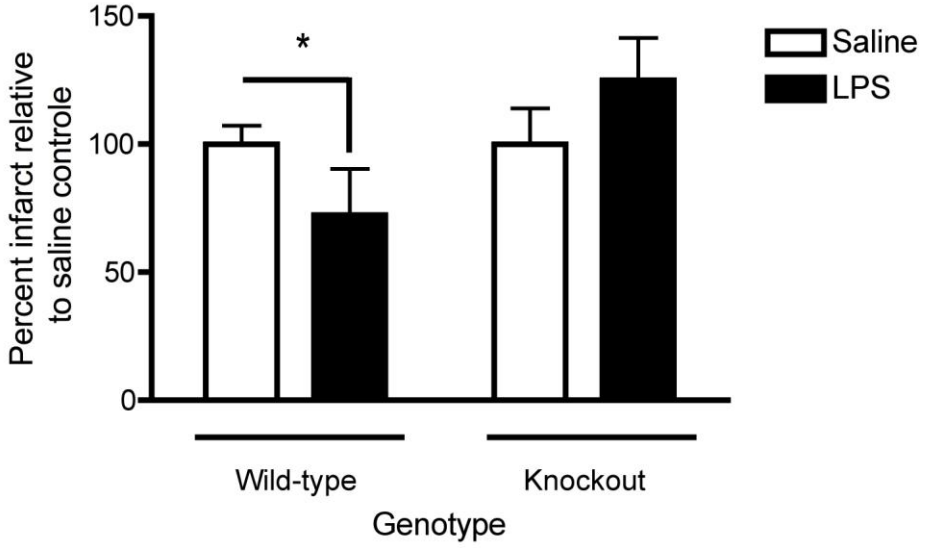


Figure 5.11. LPS preconditioning does not protect TRIF knockout mice from cerebral ischemic injury. TRIF knockout mice and wild-type controls were administered LPS (1mg/km) or saline, i.p., 72 hours prior to 40 minute MCAO. Infarct volume was determined 24 hrs following MCAO by TTC staining. Values are group means ± SEM. *$p<0.05$ by Students T test.

Chapter 6

Summary, Conclusions and Prospectives

Summary and conclusions

The studies presented in this thesis demonstrate that preconditioning the brain with TLR ligands reprograms the endogenous response to stroke. Such reprogramming entails a redirection of stroke-induced TLR signaling that transforms the response of these receptors from one that increases cell death to one that actively protects the brain from further injury. Although TLR4 and TLR9 are expressed by different sets of cells, stimulation of these receptors with LPS and CpG, respectively, appears to reprogram the brain in parallel ways. Each ligand protects the brain for a similar time interval following administration and each requires the proinflammatory cytokine TNFα for its protective effects. Intriguingly, both LPS and CpG preconditioning induce ISG expression in the brain following the ischemic event. IFNβ is the most well characterized inducer of ISGs. The neuroprotective potential of ISG activation is demonstrated by the fact that i.c.v. administration of IFNβ protects the brain from stroke injury. Hence, the final effectors of TLR-induced neuroprotection are likely ISGs within the brain. However, the molecular

mechanisms by which these ISGs are induced may depend upon the TLR involved in preconditioning.

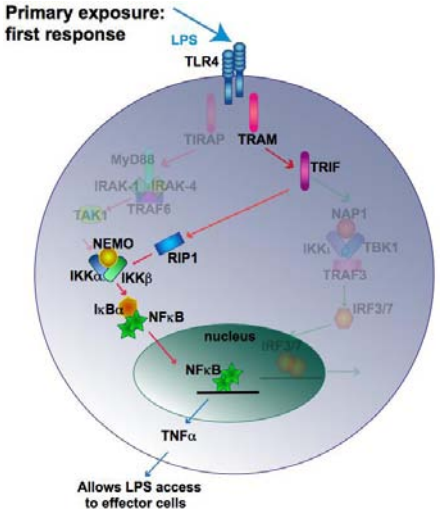
LPS preconditioning mirrors the brain's endogenous response to stroke, but it induces this response early after stroke, thereby preventing much of the damaging early inflammatory response. Ischemic stroke, without preconditioning, causes the release of molecules that stimulate TLR4, which signals through TRIF to activate NFκB. Activation of NFκB following stroke worsens ischemic damage by producing inflammatory mediators and initiating early inflammatory responses. Subsequently, anti-inflammatory molecules are produced to control the inflammatory response. These molecules dampen further TLR signaling to NFκB and increase TLR signaling to IRF3. LPS preconditioning induces the same sequence of events, but is timed such that the anti-inflammatory molecules are present at the time of stroke. In this manner, TLR signaling to NFκB at the time of stroke is blocked, and signaling to IRF3 is increased, constituting a redirection of stroke-induced TLR signaling. The redirected response is evidenced by a suppression of stroke-induced NFκB activity and an increase in IRF3-mediated transcription. Both aspects of redirected signaling have neuroprotective potential.

CpG preconditioning may induce a different series of events that also lead to a redirection of stroke-induced TLR signaling and an increase in Type I IFN gene expression in the brain. Systemic CpG administration stimulates TLR9 on natural killer (NK) cells. NK cells then redirect subsequent TLR signaling and respond to stroke-induced TLR ligands by increasing signaling to IRF3. IRF3 activity increases NK cell responsiveness to IL-12,

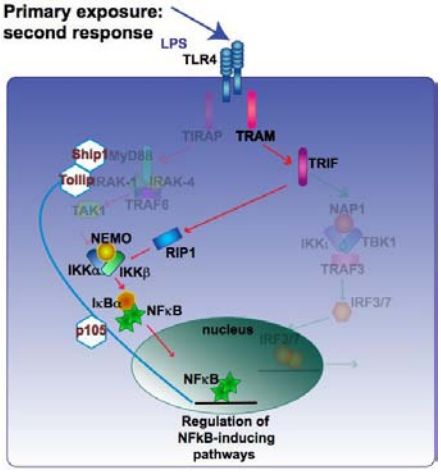
thereby increasing NK cell feedback to pDCs. Activated pDCs are potent producers of IFNα, which can cross the compromised BBB after stroke and affect a Type I IFN transcriptional response. It thus appears that LPS and CpG cause a redirection in stroke-induced TLR signaling, in the brain and in the periphery, respectively. This new response leads to the generation of a protective Type I IFN transcriptional profile in the brain following stroke which constitutes a reprogrammed response to cerebral ischemia.

These results advance the hypothesis that redirection of stroke-induced TLR signaling plays an important role in driving the reprogrammed response to stroke following TLR preconditioning. The reprogrammed response may be responsible for the neuroprotective effects of TLR preconditioning. These findings provide a strong foundation for future investigation of TLR ligands as stroke prophylactics.

A.


Primary exposure:
first response
LPS
TLR4
TIRAP
TRAM
MyD88
TRIF
IRAK-1
IRAK-4
TAK1
TRAF6
NAP1
IKKi
TBK1
NEMO
RIP1
IKKα
IKKβ
TRAF3
IκBα
NFκB
IRF3/7
nucleus
NFκB
TNFα
Allows LPS access
to effector cells

B.


Primary exposure:
second response
LPS
TLR4
TIRAP
TRAM
Ship1
MyD88
TRIF
Tollip
IRAK-1
IRAK-4
TAK1
TRAF6
NAP1
IKKi
TBK1
NEMO
RIP1
IKKα
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TRAF3
IκBα
NFκB
IRF3/7
p105
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Regulation of
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C.

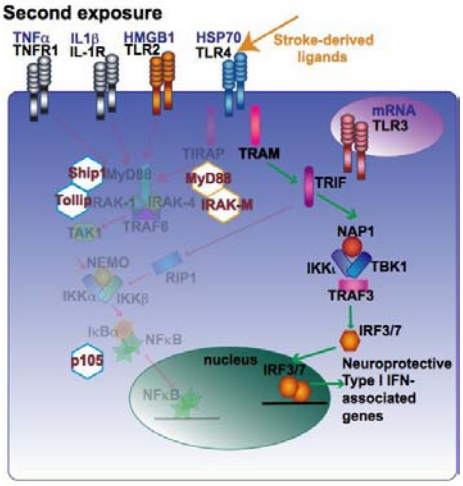
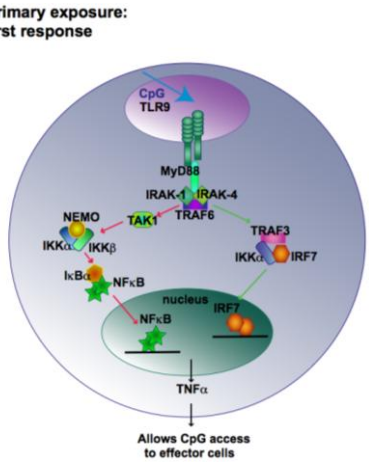
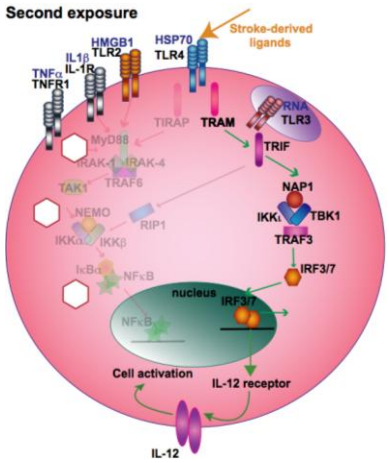


Figure 6.1 LPS preconditioning redirects stroke-induced TLR signaling. A. Systemic administration of LPS induces TNFα, which is required for neuroprotection. TNFα may allow LPS to bind to endothelial cells. B. Stimulation of TLR4 on endothelial cells causes the up-regulation of inhibitors of NFκB-inducing pathways at the time of stroke or early thereafter. C. NFκB-inducing pathways are inhibited following stroke and IRF3-inducing pathways are enhanced, leading to an up-regulation of Type I IFN-associated genes.

A.



C.



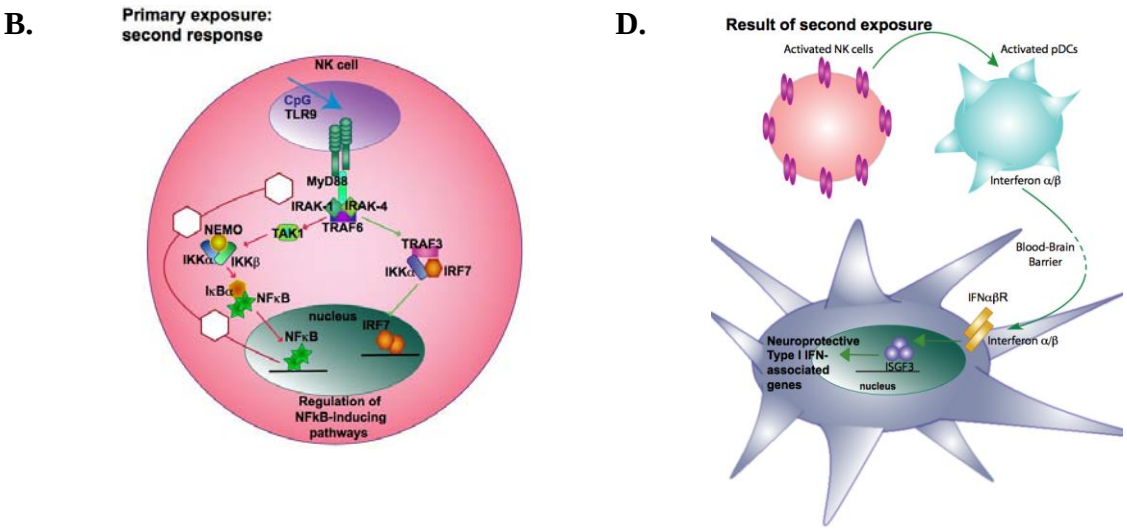


Figure 6.2 CpG preconditioning redirects stroke-induced TLR signaling. A. Systemic administration of CpG induces TNFα, which is required for neuroprotection. TNFα may allow CpG to bind to or activate effector cells. B. Stimulation of TLR4 on NK cells causes the up-regulation of inhibitors of NFκB-inducing pathways at the time of stroke or early thereafter. C. NFκB-inducing pathways are inhibited following stroke and IRF3-inducing pathways are enhanced, leading to the up-regulation of IL-12R and the enhancement of NK cell activity. D. Activated NK cells prompt IFNα production by pDCs. Following stroke, IFNα may be able to cross the BBB and induce the production of Type I IFN-associated genes from astrocytes and microglia.

1. Multiple TLR ligands can precondition the brain and protect it from ischemic injury

An effective response to pathogen invasion requires the detection of microbes and the coordination of an appropriate inflammatory response. TLRs perform both of these functions. The extracellular domain of plasma membrane TLRs and the intraluminal

domain of endocytic TLRs detect pathogen-associated molecules and the cytoplasmic domains of these receptors react by initiating intracellular signaling pathways. The cytoplasmic structures of these molecules are very similar in that each contains the Toll/IL-1 Receptor (TIR) domain. It appears that, evolutionarily, TLR extracellular and intraluminal domains have differentiated to detect multiple pathogens while the cytoplasmic domains have remained largely identical. This allows each TLR to signal through a similar intracellular pathway. In this manner the TLR family is able to detect a wide range of pathogenic molecules and launch similar, though not identical, inflammatory responses.

Similarities among TLR signaling pathways are evidenced by the fact that primary stimulation of many TLRs results in an inflammatory state, characterized by the production of cytokines and chemokines, followed by a state of tolerance to subsequent activation. For example, priming of TLR2, TLR4, TLR5, or TLR9 with their respective ligands causes macrophages to produce TNFα, IL-1β and CXCL8. For a short time thereafter, macrophages then become hypo-responsive to subsequent stimulation of these receptors. In this state of self-tolerance, many of the pro-inflammatory cytokines and cytotoxic mediators that are normally elicited by TLR activation fail to be induced by a second exposure to the TLR ligand [94, 123-125]. Cross-tolerance between two different receptors has also been reported, as ligands for TLR2, TLR5 and TLR9 induce tolerance against a subsequent exposure to LPS[93, 94, 123-125].

Similarities in the signaling pathways initiated by TLRs and in their ability to induce self- and cross-tolerance suggested that TLRs other than TLR4 may also induce tolerance to brain ischemia. Activation of TLR9 by CpG ODNs increases serum levels of TNFα, an essential mediator of LPS-induced neuroprotection [131, 189]. CpG ODNs are currently approved for human trials as vaccines and cancer immunotherapies, which makes them particularly well suited for development as stroke prophylactics. For these reasons, CpG was the next TLR ligand to be examined for its preconditioning potential.

Assessment of the neuroprotective potential of CpG ODNs began by determining if pretreatment of primary murine cortical cell cultures would protect them from a transient exposure to oxygen and glucose deprivation (OGD). OGD is a cell culture model of cerebral ischemia that allowed us to decipher the direct effects of CpG on brain cells without in vivo factors such as the BBB or systemic cytokines that could potentially confound our results. Pretreatment with CpG significantly protected cortical cells from OGD-induced cell death (Figure 2.1A). Concurrent administration of the TLR9 specific antagonist ODN2088 blocked this protection, verifying the requirement for TLR9 in CpG-induced neuroprotection (Figure 2.1B). TLR9 is expressed by murine microglia and astrocytes [190-192], hence it is likely these cells that mediate the protective effects of CpG preconditioning *in vitro*.

One advantage of LPS preconditioning over other preconditioning strategies is that LPS can be administered systemically, providing a more convenient therapeutic option for patients. It was therefore determined whether systemically administered CpG might also

confer protection against cerebral ischemia in a mouse model of stroke. CpG conferred protection when administered through several different systemic routes, including intraperitoneal, subcutaneous, and intranasal routes (Figure A1). Our LPS studies have taken advantage of intraperitoneal administration, thus this route of administration was used to further characterize CpG-induced protection. Intraperitoneal injection of CpG protected animals against subsequent stroke injury in a dose- and time- dependent manner. Twenty and forty micrograms (0.8-1.6mg/kg) of CpG conferred significant protection when MCAO was performed within 1 day of CpG administration (Figure 2.2). The neuroprotective effect remained evident when MCAO was performed within 3 days but diminished by 7 days. Fourteen days after administration, the protective effect of CpG administration was completely gone (Figure 2.3). Notably, this is a similar time window to that in which LPS administration confers protection. Together, these studies demonstrate that CpG ODNs have substantial potential for translational development as stroke prophylactic therapy.

The observation that CpG and LPS provide robust neuroprotection over similar time windows supports the notion that the two TLR agonists confer protection through similar mechanisms. I therefore postulated that, as for LPS, protection afforded by CpG preconditioning requires the activation of TNFα. Serum levels of TNFα were significantly increased 1 hour after i.p. administration of CpG or LPS, but CpG induced substantially less TNFα than LPS (Figure 2.5). Nevertheless, animals lacking TNFα were not protected by CpG preconditioning. Hence, neuroprotection conferred by CpG preconditioning requires TNFα. The required role for TNFα in the context of LPS

preconditioning has been shown to occur between the time of LPS administration and the time of MCAO. Inhibition of TNFα either systemically or within the brain during this time interval abolishes the protective effects of LPS pretreatment [79, 82]. It is not clear why TNFα is required during this time window, but several possibilities exist and each may provide insight into the required role of TNFα in CpG-induced protection. One reason why TNFα might be required is that it helps endothelial cells detect and respond to LPS in the plasma. Systemic TNFα induces an "acute phase response" from the liver, during which time anti-pathogenic proteins are released into the plasma. Two such proteins are LPS binding protein (LBP) and soluble CD14 (sCD14). LBP binds to LPS in the plasma and delivers it to the TLR4 receptor complex, which, on endothelial cells, requires sCD14. In this manner, TNFα allows endothelial cells to detect and respond to LPS. As cerebral endothelial cells lie at the interface between the blood and the brain parenchyma, it may be these cells that transmit the neuroprotective effects of LPS preconditioning. CpG may have a similar need for acute phase proteins to deliver it to the cerebral endothelium. Another potential reason for the requirement of TNFα is that it may allow LPS and CpG to cross the BBB. TNFα has been shown to disrupt the BBB through activation of TNFR1 [57]. TNFR1 causes endothelial cells to constrict thereby disrupting the tight junctions between them. This would give both LPS and CpG direct access to cells within the brain itself. Finally, TNFα might be required as a preconditioning agent in its own right. TNFα pretreatment has been shown to provide significant protection against subsequent stroke [143]. Therefore, the possibility exists that LPS and CpG act as TNFα-inducing agents, and that TNFα itself is the neuroprotective mediator.

