Studies on poly-ICLC treatment as a potent neuroprotective therapy against ischemic brain injury

By

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List of Abbreviations

aCSF	Artificial cerebral spinal fluid
ANOVA	Analysis of variance
CMC	Carboxymethylcellulose
CNS	Central nervous system
CpG	Unmethylated cytosine-guanine-rich DNA oligonucleotides
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
HIF	Hypoxia-inducible factor
ICAM	Intracellular adhesion molecule
IFIT	IFN-induced protein with tetratricopeptide repeat motifs
IFN	Interferon
IFNAR	Type-1 IFN receptor
IκB	Inhibitor of NFκB
IKK	Inhibitor of NFκB kinase
IKKε	Non-canonical IκB kinases
IL	Interleukin
i.p.	Intraperitoneal
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ISRE	Interferon-sensitive response element
LPS	Lipopolysaccharide

MAL	MyD88 adapter-like
MCAO	Middle cerebral artery occlusion
mda-5	Melanoma differentiation-associated protein 5
MIP1α	Macrophage inflammatory protein1α
MMP9	Matrix metalopeptidase 9
MyD88	Myeloid differentiation factor 88
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa-B
NHP	Non-human primate
NK	Natural killer cell
OGD	Oxygen-glucose-deprivation
OTS	Oligonucleotide transport system
PAINT	Promoter Analysis and Interaction Network Toolset
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
Poly-IC	Polyinosinic-polycytidylic acid
Poly-ICLC	Poly-IC stabilized with poly-L-lysine and carboxymethylcellulose
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
RIP	Receptor interacting protein
RIPC	Remote ischemic preconditioning
RLR	retinoic acid-inducible gene1-like-receptors

rt-PA	recombinant tissue plasminogen activator
SAINT	Stroke-Acute Ischemic NXY Treatment
s.c.	Subcutaneous
SEM	Standard error of the mean
STAIR	Stroke Therapy Academic Industry Roundtable
TAB	Transforming growth factor-β binding protein
TAK	Transforming growth factor-β activated kinase
TBK1	TRAF-family member associated NFκB activator-binding kinase 1
TGFβ	Tumor growth factor-beta
TIA	Transient ischemic attack
TIR	Toll-interleukin 1 receptor
TIRAP	TIR domain-containing Adaptor Protein
TLR	Toll-like receptor
TLR9KO	TLR9 deficient mice
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRE	Transcriptional regulatory element
TRIF	TIR domain-containing adapter inducing interferon-beta
TTC	2,3,5 triphenyltetrazolium chloride

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Forward

Lost in Translation

Imagine waking up one day in a strange hospital room unable to move, unable to speak, surrounded by strangers, trapped inside your body fully conscious and aware of the world around you. This was the reality for Jean-Dominique Bauby who experienced a stroke at the age of 43 and suffered from “locked-in” syndrome. Amazingly, he was able to dictate his memoir, *The Diving Bell and the Butterfly,* by blinking his good eye, his only means of communicating to the world. He died of lung failure 15 months after his stroke and two days after the publication of his book... Before the birth of her son, Heather experienced a series of 7 strokes. Tragically, at the young age of 25, this left Heather disabled. Though she can now speak, walk and enjoy a good party, she needs help with simple things, like taking a shower and getting dressed in the morning. But more difficult for her husband to deal with is the change in her personality and her inability to make rational decisions, like knowing when to stop shopping on the Internet… My grandmother, who suffered a stroke in her late 60s, luckily recovered without any obvious signs of deficit. However, she has never been quite the same and seems to have a lack of inhibition and loss of memory. Now in her mid-80s she is showing many common signs of aging such as memory loss, depression, difficulty completing tasks, and increased falling. Though these symptoms might be explained by other causes of aging, it is unknown how much is due to the accumulation of ischemic brain damage over time.

Stroke affects many people, one person every 40 seconds in the United States, and the outcomes can vary largely from patient to patient. Though age is one of the most significant risk factors for stroke with 95% of strokes occurring in people 45 years of age and older and 65% of strokes occurring in people over the age of 65, stroke affects people of all ages and all walks of life. rt-PA, a thrombolytic, is the only available stroke treatment. Unfortunately, it is effective for only a small percentage of patients due to the short time window during which this drug can be safely and effectively used. Numerous promising treatments developed in scientific laboratories have failed to translate into effective clinical approaches. Therefore I have taken a novel approach to drug discovery by studying the body's own endogenous methods of neuroprotection through the phenomenon of ischemic tolerance. This work is dedicated to finding new promising stroke therapeutics.

Abstract

Preconditioning induces ischemic tolerance, which confers robust protection against ischemic damage. Ischemic tolerance is a biologic process that can be utilized to enhance the brain's own endogenous protection mechanisms and as such, holds true promise for patients at risk of ischemic injury. Experimentally, preconditioning with various Toll-like receptor agonists successfully attenuates ischemic damage, in part through genomic reprogramming of the brain's response to stroke. This treatment diminishes certain damaging inflammatory responses to stroke and at the same time, promotes the production of neuroprotective mediators.

Many of the currently identified preconditioning stimuli are not appropriate for regular use in human patients. Therefore, our laboratory sought to identify novel pharmacological preconditioning stimuli that would be useful in a clinical setting. Polyinosinic polycytidylic-acid (poly-IC) treatment leads to robust production of interferon and interferon-related genes, both neuroprotective mediators. Here, we show marked protection with poly-IC preconditioning in three models of murine ischemia-reperfusion injury. Poly-IC preconditioning induced protection against ischemia modeled *in vitro* in brain cortical cells and *in vivo* in models of brain ischemia and renal ischemia. Further, unlike other Toll-like receptor ligands, which generally induce significant inflammatory responses, a preconditioning dose of poly-IC elicits only modest systemic inflammation. These results

demonstrate that poly-IC is a new, powerful, prophylactic treatment that offers promise as a clinical therapeutic strategy to minimize damage in patient populations at risk of ischemic injury.

To investigate endogenous mechanisms of neuroprotection we sought to compare the mechanisms of action of multiple preconditioning stimuli to poly-ICLC. Poly-ICLC preconditioning induces interferon related genes following ischemic challenge that are common to other preconditioning stimuli: LPS, CpG and ischemic preconditioning. This feature suggests that poly-ICLC preconditioning also reprograms the response to stroke. We also identified downstream effectors interferon regulatory factor 7 and type-1 interferon signaling as critical mediators of poly-ICLC neuroprotection.

Though we have previously identified key mechanistic components of neuroprotection mediated by preconditioning, little is known about the sites of action of preconditioning stimuli that initiate reprogramming to generate an ischemic tolerant state. Preconditioning with the TLR9 ligand CpG also reduces damage following ischemic injury. TLR9 expression is widespread; therefore, a broad range of potential target cell populations exists. To address this question we focused on the contribution of TLR9-expressing hematopoietic cells and created TLR9KO reciprocal bone marrow chimeric mice lacking TLR9 on either hematopoietic or parenchymal cells. CpG preconditioning did not protect either form of TLR9KO chimeric mice, demonstrating that the expression of TLR9 on

hematopoietic and parenchymal cells are both necessary for the protective effects of CpG. Consistent with the critical role of the cytokine TNFα in CpG-induced neuroprotection, we found that both forms of TLR9KO chimeric mice lacked the TNFα mRNA response centrally. These results indicate that TLR9 expression on hematopoietic cells is required but not sufficient to induce preconditioning neuroprotection and that this loss of protection correlates with loss of TNFα mRNA response to CpG in the brain.

Chapter 1:

Introduction

1. Stroke: a deadly and debilitating disease

Stroke is caused by the disruption of blood supply to the brain that leads to tissue damage and the loss of brain functions. Hippocrates first described the disease over 2400 years ago in ancient Greece. At that time stroke was called apoplexy meaning “struck with violence” referring to the sudden onset of symptoms that include paralysis and impaired speech as a result of injury to the brain (Nilsen, 2010). Since that time we have greatly refined our understanding of stroke pathology. There are two categories of stroke: ischemic, caused by a blockage in a vessel that serves the brain, and hemorrhagic, caused by leakage of blood into the brain following vessel rupture. The vast majority of strokes are ischemic, comprising 87% of the nearly 800,000 strokes experienced in the US each year (Roger *et al,* 2011).

Stroke is the leading cause of disability and the third leading cause of death in the United States yet there is but a single treatment for stroke, recombinant tissue plasminogen activator (rt-PA)– a drug that breaks down blood clots. Unfortunately, rt-PA is safe only for ischemic stroke patients, not hemorrhagic stroke, and may only be administered for a limited time (3 to 4.5 hours) following stroke onset (van der Worp and van Gijn, 2007). As many stroke patients do not receive timely treatment or are ineligible, currently less than 5% of acute ischemic stroke patients receive rt-PA treatment (Adeoye *et al,* 2011). Thus, there is an overwhelming need for the development of novel stroke therapeutics.

Numerous promising stroke treatments developed at the laboratory bench have failed to translate into effective clinical approaches. This failure has led to formation of the Stroke Therapy Academic Industry Roundtable (STAIR) which has developed guidelines for preclinical investigation of stroke therapeutics (STAIR (Fisher M. Chair), 1999). The goal of these guidelines is to facilitate and improve the process of translating basic research findings into clinical practice. These guidelines were recently updated and expanded as follows (Fisher *et al,* 2009; Fisher, 2011):

1. Adequate dose-response curve
2. Document that the drug accesses the target organ, the brain
3. Define the therapeutic time window in well-characterized animal stroke models
4. All animal treatment experiments should be done in a blinded, randomized manner with control of physiological variables with predefined inclusion/exclusion criteria using an adequate sample size based on an appropriate sample size estimate
5. Both histological and functional outcomes should be assessed acutely and long term
6. Efficacy studies should be performed initially in young healthy male animals using permanent occlusion modeling in most cases
7. Initial studies should be performed in rodents and then studies in gyrencephalic species should be considered
8. Additional studies with promising agents should be performed in female

animals, aged animals, and animals with comorbid conditions such as hypertension, diabetes, and hypercholesterolemia

9. Relevant biomarker endpoints such as diffusion/perfusion MRI and serum tissue injury markers should be considered
10. Interaction studies with commonly used medications should be performed

Now more than a decade after these guidelines were first published, there have been no neuroprotective drugs developed that have demonstrated efficacy and passed the approval process. This deficit may be due to inadequacies in preclinical testing that failed to meet the STAIR criteria and poor design or implementation of clinical trials which evaluated neuroprotective drugs (Fisher, 2011). However these updated criteria should prove helpful in testing new neuroprotective therapies if these guidelines are followed in earnest. Therefore, we must consider novel approaches to identify promising new therapeutic targets for stroke therapy.