My primary hypothesis is that prior treatment with TLR agonists confers ischemic tolerance in the same manner that they confer endotoxin tolerance, by redirecting subsequent TLR signaling. I thus postulated that CpG pretreatment would not protect mice lacking TLR4. I found, however, that these mice display significant protection when preconditioned with CpG (Figure A2). This critical observation reveals two key pieces of information regarding CpG preconditioning. The first is that the effects of CpG preconditioning are not due to endotoxin contamination of CpG ODNs. Endotoxin contamination has been suggested to be a confounding factor in several studies of endogenous TLR4 ligands [193], and our finding refutes this possibility in our system. The second key point is that CpG –induced ischemic tolerance does not require the activity of TLR4. It is clear that, in the context of endotoxin tolerance, CpG pretreatment redirects TLR4 such that subsequent signaling through the TLR4-NFκB pathway is suppressed and signaling through the TLR4-IRF3 pathway is enhanced. While this same redirection may occur in the context of cerebral ischemia, it is not required for protection. MyD88-NFκB signaling occurs downstream of TLR2 and the IL-1 receptor by way of these receptors' cytoplasmic TIR domains. TLR2 exacerbates ischemic injury, as TLR2 knockout mice demonstrate smaller infarcts than wild-type controls. Similarly, IL-1R worsens ischemic injury. IL-1β, which activates this receptor, has been shown to play a detrimental role in cerebral ischemia; mice lacking IL-1β have smaller infarcts than wild-type mice and inhibition of the receptor through administration of IL-1 Receptor Antagonist (IL1Ra) reduces infarct size. Hence CpG may afford protection by suppressing MyD88- NFκB signaling downstream of TLR2 and IL-1R. Notably, this

possibility does not negate a role for TLR4 in CpG-induced neuroprotection, but suggests that such a role may be minor in comparison.

In addition to CpG, other TLR ligands offer promise as agents of stroke prophylaxis. Poly(I:C) mimics dsRNA and is a synthetic ligand of TLR3. Among the TLR family, TLR3 is the only member that does not signal through MyD88 but instead signals exclusively through TRIF. It follows that pretreatment of macrophage with poly(I:C) does not induce cross-tolerance to LPS [93], presumably because it does not up-regulate negative feedback inhibitors of NFκB-inducing pathways (notably, pretreatment with poly(I:C) causes an *increase* in IL-12 production upon subsequent TLR4 stimulation[93]). However, stimulation of TLR3 with poly(I:C) results, through TRIF-dependent signaling, in a significant up-regulation of the neuroprotective cytokine IFNβ. I therefore postulated that pre-exposure to poly(I:C) would protect animals against cerebral ischemic injury. Indeed, systemic administration of poly(I:C) up-regulated serum levels of IFNβ and protected the brain against stroke damage in a dose-dependent manner when administered 72 hours prior to MCAO (Figure A3).

Of all of the TLRs, TLR3 has the most promise as a mediator of *acute* neuroprotection. Stimulation of astrocytes with poly(I:C) causes these cells to express multiple neuroprotective factors, including brain-derived neurotrophic factor, neurotrophin 4, pleiotrophin, ephrin type B receptor and TGFβ2. Poly(I:C)-conditioned media from these cultures increases neuronal survival and suppresses astrocyte growth in human brain slice cultures[27], further supporting a neuroprotective role for TLR3-mediated TRIF signaling

in the brain. I therefore postulated that *acute* administration of poly(I:C), by inducing the immediate production of neuroprotective factors by astrocytes, would also protect against cerebral ischemic damage. However, i.p. administration of poly(I:C) 30 minutes before or after MCAO failed to protect animals from injury (Figure A4). Systemically administered poly(I:C) may not cross the BBB, hence it may not have direct access to astrocytes within the brain parenchyma. My data indicate that systemic administration of poly(I:C) may be another powerful means of *preconditioning* the brain from subsequent ischemic injury and not a means of *acutely* providing protection. The possibility exists, however, that administration of poly(I:C) i.c.v. at the time of stroke would, by bypassing the BBB, directly stimulate astrocytes to produce the aforementioned neuroprotective molecules. This route may afford immediate protection that systemic administration does not. This highlights the importance of examining the timing and route of administration in both the development of therapeutics and in deciphering the mechanisms of TLR-induced ischemic tolerance.

In conclusion, I have shown that preconditioning with a number of TLR ligands can protect the brain from ischemic injury. As such, TLRs are proving to be a rich source of therapeutic targets in the search for stroke prophylactics. However, the mechanisms by which these diverse receptors provide protection remain unclear. Stimulation of both MyD88-dependent (TLR9) and MyD88-independent (TLR3) receptors precondition the brain to tolerate ischemic injury, as does stimulation of plasma membrane receptors (TLR4) and endocytic receptors (TLR3 and TLR9). These receptors are expressed by multiple cell types and respond to stimulation by generating a diverse cytokine response

dominated by TNFα (TLR4), IFNβ (TLR3) and IFNα (TLR9). Despite these differences, one characteristic common to these three receptors is the ability to change the cellular response to subsequent TLR stimulation. This powerful ability may form the basis of a common mechanism by which TLR ligands induce ischemic tolerance. Pretreatment of macrophages with TLR ligands decreases TNFα generation upon subsequent stimulation of TLR2 or TLR4 [96] and increases IFNβ generation upon subsequent stimulation of TLR3 or TLR4 [93]. Similarly, preconditioning with TLR ligands may decrease TNFα generation upon subsequent stimulation of TLR2 or TLR4 and increase IFNβ generation upon subsequent stimulation of TLR3 or TLR4 with stroke-induced ligands. Either of these cytokine changes would be expected to protect the brain from damage. I have shown that CpG does not require TLR4 for its protective effects although LPS does (Figures 5.9 and A2). This finding, and the studies described above, suggest that stroke-induced signaling from *other* TLRs may be altered by CpG preconditioning. For example, redirected TLR signaling may change the brain's transcriptional response to stroke by suppressing NFκB activity downstream of TLR2 and increasing IRF3 activity downstream of TLR3. Hence, preconditioning with LPS, CpG, or poly(I:C) may all lead to neuroprotection by redirecting TLR signaling thereby reprogramming the transcriptional response to stroke.

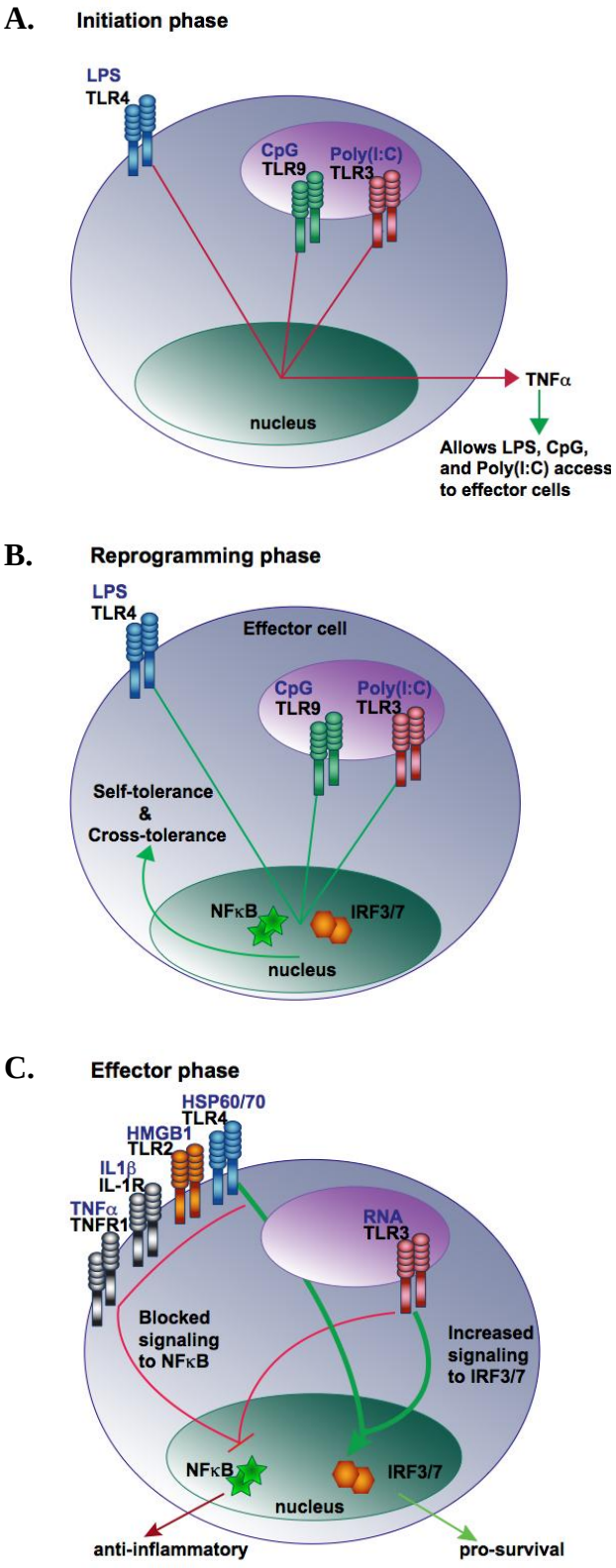


Figure 6.3 Multiple TLR ligands may protect the brain by redirecting stroke-induced TLR signaling. A. LPS and CpG (and potentially poly(I:C)) require TNFα for their neuroprotective effect. TNFα may give these molecules access to effector cells. B. TLR ligands induce self- and cross-tolerance to subsequent TLR stimulation. C. Tolerant cells suppress subsequent signaling to NFκB and increase signaling to IRF3.

2. TLR preconditioning reprograms the endogenous response to stroke

Reprogramming is defined as a change in the cellular environment that results in a novel genomic response to a known stimulus. In the context of preconditioning, reprogramming has been described as up-regulation of distinct gene sets by ischemia following preconditioning. Genomic reprogramming may be a common strategy by which all preconditioning stimuli confer neuroprotection. It has been postulated that, although each preconditioning stimulus leads to gene up-regulation following stroke, the identities of the up-regulated genes differ from one stimulus to another. As such, the phenotype of neuroprotection may be expressly tailored by the nature of the preconditioning stimulus [145]. For example, preconditioning events that deprive the brain of oxygen or glucose for a short time lead to the up-regulation of gene sets associated with energy conservation and mitochondrial integrity following the injurious ischemic episode [153, 194]. Preconditioning with TLR ligands induces a small inflammatory response prior to the ischemic event [79, 80, 82, 83]. Pretreatment with these ligands would thus be expected to cause an up-regulation of genes involved in inflammation and defense, evincing a reprogrammed response to stroke.

CpG and LPS have both been shown to reprogram the cellular response to subsequent TLR stimulation. In this context, CpG and LPS alter the genomic response to subsequent TLR3 and TLR4 stimulation, causing an up-regulation of Type I IFN-associated molecules [93]. As CpG and LPS are administered systemically and have neuroprotective effects on the brain, I hypothesized that systemic TLR preconditioning would reprogram

both brain cells and blood leukocytes, resulting in an up-regulation of Type I IFN-associated signaling following stroke.

2.1 TLR preconditioning reprograms the brain's endogenous response to stroke

Comparison of the brain's transcriptional response to stroke in the context of either CpG or LPS preconditioning revealed that each induced a similar pattern of gene regulation following stroke. Microarray analysis of ischemic brain tissue demonstrated that 223 genes differed between CpG pretreated animals and saline controls 24 hours after MCAO (Figure A5). Of these genes, greater than 60% were up-regulated. Similarly, 176 genes differed between LPS pretreated animals and saline controls at this time, and 90% of these genes were up-regulated (Figures 4.1, 4.2). Systemic administration of these TLR ligands induces novel responses to stroke that are characterized by up-regulated gene expression 24 hours following the ischemic event. Hence TLR preconditioning reprograms the brain's endogenous response to stroke.

Analysis of the reprogrammed response indicated that, in both CpG and LPS preconditioned animals, many of the genes uniquely up-regulated in the brain 24 hours after stroke were associated with Type I IFN signaling. Literature review revealed that greater than 5% of the genes up-regulated by CpG preconditioning and greater than 20% of the genes up-regulated by LPS preconditioning were associated with Type I IFNs. This pattern suggested the presence of Type I IFNs within the brain following stroke. Examination of the transcriptional regulatory elements (TREs) within the promoter regions of the up-regulated genes provided further evidence of IFN-associated

transcription. Using the web-based PAINT program, over-represented TREs within the 2 clusters of up-regulated genes were identified. In those genes up-regulated by CpG preconditioning, four TREs were identified as over-represented—IRF, IRF8, ISRE, and HMG IY (Figure 3.2). Each of these TREs is associated with Type I IFN-related transcription. The IRF identifier encompasses regulatory sequences associated with the transcription factors IRF 1-8. ISRE denotes the regulatory element recognized by the transcriptional regulators IRF3 and ISGF3. The IRF8 TRE is recognized by the transcription factor IRF8, which has been reported to magnify IFN transcription in dendritic cells [195]. Finally, HMG IY is the DNA binding site of a high mobility group protein that stabilized the IFNβ enhanceosome resulting in increased transcription [196]. These regulatory elements were found in 80% of the genes for which 5'-sequence was available for analysis. In those genes up-regulated by LPS preconditioning, 5 of the 14 over-represented TREs were IFN-associated—IRF8, ISRE, 2 versions of IRF, and IRF7 (Figure 4.3). IRF7 denotes the DNA cis-element to which the IRF7 transcription factor binds. These regulatory elements were found in 50% of the genes for which 5' sequence was available for analysis. Hence, TLR preconditioning reprograms the brain's response to stroke by causing the up-regulation of Type I IFN-associated genes after the ischemic event.

2.2 TLR preconditioning reprograms the endogenous systemic response to stroke

Systemic administration of TLR ligands not only has the potential to reprogram the *brain's* response to stroke, but also has the potential to reprogram the *peripheral* response to stroke. The endogenous peripheral response to stroke is a profound

immunosuppression that puts patients at risk for post-stroke infection. Stroke-induced immune dysfunction has been recognized in clinical settings for over 30 years—infectious complications are reported in 12-24% of patients within the first days after stroke [197-200]. Experimental stroke models have revealed the cause of this immunosuppression to be a profound lymphopenia that occurs as early as 12 hours following ischemia and persists for at least 5 days [201]. T cells, B cells, and NK cells all undergo extensive apoptosis within lymphatic organs, resulting in diminished cell numbers both in the spleen and in the blood [201]. Replenishment of T and NK cells provides protection against post-MCAO infection, emphasizing the importance of normalized immune responses in the post stroke habitus. Intriguingly, CpG has been shown to activate both T cells and NK cells via IL-12 and Type I IFNs secreted from TLR9-expressing cells [202, 203]. This observation invites the possibility that CpG preconditioning may act to counter stroke-induced immunosuppression and maintain systemic immune integrity following stroke. Thus, CpG preconditioning may change the systemic response to stroke by reprogramming peripheral leukocytes. Such a reprogramming would be expected to mitigate stroke-induced immunosuppression.

The potential for TLR preconditioning to reprogram the systemic response to stroke was assessed by analyzing the transcriptional profile of peripheral leukocytes following stroke. Microarray analysis detected 422 gene differentially expressed between CpG and saline pretreated mice 24 hours after MCAO (Figure A6). Of these genes, greater than 60% were up-regulated. Hence CpG pretreatment induces a novel systemic response to stroke characterized by a pattern of uniquely up-regulated gene expression 24 hours

following the ischemic event. To better understand the distinctive components of this unique systemic genomic response, those genes up-regulated 24 hours following ischemia in CpG pretreated animals were analyzed. Literature review revealed that approximately 10% of the up-regulated genes were associated with NK cells. This observation was supported by analysis of over-represented TREs in this gene cluster. Via PAINT analysis, a single TRE, GATA-3, was identified as over-represented within this cluster (Figure 3.2). GATA-3 denotes the DNA cis-element to which the GATA-3 transcription factor binds. This transcription factor is required for NK cell development and was found in 50% of the genes for which 5' sequence was available for analysis. Moreover, serum levels of the NK cell stimulatory factor IL-12 were up-regulated following stroke in preconditioned animals, further supporting the notion of enhanced NK cell activity (Figure A7). Together, this data indicates that TLR preconditioning reprograms the systemic response to stroke by leading to a relative increase in the expression of NK-cell associated genes.