Identifying Novel Therapeutic Targets for Stroke

Damage from stroke is multifaceted and occurs not only during the ischemic period while blood flow is occluded, but also upon restoration of blood flow termed reperfusion. Ischemia-reperfusion injury induces neuronal damage by disrupting the delicate electrical and chemical balance in the brain. Multiple pathways involving diverse cell types both in the brain and the periphery cause

damage during and following ischemia including metabolic failure, membrane failure, excitotoxicity, acidotoxicity, apoptosis, oxidative/nitrative stress, neurovascular unit dysfunction, and inflammation (Doyle *et al,* 2008). Energy demands in the brain are high in order for neurons to maintain a tightly regulated ionic gradient across the membrane, for example after depolarization. Therefore, when stroke disrupts the delivery of oxygen and nutrients into the brain neuronal homeostasis is quickly thrown out of balance. Energy failure leads to membrane depolarization, which results in dangerously high levels of intracellular calcium, increased release of excitatory neurotransmitters, disruption of neurotransmitter reuptake processes and increased intracellular acidity. Together these processes can swiftly lead to catabolism and necrosis. The threat of tissue damage gradually spreads to surrounding brain tissue, which faces new hazardous mediators over time including peri-infarct depolarization, oxygen radicals, apoptosis and inflammation (Doyle *et al,* 2008). Our focus for therapeutic intervention is the immune response to stroke, as this is readily modifiable and occurs over an extended period of time.

Stroke elicits an inflammatory response that contributes to neuronal damage through activation of microglia, endothelial cells and astrocytes in the central nervous system (CNS) and the infiltration of peripheral lymphocytes, macrophages, and neutrophils across an activated and disrupted blood brain barrier. The inflammatory response is a dual-edged sword in the recuperation process from tissue damage as it plays an essential role in eliminating dead cells

and promoting repair and regeneration. However, inflammation without sufficient regulation can exacerbate and extend stroke damage.

As the resident immune cell in the brain, microglia play a primary role in initiating and modulating the immune response to stroke (Price *et al,* 2006; Schilling *et al,* 2003; Wake *et al,* 2009). Depending on the conditions, microglia may exert both damaging and protective effects in the brain (Colton, 2009; Kraft *et al,* 2009; Lai and Todd, 2006; Neumann *et al,* 2008; Rivest, 2009). To aid in recovery, they engulf dead cells and produce neurotrophic factors, growth factors, and regulatory cytokines such as tumor growth factor-beta (TGFβ) and Interleukin-6 (IL-6). However, they also incite damaging inflammation with the production of pro-inflammatory cytokines such as tumor necrosis factorα (TNFα) and IL-1, oxygen-free radicals, and chemokines such as macrophage inflammatory protein1α (MIP1α), which work to attract the infiltration of peripheral immune cells, including neutrophils, lymphocytes and monocytes. Infiltration continues over time and extends the inflammatory response and may exacerbate stroke damage. Monocytes are the first cells to enter the ischemic region, followed closely by a large infiltration of neutrophils and a much smaller presence of lymphocytes (Gelderblom *et al,* 2009). These activated immune cells also produce proinflammatory cytokines and oxygen radicals that damage neurons. They also promote upregulation of cellular adhesion molecules further aiding infiltration into the brain and exacerbating ischemic injury. However, there is also infiltration of regulatory immune cells such as regulatory T-cells and natural killer

T-cells that may help to limit damaging inflammation (Gelderblom *et al,* 2009). Our goal with therapeutic intervention is to suppress the negative consequences of inflammation, while enhancing the protective and regulatory aspects of the immune response.

Following stroke, ischemic brain regions can be classified in two categories: an ischemic core of terminally damaged tissue in close proximity to the site of blood flow disruption where cells begin to die within minutes by necrosis and excitotoxicity, and a surrounding penumbra, which receives some collateral blood flow and where cell death is delayed and occurs via apoptosis and inflammation over an extended period of time. A report by Astrup et al. (Astrup *et al,* 1977) identified the penumbral region through abnormal electrical function adjacent to the central area of ischemic damage that could be restored by increasing blood flow. The ischemic penumbra has also been defined as a region of reduced cerebral blood flow down to 20-40% of baseline, whereas core flow is reduced to 5-20% (Ginsberg, 2003). There are also molecular indicators that can be used to identify the penumbra region, such as heat shock protein 70 and hypoxia-inducible factor (HIF) and these proteins can contribute to endogenous repair mechanisms (Sharp *et al,* 2000). For instance, heat shock protein 70 helps proteins denatured due to injury, regain structural conformation, and increased expression of this protein protects cells from injury including ischemia (Sharp *et al,* 2000). Anti-apoptotic genes such as Bcl-2 and Bcl-xl are also expressed in the penumbra and may function to save cells in this vulnerable region (Sharp *et*

al, 2000). The ischemic penumbra provides an opportunity for intervention whereby this tissue, at risk of dying following stroke, may be salvaged. (Lo, 2008). It is unknown which processes in the penumbra region contribute to damage and which represent endogenous repair mechanisms that help to salvage tissue in this region. Furthermore, the time point at which recovery and repair processes may begin is unclear, making it difficult to know how and when to intervene with stroke therapies (Lo, 2008). An effective neuroprotectant for stroke treatment must be a therapy that has a clinically relevant time window of effectiveness, is able to combat multiple injury mechanisms, and promotes endogenous repair mechanisms (Doyle *et al,* 2008; Endres *et al,* 2008; Lo, 2008). Preconditioning may be just such an approach as it could be administered prior to stroke and tap into the brain's endogenous protection mechanisms on a number of fronts to combat ischemic damage.

Preconditioning to induce ischemic tolerance

Preconditioning is a phenomenon whereby exposure to a modest amount of an otherwise harmful stimulus can protect against a subsequent, more severe injury. In the case of ischemia-reperfusion injury, the preconditioning stimulus lessens damage through the establishment of ischemic tolerance, a state where the tissue is resistant to ischemic damage. The first demonstration of a prophylactic treatment providing protection from focal ischemic stroke utilized brief hypothermia as the preconditioning stimulus (Chopp *et al,* 1989). Since then,

numerous preconditioning stimuli including mild epilepsy, brief exposure to hypoxia, ischemia, spreading depression, hypo-perfusion and moderate inflammatory activation have been used to effectively protect against cerebral ischemia (Gidday, 2006).

The protective effect of delayed preconditioning requires several hours to develop and provides a neuroprotective window that lasts several days. In order for preconditioning to be effective it must be initiated prior to stroke, therefore, one must have prior knowledge that cerebral ischemia will occur. There are several risk factors that identify patient populations that could benefit from antecedent therapy to protect against ischemia. A transient ischemic attack (TIA) is a small ischemic event that is thought to be relatively benign but carries with it a risk of having a subsequent stroke. There is a 5% chance of having a stroke in the first 48 hours after a TIA and a 10-15% chance of having a stroke in the next 3 months. In the long term by 10 years out, the risk rises to 44% (Giles and Rothwell, 2008). Similar to TIA patients, patients who have had a stroke have a 10-25% risk of recurrent stroke within 5 years (Roger *et al,* 2011). Additionally, perioperative stroke is a serious complication of surgery that occurs in approximately 0.7% of general surgery patients; however, the risk increases greatly following vascular or cardiac surgery to upwards of 10% risk of stroke (Selim, 2007). Beyond stroke, perioperative ischemia causes other complications. For instance, coronary artery bypass patients have a 50% risk of iatrogenically induced cognitive deficits (Selim, 2007). Patients undergoing

cardiac surgery have a 50% chance of incurring ischemic injury to the brain and for cardiac patients the risk of ischemic injury also extends to other organs including the kidneys (30% occurrence) and lungs (18% occurrence) (Barber *et al,* 2008; Fukada *et al,* 2004; Mehta *et al,* 2010). These complications are known to increase post-operative decline and mortality. All of these patient populations could benefit from a prophylactic intervention such as preconditioning to protect from the devastating consequences of ischemia-reperfusion injury.

Many types of preconditioning stimuli are not reasonable treatments for patients, as they might require surgery or would carry high risk for patients. However, pharmacological approaches utilizing Toll-like receptor (TLR) ligands, which induce a moderate inflammatory activation, provide robust protection against stroke (Rosenzweig *et al,* 2007). Importantly, TLR ligands hold great potential for clinical use as they have been used to safely treat other diseases in humans. Potentially, preconditioning can salvage tissue in the ischemic penumbra by suppressing the damaging inflammatory response following ischemia while enhancing the protective immune response thereby improving outcomes for stroke patients. The following sections provide background on TLRs, describe their role in ischemic injury, their use as preconditioning agents and the potential mechanisms of TLR-mediated neuroprotection.

2. The Toll-like receptor family

The TLRs are a family of pattern recognition receptors involved in the innate immune system functions. Structurally, TLRs have an ectodomain with leucine-rich repeat motifs and an intracellular Toll-interleukin 1 receptor (TIR) domain (Bowie and O'Neill, 2000). Functionally, TLRs recognize common pathogen molecules, or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin. More recent discoveries show that TLRs also recognize endogenous ligands known as damage-associated molecular patterns (DAMPs) that are released from dead or injured cells, such as nucleic acids, heat shock proteins or fibrinogen. Therefore, TLRs alert the immune system to invading pathogens and also act as sentinels of tissue damage.