In addition to their known role in protecting against post-MCAO infection, NK cells may also play a role in CpG-induced neuroprotection. NK cells are evolutionarily primitive lymphocytes that distinguish self from non-self through the detection of MHC class I receptors ubiquitously expressed on healthy cells. Cells that do not express MHC class I receptors are killed by NK cells via contact-dependent pathways involving perforin/granzyme, Fas/FasL, and TRAIL/TRAIL-ligand interactions. NK cells also produce proinflammatory cytokines such as IFNγ thereby activating other immune cells.

In seeming contrast to their pro-inflammatory cytotoxic profile, NK cells play a major role in restraining neuroinflammation. A number of researchers have demonstrated that NK cells limit the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) [148, 204, 205]. The authors suggest that this protective effect is due to both NK cell regulation of autoreactive T-cells and to the directly neuroprotective actions of brain-infiltrating NK cells, which have been shown to express brain derived neurotrophic factor, NT-2, and glial cell derived neurotrophic factor [148]. Notably, administration of CpG prior to EAE induction significantly reduces disease severity [149, 206]. Hence, in EAE, and potentially in stroke, CpG-activated NK cells limit neuroinflammation and protect neurons. CpG administration has also been shown to inhibit inflammatory arthritis by stimulating NK cells, via IL-12 released from activated DCs, to produce IFNγ. Serum IFNγ suppresses neutrophil infiltration into the inflamed joint [150]. Similarly, CpG may cause NK cells to produce IFNγ and thereby prevent neutrophil infiltration into the ischemic brain. Together, these data suggest that a CpG-induced increase in NK cell activity may be neuroprotective in the context of cerebral ischemia.

2.3 TLR preconditioning reprograms the brain and the blood by redirecting TLR signaling

Preconditioning-induced reprogramming of brain cells and peripheral leukocytes may occur by redirected TLR signaling both in the brain and the blood. In the brain, LPS preconditioned animals demonstrate a significant up-regulation of IFN-associated genes following stroke which, based our microarray data, is likely to be produced via the TLR-

TRIF-IRF3 pathway. Several interferon-associated genes previously shown to be induced by TRIF signaling, including Ifit1, Ifit3, and Oasl2, were found to be up-regulated 24 hours following stroke. Ifit1 and Ifit3 are up-regulated following LPS treatment of peritoneal macrophages from MyD88-deficient mice [158]. Similarly, Ifit2 and Oasl1, close family members of the genes found on our array, are also up-regulated in these cells, suggesting that these genes are all products of TRIF-dependent signaling. Together, this data indicates that the LPS-induced Type I IFN "fingerprint" is generated downstream of the TLR-TRIF-IRF3 signaling axis. This supports the concept of TLR reprogramming within the brain and implicates increased TRIF-IRF3 signaling from TLR4 and/or TLR3, the only two TLRs that utilize TRIF.

CpG preconditioning may reprogram the systemic response to stroke by redirecting stroke-induced TLR signaling in leukocytes. NK cells express TLR2, TLR3, TLR4, and TLR9, [207, 208] and become hyporesponsive following exposure to LPS *in vitro* and *in vivo* [209, 210]. Hence, these cells can be directly stimulated by poly(I:C), LPS, and CpG, and they can become tolerant to TLR stimulation. Tolerant cells respond to TLR4 stimulation by signaling through the TRIF-IRF3 axis. IRF3 has been shown to increase IL-12 Receptor B1 (IL-12RB1) expression by NK cells. Redirected TLR signaling would therefore cause NK cells to respond to stroke-induced TLR ligands by up-regulating IL-12RB1. This would increase NK cell response to IL-12, amplifying it's stimulating effect and increasing the expression of the NK cell transcription factor GATA-3. Furthermore, TNFα has been shown to be a significant contributor to NK cell activation by CpG, which may explain its requirement for CpG-induced neuroprotection. These findings are

consistent with the idea that, both within the brain and throughout the periphery, TLR preconditioning reprograms the response to stroke by redirecting stroke-induced TLR signaling toward the TRIF-IRF3 axis.

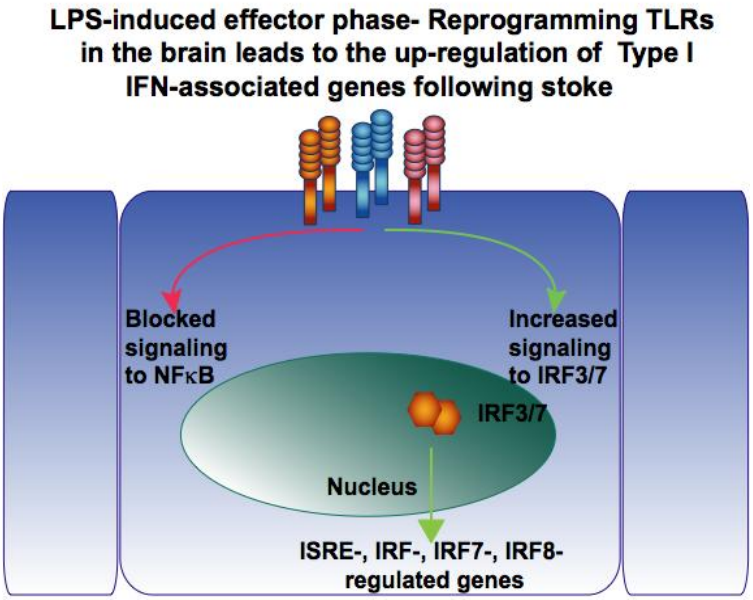


Figure 6.4. LPS preconditioning reprograms cells within the brain. Reprogrammed brain cells redirect stroke-induced TLR signaling towards an up-regulation of Type I IFN-associated genes.

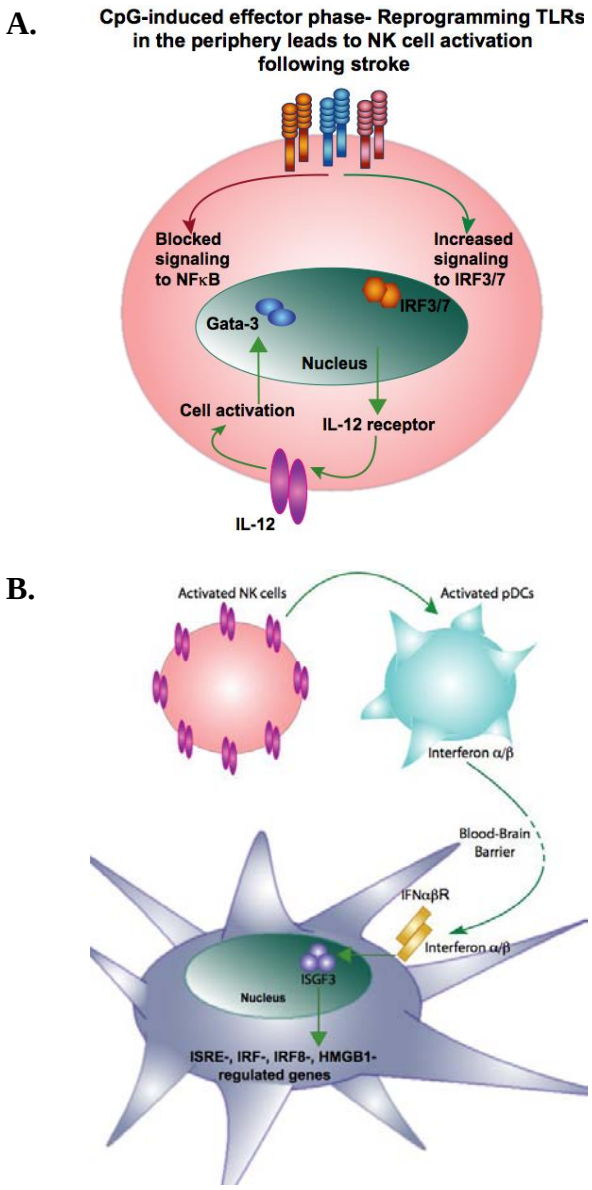


Figure 6.5. CpG preconditioning reprograms cells within the periphery. A. Reprogrammed NK cells may redirect stroke-induced TLR signaling towards an up-regulation of IL-12. B. IL-12-activated NK cells prompt pDCs to produce IFNα, which may cross the compromised BBB and initiate the production of Type I IFN-associated genes.

3. TRIF and IRF3, but not IFNβ, are required for TLR-induced neuroprotection

Our microarray data contain compelling evidence of a preconditioning-induced switch in TLR signaling following stroke that results in the up-regulation of Type I IFN-associated genes. Both CpG and LPS preconditioning elicit this genomic response within the brain, and these up-regulated transcripts compose a large portion of the genes differentially regulated between preconditioned and control mice. This suggests that the switch toward IFN-associated signaling may be neuroprotective and that ISGs themselves may have a role in protecting the brain from ischemic injury.

The potential for ISGs to confer neuroprotection is evidenced by the protective actions of IFNβ. In the context of cerebral ischemia, IFNβ has been shown to stabilize the BBB [23, 102], and to reduce cellular infiltration into brain regions damaged by stroke [25]. Systemic administration of IFNβ reduces tissue damage in rat and rabbit models of ischemic stroke [24, 25]. It should be noted that systemic administration of IFNβ failed to confer protection in one model of cerebral ischemia [211]. The authors propose that their occlusion model caused less disruption of the BBB and thus IFNβ was not able to reach the affected brain. Hence, the presence of IFNβ *within* the brain appears to be critical for its neuroprotection functions. IFNβ activates the ISGF3 transcription factor. ISGF3 binds to ISRE motifs within the promoters of ISGs, increasing their expression. It is reasonable to predict that the protection afforded by IFNβ is mediated by the ISGs it induces.

The TLR-induced transcription factor IRF3 can also induce ISG expression. IRF3 binds to several IFN-associated TREs, including ISRE and IRF. IRF3 binds to ISRE motifs within the promoters of ISGs, increasing their expression. Hence, similar to IFNβ, IRF3 may afford neuroprotection by up-regulating ISGs within the brain. IRF3 also promotes transcription by binding to IRF motifs. Importantly, IRF3 binds to several IRF motifs within the IFNβ promoter and is required for TLR-induced production of IFNβ. It thus appears that IRF3 acts to kindle a Type I IFN genomic response by inducing transcription of ISGs. IRF3 also up-regulates IFNβ, which acts in an autocrine and paracrine manner to amplify and spread the IFN response.

We hypothesized that the genomic IFN "fingerprint" was evidence of a protective Type I IFN response within the brain following stroke in the context of preconditioning. IRF3 and IFNβ, molecules downstream of redirected TLR signaling, both have the capacity to elicit such a transcriptional response. As IFNβ has known neuroprotective effects, we began to test our hypothesis by examining the ability of IFNβ to mediated TLR-induced ischemic tolerance. We found that LPS preconditioning did not increase IFNβ transcripts within the brain following injection, but significantly increased transcript levels after stroke (Figure 4.4A). This pattern is consistent with the idea that LPS pretreatment, which itself does not induce IFNβ, reprograms subsequent TLR signaling such that stroke-induced ligands initiate signaling through TRIF-IRF3 and generate IFNβ. We found a different pattern of IFNβ induction within the brains of CpG preconditioned mice. Unlike LPS pretreated mice, CpG pretreated mice demonstrated an increase in IFNβ following injection, and no increase following stoke (Figure A8). The difference in

IFNβ induction between the two TLR ligands may be due to differences in the cell types that express each TLR. Alternatively, it may be due to differences in the signaling pathways initiated by each TLR. Nonetheless, both LPS and CpG preconditioning induced IFNβ transcripts within the brain. We then tested whether this increase in IFNβ might confer neuroprotection.

Mice lacking IFNβ incurred infarcts of similar size to wild type mice, suggesting that endogenous IFNβ does not protect the brain from ischemic injury (Figure 4.5A). However, exogenous administration of IFNβ i.c.v. at the time of stroke conferred significant protection against ischemic damage, indicating that local up-regulation of this cytokine may be neuroprotective (Figure 4.4B). Nevertheless, IFNβ was *not required* for either LPS- or CpG-induced protection, as preconditioning with either TLR ligand protected IFNβ-deficient mice from stroke damage (Figures 4.5B and A9, respectively). We concluded that IFNβ is sufficient but not necessary for LPS- and CpG-induced ischemic tolerance.

The above findings indicated that a Type I IFN-associated factor other than IFNβ might be involved in effecting TLR-induced neuroprotection. I thus postulated that IRF3 might be a critical mediator of preconditioning. I first tested whether IRF3 is involved in the brain's natural response to stroke. Mice lacking IRF3 incurred infarcts of similar size to wild type mice, suggesting that IRF3 is not part of the brain's endogenous response to ischemia (Figure 4.6A). However, LPS preconditioning failed to protect IRF3-deficient

mice from ischemic damage (Figure 4.6B). Hence IRF3 is necessary for the neuroprotective effects of LPS preconditioning.

The neuroprotective function of IRF3 in the context of LPS preconditioning may lie in its ability to up-regulate ISGs. In this context, IRF3 induction of IFNβ may act to amplify ISRE-regulated gene expression, causing a feed-forward loop of ISG production. IRF3 alone is required for LPS-induced neuroprotection, suggesting that post-stroke activation of IRF3 is sufficient to induce a protective response and does not require amplification via IFNβ. The up-regulation of IFNβ we observed following stroke in LPS-preconditioned animals may be an indicator of enhanced IRF3 activity and may afford an ancillary means of neuroprotection.

CpG preconditioning did not up-regulate IFNβ transcripts following stroke. As IRF3 is required for IFNβ transcription, this finding suggests that IRF3 activity may not be increased following stroke in these animals. CpG may therefore utilize a different means of generating protective ISGs. We have shown that CpG preconditioning increases NK cell-associated gene expression in blood leukocytes following stroke. The intricate relationship between NK cells and IFN-producing plasmacytoid DCs (pDCs) has yet to be worked out in detail, but recent studies suggest that cross-talk between these two cell types plays a major role is shaping immune responses. Importantly, it has recently been shown that NK cells promote the release of IFNα from pDCs in a CpG- or IL-12-dependent manner [151, 152]. These studies indicate that pretreatment with CpG may activate pDCs to produce IL-12, which activates NK cells that, in the continued presence of IL-

12, feedback to induce IFNα generation from pDCs. Serum IFNα does not normally cross the BBB. However, cerebral ischemia causes a transient loss of BBB function, allowing molecules and cells into the brain that would otherwise not pass through. In this manner, a systemic increase in IFNα following stroke could lead to an increase in this cytokine within the brain and thus induce the brain-specific interferon "fingerprint". IRF3 does not appear to be critical for the production of IFNα. However, as described earlier, IRF3 activity may be required for full activation of NK cells by IL-12. I would thus posit that CpG also requires IRF3 for its neuroprotective effects.

After establishing the requirement for IRF3 in LPS-induced ischemic tolerance, I considered the pathways by which IRF3 might be activated following stroke in the context of preconditioning. Several TLRs and other pattern-recognition receptors activate IRF3, but only TLR3 and TLR4 activate this transcription factor through recruitment of the TRIF adaptor molecule. I reasoned that, if redirected TLR4 signaling is responsible for TLR-induced ischemic tolerance, mice lacking TRIF would not be protected by TLR preconditioning. Mice lacking TRIF incurred infarcts of similar size to wild-type mice, suggesting that endogenous TRIF-dependent signaling does not protect the brain from ischemic injury (Figure 5.3). However, TRIF was *required* for LPS-induced neuroprotection, as preconditioning with LPS did not protect TRIF-deficient mice from stroke damage (Figure 5.11). As a counterpoint, MyD88 is not required for LPS-induced neuroprotection, as preconditioning with LPS protects MyD88-deficient mice from ischemic injury (Figure 5.10). I thus concluded that TRIF, but not MyD88, is necessary for LPS-induced ischemic tolerance.

The induction of the interferon "fingerprint" following stroke in CpG preconditioned animals may occur through the actions of IFNα, which does not appear to be generated downstream of TRIF-IRF3 signaling. Instead, IFNα is primarily produced via MyD88-IRF7 signaling. However, as described earlier, a switch in systemic TLR signaling may be the basis of CpG-induced neuroprotection. I would thus posit that MyD88 and TRIF are both required for CpG-induced ischemic tolerance.

The up-regulation of IFNβ and the generation of the interferon "fingerprint" in the brain following stroke suggests that systemic administration of LPS reprograms cells within the brain. TLR4 is widely expressed in the CNS [37, 38, 159] and many studies have shown that peripheral LPS induces a pro-inflammatory response within the brain [160, 161] However, it is unclear whether LPS crosses the BBB and/or if it induces peripheral cytokines which, in turn, cross into the brain. Recent evidence suggests that systemic LPS elicits TLR4 signaling in the brain independent of peripheral cytokine responses [37, 162]. However, other researchers have failed to find LPS within the brain parenchyma following systemic administration [29]. Hence, LPS may or may not have direct access to the brain parenchyma. What is clear is that LPS binds to cerebral endothelial cells [29, 163]. As these cells form the border between the systemic circulation and the brain parenchyma, they are in a position to integrate information from both compartments. Hence, LPS-induced reprogramming of TLR signaling may occur within the cerebral endothelium.

These studies constitute the first demonstration that direct, i.c.v. administration of IFNβ at the time of stroke confers ischemic protection and the first report of the neuroprotective actions of IRF3 and TRIF. Based on the data presented within this thesis, it is attractive to speculate that LPS preconditioning reprograms TLR signaling in the brain and CpG preconditioning reprograms TLR signaling in systemic leukocytes. Preconditioning with either TLR ligand leads to the up-regulation of ISGs within the brain, and these ISGs appear to be effectors of neuroprotection. TLR ligands may thus initiate different processes that have the same result—reprogramming of stroke-induced TLR signaling that leads to neuroprotection. Such reprogramming events may exemplify endogenous processes that protect the brain against further injury. By preconditioning with TLR ligands, we may therapeutically induce these endogenous protective processes *early* after ischemia, thereby preventing damage in the first place.