TLRs can be classified broadly into six subfamilies based on amino acid homologies: TLR2, TLR3, TLR4, TLR5, TLR9 and TLR11 subfamilies. The TLR2 family consists of TLR1, TLR2, and TLR6 activated by components from a variety of microorganisms, including peptidoglycan, lipoteichoic acid, diacyl lipopeptides, triacyl lipopeptides, hyaluronic acid and the synthetic ligand Pam3CSK4 (Rock *et al,* 1998; Takeda *et al,* 2003; Takeuchi *et al,* 1999). TLR3, in a family by itself, recognizes double-stranded RNA (dsRNA) produced by viruses, the synthetic nucleoside moiety, polyinosinic-polycytidylic acid (poly-IC) and the endogenous ligands self-mRNA and stathmin (Alexopoulou *et al,* 2001; Bsibsi *et al,* 2010; Kariko *et al,* 2004a). TLR5 recognizes flagellin – a predominant protein in

flagella, which are responsible for motility in some bacteria (Hayashi *et al,* 2001). TLR4 recognizes LPS from gram-negative bacteria and a range of endogenous host-derived molecules such as heat shock proteins and extracellular matrix components including fibronectin, hyaluronic acid and heparan sulfate (Johnson *et al,* 2002; Lehnardt *et al,* 2008; Smiley *et al,* 2001). The TLR9 subfamily includes TLR7 and TLR8, which are activated by single-stranded RNA present in certain classes of RNA viruses and imidazoquinolines (Chuang and Ulevitch, 2000; Du *et al,* 2000). TLR9 is activated by unmethylated CpG motifs that are found in bacterial and viral DNA, synthetic oligodeoxynucleotides and endogenous DNA complexes released from dying cells (Barrat *et al,* 2005; Diebold *et al,* 2004; Hemmi *et al,* 2000; Hemmi *et al,* 2002). Less is known about the recently identified TLR11 family in mice that consists of TLR 11-13; however, current reports suggest TLR11 responds to uropathogenic bacteria (Zhang *et al,* 2004) and a profilin-like protein from parasites (Okun *et al,* 2009; Yarovinsky *et al,* 2005) and TLR13 recognizes vesicular stomatitis virus (Shi *et al,* 2011).

Toll-like Receptor Distribution

Toll-like receptors are expressed on a wide variety of cell types. TLRs are located on immune cells such as macrophages, T cells, B cells, and dendritic cells where they play an important role in the detection of pathogens and initiation of an immune response. Aside from the immune system TLRs are also found on epithelial cells, endothelial cells, smooth muscle cells and cells in the

nervous system (Cole *et al,* 2011; El Kebir *et al,* 2009; Lundberg *et al,* 2007). As cells of the nervous system are of particular interest for stroke research the following briefly expands on TLR distribution in the brain (Table 1). Expression of TLRs 1–9 have been reported on microglia (Jack *et al,* 2005), and TLR 1-8 and 11-13 on neurons (Cameron *et al,* 2007; Jackson *et al,* 2006; Lafon *et al,* 2006; Ma *et al,* 2006; Mishra *et al,* 2006; Mishra *et al,* 2008; Tang *et al,* 2007). In addition, TLR 1-7, 9,10, and13 are expressed on astrocytes, TLR2 and TLR3 on oligodendrocytes (Bsibsi *et al,* 2002) and TLR3 and 13 on cerebral endothelial cells (Bsibsi *et al,* 2002; Bsibsi *et al,* 2006; Farina *et al,* 2005; Jack *et al,* 2005; Lundberg *et al,* 2007; Mishra *et al,* 2006; Morris *et al,* 2006). It is important to note that each cell type may induce different downstream signaling and cytokines in response to a TLR signal.

TLR Signaling Pathways

Upon ligand binding, TLRs dimerize, undergo a conformational change and initiate signaling utilizing two signaling pathways: the myeloid differentiation factor 88 (MyD88) pathway and the TIR domain-containing adaptor inducing interferon (TRIF) pathway (O'Neill and Bowie, 2007). Signaling down these pathways leads to the activation of nuclear factor κB (NFκB) with the production of pro-inflammatory cytokines and leads to the activation of interferon regulatory factors (IRFs) with the production of type-1 interferon (IFN) and other IFN-inducible genes.

Table 1-1: TLR expression in cells of the CNS

CNS CELL TYPES	TOLL-LIKE RECEPTORS
Neurons	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR11, TLR12, TLR13
Microglia	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9
Astrocytes	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, TLR10, TLR13
Oligodendrocytes	TLR2, TLR3
Cerebral Endothelial Cells	TLR3, TLR13

MyD88 pathway

For most TLRs, MyD88 is recruited directly to the TLR itself with the exception of TLR2 and TLR4, which require the adaptor protein, TIR domain-containing Adaptor Protein (TIRAP) (also known as MyD88 adapter-like (MAL)). MyD88 association is followed by recruitment of the IL-1 receptor associated kinase (IRAK) 4 to the receptor complex and phosphorylation of IRAK1. Activated IRAK1 binds with TNF receptor associated factor 6 (TRAF6), which causes its dissociation. The dissociated IRAK1-TRAF6 complex interacts with the transforming growth factor-β activated kinase (TAK) 1 and two adaptor proteins, transforming growth factor-β binding protein (TAB) 1 and TAB2 or TAB3. The subsequent phosphorylation of the TAK1 complex induces TAK1 to activate the IKK (inhibitor of NFκB kinase) complex, that then degrades IκB (inhibitor of NFκB) to release the transcription factor NFκB to translocate to the nucleus, resulting in the production of pro-inflammatory cytokines, such as TNFα and IL-6. (Figure 1-1)

Downstream of TLRs 7-9, MyD88 signaling can also lead to the activation of IRFs and the production of type-1 IFN. This occurs through the binding of TRAF3, which leads to the activation and nuclear translocation of several IRFs (e.g. IRF1, IRF5, IRF7 and IRF8) and results in the expression of type-1IFN and IFN-inducible genes (Figure 1-1).

TRIF pathway

TLR3 signals exclusively through the TRIF pathway. TLR3 dimerizes in response to ligand binding, recruits the adaptor protein TRIF, and activates TRAF-family member associated NFκB activator binding kinase (TBK1) and non-canonical IκB kinases (IKKε). Activation of IRF1, IRF3, IRF5 and IRF7 ensues, which leads to induction of type-1 IFN and IFN-inducible genes. TRIF can also activate TRAF6 directly or via receptor interacting protein-1 (RIP-1), leading to the activation of the IKK complex and subsequent NFκB activation (Figure 1-1).

TLR4 signals via both the MyD88 and TRIF pathways. The TLR4-TRIF pathway is largely similar to that described for TLR3, however, TLR4 associates directly with TRIF-related adapter molecule (TRAM) and in turn, indirectly to TRIF. Subsequent induction of IFNβ and IFN-inducible genes occurs through the transcription factors IRF1, IRF3, and IRF5 (Figure 1-1).

3. Preconditioning with TLR ligands reduces ischemic damage

Activation of certain TLRs prior to ischemia provides robust protection against injury. LPS preconditioning was first shown to protect against ischemia in myocardial tissue (Brown *et al,* 1989). Since then, LPS preconditioning has been shown to protect numerous organs from ischemic damage including the brain, liver, kidneys, retina and lungs (Colletti *et al,* 1994; Franco *et al,* 2008; Heemann *et al,* 2000; Merry *et al,* 2010; Tasaki *et al,* 1997). Studies in mice demonstrated

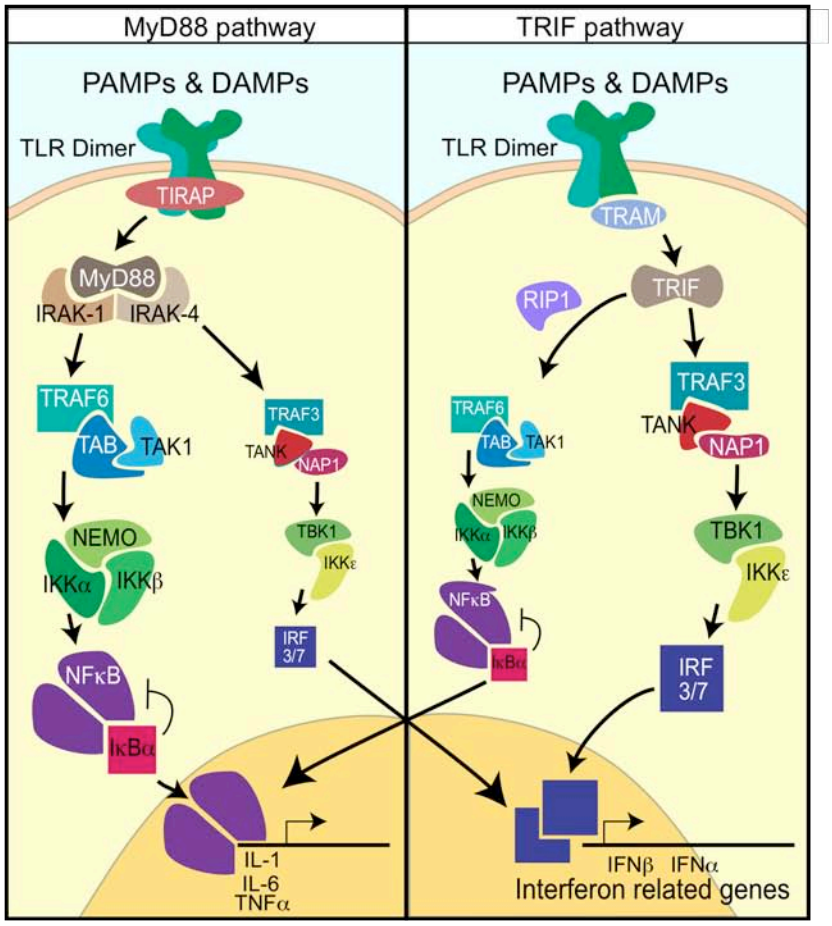

Figure 1-1: TLR signaling pathways

TLR signaling is mediated by either the MyD88 (left panel) or the TRIF (right panel) pathway. All TLRs, with the exception of TLR3, signal via the MyD88 pathway. MyD88 primarily leads to the release of the transcription factor NFκB, directing production of proinflammatory cytokines such as IL-1, IL-6 and TNFα. Downstream of certain TLRs, MyD88 signaling via the signaling molecule TRAF3, can also lead to the activation of IRFs and the expression of IFN-related genes. The TRIF pathway is utilized by TLR3 and TLR4 and TRIF recruits TRAF3 leading to the activation of IRF and the expression of IFN-related genes. Alternatively TRIF may also activate NFκB by the recruitment of TRAF6 or RIP-1 leading to the production of inflammatory cytokines.

that neuroprotection against stroke, induced by LPS preconditioning, occurs within 1 day of exposure and lasts approximately 1 week (Rosenzweig et al, 2007). As with other delayed preconditioning stimuli, the observed tolerance to ischemia by LPS depends on de novo protein synthesis and involves new gene expression (Bordet et al, 2000). Two other TLR family members, TLR2 and TLR9, also induce neuroprotection against an ischemic injury when administered prior to stroke in mouse models (Hua et al, 2008; Stevens et al, 2008). In conclusion, multiple TLR ligands are effective preconditioning stimuli against cerebral ischemic damage and the TLR4 ligand, LPS, provides protection from ischemic injury to multiple organs. Thus, to develop new stroke therapies, it is important to understand the mechanisms that govern TLR-preconditioning induced protection against ischemia.