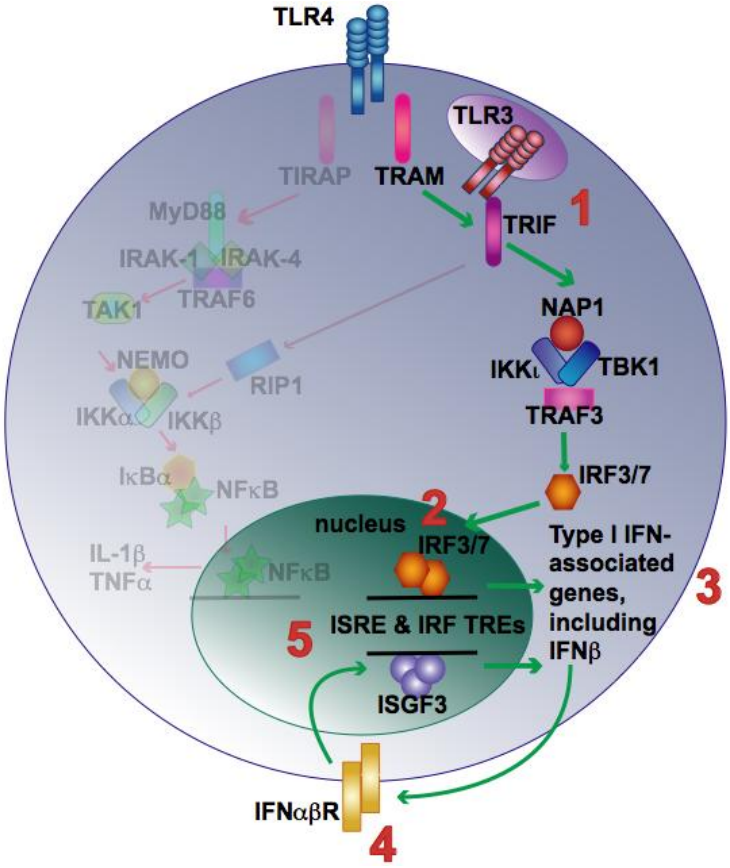


Figure 6.6. Redirected TLR signaling. 1. TR3 and TLR4 signal through TRIF to the non-canonical IKKs, IKKι and TBK. 2. IKK activation leads to the nuclear translocation of the IRF transcription factors. 3. IRF3 up-regulates transcription of Type I IFN-associated genes, including IFNβ by binding to the ISRE and IRF elements in their promoters. 4. IFNβ binds to the IFNαβR in an autocrine and paracrine manner to activate the transcription factor ISGF3. 5. ISGF3 binds to ISRE elements in gene promoters, amplifying the production of Type I IFN-associated genes.

4. The MyD88 signaling axis is suppressed early after stroke following LPS preconditioning

Several pieces of evidence suggest that the detrimental effects of TLR4 signaling following stroke occur through the TLR4-TRIF-NFκB axis. First, signaling from TLR4 to NFκB exacerbates ischemic damage. C57Bl/10ScN mice, which carry a large genomic deletion encompassing the TLR4 gene, have significantly smaller infarcts than their wild-type counterparts (Figure 5.1). This difference is "dose" dependent, as the TLR4 knockout mice have significantly smaller infarcts when MCAO lasts for 40 or 50 minutes but have infarcts of similar size to wild-type mice when MCAO lasts for 60 minutes. Conversely, mice lacking TLR3 incur significantly larger infarcts than wild-type mice. TLR4 signals through two adaptor molecules—TRIF and MyD88—that result in the activation of the transcription factors IRF3 and NFκB. TLR3 signals exclusively through TRIF. Notably, mice lacking the TRIF adaptor molecule have infarcts of similar size to wild-type mice, suggesting that the beneficial effects of TLR3-TRIF signaling following stroke may be counterbalanced by the deleterious effect of TLR4-TRIF signaling. Mice lacking IRF3 have infarcts of similar size to wild-type mice, suggesting that beneficial TLR3-TRIF signaling following stroke may result in IRF7 activation rather than IRF3 activation. Similarly, that the detrimental effects of TLR4-TRIF signaling following stroke may be due to NFκB activity. It is known that mice lacking the p50 subunit of the NFκB transcription factor suffer significantly smaller infarcts than wild-type mice and that inhibition of NFκB at the time of stroke protects animals from injury [178-181]. Thus, TLR4 signaling in response to stroke may occur either through the TRIF - NFκB axis.

Part of the brain's delayed response to stroke appears to be a redirection of TLR signaling in an endogenous attempt to prevent further injury. I have shown that part of the endogenous response to stroke is a suppression of the MyD88 protein within the brain 24 hours after ischemia and an increase in IRAK-M, a non-functional IRAK decoy, at the same time (Figure 5.6). It is also at this time that a small increase in IFNβ transcription was detected in *non*-preconditioned brains. Together, these data indicate that part of the endogenous response to stroke within the brain is a delayed attempt to suppress signaling through the TLR-NFκB axis and to switch signaling toward the TLR-IRF3 axis.

Preconditioning with LPS elicited an early increase in IFNβ (3 hours post MCAO) that suggests preconditioning initiates this endogenous redirection *early* and thereby prevents injury. In the context of endotoxin tolerance, the priming dose of LPS causes the up-regulation of inhibitors of the MyD88-NFκB signaling axis. Those inhibitors are present upon subsequent exposure to LPS, blocking signaling through this pathway. I postulated that preconditioning with LPS similarly up-regulates inhibitors of the MyD88-NFκB signaling axis within the brain that are present *at the time of stroke*. I further posited that this early inhibitor induction, relative to the brain's endogenous induction of inhibitors 24 hours after stroke, contributes to neuroprotection by blocking the deleterious arm of TLR4 signaling following stroke.

To begin to examine the potential for LPS preconditioning to up-regulate inhibitors of TLR-MyD88-NFκB signaling in the brain, I first determined if systemic administration

of LPS could induce transcriptional changes within the brain, and during what time window these changes might occur. I therefore assessed the activity of NFκB in the brain after i.p. LPS administration. Systemic LPS injection significantly increased the DNA-binding capacity of cortical NFκB as early as 24 hours after administration, and this increase lasted out to 72 hours (Figure 5.7). Curiously, I found a significant *decrease* in NFκB activity early (3 hours) after LPS administration. NFκB is a unique transcription factor in that it up-regulates transcription of its own inhibitor, IκBα. Because of this, NFκB is known to have an oscillating pattern of activation, which may explain the decreased activity observed at 3 hours. Although I examined NFκB activity as early as 1 hour after LPS administration, I was unable to find an earlier increase in DNA binding capacity that could cause an increase in IκBα and explain the 3-hour results. Hence LPS may induce a very early increase in NFκB activity that initiates the observed oscillation pattern.

Having determined that systemic LPS administration increased NFκB activity within the brain, I postulated that NFκB-induced inhibitors of the TLR-MyD88-NFκB signaling axis might also be increased within the brain following LPS administration. Indeed, I found that several such inhibitors were increased 72 hours after injection, that is, at the time of MCAO (Figure 5.8). Among the up-regulated molecules were Ship-1, a phosphatase that inhibits TLR4-MyD88 interaction [212], Tollip, a kinase that binds MyD88 and the IRAK complex and inhibits NFκB activation [213], Socs-1, a suppressor of LPS-induced NFκB activity [214], and p105, a non-canonical IκB protein. Ship-1, Tollip and p105 were also significantly up-regulated 24 hours after MCAO in preconditioned

animals. Interestingly, MyD88 and IRAK-M, the two inhibitors endogenously regulated following stroke in control animals, were *not* regulated following stroke in preconditioned animals. Collectively these findings suggest that part of the brain's endogenous response to stroke is to suppress damaging TLR-MyD88-NFκB signaling 24 hours after ischemia by decreasing MyD88 and increasing IRAK-M. LPS preconditioning causes an earlier up-regulation of a different set of inhibitors, namely Ship-1, Tollip, Socs-1, and p105 which may act to suppress TLR-MyD88-NFκB signaling *at the time of stroke* or early thereafter, thereby negating the need for subsequent regulation of MyD88 and IRAK-M.

The effect of suppressing TLR-MyD88-NFκB signaling in the brain would be a decrease in damaging NFκB activity following stroke. I first determined that stroke itself caused a significant increase in NFκB DNA-binding activity 24 hours after the ischemic event. I then found that LPS preconditioning significantly suppressed stroke-induced NFκB activity (Figure 5.7). Together, these experiments indicate that LPS preconditioning activates NFκB within the brain, causing the up-regulation of inhibitors of TLR-MyD88-NFκB signaling. These inhibitors are present at the time of the subsequent stroke, such that stroke-induced NFκB activity is suppressed. Given that suppression of NFκB during cerebral ischemia has been shown to protect the brain from damage, it is reasonable to posit that suppression of stoke-induced NFκB activity is one mechanism by which LPS preconditioning confers neuroprotection.

Suppression of MyD88-dependent pathways appears to be a theme in both the delayed endogenous response to stroke and the early reprogrammed response to stroke. This suggests that MyD88 may play a deleterious role in cerebral ischemic damage. I thus postulated that mice lacking MyD88 would incur less damage following MCAO than wild-type mice. I found, however, that MyD88 knockout mice incurred infarcts of similar size to wild-type mice (Figure 5.4). This finding indicates that MyD88 either plays no part in the endogenous response to stroke or that it has multiple functions that counteract each other in this setting.

The results of this experiment are confounded by the fact that MyD88-dependent TLRs are active within the developing brain and influence neurogenic stem cell development. TLR2 and TLR4 are both expressed by these cells. Endogenous stimulation of TLR2 has been shown to guide neurogenic stem cells toward a neuronal cell fate while endogenous stimulation of TLR4 guides them towards an astrocytic cell fate [48]. Deletion of MyD88 would abolish signaling from TLR2 completely, but might allow TLR4 signaling to remain intact. In this scenario, neurogenic cells would be preferentially guided toward an astrocytic fate. Hence, developmental MyD88 deficiency may lead to an over-abundance of astrocytes within the brain. Astrocytes play a dual role in cerebral ischemia, clearing extracellular glutamate when their ATP stores are high, and releasing glutamate into the extracellular space when their ATP stores are low. During severe ischemia, a substantial decrease in energy stores in the presence of increased astrocyte numbers may increase excitotoxic cell death and worsen injury. This exacerbation of excitotoxic injury would

be expected to counter the beneficial effects of MyD88 deficiency on stroke-induced NFκB activity and may explain the above results.

CpG has been shown to induce tolerance to subsequent LPS by down-regulating IRAK-1 and up-regulating IRAK-M. Similarly, CpG preconditioning may cause an early up-regulation of MyD88-NFκB inhibitors. However, data presented earlier in this thesis indicate that CpG pretreatment may redirect stroke-induced TLR signaling in blood leukocytes rather that in brain cells. Hence, inhibitors of the TLR-MyD88-NFκB signaling axis would be expected to be up-regulated systemically following CpG administration.

These studies constitute the first demonstration that the preconditioned brain induces some of the same protective mechanisms that the non-preconditioned brain does, but induces them at a critical, early time point. Together, these studies support a model in which TLR preconditioning causes a small initial inflammatory response [1], which is followed by the up-regulation of inhibitors of that response, namely inhibitors of NFκB-inducing pathways. These inhibitors change the intracellular milieu such that subsequently activated TLRs are unable to signal to NFκB. Instead, signaling is shunted towards IRFs. Either of these changes has the potential to confer neuroprotection. Suppression of NFκB activity has been shown to protect the brain from ischemic damage. Similarly, activation of IRFs, and the subsequent generation of ISGs, has the potential to protect the brain in the same way that i.c.v. administration of IFNβ does. TLRs appear to be unique preconditioning agents in that they not only suppress damage-inducing

processes, but they up-regulate survival-inducing processes that actively protect the brain following stroke.

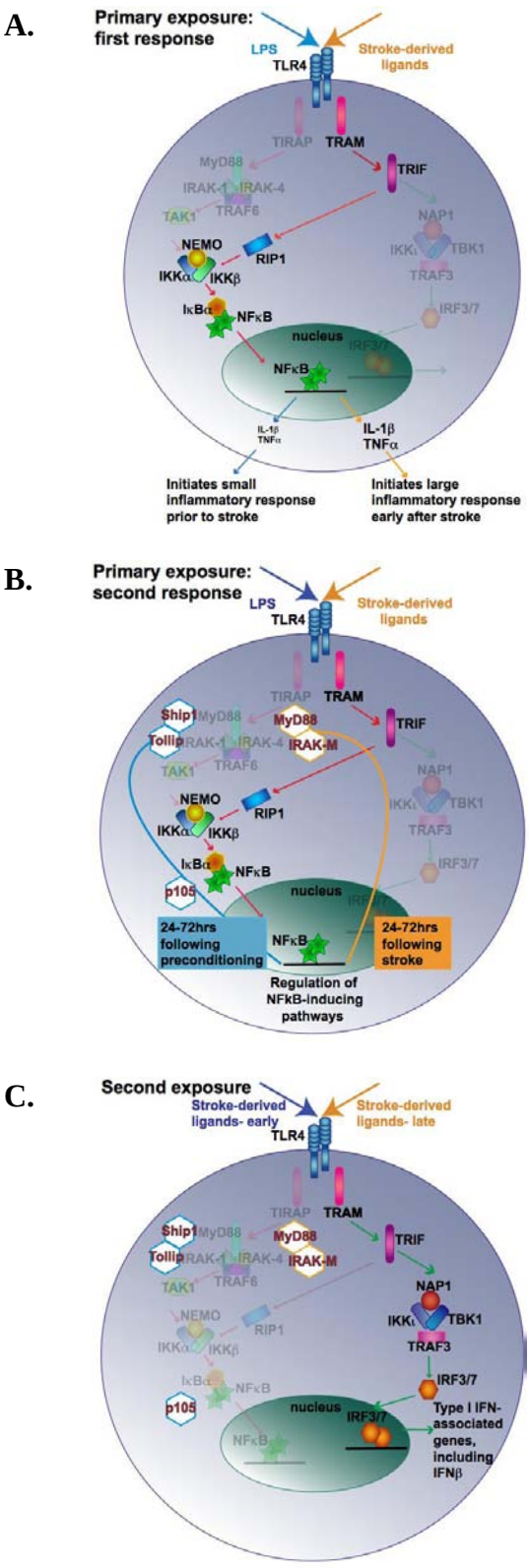


Figure 6.7. LPS preconditioning initiates endogenous mechanisms of protection early after ischemia. A. Upon initial exposure to either LPS or stroke-derived ligands, TLR4 signaling results in an increase in pro-inflammatory molecules [1, 82]. B. Subsequently, NFκB-inducing pathways are down-regulated. C. Upon secondary exposure to stroke-derived ligands, TLR4 signaling through NFκB-inducing pathways is inhibited, shunting signaling towards IRF-inducing pathways with a resultant increase in the transcription of Type I IFN-associated genes.

5. Reprogramming TLRs as a common neuroprotective mechanism

The brain has evolved numerous mechanisms that allow it to withstand the shortage of energy and the oxidative stress caused by ischemia. This tolerant state can be induced therapeutically by prior exposure to LPS or CpG, or by prior exposure to other non-damaging (i.e., sub-threshold) noxious stimuli. For example, mild exposure to ischemia, excitotoxic stimuli, or inflammatory mediators can precondition the brain to better tolerate a subsequent injurious ischemic event. These mild preconditioning exposures herald impending danger and, as such, induce endogenous protective strategies in anticipation of injury.

Though the final outcome of tolerance induction is the same--protection of brain tissue from ischemic injury—the effector mechanisms employed by the brain are as diverse as the preconditioning stimuli that induce them. In fact, the phenotype of neuroprotection may be specifically tailored by the nature of the preconditioning stimulus [145]. However, there is evidence to suggest that reprogrammed TLR signaling may be involved in shaping each of these distinct responses.

The first demonstration that a short period of oxygen deprivation could protect the brain from a subsequent extended period of hypoxia occurred in 1943 [215]. Since then, hundreds of studies have been undertaken to better understand the underlying mechanisms of "ischemic preconditioning." Though several endogenously protective pathways are induced by the initiating ischemic event, one particular theme is emerging—that of

mitochondrial maintenance and energy conservation [216]. The priming ischemic episode induces cellular pathways that protect mitochondria against stroke-induced deficits in the electron transport chain [217]. These pathways protect mitochondrial membrane potential [218] preserve mitochondrial cytochrome c [219], increase mitochondrial sequestration of Ca^{+} and increase Ca^{+}-ATPase activity. The priming event also suppresses molecules that regulate ion channels, leading to channel arrest—i.e. reduction in ion permeability through the plasma membrane—that has been shown to reduce the amount of ATP required to maintain ionic homeostasis [153, 220]. Finally, the preconditioning stimulus suppresses the expression of genes involved in protein turnover, proteasomal degradation, and energy metabolism [153]. This decrease in the overall cellular metabolic rate further limits the stressful effects of oxygen deprivation.

Several studies have shown that the priming ischemic event induces HSP70 within the brain [221]. In addition to its role in stabilizing protein structure, HSP70 acts as an endogenous ligand of TLR4. In fact, extracellular HSP70 can induce tolerance to endotoxin [222]. Hence TLRs may be stimulated in the course of ischemic preconditioning, resulting in a reprogrammed TLR response to subsequent injurious ischemia. One of the molecular consequences of reprogrammed TLR signaling is an increase in IFNβ. Notably, IFNβ has been shown to aid in the maintenance of mitochondrial integrity. For example, treatment of astrocytes with IFNβ prevents neuronal mitochondrial respiratory chain damage [106] and mitigates IFNγ induced nitric oxide synthase [223]. Thus reprogrammed TLR signaling may help shape the phenotype of energy conservation in ischemia-induced tolerance.

The observation that a small inflammatory reaction could induce tolerance to brain ischemia was first made by Nawashiro and colleagues (1997). The authors found that intracisternal administration of TNFα protects the brain from subsequent ischemic challenge [143]. This protection is correlated to a decrease in CD11b immunoreactivity, suggesting a decrease in the inflammatory response to ischemia in the setting of preconditioning. Consistent with this observation, TNFα pretreatment of astrocytes and endothelial cells, through its signaling intermediate ceramide, produces a state of hypo-responsiveness as pretreated cells fail to up-regulate ICAM-1 during subsequent hypoxia [224]. The decrease in ICAM-1 does not reflect global transcriptional suppression, but is instead evidence of a reprogrammed genomic response to stroke, as the hypoxia-induced expression of cytoprotective MnSOD is not affected by preconditioning. This evidence is supported by the observation that TNFα preconditioning prevents hypoxia-induced phosphorylation of NFκBp65, thereby preventing its interaction with, and activation by, p300. Taken together, these data indicate that pretreatment with TNFα reprograms the cellular environment and hence alters inflammatory reactions in response to ischemia.

Just as TNFα can induce tolerance to subsequent ischemic exposure, it can induce tolerance to subsequent LPS exposure [225-227]. Hence TNFα preconditioning has the potential to induce a state of cross-tolerance to TLR ligands, and thereby reprogram the TLR response to stroke. IFNβ has been shown to cause many of the effects observed in TNFα-induced ischemic tolerance, such as suppression of inflammatory cytokine production, including TNFα itself, and reduction of cellular infiltration into ischemic

brain regions [25]. Thus reprogrammed TLR signaling may help alter the inflammatory response to stroke following TNFα preconditioning.

Together, these studies suggest that multiple preconditioning stimuli may cause a reprogrammed TLR response to stroke. IFNβ, produced secondary to this reprogrammed response, may aid in maintaining energy stores and in dampening the inflammatory responses to injurious ischemia. Assuming the protective effects IFNβ are mediated by the ISGs it induces, then other ISG-inducing molecules, such as IRF3 and IFNα, may also play critical roles in effecting neuroprotection in multiple models of preconditioning.

Prospective

The studies described within this thesis demonstrate an emerging role for TLRs in both ischemic damage and ischemic prophylaxis. Clarification of the molecular mechanisms that underlie TLR-induced neuroprotection has provided a powerful new paradigm for stroke therapeutics. Within this paradigm, preconditioning causes the early induction of endogenous protective mechanisms following stroke. These protective mechanisms include a transformation of TLR signaling from one that worsens injury to one that protects against it.

There is considerable potential for prophylactic ischemic treatment. Approximately 30% of first-time stroke survivors will suffer from another stroke within their lifetime, and up to 1% of all surgical patients will suffer from peri-operative strokes (reviewed in reference [228]). Systemic pretreatment of these patients, be it by repeated administration in anticipation of an impending stroke or by a single acute treatment in preparation for surgery, has the potential to improve the quality of life of thousands of high-risk patients each year. By setting the stage for improved outcome, should an ischemic event occur, TLR pretreatment offers a low-risk, high-benefit opportunity to combat cerebral ischemia.

Appendix

Additional Data Figures

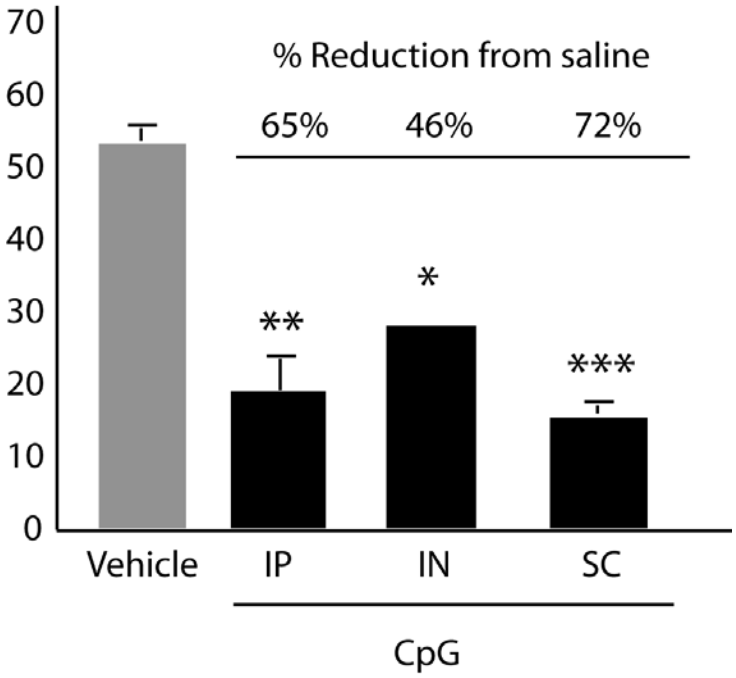


Figure A1. CpG conditions against ischemic injury via multiple routes of administration. C57Bl/6 mice received CpG or vehicle via either an i.p. injection, subcutaneous injection (0.8mg/kg) or intranasal administration (3.2mg/kg) 72hr prior to 60min MCAO. Infarct volume was determined 24hr following MCAO by TTC staining. Values are group means±SEM;*$p<0.05$,**$p<0.005$,***$p<0.001$ comparison to vehicle controls

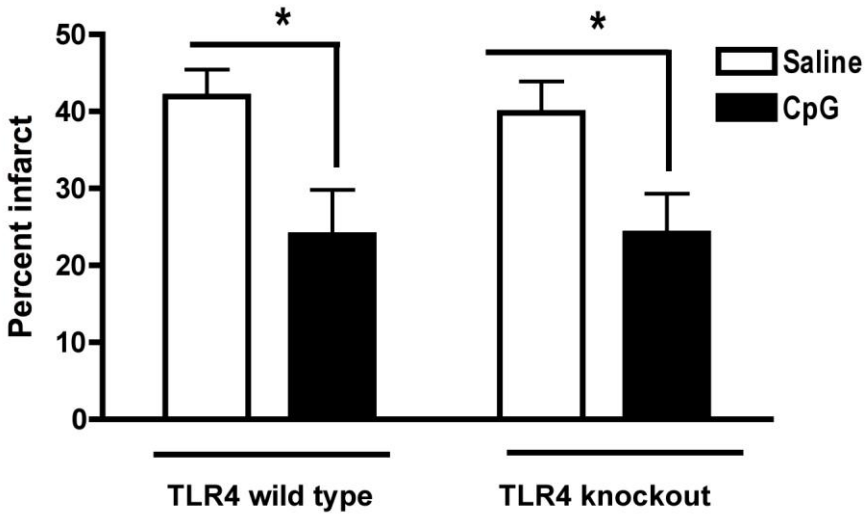
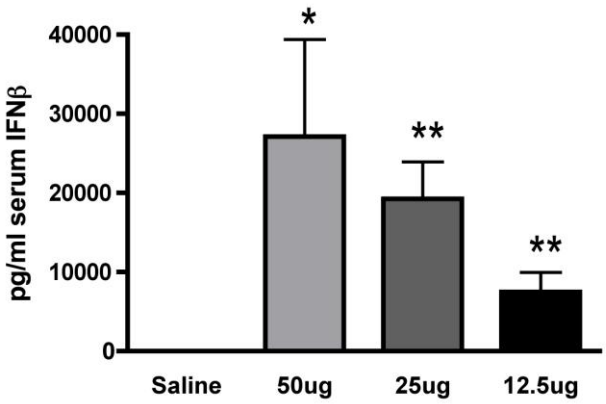


Figure A2. CpG protects TLR4 knockout mice from ischemic damage.

C57Bl/10ScSn (TLR4 wild-type) mice received CpG (1.6mg/kg) or vehicle 72hr prior to 40min MCAO. C57Bl/10ScN (TLR4 knockout) mice received CpG (1.6mg/kg) or vehicle 72hr prior to 50min MCAO. Infarct volume was determined 24hr following MCAO by TTC staining. Values are group means±SEM;*$p<0.05$ comparison to vehicle controls.

A.



B.

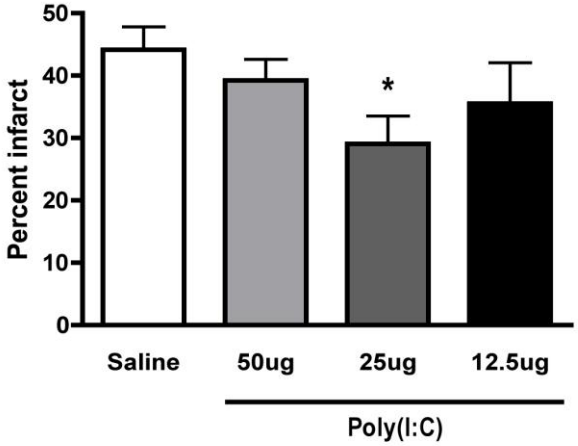


Figure A3. Poly (I:C) increases serum IFNβ in the blood and preconditions against cerebral ischemic damage. A**.** IFNβ ELISA analysis was performed on serum collected 2 hours following administration of either Poly(I:C) or saline. Results are presented as pg/ml. N= 6-8 mice/group; data are group means ± SEM. B. C57Bl/6 mice received Poly(I:C) or saline 72hr prior to 40min MCAO. Infarct volume was determined 72hr following MCAO by TTC staining. N= 8-10 mice/group; data are group means±SEM; *p<0.05, **p<0.01, comparison to saline controls.

A.

B.

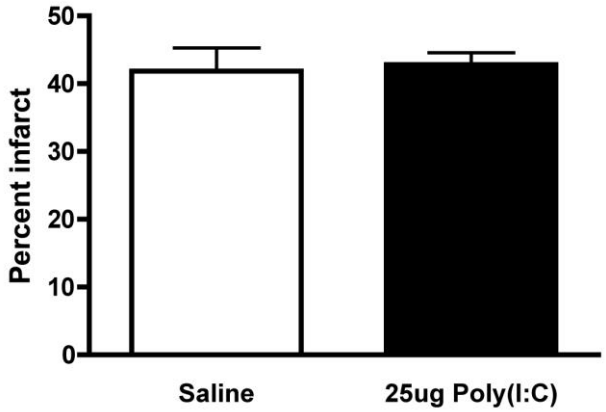


Figure A4. Acute, systemic administration of Poly(I:C) does not protect against cerebral ischemic damage. A. C57Bl/6 mice received Poly(I:C) (1mg/kg) or saline immediately prior to 45min MCAO. Infarct volume was determined 72hr following MCAO by TTC staining. N= 8 mice/group; data are group means±SEM. B. C57Bl/6 mice received Poly(I:C) (1mg/kg) or saline immediately after 45min MCAO. Infarct volume was determined 72hr following MCAO by TTC staining. N= 9-10 mice/group; data are group means±SEM.

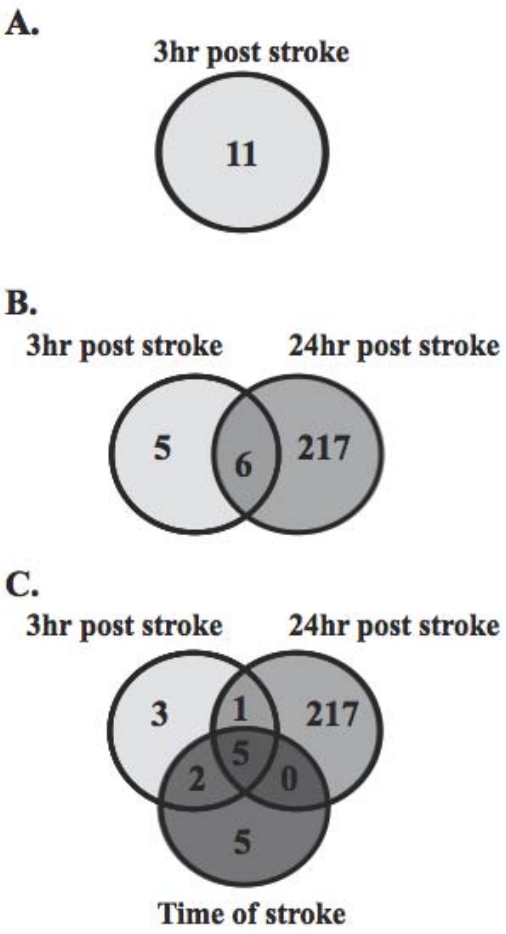


Figure A5. Microarray analysis of ipsilateral cortex reveals a unique, increasingly diverse, transcriptional profile in CpG preconditioned mice following stroke. A. At 3 hours following MCAO, 11 genes are differentially regulated in the cortices of animals pretreated with CpG relative to those pretreated with saline. B. By 24 hours, the number of genes differentially regulated in CpG pretreated animals has risen to 223, over 95% of which are uniquely regulated at this time point C. Approximately 95% of the genes differentially regulated in the cortices of CpG pretreated animals following stroke are uniquely regulated at these time points- only 5% are also regulated at the time of stroke (72 hours after injection).

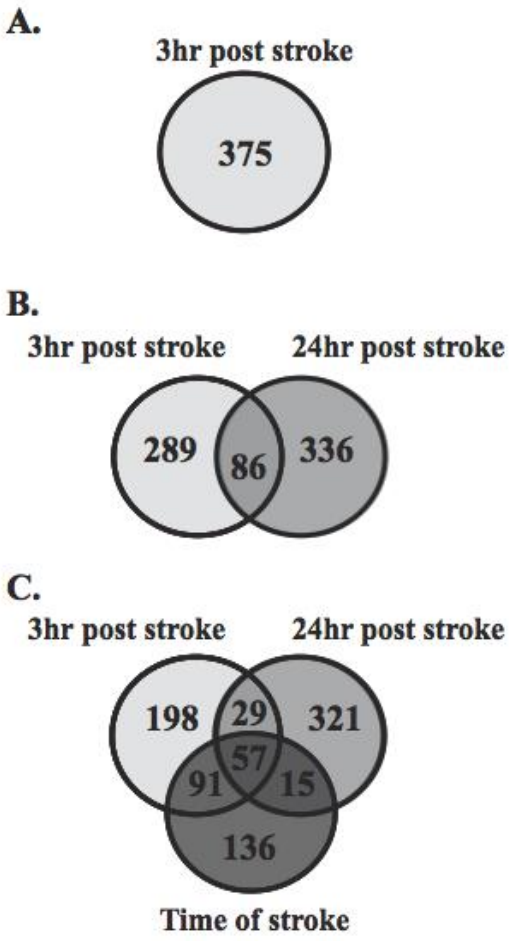


Figure A6. Microarray analysis of leukocytes reveals a unique, increasingly diverse, transcriptional profile in CpG preconditioned mice following stroke. A. At 3 hours following MCAO, 375 genes are differentially regulated in leukocytes from animals pretreated with CpG relative to those pretreated with saline. B. By 24 hours, the number of genes differentially regulated in CpG pretreated animals has risen to 422, 80% of which are uniquely regulated at this time point. C. Approximately 75% of the genes differentially regulated in CpG pretreated animals following stroke are uniquely regulated at these time points- only 25% are also regulated at the time of stroke (72 hours after injection).

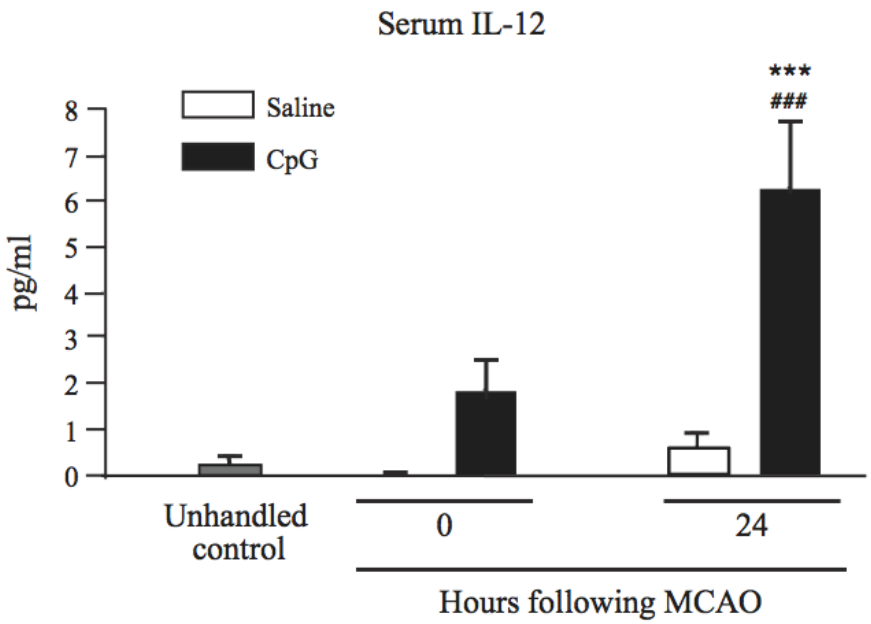


Figure A7. CpG preconditioned mice up-regulate IL-12 following MCAO. Graph is representative of 2 replicate experiments. Data shown are mean cytokine levels ± SEM. N = 3-4 / treatment group. Significance was determined by Multivariant ANOVA with Bonferonni post hoc analysis. * significant difference from saline, ***$p<0.001$, # significant difference from unhandled control, ###$p<0.001$.