Potential Mechanisms of TLR-induced protection

The phenomenon of preconditioning provides a window into the endogenous mechanisms of neuroprotection. Unlike with acute neuroprotectants where the drug itself is providing direct protection, with preconditioning the stimulus causes a reprogramming event that enables endogenous protective factors following stroke. Understanding the molecular mechanisms responsible for preconditioning protection will both provide insight into survival pathways and guidance for future stroke treatment strategies. We have modeled our investigation of the mechanisms of TLR-mediated neuroprotection on the established field of TLR

tolerance (described below), whereby with stroke TLRs can contribute to stroke injury, but can also be utilized as targets for preconditioning.

TLRs in ischemia-reperfusion injury

Cerebral ischemia induces a profound inflammatory response that contributes significantly to damage. TLRs play a role in this inflammatory response through their ability to recognize DAMPs released following injury such as heat shock proteins and extracellular matrix components. TLR2 and TLR4 directly contribute to ischemic injury in the brain, as evidenced by TLR2 and TLR4 deficient mice, which have significantly smaller infarcts in response to stroke than their wild-type counterparts (Cao *et al,* 2007; Oyama *et al,* 2004; Tang *et al,* 2007). This suggests TLR2 and TLR4 are activated following stroke, triggering an immune response that exacerbates stroke damage. Not all TLRs contribute to stroke injury as similar experiments utilizing TLR3 and TLR9 deficient mice had no difference in infarct volume following stroke compared to wild-type controls (Hua *et al,* 2009; Hyakkoku *et al,* 2010). Analogous to the detrimental role of some TLRs in brain ischemia, TLR2 and TLR4 contribute to ischemic injury in the kidney and the heart (Arumugam *et al,* 2009; Lu *et al,* 2007), and TLR4 contributes to ischemic injury in the liver (Zhai *et al,* 2008). TLR3 contributes to ischemic injury in the gut (Cavassani *et al,* 2008). In contrast, TLR2 has a *protective* effect against ischemic injury in the gut (Aprahamian *et al,* 2008). Therefore, the outcome of TLR-mediated inflammatory responses are not

consistent from one organ system to the next. The role of TLRs in various ischemia-reperfusion injury models has been extensively reviewed by Arumugam (2009).

Several studies have also found a relationship between TLR expression and clinical outcome in stroke patients. Expression of TLR2 and TLR4 in peripheral blood leukocytes correlated with cytokine levels, poor outcomes and stroke severity (Brea *et al,* 2011a; Park *et al,* 2011; Yang *et al,* 2008). Expression by peripheral blood leukocytes of TLR7 and 8, but not TLR 3 or 9, is also associated with poor outcome and inflammation in acute ischemic stroke (Brea *et al,* 2011b). Taken together with evidence from animal stroke models, TLR signaling modulates ischemic injury likely through the immune response. Therefore, targeting TLR-mediated inflammation is a potential therapeutic opportunity. The phenomenon of TLR tolerance may help to explain how TLR preconditioning redirects TLR inflammation post-stroke.

TLR Tolerance

TLR tolerance is a phenomenon whereby pre-exposure to a low dose of a TLR ligand protects against subsequent exposure to a lethal dose. This was first described with homotolerance of the TLR4 ligand LPS, whereby pretreatment with LPS tolerizes the response to future LPS challenge. Work in this field has found that initial stimulation of TLR4 leads to the activation of NFκB and

inflammatory mediators that induce a refractory state to further TLR4 activation (called homotolerance). In this alternative, tolerant state, proinflammatory responses are downregulated while IFN and anti-inflammatory responses are upregulated (Biswas and Lopez-Collazo, 2009). Pre-exposure to other TLR ligands can also protect from a lethal dose of LPS in a process called heterotolerance (Broad *et al,* 2007). Due to the similarities with TLR tolerance our laboratory and others believe that the mechanism of TLR preconditioning in ischemia may be analogous to the mechanism of TLR tolerance (Kariko *et al,* 2004b; Vartanian and Stenzel-Poore, 2010). Hence a preconditioning dose of a TLR ligand protects against an ischemic challenge, potentially by inducing TLR tolerance and redirecting the damaging contributions of TLR2 and TLR4 activation following stroke. The following sections provide further details of this reprogramming process.

Characteristics of preconditioning protection

Our laboratory has performed large-scale transcriptomic gene profiling analyses and molecular-level investigations of TLR signaling to begin to define the elements critical for TLR-induced neuroprotection (Marsh *et al,* 2009a; Stenzel-Poore *et al,* 2007; Stevens *et al,* 2011; Vartanian *et al,* 2011). Based on our studies we have identified characteristics of preconditioning prior to stroke and following stroke.

Prior to stroke: inflammation and TNFα

Genomic analyses have revealed that LPS preconditioning by peripheral administration, induces changes in gene regulation in the brain acutely following administration, that largely return to baseline by 72 hours (Marsh *et al,* 2009a). The majority of the genes regulated are related to defense and inflammation responses (Marsh *et al,* 2009a). Further analysis comparing gene expression in the brain following LPS, CpG and ischemic preconditioning, revealed that all three stimuli regulate genes related to TLR signaling, cytokine signaling and apoptosis (Stevens *et al,* 2011). Together these studies demonstrate inflammation-related signaling in the brain following preconditioning.

The pro-inflammatory response that is initiated following treatment with a TLR ligand may be a critical component of preconditioning. TNFα is a key pro-inflammatory cytokine involved in preconditioning that is induced shortly after administration of TLR ligands CpG and LPS. TNFα is required for LPS and CpG preconditioning, as inhibiting TNFα following LPS administration nullifies protection (Tasaki *et al,* 1997) and TNFα-deficient mice cannot be protected by either LPS or CpG preconditioning (Rosenzweig *et al,* 2007; Stevens *et al,* 2008). TNFα itself is an effective preconditioning stimulus when given directly into the CNS prior to insult, however systemic administration of TNFα is not protective (Nawashiro *et al,* 1997b). Taken together these findings suggest inflammation and TNFα play a significant role in preconditioning.

Following stroke: Inhibition of NFκB

NFκB plays a damaging role in ischemia as demonstrated by studies that inhibited NFκB action with systemic administration of a 5-lipoxygenase inhibitor or with TAT-NBD that lead to protection from ischemia (Jatana *et al,* 2006; Nijboer *et al,* 2008a; Nijboer *et al,* 2008b). Of note is that preconditioning with LPS also results in a suppression of NFκB activity following ischemic insult and thus could be a means of reducing injury (Vartanian *et al,* 2011). The inhibition of NFκB is most likely through the induction of the MyD88-NFκB pathway inhibitors, Ship1, Tollip, Trim30 and p105, which are induced in the brain following ischemia in mice that have been preconditioned with LPS (Vartanian *et al,* 2011). These inhibitors may serve to redirect signaling towards a neuroprotective response following ischemia.

TNFα is an inflammatory cytokine induced by NFκB activation that contributes to damage following stroke as inhibiting this cytokine reduces focal ischemic damage (Nawashiro *et al,* 1997a; Nawashiro *et al,* 1997b). Following stroke, mice that have been preconditioned display reduced levels of TNFα in the systemic circulation and in the brain diminished levels of cellular TNFR1 with increased levels of neutralizing soluble TNFR1 (Rosenzweig *et al,* 2007). A disrupted inflammatory response following stroke is also evinced by a decrease in microglial activation and neutrophil infiltration into the brain and reduced activation of monocytes in the periphery (Rosenzweig *et al,* 2004).

Following stroke: Enhancement of interferon pathways

Coupled with the suppression of NFκB is an enhancement of the IFN response. Anti-inflammatory and type-1 IFN-induced cytokines and chemokines are enhanced in the brain and blood of LPS-preconditioned mice following stroke. TGFβ, RANTES, IFNβ and IFIT1 expression are significantly upregulated in the brain and IL-10 and RANTES protein levels were significantly higher in the blood in LPS-preconditioned animals following stroke (Marsh *et al,* 2009a; Vartanian *et al,* 2011). Evidence of an enhanced IFN response also includes an "IFN fingerprint" revealed in genomic microarray studies following ischemic insult after TLR4, TLR9 or ischemic preconditioning (Marsh *et al,* 2009a; Stevens *et al*, 2011). This IFN fingerprint is comprised of 13 genes upregulated in the brain 24 hours after stroke in all three preconditioning paradigms. Promoter region analysis of the commonly regulated genes identified IFN regulatory sequences as overrepresented in the 5' upstream sequence of this population of genes, implicating IRF and ISRE transcription factor activity at this timepoint (Stevens *et al,* 2011; Vartanian, in press). Further evidence for an important role for IRFs includes increased expression of IRF7 and increased activity of IRF3 following stroke (Stevens *et al,* 2011; Vartanian *et al,* 2011). IRF3 and IRF7 are both required for LPS and CpG preconditioning as mice deficient in either of these factors are not protected by LPS or CpG (Stevens *et al,* 2011). This enhanced IFN-related response may be a key element of preconditioning neuroprotection.

Several lines of evidence suggest the neuroprotective potential of type-1 IFN. IFNβ administered subcutaneously at the time of stroke or after stroke significantly reduces infarct volume (Liu *et al,* 2002; Veldhuis *et al,* 2003a). This IFN treatment is associated with improved integrity of the blood brain barrier consisting of decreased matrix metalopeptidase 9 (MMP9), limited neutrophil or macrophage entry into the brain and reduced leakage of contrast agent in MRI studies (Jin *et al,* 2007; Veldhuis *et al,* 2003b). In addition, IFNβ given directly into the brain at the time of stroke is protective (Marsh *et al,* 2009a). Thus, increased type-1 IFN or IFN associated genes in the brain appears to be beneficial in the setting of ischemic injury.