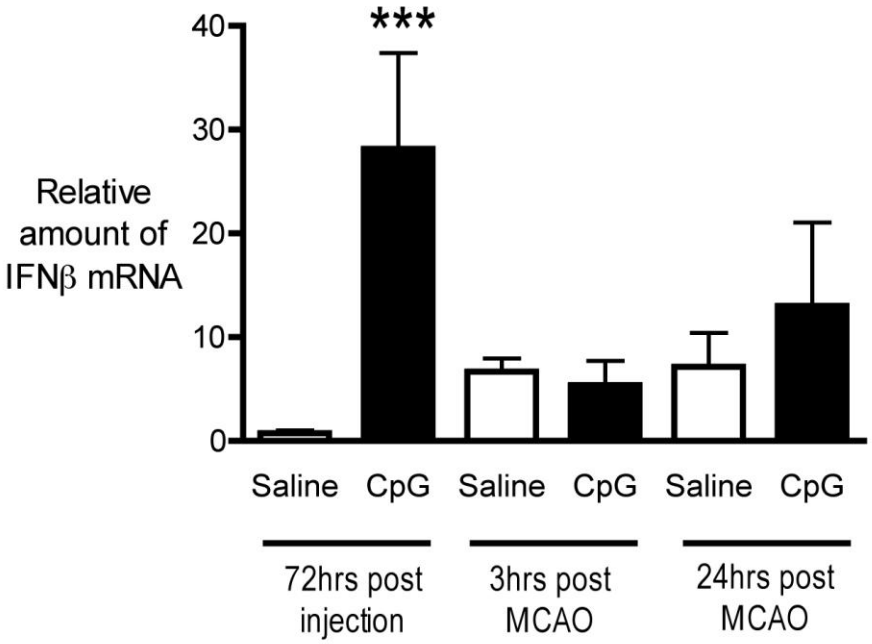


Figure A8. Increased levels of IFNβ prior to, but not after, MCAO in CpG preconditioned mice. Real-time PCR analysis was performed on RNA derived from the cortices at the time of MCAO (72 hrs post injection) or following MCAO (3 and 24hr) of mice either preconditioned with CpG or saline. β-actin was used as a loading control. Results are presented as fold increase relative to unhandled controls. N= 3-4 mice/group; data are group means ± SEM; ***$p<0.001$.

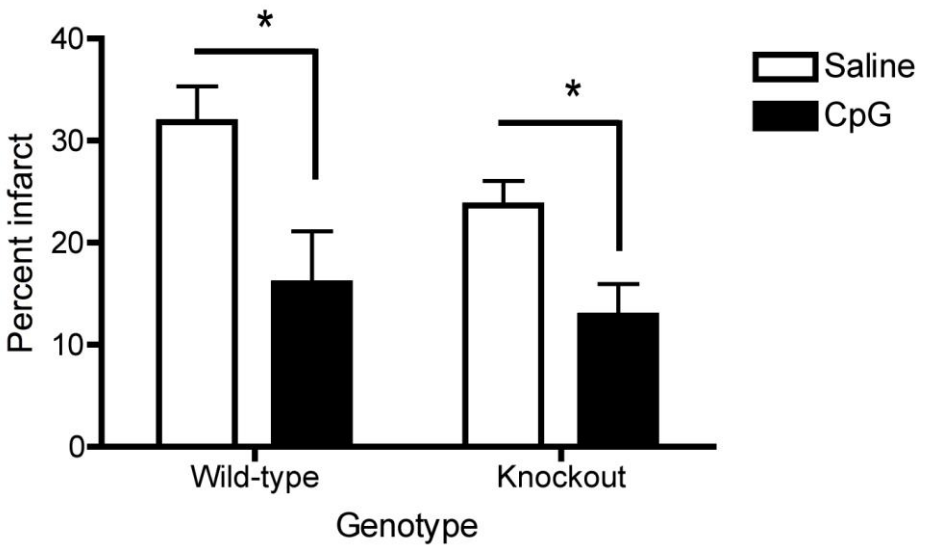


Figure A9. CpG preconditioning protects IFNβ knockout mice against cerebral ischemic injury. IFNβ wild-type and knockout mice received CpG (1.6mg/kg) or vehicle 72hr prior to 40min MCAO.. Infarct volume was determined 24hr following MCAO by TTC staining. Values are group means±SEM; *$p<0.05$ comparison to vehicle controls.

Literature Cited

1. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, Lessov NS, Simon RP, Stenzel-Poore MP. Toll-like receptor 9: A new target of ischemic preconditioning in the brain. *J Cereb Blood Flow Metab*. 2008;28:1040-1047
2. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y. Heart disease and stroke statistics 2008 update. A report from the american heart association statistics committee and stroke statistics subcommittee. *Circulation*. 2007
3. Hankey GJ. Long-term outcome after ischaemic stroke/transient ischaemic attack. *Cerebrovasc Dis*. 2003;16 Suppl 1:14-19
4. Kleindorfer D, Panagos P, Pancioli A, Khoury J, Kissela B, Woo D, Schneider A, Alwell K, Jauch E, Miller R, Moomaw C, Shukla R, Broderick JP. Incidence and short-term prognosis of transient ischemic attack in a population-based study. *Stroke*. 2005;36:720-723
5. Johnston SC, Sidney S, Bernstein AL, Gress DR. A comparison of risk factors for recurrent tia and stroke in patients diagnosed with tia. *Neurology*. 2003;60:280-285

6. Lisabeth LD, Ireland JK, Risser JM, Brown DL, Smith MA, Garcia NM, Morgenstern LB. Stroke risk after transient ischemic attack in a population-based setting. *Stroke*. 2004;35:1842-1846

7. Coull AJ, Lovett JK, Rothwell PM. Population based study of early risk of stroke after transient ischaemic attack or minor stroke: Implications for public education and organisation of services. *Bmj*. 2004;328:326

8. Johnston SC. Towards faster treatment of tia and minor stroke. *Lancet Neurol*. 2007;6:941-943

9. Deplanque D, Masse I, Lefebvre C, Libersa C, Leys D, Bordet R. Prior tia, lipid-lowering drug use, and physical activity decrease ischemic stroke severity. *Neurology*. 2006;67:1403-1410

10. Kam PC, Calcroft RM. Peri-operative stroke in general surgical patients. *Anaesthesia*. 1997;52:879-883

11. Bucerius J, Gummert JF, Borger MA, Walther T, Doll N, Onnasch JF, Metz S, Falk V, Mohr FW. Stroke after cardiac surgery: A risk factor analysis of 16,184 consecutive adult patients. *Ann Thorac Surg*. 2003;75:472-478

12. Gutierrez IZ, Barone DL, Makula PA, Currier C. The risk of perioperative stroke in patients with asymptomatic carotid bruits undergoing peripheral vascular surgery. *Am Surg*. 1987;53:487-489

13. Nosan DK, Gomez CR, Maves MD. Perioperative stroke in patients undergoing head and neck surgery. *Ann Otol Rhinol Laryngol*. 1993;102:717-723

14. McKhann GM, Grega MA, Borowicz LM, Jr., Baumgartner WA, Selnes OA. Stroke and encephalopathy after cardiac surgery: An update. *Stroke*. 2006;37:562-571

15. Bond R, Rerkasem K, Shearman CP, Rothwell PM. Time trends in the published risks of stroke and death due to endarterectomy for symptomatic carotid stenosis. *Cerebrovasc Dis*. 2004;18:37-46

16. Lund C, Nes RB, Ugelstad TP, Due-Tonnessen P, Andersen R, Hol PK, Brucher R, Russell D. Cerebral emboli during left heart catheterization may cause acute brain injury. *Eur Heart J*. 2005;26:1269-1275

17. Restrepo L, Wityk RJ, Grega MA, Borowicz L, Jr., Barker PB, Jacobs MA, Beauchamp NJ, Hillis AE, McKhann GM. Diffusion- and perfusion-weighted magnetic resonance imaging of the brain before and after coronary artery bypass grafting surgery. *Stroke*. 2002;33:2909-2915

18. Bendszus M, Stoll G. Silent cerebral ischaemia: Hidden fingerprints of invasive medical procedures. *Lancet Neurol*. 2006;5:364-372

19. Kapinya K, Lowl K, Futterer C, Maurer M, Waschke K, Isaev N, Dirnagle U. Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible no synthase dependent. *Stroke*. 2002;333:1889-1898

20. Towfighi J, Housman C, Mauger D, Vannucci R. Effect of seizures on cerebral hypoxic-ischemic lesions in immature rats. *Brain Res Dev Brain Res*. 1999;113:83-95

21. Kobayashi S, Harris V, Welsch F. Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. *Journal of Cerebral Blood Flow & Metabolism*. 1995;15:721-727

22. Simon RP, Niiro M, Gwinn R. Prior ischemic stress protects against experimental stroke. *Neuroscience Letters*. 1993;163:135-137

23. Veldhuis WB, Floris S, van der Meide PH, Vos IM, de Vries HE, Dijkstra CD, Bar PR, Nicolay K. Interferon-beta prevents cytokine-induced neutrophil infiltration and attenuates blood-brain barrier disruption. *J Cereb Blood Flow Metab*. 2003;23:1060-1069

24. Liu H, Xin L, Chan BPL, Teoh R, Tang BL, Tan YH. Interferon beta administration confers a beneficial outcome in a rabbit model of thromboembolic cerebral ischemia. *Neurosci Lett*. 2002;327:146-148

25. Veldhuis W, Derksen J, Floris S, van der Meide P, de Vries H, Schepers J, Vos I, Dijkstra C, Kappelle L, Nicolay K, Bar P. Interferon-beta blocks infiltration of inflammatory cells and reduces infarct volume after ischemic stroke in the rat. *J Cereb Blood Flow & Metab*. 2003;23:1029-1039

26. Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP. Tlr signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol*. 2005;175:4320-4320

27. Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwsen S, Ravid R, Van Noort JM. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. *Glia*. 2006;53:688-695

28. Bsibsi M, Ravid R, Gveric D, Noort JMv. Broad expression of toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol*. 2002;61:1013-1021

29. Singh AK, Jiang Y. How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology*. 2004;201:197-207

30. Vogel SN, Fitzgerald KA, Fenton MJ. Tlrs: Differential adapter utilization by toll-like receptors mediates tlr-specific patterns of gene expression. *Mol Interv*. 2003;3:466-477

31. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol*. 2005;17:1-14

32. Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Hacker G, Mann M, Karin M. Specificity in toll-like receptor signalling through distinct effector functions of traf3 and traf6. *Nature*. 2006;439:204-207

33. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T. Spatiotemporal regulation of myd88-irf-7 signalling for robust type-i interferon induction. *Nature*. 2005;434:1035-1040

34. Tanimura N, Saitoh S, Matsumoto F, Akashi-Takamura S, Miyake K. Roles for lps-dependent interaction and relocation of tlr4 and tram in trif-signaling. *Biochem Biophys Res Commun*. 2008;368:94-99

35. Laflamme N, Soucy G, Rivest S. Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding toll-like receptor 2 in the cns. *J Neurochem*. 2001;79:648-657

36. Laflamme N, Rivest S. Toll-like receptor 4: The missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *Faseb J*. 2001;15:155-163

37. Chakravarty S, Herkenham M. Toll-like receptor 4 on nonhematopoietic cels sustains cns inflammation during endotoxemia, independent of systemic cytokines. *J Neurosci*. 2005;25:1788-1796

38. Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple tlrs. *J Immunol*. 2004;173:3916-3924

39. McKimmie CS, Fazakerley JK. In response to pathogens, glial cells dynamically and differentially regulate toll-like receptor gene expression. *J Neuroimmunol*. 2005;169:116-125

40. Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ, Miller SD. Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia*. 2005;49:360-374

41. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. Tram couples endocytosis of toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*. 2008;9:361-368

42. Constantin D, Cordenier A, Robinson K, Ala'Aldeen DA, Murphy S. Neisseria meningitidis-induced death of cerebrovascular endothelium: Mechanisms triggering transcriptional activation of inducible nitric oxide synthase. *J Neurochem*. 2004;89:1166-1174

43. Ziegler G, Harhausen D, Schepers C, Hoffmann O, Rohr C, Prinz V, Konig J, Lehrach H, Nietfeld W, Trendelenburg G. Tlr2 has a detrimental role in mouse transient focal cerebral ischemia. *Biochem Biophys Res Commun*. 2007;359:574-579

44. Zhou ML, Shi JX, Hang CH, Zhang FF, Gao J, Yin HX. Expression of toll-like receptor 4 in the brain in a rabbit experimental subarachnoid haemorrhage model. *Inflamm Res*. 2007;56:93-97

45. Lloyd-Jones KL, Kelly MM, Kubes P. Varying importance of soluble and membrane cd14 in endothelial detection of lipopolysaccharide. *J Immunol*. 2008;181:1446-1453

46. Lafon M, Megret F, Lafage M, Prehaud C. The innate immune facet of brain: Human neurons express tlr-3 and sense viral dsrna. *J Mol Neurosci*. 2006;29:185-194

47. Ma Y, Li J, Chiu I, Wang Y, Sloane JA, Lu J, Kosaras B, Sidman RL, Volpe JJ, Vartanian T. Toll-like receptor 8 functions as a negative regulator of neurite outgrowth and inducer of neuronal apoptosis. *J Cell Biol*. 2006;175:209-215

48. Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M. Toll-like receptors modulate adult hippocampal neurogenesis. *Nat Cell Biol*. 2007;9:1081-1088

49. Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis*. 2002;186:798-806

50. Mun HS, Aosai F, Norose K, Chen M, Piao LX, Takeuchi O, Akira S, Ishikura H, Yano A. Tlr2 as an essential molecule for protective immunity against toxoplasma gondii infection. *Int Immunol*. 2003;15:1081-1087

51. Thuong NT, Hawn TR, Thwaites GE, Chau TT, Lan NT, Quy HT, Hieu NT, Aderem A, Hien TT, Farrar JJ, Dunstan SJ. A polymorphism in human tlr2 is associated with increased susceptibility to tuberculous meningitis. *Genes Immun*. 2007;8:422-428

52. Stenzel W, Soltek S, Sanchez-Ruiz M, Akira S, Miletic H, Schluter D, Deckert M. Both tlr2 and tlr4 are required for the effective immune response in staphylococcus aureus-induced experimental murine brain abscess. *Am J Pathol*. 2008;172:132-145

53. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. Role of toll-like receptor signalling in abeta uptake and clearance. *Brain*. 2006;129:3006-3019

54. Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM. Activation of toll-like receptor 2 on microglia promotes cell uptake of alzheimer disease-associated amyloid beta peptide. *J Biol Chem*. 2006;281:3651-3659

55. Richard KL, Filali M, Prefontaine P, Rivest S. Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1-42 and delay the cognitive decline in a mouse model of alzheimer's disease. *J Neurosci*. 2008;28:5784-5793

56. Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW. Herpes simplex virus 1 interaction with toll-like

receptor 2 contributes to lethal encephalitis. *Proc Natl Acad Sci U S A*. 2004;101:1315-1320

57. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates west nile virus entry into the brain causing lethal encephalitis. *Nat Med*. 2004;10:1366-1373

58. Visser L, Jan de Heer H, Boven LA, van Riel D, van Meurs M, Melief MJ, Zahringer U, van Strijp J, Lambrecht BN, Nieuwenhuis EE, Laman JD. Proinflammatory bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease. *J Immunol*. 2005;174:808-816

59. Kerfoot SM, Long EM, Hickey MJ, Andonegui G, Lapointe BM, Zanardo RC, Bonder C, James WG, Robbins SM, Kubes P. Tlr4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. *J Immunol*. 2004;173:7070-7077

60. So EY, Kang MH, Kim BS. Induction of chemokine and cytokine genes in astrocytes following infection with theiler's murine encephalomyelitis virus is mediated by the toll-like receptor 3. *Glia*. 2006;53:858-867

61. Jou I, Lee JH, Park SY, Yoon HJ, Joe EH, Park EJ. Gangliosides trigger inflammatory responses via tlr4 in brain glia. *Am J Pathol*. 2006;168:1619-1630

62. Yoon HJ, Jeon SB, Suk K, Choi DK, Hong YJ, Park EJ. Contribution of tlr2 to the initiation of ganglioside-triggered inflammatory signaling. *Mol Cells*. 2008;25:99-104

63. Blanco AM, Perez-Arago A, Fernandez-Lizarbe S, Guerri C. Ethanol mimics ligand-mediated activation and endocytosis of il-1ri/tlr4 receptors via lipid rafts caveolae in astroglial cells. *J Neurochem*. 2008

64. Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. *Surg Neurol*. 2006;66:232-245

65. Yamasaki Y, Matsuo Y, Zagorski J, Matsuura N, Onodera H, Itoyama Y, Kogure K. New therapeutic possibility of blocking cytokine-induced neutrophil chemoattractant on transient ischemic brain damage in rats. *Brain Res*. 1997;759:103-111