Reprogramming the response to stroke

We have learned that prior to stroke, preconditioning is characterized by moderate inflammation and an increase of the inflammatory cytokine TNFα. Following stroke, preconditioning causes a shift, or reprogramming, from the normally pro-inflammatory, damaging response to stroke to an IFN-driven, protective response. This may be achieved by the redirection of TLR signaling pathways such as TLR2 and TLR4 that contribute to ischemic injury analogous to the process of TLR tolerance (Cao *et al,* 2007; Oyama *et al,* 2004; Tang *et al,* 2007). Initial stimulation of TLR4 by LPS preconditioning activates NFκB and the production of pro-inflammatory cytokines such as TNFα. This initial moderate TLR4 stimulation reprograms the subsequent TLR response to ischemia through inhibition of the NFκB pathway and the activation of IRFs and production of IFN

and IFN-inducible genes (Figure 1-2). Likewise by TLR heterotolerance, preconditioning with other TLR ligands may also redirect the damaging TLR4 response to stroke towards the protective response. Preconditioning reprograms the damaging inflammatory response to a protective immune response following stroke and this results in the preservation of brain tissue in the vulnerable penumbra region.

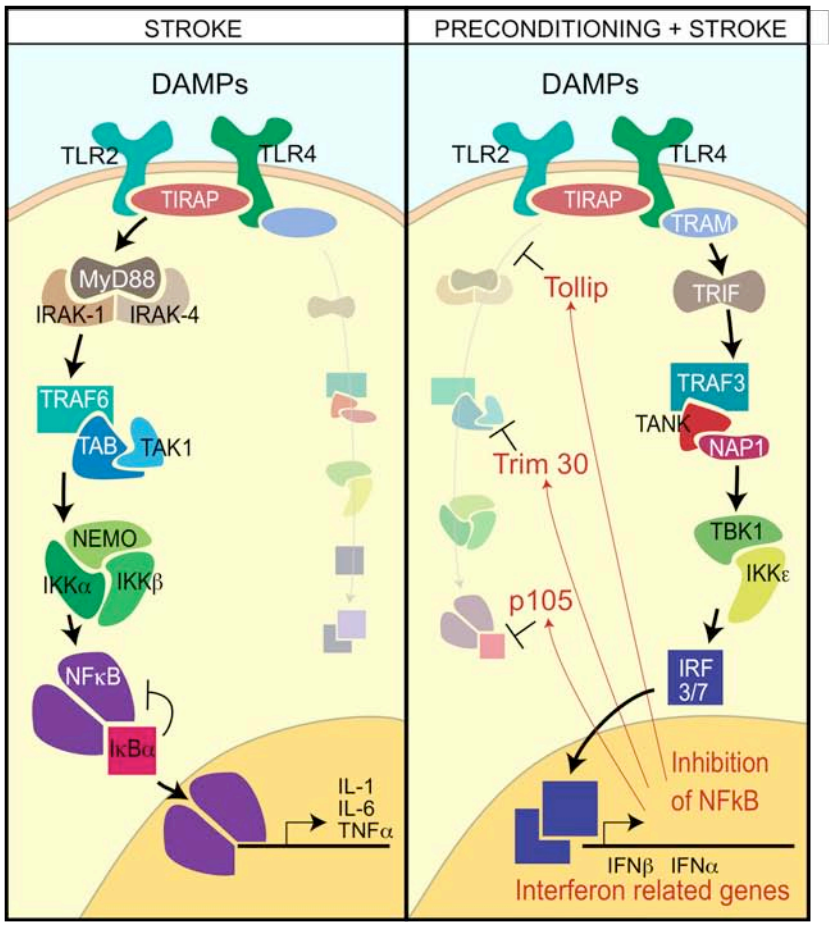

Figure 1-2: TLR preconditioning reprograms the response to stroke

TLR2 and TLR4 recognize DAMPs released by damaged cells following stroke. These TLRs trigger damaging inflammatory responses via activation of NFκB and the production of inflammatory cytokines (left panel). Initial stimulation of TLR4 by LPS preconditioning activates NFκB and the production of pro-inflammatory cytokines such as TNFα. This initial moderate TLR4 stimulation reprograms the subsequent TLR response to stroke through inhibition of the NFκB pathway and the enhancement of IRF activity and production of IFN and IFN-inducible genes (right panel). (This figure was adapted from (Vartanian and Stenzel-Poore, 2010)).

4. Research Questions

Innovative and exciting data on TLRs and their roles in ischemic damage and ischemic tolerance have recently emerged. Gene expression studies have identified key effector pathways such as IFN and the IFN related signaling cascade. These studies suggest that neuroprotection involves fundamental genomic reprogramming of the response to ischemia, which redirects signaling away from cell death and toward cell survival, preserving tissue integrity in the ischemic penumbra. Investigation of preconditioning processes can be used to elucidate molecular mechanisms of endogenous neuroprotection in order to broaden our development of stroke therapeutics.

The use of TLR agonists to prevent ischemic injury offers real promise as a neuroprotective therapy that involves treatment prior to an ischemic event. This approach could be useful to patients at high risk of ischemia, which includes those with a transient ischemic attack, those who undergo cardiac surgery and those who have had a stroke and risk recurrent stroke. Antecedent treatment of these high-risk populations has enormous potential to protect patients from devastating neurological complications and death.

The goal of my research is to develop therapeutics to protect against ischemic damage and to investigate the mechanisms of neuroprotection utilized by these drugs in the context of cerebral ischemia. We are interested in developing novel preconditioning stimuli that can be safely used in patient populations. The

synthetic dsRNA poly-IC is a candidate neuroprotectant that has been used to treat a wide range of diseases in animal models and human patient populations safely and successfully. More importantly, poly-IC treatment elicits a unique transcription profile characterized by activity of IRF3 and IRF7 leading to robust production of type-1 IFN and IFN-inducible genes, all factors that appear critical to preconditioning neuroprotection (Sen and Sarkar, 2005; Vogel *et al,* 2003). Poly-IC activates TLR3, unique among the TLR family as the only TLR to signal exclusively through the adapter TRIF (Figure 1-1). We have found that TRIF is required for TLR4-mediated preconditioning, suggesting that preconditioning with poly-IC through the TRIF pathway would also be protective (Vartanian *et al,* 2011). Further poly-IC treatment targets the IRF/IFN pathway directly avoiding potentially damaging inflammation that occurs following TLR4 activation; therefore, poly-IC preconditioning may be a superior preconditioning approach therapeutically. I hypothesize that preconditioning with poly-IC protects against a subsequent ischemic insult via an IFN-driven mechanism. In chapter two I determine the protective potential of poly-IC in multiple models of ischemia and in chapter three I investigate the molecular mechanisms that underlie poly-IC neuroprotection and compare these findings to the current model of TLR-mediated protection.

To further examine the mechanisms of preconditioning neuroprotection, I also investigated the site of action of TLR ligands to induce protection. A number of diverse cell types throughout the body express TLRs and the critical site of TLR

action in the context of neuroprotection is unknown. Systemic preconditioning with CpG, the TLR9 ligand, protects against cerebral ischemia in both murine and non-human primate models (Bahjat *et al,* 2011; Stevens *et al,* 2008) and I chose to focus on CpG for these studies. Several lines of evidence suggest that CpG can directly target central cells to induce neuroprotection: direct CpG preconditioning of primary cortical culture protects against modeled ischemia, peripheral CpG administration affects gene expression in the brain, and central administration of TNFα, a critical cytokine for CpG preconditioning, is sufficient to induce protection. Therefore, I hypothesize that the hematopoietic compartment is not required for CpG neuroprotection. In chapter four I investigate whether CpG's site of action is peripheral or central using TLR9-deficient reciprocal bone marrow chimeric mice. This study is significant not only because it will help us to focus our investigation into the mechanisms of preconditioning, but it will also help to direct future potential clinical applications.

Chapter 2:

Poly-IC preconditioning protects against cerebral and renal ischemia-reperfusion injury

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Introduction:

Patients undergoing cardiac surgery risk ischemic injury to the brain (50% occurrence), kidneys (30% occurrence) and lungs (18% occurrence) (Barber *et al,* 2008; Fukada *et al,* 2004; Mehta *et al,* 2010). Despite these outcomes, there remain few treatments for surgically-induced ischemia or preventive measures to lessen tissue damage. A promising therapeutic approach exploits the phenomenon of ischemic tolerance, whereby brief exposure to a harmful stimulus, such as ischemia, volatile anesthetics, hypothermia, hypoxia or inflammation, when given prior to an ischemic challenge provides protection against lethal ischemia (Dirnagl *et al,* 2003). Toll-like receptors (TLR), sentinels of the innate immune system, recognize invading pathogens and endogenous damage signals. Preconditioning with TLR ligands including lipopolysaccharide (LPS; TLR4) confers robust protection from ischemic injury (Heemann *et al,* 2000; Rosenzweig *et al,* 2007; Stevens *et al,* 2008). However, LPS may not be a viable option for therapeutic consideration due to its known harmful side effects, including a robust inflammatory response that can lead to serum sickness or a sepsis-like syndrome. Thus, we sought an alternate therapeutic that would have the widespread protective effects of LPS preconditioning on those organs at greatest risk, brain and kidneys, but would not induce potentially deleterious inflammation.

Polyinosinic polycytidylic acid (poly-IC) is a synthetic double-stranded RNA that activates a complex immune response via TLR3, retinoic acid-inducible gene1-like-receptors (RLRs), oligoadenylate synthetase, and protein kinase RNA-activated (PKR). Poly-ICLC (Hiltonol®) is a version of poly-IC stabilized with poly-L-lysine and carboxymethylcellulose (CMC) moieties that have been added to improve pharmacokinetics. It has shown clinical promise in humans for various indications (e.g. vaccines, multiple sclerosis, cancer, viral infections) (Markosian and Hyde, 2005; Rosenfeld *et al,* 2010). Here we show that poly-ICLC preconditioning protects against cerebral ischemic damage in an *in vitro* system of modeled ischemia and in an *in vivo* experimental mouse model of stroke*.* Additionally, we show that poly-IC preconditioning protects against damage in a model of renal ischemia. We report that unlike LPS preconditioning which produces a robust inflammatory response, poly-ICLC preconditioning induces very modest levels of pro-inflammatory cytokines mitigating secondary effects. These results emphasize the promise of poly-ICLC as a novel therapeutic approach to reduce perioperative ischemic damage.