66. Garau A, Bertini R, Colotta F, Casilli F, Bigini P, Cagnotto A, Mennini T, Ghezzi P, Villa P. Neuroprotection with the cxcl8 inhibitor repertaxin in transient brain ischemia. *Cytokine*. 2005;30:125-131

67. Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K. Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke*. 1995;26:676-681

68. Stevens S, Bao J, Hollis J, Lessov NS, Clark WM, Stenzel-Poore M. The use of flow cytometry to evaluate temporal changes in inflammation. *Brain Res*. 2002;932:110-119

69. Zarember KA, Godowski PJ. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mrnas in leukocytes in response to microbes, their products, and cytokines. *J Immunol*. 2002;168:554-561

70. Hua F, Ma J, Ha T, Xia Y, Kelley J, Williams DL, Kao RL, Browder IW, Schweitzer JB, Kalbfleisch JH, Li C. Activation of toll-like receptor 4 signaling

contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. *J Neuroimmunol*. 2007;190:101-111

71. Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG, Lathia JD, Siler DA, Chigurupati S, Ouyang X, Magnus T, Camandola S, Mattson MP. Pivotal role for neuronal toll-like receptors in ischemic brain injury and functional deficits. *Proc Natl Acad Sci U S A*. 2007;104:13798-13803

72. Lehnardt S, Lehmann S, Kaul D, Tschimmel K, Hoffmann O, Cho S, Krueger C, Nitsch R, Meisel A, Weber JR. Toll-like receptor 2 mediates cns injury in focal cerebral ischemia. *J Neuroimmunol*. 2007;190:28-33

73. Caso JR, Pradillo JM, Hurtado O, Leza JC, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in subacute stress-induced neuroinflammation and in the worsening of experimental stroke. *Stroke*. 2008;39:1314-1320

74. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation*. 2007;115:1599-1608

75. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in toll-like receptor 4 deficient mice. *Biochem Biophys Res Commun*. 2007;353:509-514

76. Lehnardt S, Schott E, Trimbuch T, Laubisch D, Krueger C, Wulczyn G, Nitsch R, Weber JR. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the cns. *J Neurosci*. 2008;28:2320-2331

77. Kinouchi H, Sharp FR, Hill MP, Koistinaho J, Sagar SM, Chan PH. Induction of 70-kda heat shock protein and hsp70 mrna following transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab*. 1993;13:105-115

78. Faraco G, Fossati S, Bianchi ME, Patrone M, Pedrazzi M, Sparatore B, Moroni F, Chiarugi A. High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. *J Neurochem*. 2007;103:590-603

79. Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM. Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. *Brain Res*. 1997;748:267-270

80. Rosenzweig HL, Lessov NS, Henshall DC, Minami M, Simon RP, Stenzel-Poore MP. Endotoxin preconditioning prevents the cellular inflammatory response during ischemic neuroprotection in mice. *Stroke*. 2004;35:2576-2581

81. Hickey EJ, You X, Kaimaktchiev V, Stenzel-Poore M, Ungerleider RM. Lipopolysaccharide preconditioning induces robust protection against brain injury resulting from deep hypothermic circulatory arrest. *J Thorac Cardiovasc Surg*. 2007;133:1588-1596

82. Rosenzweig HL, Minami M, Lessov NS, Coste SC, Stevens SL, Henshall DC, Meller R, Simon RP, Stenzel-Poore MP. Endotoxin preconditioning protects against the cytotoxic effects of tnfa after stroke: A novel role for tnfa in lps-ischemic tolerance. *J Cereb Blood Flow & Metab*. 2007;27:1663-1674

83. Kunz A, Park L, Abe T, Gallo EF, Anrather J, Zhou P, Iadecola C. Neurovascular protection by ischemic tolerance: Role of nitric oxide and reactive oxygen species. *J Neurosci*. 2007;27:7083-7093

84. Furuya K, Zhu L, Kawahara N, Abe O, Kirino T. Differences in infarct evolution between lipopolysaccharide-induced tolerant and nontolerant conditions to focal cerebral ischemia. *J Neurosurg*. 2005;103:715-723

85. Bordet R, Deplanque D, Maboudou P, Puisieux F, Pu Q, Robin E, Martin A, Bastide M, Leys D, Lhermitte M, Dupuis B. Increase in endogenous brain superoxide dismutase as a potential mechanism of lipopolysaccharide-induced brain ischemic tolerance. *J Cereb Blood Flow Metab*. 2000;20:1190-1196

86. Ahmed S, He Y, Nassief A, Xu J, Xu X, Hsu C. Effects of lipopolysaccharide priming on acute ischemic brain injury. *Stroke*. 2000;31:193-199

87. Dawson DA, Furuya K, Gotoh J, Nakao Y, Hallenbeck JM. Cerebrovascular hemodynamics and ischemic tolerance: Lipopolysaccharide-induced resistance to focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. *Journal of Cerebral Blood Flow and Metabolism*. 1999;19:616-623

88. Ginis I, Schweizer U, Brenner M, Liu J, Azzam N, Spatz M, Hallenbeck J. Tnf-alpha pretreatment prevents subsequent activation of cultured brain cells with tnf-alpha and hypoxia via ceramide. *Am J Physiol*. 1999;276:C1171

89. Medvedev AE, Lentschat A, Wahl LM, Golenbock DT, Vogel SN. Dysregulation of lps-induced toll-like receptor 4-myd88 complex formation and il-1 receptor-

associated kinase1 activation in endotoxin-tolerant cells. *J Immunol*. 2002;169:5209-5216

90. Sly LM, Rauh MJ, Kalesnikoff J, Song CH, Krystal G. Lps-induced upregualtion of ship is essential for endotoxin tolerance. *Immunity*. 2004;21:227-239

91. Kobayashi K, Hernandex L, Galan J, CA Janeway J, Medzhitov R, Flavell R. Irak-m is a negative regulator of toll-like receptor signaling. *Cell*. 2002;110:191-202

92. Shi M, Deng W, Bi E, Mao K, Ji Y, Lin G, Wu X, Tao Z, Li Z, Cai X, Sun S, Xiang C, Sun B. Trim30 alpha negatively regulates tlr-mediated nf-kappa b activation by targeting tab2 and tab3 for degradation. *Nat Immunol*. 2008;9:369-377

93. Broad A, Kirby JA, Jones DE. Toll-like receptor interactions: Tolerance of myd88-dependent cytokines but enhancement of myd88-independent interferon-beta production. *Immunology*. 2007;120:103-111

94. Dalpke AH, Lehner MD, Hartung T, Heeg K. Differential effects of cpg-DNA in toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology*. 2005;116:203-212

95. Kim YI, Park JE, Martinez-Hernandez A, Yi AK. Cpg DNA prevents liver injury and shock-mediated death by modulating expression of interleukin-1 receptor-associated kinases. *J Biol Chem*. 2008;283:15258-15270

96. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, Hellman J. Myd88-dependent and myd88-independent pathways in synergy, priming, and tolerance between tlr agonists. *J Immunol*. 2007;178:1164-1171

97. Sakaguchi S, Negishi H, Asagiri M, Nakajima C, Mizutani T, Takaoka A, Honda K, Taniguchi T. Essential role of irf-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. *Biochem Biophys Res Commun*. 2003;306:860-866

98. Escalante CR, Nistal-Villan E, Shen L, Garcia-Sastre A, Aggarwal AK. Structure of irf-3 bound to the prdiii-i regulatory element of the human interferon-beta enhancer. *Mol Cell*. 2007;26:703-716

99. Grandvaux N, Servant MJ, tenOever B, Sen GC, Balachandran S, Barber GN, Lin R, Hiscott J. Transcriptional profiling of interferon regulatory factor 3 target genes: Direct involvement in the regulation of interferon-stimulated genes. *J Virol*. 2002;76:5532-5539

100. Yamada T, Yamanaka I. Microglial localization of alpha-interferon receptor in human brain tissues. *Neurosci Lett*. 1995;189:73-76

101. Traugott U, Lebon P. Demonstration of alpha, beta, and gamma interferon in active chronic multiple sclerosis lesions. *Ann N Y Acad Sci*. 1988;540:309-311

102. Kraus J, Ling AK, Hamm S, Voigt K, Oschmann P, Engelhardt B. Interferon-beta stabilizes barrier characteristics of brain endothelial cells in vitro. *Ann Neurol*. 2004;56:192-205

103. Liuzzi GM, Latronico T, Fasano A, Carlone G, Riccio P. Interferon-beta inhibits the expression of metalloproteinases in rat glial cell cultures: Implications for multiple sclerosis pathogenesis and treatment. *Mult Scler*. 2004;10:290-297

104. Seguin R, Moditi Z, Rotondo R, Biernacki K, Wosik K, Prat A, Antel JP. Human brain endothelial cells supply support for monocyte immunoregulatory functions. *J Neuroimmunol*. 2003;135:96-106

105. Hua L, Kim M, Brosnan C, Lee S. Modulation of astrocyte inducible nitric oxide synthase and cytokine expression by interferon β is associated with induction and inhibition of interferon γ- activated sequence binding activity. *Journal of Neurochemistry*. 2002;83:1120-1128

106. Stewart VC, Land JM, Clark JB, Heales SJ. Pretreatment of astrocytes with interferon-alpha/beta prevents neuronal mitochondrial respiratory chain damage. *J Neurochem*. 1998;70:432-434

107. Lopez-Collazo E, Hortelano S, Rojas A, Bosca L. Triggering of peritoneal macrophages with ifn-alpha/beta attenuates the expression of inducible nitric oxide synthase through a decrease in nf-kappab activation. *J Immunol*. 1998;160:2889-2895

108. Bosca L, Bodelon OG, Hortelano S, Casellas A, Bosch F. Anti-inflammatory action of type i interferons deduced from mice expressing interferon beta. *Gene Ther*. 2000;7:817-825

109. Palmer G, Mezin F, Juge-Aubry CE, Plater-Zyberk C, Gabay C, Guerne PA. Interferon beta stimulates interleukin 1 receptor antagonist production in human articular chondrocytes and synovial fibroblasts. *Ann Rheum Dis*. 2004;63:43-49

110. Boutros T, Croze E, Yong VW. Interferon-beta is a potent promoter of nerve growth factor production by astrocytes. *J Neurochem*. 1997;69:939-946

111. Jin S, Kawanokuchi J, Mizuno T, Wang J, Sonobe Y, Takeuchi H, Suzumura A. Interferon-beta is neuroprotective against the toxicity induced by activated microglia. *Brain Res*. 2007;1179:140-146

112. Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol*. 2003;171:1393-1400

113. Stunz LL, Lenert P, Peckham D, Yi AK, Haxhinasto S, Chang M, Krieg AM, Ashman RF. Inhibitory oligonucleotides specifically block effects of stimulatory cpg oligonucleotides in b cells. *Eur J Immunol*. 2002;32:1212-1222

114. Clark WM, Lessov NS, Dixon MP, Eckenstein F. Monofilament intraluminal middle cerebral artery occlusion in the mouse. *Neurological Research*. 1997;19:641-648

115. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with imagej. *Biophotonics International*. 2004;11:36-42

116. Swanson RA, Morton MT, Tsao-Wu G. A semiautomated method for measuring brain infarct volume. *Journal of Cerebral Blood Flow & Metabolism*. 1990;10:290-293

117. Irizarry RA, Gautier L, Cope LM. An r package for oligonucleotide array statisitical analysis. In: Giovanni Parmigiani ESG, Rafael A Irizarry, Scott L Zeger, ed. *The analysis of gene expression data: Methods and software.* NY: Springer; 2003.

118. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med*. 1990;9:811-818

119. Vadigepalli R, Chakravarthula P, Zak DE, Schwaber JS, Gonye GE. Paint: A promoter analysis and interaction network generation tool for gene regulatory network identification. *Omics*. 2003;7:235-252

120. Meller R, Stevens SL, Minami M, Cameron JA, King S, Rosenzweig H, Doyle K, Lessov NS, Simon RP, Stenzel-Poore MP. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow & Metab*. 2005;25:217-225

121. West M, Heagy W. Endotoxin tolerance: A review. *Crit Care Med*. 2002;30:S64-S73

122. Fan H, Cook JA. Molecular mechanisms of endotoxin tolerance. *Journal of Endotoxin Research*. 2004;10:71-84

123. Mizel SB, Snipes JA. Gram-negative flagellin-induced self-tolerance is associated with a block in interleukin-1 receptor-associated kinase release from toll-like receptor 5. *J Biol Chem*. 2002;277:22414-22420

124. Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, Akira S. Synergy and cross-tolerance between toll-like receptor (tlr) 2- and tlr4-mediated signaling pathways. *J Immunol*. 2000;165:7096-7101

125. Yeo SJ, Yoon JG, Hong SC, Yi AK. Cpg DNA induces self and cross-hyporesponsiveness of raw264.7 cells in response to cpg DNA and lipopolysaccharide: Alterations in il-1 receptor-associated kinase expression. *J Immunol*. 2003;170:1052-1061

126. Lehner MD, Morath S, Michelsen KS, Schumann RR, Hartung T. Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via

different toll-like receptors independent of paracrine mediators. *J Immunol*. 2001;166:5161-5167

127. Dobrovolskaia MA, Medvedev AE, Thomas KE, Cuesta N, Toshchakov V, Ren T, Cody MJ, Michalek SM, Rice NR, Vogel SN. Induction of in vitro reprogramming by toll-like receptor (tlr)2 and tlr4 agonists in murine macrophages: Effects of tlr "Homotolerance" Versus "Heterotolerance" On nf-kappa b signaling pathway components. *J Immunol*. 2003;170:508-519

128. Heemann U, Szabo A, Hamar P, Muller V, Witzke O, Lutz J, Philipp T. Lipopolysaccharide pretreatment protects from renal ischemia/reperfusion injury: Possible connection to an interleukin-6-dependent pathway. *American Journal of Pathology*. 2000;156:287-293

129. Rowland RT, Meng X, Cleveland JC, Meldrum DR, Harken AH, Brown JM. Lps-induced delayed myocardial adaptation enhances acute preconditioning to optimize postischemic cardiac function. *Am J Physiol*. 1997;272:H2708-2715

130. Toyoda T, Kassell NF, Lee KS. Induction of tolerance against ischemia/reperfusion injury in the rat brain by preconditioning with the endotoxin analog diphosphoryl lipid a. *Journal of Neurosurgery*. 2000;92:435-441

131. Vasilakos JP, Smith RM, Gibson SJ, Lindh JM, Pederson LK, Reiter MJ, Smith MH, Tomai MA. Adjuvant activities of immune response modifier r-848: Comparison with cpg odn. *Cell Immunol*. 2000;204:64-74

132. Vollmer J, Weeratna RD, Jurk M, Davis HL, Schetter C, Wullner M, Wader T, Liu M, Kritzler A, Krieg AM. Impact of modifications of heterocyclic bases in

cpg dinucleotides on their immune-modulatory activity. *J Leukoc Biol*. 2004;76:585-593

133. Applequist SE, Wallin RP, Ljunggren HG. Variable expression of toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol*. 2002;14:1065-1074

134. Krieg AM. Therapeutic potential of toll-like receptor 9 activation. *Nat Rev Drug Discov*. 2006;5:471-484

135. Horng T, Barton GM, Medzhitov R. Tirap: An adapter molecule in the toll signaling pathway. *Nat Immunol*. 2001;2:835-841

136. Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. Recognition of cpg DNA is mediated by signaling pathways dependent on the adaptor protein myd88. *Curr Biol*. 2000;10:1139-1142

137. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB. Human tlr9 confers responsiveness to bacterial DNA via species-specific cpg motif recognition. *Proc Natl Acad Sci U S A*. 2001;98:9237-9242

138. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A toll-like receptor recognizes bacterial DNA. *Nature*. 2000;408:740-745

139. Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M. Tlr9-dependent recognition of mcmv by ipc and dc generates coordinated cytokine responses that activate antiviral nk cell function. *Immunity*. 2004;21:107-119

140. Krieg AM. Cpg motifs: The active ingredient in bacterial extracts? *Nat Med*. 2003;9:831-835

141. Elkins KL, Rhinehart-Jones TR, Stibitz S, Conover JS, Klinman DM. Bacterial DNA containing cpg motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J Immunol*. 1999;162:2291-2298

142. Krieg AM, Love-Homan L, Yi AK, Harty JT. Cpg DNA induces sustained il-12 expression in vivo and resistance to listeria monocytogenes challenge. *J Immunol*. 1998;161:2428-2434

143. Nawashiro H, Tasaki K, Ruetzler CA, Hallenbeck JM. Tnf-alpha pretreatment induces protective effects against focal cerebral ischemia in mice. *Journal of Cerebral Blood Flow & Metabolism*. 1997;17:483-490

144. Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. *Glia*. 2003;43:281-291

145. Stenzel-Poore MP, Stevens SL, King JS, Simon RP. Preconditioning reprograms the response to ischemic injury and primes the emergence of unique endogenous neuroprotective phenotypes: A speculative synthesis. *Stroke*. 2007;38:680-685

146. Newman MF, Mathew JP, Grocott HP, Mackensen GB, Monk T, Welsh-Bohmer KA, Blumenthal JA, Laskowitz DT, Mark DB. Central nervous system injury associated with cardiac surgery. *Lancet*. 2006;368:694-703

147. Rice L, Orlow D, Ceonzo K, Stahl GL, Tzianabos AO, Wada H, Aird WC, Buras JA. Cpg oligodeoxynucleotide protection in polymicrobial sepsis is dependent on interleukin-17. *J Infect Dis*. 2005;191:1368-1376