Materials and Methods:

Mice: Animal procedures were conducted using C57/Bl6 male mice, 8-12 weeks old (Jackson Laboratory, West Sacramento, CA), according to Oregon Health & Science University (OWLAW #A3304-01) Institutional Animal Care and Use

Committee and National Institute of Health Guidelines. The American Association for Laboratory Animal Care accredits the housing facility.

Reagents: CMC (Sigma Aldrich, St. Louis, MO), poly-ICLC (Oncovir, Washington, DC), saline, poly-IC high molecular weight (Invivogen, San Diego, CA), and LPS [*Escherichia coli* serotype *0111:B4*; phenol extraction purified, protein content 3% (Sigma Aldrich)] were used. Agents were delivered by either intraperitoneal (i.p.) or subcutaneous (s.c.) administration as noted in the methods and figure legends. We have found both routes provide equivalent levels of protection.

Oxygen Glucose Deprivation (OGD): Primary mixed cortical cultures were prepared from E15-E17 mouse fetuses (1litter/experiment) as previously published (Stevens *et al,* 2008). During OGD, medium was replaced with D-PBS (Gibco, Carlsbad, CA) and cells were incubated in an anaerobic atmosphere of 85% N_2, 10% CO_2, 5% H_2 at 37°C for 3 hours (Coy Laboratories, Grass Lake, MI). Following OGD, D-PBS was replaced with medium and cells returned to a normoxic incubator. Control plates remained in the normoxic incubator during OGD. Cell death was determined 24-hours following OGD using propidium iodide staining (Sigma Aldrich) and quantified with Metmorph7 software (Molecular Devices Corp., Downington, PA). Within an experiment, each treatment was performed in triplicate and 3 independent experiments were performed.

Middle Cerebral Artery Occlusion (MCAO): Mice were treated with poly-ICLC (0.4, 0.8 or 1.6 mg/kg), LPS (1 mg/kg), or appropriate vehicle (s.c.; n=9-10/group) 72 hours prior to right MCAO (45 minute) performed as previously described (Stevens *et al,* 2008). Laser Doppler (Transonic Systems Incorporated, Ithaca, NY) was used to measure cerebral blood flow beneath the skull adjacent to the MCA to insure occlusion reduced flow to ≤20% of baseline. In blinded fashion 24 hours following MCAO, the neurological testing was administered and indirect infarct volume was subsequently determined by 2,3,5 triphenyltetrazolium chloride (TTC) stain. There are two categories of neurological scoring, each based on a 28 point scale, which correlates with infarct volume (Clark *et al,* 1997). The general score evaluates activity, posture and grooming. The focal score evaluates body symmetry, gait and traits characteristic of unilateral MCAO damage. The corner test evaluates body function and asymmetry by noting direction turned when mice approach a 45˚ corner. Following MCAO mice tend to favor their ipsilateral (right) side turning preferentially right when exiting while naive mice turn equally to either side. Each mouse was scored ten consecutive times to calculate the percentage of right hand turns (Zhang *et al,* 2002). For extended recovery, mice were treated with either poly-IC (i.p.; 1 mg/kg) or vehicle (n=8-10/group) and infarct volume was assessed 72 hours following MCAO.

Renal Ischemia Procedure: Mice were given vehicle, LPS (1.6 mg/kg) or poly-IC (i.p.; 1.6 mg/kg) 48 hours prior to surgery (n=6/group). Renal ischemia was induced in anesthetized mice (isoflurane 1.5-2%) by performing a midline

incision, isolating the renal vessels bilaterally, clamping the renal vessels with small bulldog clamps (45 minutes), and confirming occlusion and reperfusion by Parks Ultrasonic 811-B Doppler (Kroslak Enterprises, Riverview, FL). Blood samples were taken via saphenous vein prior to renal ischemia surgery and 48 hours following reperfusion for creatinine level determination by the Jaffe rate method using a Beckman Coulter DXC800 (Brea, CA).

Plasma Cytokine Evaluation: Blood was collected via cardiac puncture under isoflurane anesthesia 3 hours following s.c. administration of vehicle, LPS (1 mg/kg) or poly-ICLC (1.6 mg/kg) (n=3/group). Plasma cytokine levels were evaluated by custom multiplex ELISA for IL-1β, IL-12, IFNγ (Quansys Biosciences, Logan, UT) and cytometric bead array for IL-6 and TNFα (BD Biosciences, Franklin Lakes, NJ).

Data Analysis: Researchers were blinded to treatment during analyses. Significance ($p<0.05$) was determined using Student's t-test or One-way analysis of variance (ANOVA) with Bonferroni's post-hoc test as noted using Prism 5 software (GraphPad, LaJolla, CA).

Results:

Poly-ICLC preconditioning confers neuroprotection in experimental models of stroke *in vitro* and *in vivo*

Mixed cortical cultures were used to test whether poly-IC preconditioning provides protection against modeled ischemia *in vitro*. Preconditioning with poly-ICLC (1-100 ng/ml) produced a marked attenuation of cell death following OGD compared to controls. Cells pretreated with poly-ICLC showed little cell death following OGD with the greatest protection exhibited at 100 ng/ml with 5.6 ± 2.4% cell death compared to 80.6 ± 3.1% cell death in vehicle-treated controls (Figure 2-1A). These data show that poly-ICLC preconditioning protects mixed cortical cells from OGD-induced death *in vitro*.

We next examined whether poly-ICLC preconditioning protects the brain from ischemic injury in an *in vivo* mouse model of stroke. Poly-ICLC (0.4, 0.8 or 1.6 mg/kg) was administered 3 days prior to MCAO as this time point has shown consistent protection with other preconditioning stimuli including LPS. The infarct volume determined 24 hours post-MCAO was significantly smaller in poly-ICLC-treated animals (1.6 mg/kg 17.64% ± 3.89%) compared to vehicle-treated controls (35.29% ± 2.41%; $p<0.05$; Figure 2-1B & C). The degree of protection was similar to the known preconditioning agent, LPS (Figure 2-1C). As with other preconditioning stimuli, the reduction in infarct size occurred in a dose-dependent

manner. Poly-IC administered at a higher dose (4 mg/kg) neither conferred neuroprotection nor exacerbated damage (vehicle 41.58 ± 3.06% poly-IC 43.72 ± 2.96% $p>0.05$). Neurological and motor deficits were attenuated in poly-ICLC preconditioned mice as evidenced by reduced neurological scores and improved performance in the corner test. Preconditioned animals showed a decreased tendency to turn to the ipsilateral side compared to vehicle-treated controls (Figure 2-1D & E). Poly-IC preconditioning has a lasting effect demonstrating protection for 72 hours following MCAO (44.1% ± 3.70% vehicle versus 29.1 ± 4.48% poly-IC; Figure 2-1F). Thus, as hypothesized, poly-ICLC treatment prior to exposure to ischemia significantly reduced injury in both *in vitro* and *in vivo* models of cerebral ischemia.

Poly-IC preconditioning protects from renal ischemic damage

We hypothesized that poly-IC preconditioning would also shield the kidneys from ischemic damage. To test this, mice were preconditioned with LPS, poly-IC or vehicle two days prior to bilateral renal ischemia. Serum creatinine levels were assessed as a measure of kidney function two days following reperfusion and compared to baseline samples taken prior to ischemia. As with LPS, creatinine levels were significantly attenuated (74% reduction) in poly-IC preconditioned mice (1.26 ± 0.036 fold increase) compared to vehicle (3.98 ± 0.523 fold increase; $p<0.001$; Figure 2-1G). Thus poly-IC preconditioning protects from functional deficits incurred in the setting of renal ischemia.

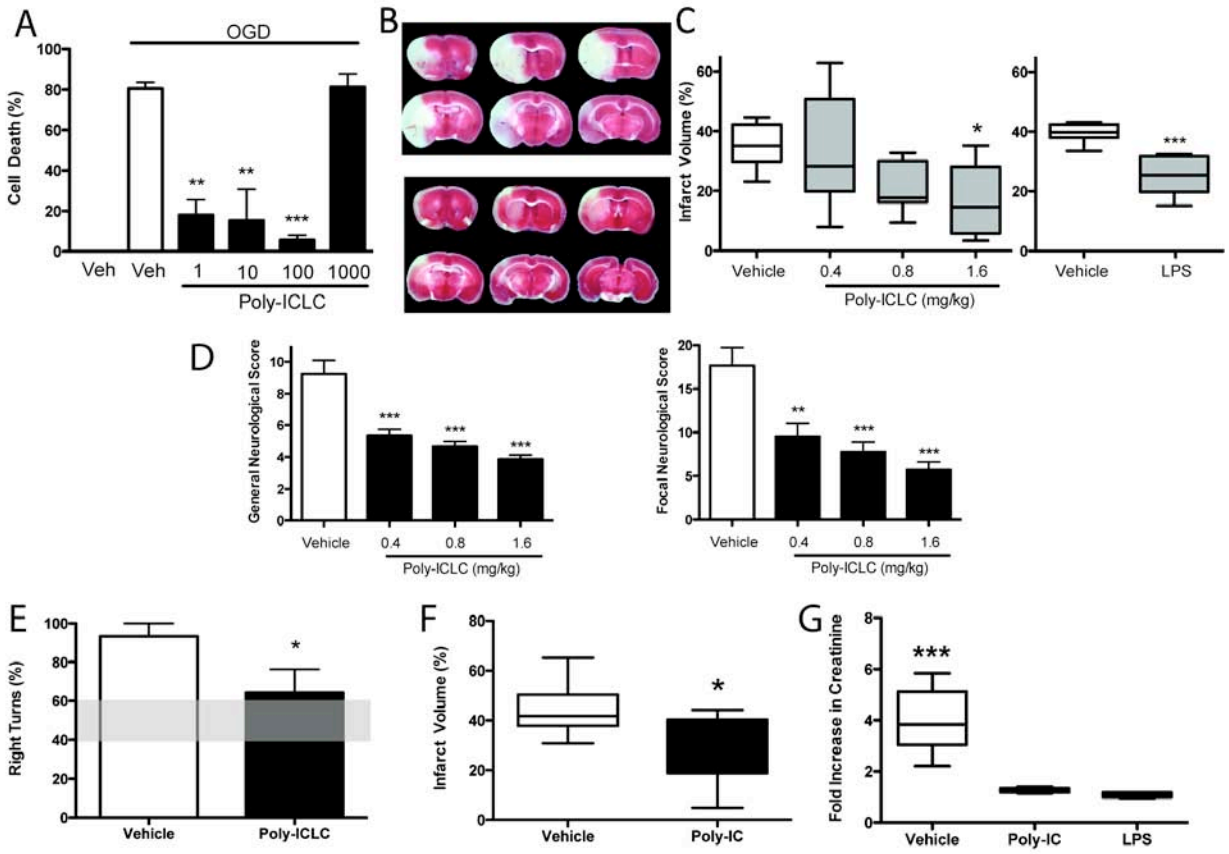

Figure 2-1: Poly-IC preconditioning protects against cerebral and renal ischemia-reperfusion injury

(A) Poly-ICLC preconditioning is neuroprotective in modeled ischemia *in vitro*. Mixed cortical cultures were preconditioned with poly-ICLC (1-1000 ng/ml) 24 hours prior to 3 hour OGD. Cell death was determined by PI staining 24 hours following OGD. A representative example of 3 independent experiments is shown. Values are group means ± SEM; **p<0.01, ***p<0.001 versus vehicle control by ANOVA and Bonferroni post-hoc test. **(B-F) Poly-ICLC preconditioning is neuroprotective in an experimental model of stroke *in vivo*.** Mice were administered poly-ICLC (0.4, 0.8 or 1.6 mg/kg), LPS (1 mg/kg) or appropriate vehicle s.c. 3 days prior to 45-minute MCAO (n=9-10/group).

Extent of brain injury was assessed by measuring infarct volume 24 hours following MCAO. Neurological outcomes were evaluated and motor deficits were quantified using the corner test. Representative images of TTC stained brain sections (rostral surface) from vehicle treated (B top) and poly-ICLC treated (B bottom) animals. Collective infarct volume (C) is shown as group box and whisker (min/max) while neurological score (D) and corner test score (E) values reflect means ± SEM. (E) Shaded horizontal bar shows corner test score range for naïve mice. For the extended recovery experiment mice were administered poly-IC (1 mg/kg) or vehicle i.p. 3 days prior to 45-minute MCAO (n=8-10/group). Damage to the brain was assessed by measuring infarct volume 72 hours following MCAO. Collective infarct volume (F) values represent group box and whisker (min/max); *p<0.05, **p<0.01, ***p<0.001 by student's t-test and 1-way ANOVA with Bonferroni post-hoc test. **(G) Poly-IC preconditioning treatment improves renal function following ischemia.** Mice were administered poly-IC (1.6 mg/kg), LPS (1.6 mg/kg) or vehicle i.p. 48 hours prior to renal ischemia (n=6/group). Creatinine levels were measured in whole blood samples taken prior to and 48 hours following renal ischemia challenge. Values given as fold-increase over baseline; ***p<0.001 by ANOVA and Bonferroni post-hoc test.

Poly-ICLC treatment elicits reduced systemic plasma cytokine levels compared to LPS

The magnitude of the pro-inflammatory response to LPS limits its use in a therapeutic setting, despite the fact that LPS preconditioning induces ischemic tolerance in multiple organs. To test whether preconditioning doses of poly-ICLC elicits systemic cytokines, we compared plasma cytokine levels 3 hours following administration of protective doses of poly-ICLC or LPS. Mice treated with poly-ICLC had significantly decreased plasma levels of IL-1β, IL-6, IL-12, TNFα and IFNγ compared to mice treated with LPS ($p<0.01$; Figure 2-2). Thus poly-ICLC provides protection without the robust and considerably detrimental inflammatory response observed following LPS treatment.

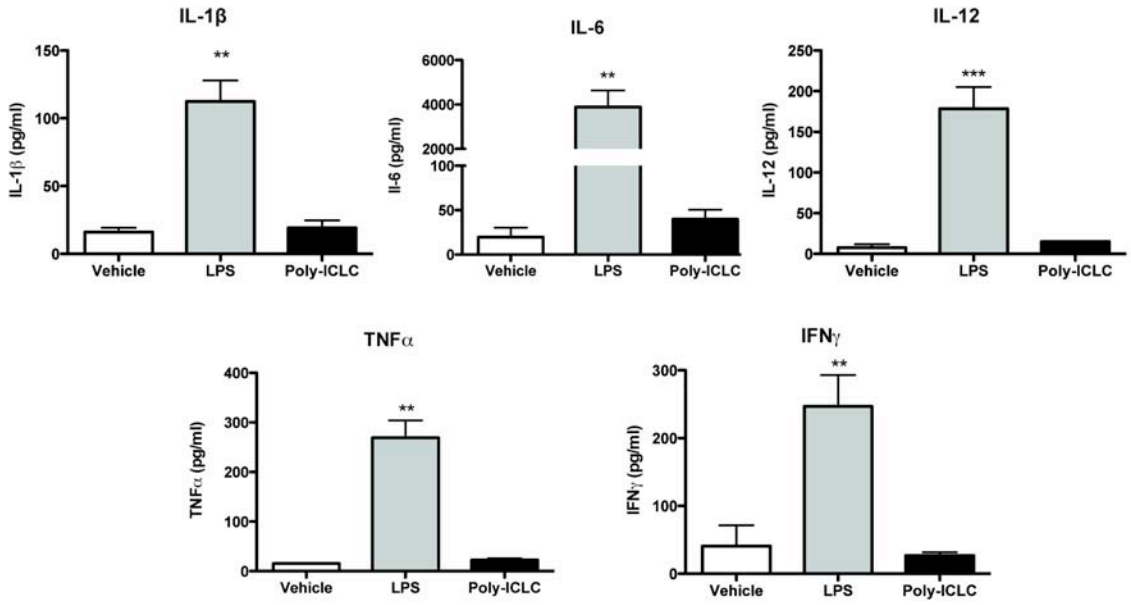

Figure 2-2: Poly-ICLC treatment resulted in reduced systemic plasma cytokine levels at 3 hours following administration compared to LPS treatment.

Plasma cytokine levels were measured from samples taken at 3 hours following s.c. injection of vehicle, LPS (1 mg/kg) or poly-ICLC (1.6 mg/kg) (n=3/treatment). Plasma cytokine levels were evaluated by custom multiplex ELISA for IL-1β, IL-12, and IFNγ and cytometric bead array for IL-6 and TNFα. Cytokine levels are significantly lower in poly-ICLC treated animals compared with LPS treatment: **p<0.01, *** p<0.001 by ANOVA and Bonferroni post-hoc test.

Discussion:

We have discovered that preconditioning with poly-IC induces tolerance to both cerebral and renal ischemic injury. We show decreased tissue damage following cerebral ischemia and improved function based on neurological testing and measurement of creatinine levels respectively. Numerous patients at risk of ischemic damage due to surgical procedures would benefit from antecedent treatment that induces protection against ischemia-reperfusion injury. In addition to the high risk of ischemic injury following cardiac surgery, cognitive dysfunction following major non-cardiac surgery affects approximately 40% of patients (Monk *et al,* 2008). Importantly, poly-IC preconditioning may provide a benefit to patients who undergo major surgery by reducing ischemic damage and improving functional outcomes.

Additionally, prophylactic ischemic tolerance may protect organs from transplant ischemia-reperfusion damage that is inherent in this procedure. Despite advances in transplant technologies there remains high demand for transplanted organs and the ability to utilize organs under sub-optimal conditions, such as prolonged ischemia, could increase the available organ pool. The duration of ischemia prior to transplant predicts long-term kidney failure in humans (Salahudeen *et al,* 2004). Prophylactic ischemic preconditioning was reported to improve viability of transplanted organs such as liver and kidney (Ambros *et al,* 2007). We believe that pharmacological preconditioning using poly-IC

administration offers a more practical therapeutic approach to protect transplant organs from ischemia-reperfusion injury although further studies are needed to investigate this potential.

We show, compared to preconditioning doses of LPS, poly-ICLC produces a very modest pro-inflammatory plasma cytokine response, which lowers the risk of toxicity and thereby offers a more favorable approach to generate ischemic tolerance. LPS preconditioning requires TNFα- an inflammatory cytokine not strongly induced by poly-ICLC (Rosenzweig *et al,* 2007). This suggests that poly-ICLC may work through an entirely novel mechanism of protection that is TNFα-independent. One candidate mediator of ischemic protection may be type-1 IFN, which is induced by poly-ICLC (Markosian and Hyde, 2005) and has previously been shown to be neuroprotective (Marsh *et al,* 2009a).

Poly-ICLC, as a clinical-stage therapeutic being evaluated for multiple indications, looks promising as a safe and effective preconditioning agent for ischemic injury. More than two-dozen phase I/II clinical trials have been initiated with poly-ICLC for a wide range of indications including cancer, AIDS, malaria, hepatitis, multiple sclerosis and viral infection (Markosian and Hyde, 2005; Rosenfeld *et al,* 2010). Hundreds of patients have been safely treated with poly-ICLC 2-3 times per week for years; therefore, the use of poly-ICLC as a prophylactic treatment for patients at risk of ischemic damage holds true clinical potential.

Chapter 3:

Poly-ICLC cerebral ischemic tolerance is mediated by a novel IRF7 and IFNAR driven mechanism

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Introduction:

Polyinosinic:Polycytidiylic acid (poly-ICLC) was recently identified as a preconditioning stimulus that induces tolerance to ischemic damage in both cerebral and renal murine ischemia models, however little is known about it's mechanism of protection (Packard *et al,* 2011). This compound is one of a number of effective preconditioning stimuli against cerebral ischemic damage that have been identified, including brief ischemia, anesthetics, hyperthermia and inflammation (Gidday, 2006). A preconditioning stimulus is a small dose of an otherwise harmful stimulus, given prior to an ischemic challenge, which provides protection against subsequent tissue damage. The immune activating preconditioning stimuli lipopolysaccharide (LPS) and unmethylated cytosine-guanine-rich DNA oligonucleotides (CpG), have been studied in regards to their mechanism of induced neuroprotection. Poly-ICLC signaling shares many characteristics with LPS and CpG, therefore we sought to apply what we have learned about LPS and CpG-mediated neuroprotection to begin to characterize the mechanisms underlying protection by poly-ICLC.