148. Hammarberg H, Lidman O, Lundberg C, Eltayeb SY, Gielen AW, Muhallab S, Svenningsson A, Linda H, van Der Meide PH, Cullheim S, Olsson T, Piehl F. Neuroprotection by encephalomyelitis: Rescue of mechanically injured neurons and neurotrophin production by cns-infiltrating t and natural killer cells. *J Neurosci*. 2000;20:5283-5291

149. Boccaccio GL, Mor F, Steinman L. Non-coding plasmid DNA induces ifn-gamma in vivo and suppresses autoimmune encephalomyelitis. *Int Immunol*. 1999;11:289-296

150. Wu HJ, Sawaya H, Binstadt B, Brickelmaier M, Blasius A, Gorelik L, Mahmood U, Weissleder R, Carulli J, Benoist C, Mathis D. Inflammatory arthritis can be reined in by cpg-induced dc-nk cell cross talk. *J Exp Med*. 2007;204:1911-1922

151. Della Chiesa M, Romagnani C, Thiel A, Moretta L, Moretta A. Multidirectional interactions are bridging human nk cells with plasmacytoid and monocyte-derived dendritic cells during innate immune responses. *Blood*. 2006;108:3851-3858

152. Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, Trinchieri G. The reciprocal interaction of nk cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol*. 2005;174:727-734

153. Stenzel-Poore MP, Stevens SL, Xiong Z, Lessov NS, Harrington CA, Mori M, Meller R, Rosenzweig HL, Tobar E, Shaw TE, Chu X, Simon RP. Effect of ischemic preconditioning on genomic response to cerebral ischemia: Similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. *The Lancet*. 2003;362:1028-1037

154. Kinouchi H, Sharp FR, Koistinaho J, Hicks K, Kamii H, Chan PH. Induction of heat shock hsp 70 mrna and hsp70 kda protein in neurons in the 'penumbra' following focal cerebral ischemia in the rat. *Brain Res*. 1993;619:334-338

155. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nature Reviews Immunology*. 2005;5:446-458

156. Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, Tanaka N, Taniguchi T. Gene induction pathways mediated by distinct irfs during viral infection. *Biochem Biophys Res Commun*. 2001;283:1150-1156

157. Lang T, Mansell A. The negative regulation of toll-like receptor and associated pathways. *Immunol Cell Biol*. 2007;85:425-434

158. Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, Takeuchi O, Akira S. Regulation of lipopolysaccharide-inducible genes by myd88 and toll/il-1 domain containing adaptor inducing ifn-beta. *Biochem Biophys Res Commun*. 2005;328:383-392

159. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T. The toll-like receptor tlr4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the cns. *J Neurosci*. 2002;22:2478-2486

160. Chen R, Zhou H, Beltran J, Malellari L, Chang SL. Differential expression of cytokines in the brain and serum during endotoxin tolerance. *J Neuroimmunol*. 2005;163:53-72

161. Qin L, He J, Hanes RN, Pluzarev O, Hong JS, Crews FT. Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *J Neuroinflammation*. 2008;5:10

162. Gosselin D, Rivest S. Myd88 signaling in brain endothelial cells is essential for the neuronal activity and glucocorticoid release during systemic inflammation. *Mol Psychiatry*. 2008;13:480-497

163. Verma S, Nakaoke R, Dohgu S, Banks WA. Release of cytokines by brain endothelial cells: A polarized response to lipopolysaccharide. *Brain Behav Immun*. 2006;20:449-455

164. Bederson J, Pitts L, Germano S, Nishimura M, Davis R, Bartowski H. Evaluation of 2,3,5-triphenyltetrazoline chloride as a stain for detection of quantification of experimental cerebral infarction in rats. *Stroke*. 1986;17:1304-1308

165. Thomas KE, Galligan CL, Newman RD, Fish EN, Vogel SN. Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. *J Biol Chem*. 2006;281:31119-31130

166. Zou W, Kim JH, Handidu A, Li X, Kim KI, Yan M, Li J, Zhang DE. Microarray analysis reveals that type i interferon strongly increases the expression of immune-response related genes in ubp43 (usp18) deficient macrophages. *Biochem Biophys Res Commun*. 2007;356:193-199

167. Kolosov M, Kolosova I, Zhou A, Leu RW. Autocrine induction of macrophage synthesis of complement subcomponent c1q by endogenous interferon-alpha/beta. *J Interferon Cytokine Res*. 1996;16:209-215

168. de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol*. 2001;69:912-920

169. Rani MR, Shrock J, Appachi S, Rudick RA, Williams BR, Ransohoff RM. Novel interferon-beta-induced gene expression in peripheral blood cells. *J Leukoc Biol*. 2007;82:1353-1360

170. Miller A, Lanir N, Shapiro S, Revel M, Honigman S, Kinarty A, Lahat N. Immunoregulatory effects of interferon-beta and interacting cytokines on human vascular endothelial cells. Implications for multiple sclerosis autoimmune diseases. *J Neuroimmunol*. 1996;64:151-161

171. Njenga MK, Pease LR, Wettstein P, Mak T, Rodriguez M. Interferon alpha/beta mediates early virus-induced expression of h-2d and h-2k in the central nervous system. *Lab Invest*. 1997;77:71-84

172. Zerrahn J, Schaible UE, Brinkmann V, Guhlich U, Kaufmann SH. The ifn-inducible golgi- and endoplasmic reticulum- associated 47-kda gtpase iigp is transiently expressed during listeriosis. *J Immunol*. 2002;168:3428-3436

173. Urosevic M, Dummer R, Conrad C, Beyeler M, Laine E, Burg G, Gilliet M. Disease-independent skin recruitment and activation of plasmacytoid predendritic cells following imiquimod treatment. *J Natl Cancer Inst*. 2005;97:1143-1153

174. Min W, Pober JS, Johnson DR. Interferon induction of tap1: The phosphatase shp-1 regulates crossover between the ifn-alpha/beta and the ifn-gamma signal-transduction pathways. *Circ Res*. 1998;83:815-823

175. Su ZZ, Sarkar D, Emdad L, Barral PM, Fisher PB. Central role of interferon regulatory factor-1 (irf-1) in controlling retinoic acid inducible gene-i (rig-i) expression. *J Cell Physiol*. 2007;213:502-510

176. Longman RS, Braun D, Pellegrini S, Rice CM, Darnell RB, Albert ML. Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of ifn-alpha/beta on antigen cross-presentation. *Blood*. 2007;109:1113-1122

177. Smith RA, Young J, Weis JJ, Weis JH. Expression of the mouse fragilis gene products in immune cells and association with receptor signaling complexes. *Genes Immun*. 2006;7:113-121

178. Jatana M, Giri S, Ansari MA, Elango C, Singh AK, Singh I, Khan M. Inhibition of nf-kappab activation by 5-lipoxygenase inhibitors protects brain against injury in a rat model of focal cerebral ischemia. *J Neuroinflammation*. 2006;3:12

179. Williams AJ, Dave JR, Tortella FC. Neuroprotection with the proteasome inhibitor mln519 in focal ischemic brain injury: Relation to nuclear factor kappab (nf-kappab), inflammatory gene expression, and leukocyte infiltration. *Neurochem Int*. 2006;49:106-112

180. Phillips JB, Williams AJ, Adams J, Elliott PJ, Tortella FC. Proteasome inhibitor ps519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. *Stroke*. 2000;31:1686-1693

181. Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. Nf-kappab is activated and promotes cell death in focal cerebral ischemia. *Nat Med*. 1999;5:554-559

182. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. Hmgb1 signals through toll-like receptor (tlr) 4 and tlr2. *Shock*. 2006;26:174-179

183. Bjorkbacka H, Fitzgerald KA, Huet F, Li X, Gregory JA, Lee MA, Ordija CM, Dowley NE, Golenbock DT, Freeman MW. The induction of macrophage gene expression by lps predominantly utilizes myd88-independent signaling cascades. *Physiol Genomics*. 2004;19:319-330

184. Prehaud C, Megret F, Lafage M, Lafon M. Virus infection switches tlr-3-positive human neurons to become strong producers of beta interferon. *J Virol*. 2005;79:12893-12904

185. Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. Activation of innate immunity in the cns triggers neurodegeneration through a toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci*. 2003;100:8514-8519

186. Katoh Y, Shiba Y, Mitsuhashi H, Yanagida Y, Takatsu H, Nakayama K. Tollip and tom1 form a complex and recruit ubiquitin-conjugated proteins onto early endosomes. *J Biol Chem*. 2004;279:24435-24443

187. Huang Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Nf-kappab precursor, p105, and nf-kappab inhibitor, ikappabgamma, are both elevated in alzheimer disease brain. *Neurosci Lett*. 2005;373:115-118

188. Nishimura M, Naito S. Tissue-specific mrna expression profiles of human toll-like receptors and related genes. *Biol Pharm Bull*. 2005;28:886-892

189. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, Wader T, Tluk S, Liu M, Davis HL, Krieg AM. Characterization of three cpg oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol*. 2004;34:251-262

190. Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. *Glia*. 2003;43:281-291

191. Lee S, Hong J, Choi SY, Oh SB, Park K, Kim JS, Karin M, Lee SJ. Cpg oligodeoxynucleotides induce expression of proinflammatory cytokines and chemokines in astrocytes: The role of c-jun n-terminal kinase in cpg odn-mediated nf-kappab activation. *J Neuroimmunol*. 2004;153:50-63

192. Dalpke AH, Schafer MK, Frey M, Zimmermann S, Tebbe J, Weihe E, Heeg K. Immunostimulatory cpg-DNA activates murine microglia. *J Immunol*. 2002;168:4854-4863

193. Tsan MF, Gao B. Endogenous ligands of toll-like receptors. *J Leukoc Biol*. 2004;76:514-519

194. McFalls EO, Sluiter W, Schoonderwoerd K, Manintveld OC, Lamers JM, Bezstarosti K, van Beusekom HM, Sikora J, Ward HB, Merkus D, Duncker DJ. Mitochondrial adaptations within chronically ischemic swine myocardium. *J Mol Cell Cardiol*. 2006;41:980-988

195. Tailor P, Tamura T, Kong HJ, Kubota T, Kubota M, Borghi P, Gabriele L, Ozato K. The feedback phase of type i interferon induction in dendritic cells requires interferon regulatory factor 8. *Immunity*. 2007;27:228-239

196. Yie J, Merika M, Munshi N, Chen G, Thanos D. The role of hmg i(y) in the assembly and function of the ifn-beta enhanceosome. *Embo J*. 1999;18:3074-3089

197. Heuschmann PU, Kolominsky-Rabas PL, Misselwitz B, Hermanek P, Leffmann C, Janzen RW, Rother J, Buecker-Nott HJ, Berger K. Predictors of in-hospital mortality and attributable risks of death after ischemic stroke: The german stroke registers study group. *Arch Intern Med*. 2004;164:1761-1768

198. Langhorne P, Stott DJ, Robertson L, MacDonald J, Jones L, McAlpine C, Dick F, Taylor GS, Murray G. Medical complications after stroke: A multicenter study. *Stroke*. 2000;31:1223-1229

199. Davenport RJ, Dennis MS, Wellwood I, Warlow CP. Complications after acute stroke. *Stroke*. 1996;27:415-420

200. Kwan J, Hand P. Infection after acute stroke is associated with poor short-term outcome. *Acta Neurol Scand*. 2007;115:331-338

201. Prass K, Meisel C, Hoflich C, Braun J, Halle E, Wolf T, Ruscher K, Victorov IV, Priller J, Dirnagl U, Volk HD, Meisel A. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke t helper cell type 1-like immunostimulation. *J Exp Med*. 2003;198:725-736

202. Chace JH, Hooker NA, Mildenstein KL, Krieg AM, Cowdery JS. Bacterial DNA-induced nk cell ifn-gamma production is dependent on macrophage secretion of il-12. *Clin Immunol Immunopathol*. 1997;84:185-193

203. Sun S, Zhang X, Tough DF, Sprent J. Type i interferon-mediated stimulation of t cells by cpg DNA. *J Exp Med*. 1998;188:2335-2342

204. Matsumoto Y, Kohyama K, Aikawa Y, Shin T, Kawazoe Y, Suzuki Y, Tanuma N. Role of natural killer cells and tcr gamma delta t cells in acute autoimmune encephalomyelitis. *Eur J Immunol*. 1998;28:1681-1688

205. Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (nk) cells. *J Exp Med*. 1997;186:1677-1687

206. Lobell A, Weissert R, Eltayeb S, Svanholm C, Olsson T, Wigzell H. Presence of cpg DNA and the local cytokine milieu determine the efficacy of suppressive DNA vaccination in experimental autoimmune encephalomyelitis. *J Immunol*. 1999;163:4754-4762

207. Sawaki J, Tsutsui H, Hayashi N, Yasuda K, Akira S, Tanizawa T, Nakanishi K. Type 1 cytokine/chemokine production by mouse nk cells following activation of their tlr/myd88-mediated pathways. *Int Immunol*. 2007;19:311-320

208. Girart MV, Fuertes MB, Domaica CI, Rossi LE, Zwirner NW. Engagement of tlr3, tlr7, and nkg2d regulate ifn-gamma secretion but not nkg2d-mediated cytotoxicity by human nk cells stimulated with suboptimal doses of il-12. *J Immunol*. 2007;179:3472-3479

209. Vaknin I, Blinder L, Wang L, Gazit R, Shapira E, Genina O, Pines M, Pikarsky E, Baniyash M. A common pathway mediated through toll-like receptors leads to t- and natural killer-cell immunosuppression. *Blood*. 2008;111:1437-1447

210. Varma TK, Toliver-Kinsky TE, Lin CY, Koutrouvelis AP, Nichols JE, Sherwood ER. Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice. *Infect Immun.* 2001;69:5249-5263

211. Maier CM, Yu F, Nishi T, Lathrop SJ, Chan PH. Interferon-beta fails to protect in a model of transient focal stroke. *Stroke*. 2006;37:1116-1119

212. An H, Xu H, Zhang M, Zhou J, Feng T, Qian C, Qi R, Cao X. Src homology 2 domain-containing inositol-5-phosphatase 1 (ship1) negatively regulates tlr4-mediated lps response primarily through a phosphatase activity- and pi-3k-independent mechanism. *Blood*. 2005;105:4685-4692

213. Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by tollip. *J Biol Chem*. 2002;277:7059-7065

214. Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, Kishimoto T. Socs-1 participates in negative regulation of lps responses. *Immunity*. 2002;17:677-687

215. Noble R. The development of resistance by rats and guinea pigs to amounts of trauma usually fatal. *Am J Physio*. 1943;38:346-351

216. Dirnagl U, Meisel A. Endogenous neuroprotection: Mitochondria as gateways to cerebral preconditioning? *Neuropharmacology*. 2008

217. Dave KR, Saul I, Busto R, Ginsberg MD, Sick TJ, Perez-Pinzon MA. Ischemic preconditioning preserves mitochondrial function after global cerebral ischemia in rat hippocampus. *J Cereb Blood Flow Metab*. 2001;21:1401-1410

218. Wu LY, Ding AS, Zhao T, Ma ZM, Wang FZ, Fan M. Involvement of increased stability of mitochondrial membrane potential and overexpression of bcl-2 in enhanced anoxic tolerance induced by hypoxic preconditioning in cultured hypothalamic neurons. *Brain Res*. 2004;999:149-154

219. Zhan RZ, Fujihara H, Baba H, Yamakura T, Shimoji K. Ischemic preconditioning is capable of inducing mitochondrial tolerance in the rat brain. *Anesthesiology*. 2002;97:896-901

220. Buck LT, Hochachka PW. Anoxic suppression of na+-k+-atpase and constant membrane potential in hepatocytes: Support for channel arrest. *Am J Physiol*. 1993;265:R1020-R1025

221. Truettner J, Busto R, Zhao W, Ginsberg MD, Perez-Pinzon MA. Effect of ischemic preconditioning on the expression of putative neuroprotective genes in the rat brain. *Brain Res Mol Brain Res*. 2002;103:106-115

222. Aneja R, Odoms K, Dunsmore K, Shanley TP, Wong HR. Extracellular heat shock protein-70 induces endotoxin tolerance in thp-1 cells. *J Immunol*. 2006;177:7184-7192

223. Stewart VC, Giovannoni G, Land JM, McDonald WI, Clark JB, Heales SJ. Pretreatment of astrocytes with interferon-alpha/beta impairs interferon-gamma induction of nitric oxide synthase. *J Neurochem*. 1997;68:2547-2551

224. Ginis I, Jaiswal R, Klimanis D, Liu J, Greenspon J, Hallenbeck J. Tnfa induced tolerance to ischemic injury involves differential control of nf-kb transactivation: The role of nf-kb association with p300 adaptor. *J Cereb Blood Flow & Metab*. 2002;22

225. Ferlito M, Romanenko OG, Ashton S, Squadrito F, Halushka PV, Cook JA. Effect of cross-tolerance between endotoxin and tnf-alpha or il-1beta on cellular signaling and mediator production. *J Leukoc Biol*. 2001;70:821-829

226. Murphey ED, Traber DL. Protective effect of tumor necrosis factor-alpha against subsequent endotoxemia in mice is mediated, in part, by interleukin-10. *Crit Care Med*. 2001;29:1761-1766

227. Porter MH, Arnold M, Langhans W. Tnf-alpha tolerance blocks lps-induced hypophagia but lps tolerance fails to prevent tnf-alpha-induced hypophagia. *Am J Physiol*. 1998;274:R741-745

228. Selim M. Perioperative stroke. *N Engl J Med*. 2007;356:706-713