LPS and CpG are ligands for toll-like receptors (TLRs) 4 and 9, respectively. TLRs are a family of pattern recognition receptors that recognize both exogenous "danger" signals such as invading pathogens like viruses or bacteria and endogenous "damage" signals released from injured and dying cells. Recently, TLRs 2 and 4 have been identified as deleterious contributors to ischemic injury,

most likely through their recognition of endogenous “damage” ligands (such as heat shock proteins and fibrinogen) and the induction of inflammation (Cao *et al,* 2007; Oyama *et al,* 2004; Tang *et al,* 2007). Following preconditioning however, we believe this deleterious TLR response is redirected towards a protective interferon (IFN) related response, termed the “IFN fingerprint”, which leads to reduced ischemic damage. There are several hallmarks of this IFN fingerprint that have been identified for LPS and CpG preconditioning, which include transcription factor activity and induction of IFN related genes. Comparative microarray analysis of cerebral cortex revealed a shared group of 13 genes that are all induced 24-hours following stroke in mice preconditioned with LPS, CpG or brief ischemia, but are not evident in non-preconditioned mice (Stevens *et al,* 2011). These genes may represent a signature for the protective phenotype. Promoter region analysis of the commonly regulated genes identified IFN regulatory sequences as overrepresented in the 5’ upstream sequence of this population of genes, implicating the transcriptional response elements interferon stimulated response element (ISRE) and IFN regulatory factor (IRF) as regulators of this event (Figure 3-1) (Vartanian, in press). Corroborating this evidence of increased IRF activity at this timepoint, IRF7 mRNA and IRF3 activity were increased in the brains of LPS preconditioned mice (Stevens *et al,* 2011; Vartanian *et al,* 2011). In addition, IRF3 or IRF7 deficient mice fail to be protected against ischemic injury following LPS or CpG preconditioning, suggesting a critical role for IRFs in neuroprotection (Stevens *et al,* 2011). Does this mechanism of protection extend to poly-ICLC preconditioning?

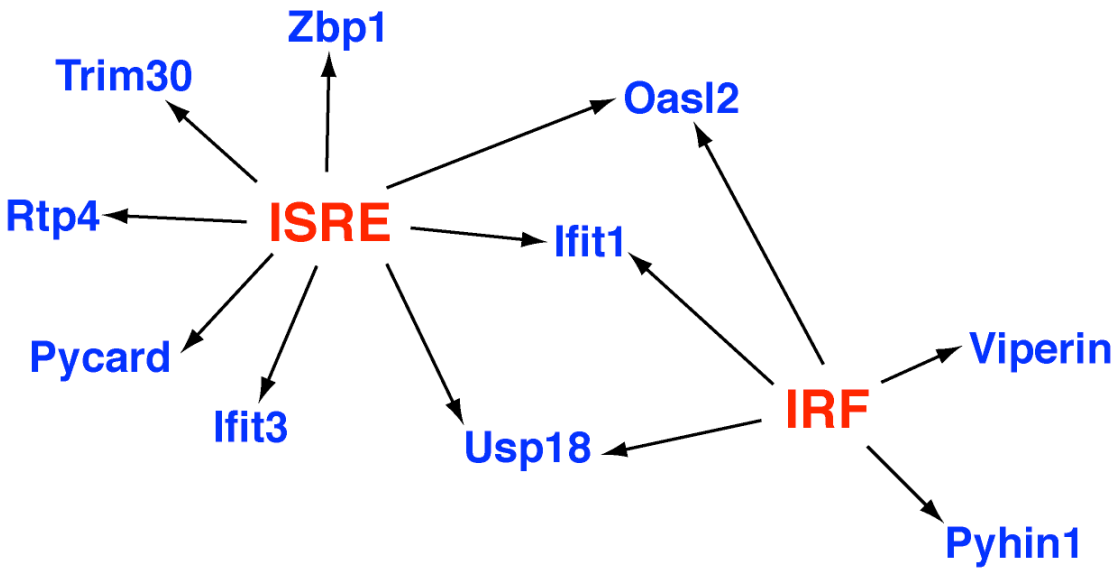

Figure 3-1: Interferon regulatory elements are overrepresented in genes upregulated following stroke in preconditioned mice.

Promoter region analysis was performed for the genes common to LPS, CpG and ischemic preconditioning paradigms 24 hours following stroke. Promoter Analysis and Interaction Network Toolset (PAINT) (http://www.dbi.tju.edu/dbi/tools/paint/) generated Hypothesis Gene-Trancriptional Regulatory Element (TRE) network showing the relationship of the over-represented TREs to the regulated genes. TREs IFN-sensitive response element (ISRE) and IRF are shown in all capital letters and red and genes are shown in lower case letters and blue. (This figure is taken from Vartanian, in press).

Poly-ICLC is a synthetic version of dsRNA that mainly signals through two types of pattern recognition receptors (PRRs), TLR3 and melanoma differentiation-associated protein 5 (mda-5), a member of the retinoic acid-inducible gene1-like receptor (RLR) family. Similar to other PRRs, TLR3 and mda-5 ligation leads to activity of both NFκB and IRF transcription factors. These common signaling pathways suggest that poly-ICLC may share similar features with CpG and LPS preconditioning. However, unlike CpG and LPS, Poly-ICLC induces a robust type-1 IFN response following administration (Longhi *et al,* 2009). We hypothesize that poly-ICLC preconditioning works through an IFN-driven mechanism of neuroprotection.

Here, we will show that poly-ICLC preconditioning, like CpG and LPS, induces IFN related genes in the brain 24 hours following stroke. Additionally, we provide evidence that the transcription factor IRF7 is required for poly-ICLC preconditioning as it is for CpG and LPS suggesting that this factor is central for inflammatory-mediated neurprotective mechanisms. We investigate the role of type-1 IFN in preconditioning and find that type-1 IFN receptor (IFNAR) is required for poly-ICLC preconditioning but not for CpG or LPS. Finally we show that poly-ICLC neuroprotection does not depend on the TLR3 pathway, suggesting this is a novel TLR-independent mechanism. Together these results indicate that while poly-ICLC neuroprotection shares IFN-related genes and IRFs with other preconditioning stimuli, poly-ICLC does have a unique mechanism of neuroprotection that is TLR-independent and requires activity of type-1 IFN.

Materials and Methods:

Animal Care and Criteria: C57Bl/6J (WT) and TLR3$^{-/-}$ mice were obtained from Jackson Laboratories (West Sacramento, CA). Dr. Ian Rifkin provided the IRF7$^{-/-}$ mice (Boston University School of Medicine, Boston, MA) (Honda *et al,* 2005) and IFNAR$^{-/-}$ mice were provided by Dr. Herbert Virgin and Dr. Anthony French (Washington University School of Medicine, St. Louis, MO) (Muller *et al,* 1994). TLR3$^{-/-}$, IRF7$^{-/-}$ and IFNAR$^{-/-}$ were backcrossed at least 8 generations onto C57Bl/6. All stroke studies were performed with male mice between 8 – 12 weeks of age. All mice were given free access to food and water and housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal protocols were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee (OWLAW# A3304-01) and met the guidelines set forth by the National Institutes of Health.

Drug treatments: Mice were given a subcutaneous injection of poly-ICLC (also known as Hiltonol® a version of poly-IC stabilized with poly-L-lysine and carboxymethyl cellulose; 1.6 mg/kg, Oncovir, Washington DC), CpG ODN 1826 (1.6 mg/kg, Invivogen, San Diego CA), LPS [*Escherichia coli* serotype *0111:B4*; phenol extraction purified, protein content 3% (1 mg/kg, Sigma Aldrich, St.Louis MO)], or vehicle in a total volume of 100 µl. The appropriate vehicle was used depending on the drug treatment in each experiment. The vehicle for poly-ICLC

is carboxymethyl cellulose and the vehicle for both LPS and CpG is saline. For ischemia experiments, mice were treated 72 hours prior to the induction of ischemia.

Ischemia-reperfusion model: Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described previously (Stevens *et al,* 2008). In brief, MCAO was performed in anesthetized mice (1.5-2% isoflurane) by threading a 7-0 silicon-coated nylon surgical filament (Docol, Redlands CA) through the external carotid artery to the internal carotid artery, blocking blood flow at the bifurcation of the MCA and anterior cerebral artery. Following 45 minutes of occlusion, the filament was removed and blood flow was restored. Cerebral blood flow was monitored throughout the procedure by laser Doppler flowmetry (Transonic System Inc., Ithaca NY) and animals were excluded if blood flow was not reduced by 80% or greater during occlusion. Body temperature was maintained at 37°C during the surgery.

Evaluation of infarct size: Twenty-four hours following MCAO, mice were deeply anesthetized with isoflurane then perfused with ice-cold saline containing 2 U/ml sodium heparin. Brains with olfactory bulbs removed were sectioned into 1 mm slices beginning from the rostral end, for a total of 7 slices. The infarct area was visualized by incubating the sections in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, St. Louis MO) in phosphate buffered saline (PBS) for 10 min at 37°C (Sigma Aldrich). Sections were then imaged and the infarct

area was measured using ImageJ software (NIH Image, Bethesda MD). Infarct volume was calculated using the indirect method [(contralateral live – ipsilateral live) / contralateral live * 100] to account for the effects of edema, and the final infarct data are given as % damage of the contralateral hemisphere normalized to genotype-matched vehicle-treated controls.

Tissue processing and quantitative real time PCR: Total RNA was isolated from the brain cortex using the Qiagen Rneasy Lipid Mini Kit (Qiagen, Valencia CA). RNA was reverse transcribed using an Omniscript Reverse Transcription kit (Qiagen). Quantitative PCR (qPCR) was performed using the TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad CA) on an ABI-prism 7700. Results were normalized to β-Actin expression and the relative quantification was determined using the comparative CT method (2^{-DDCt}). Statistics for qPCR results were performed using dCT values.

Statistical Analysis: Statistical testing was performed using Prism 5 software (GraphPad, LaJolla, CA). Values are given as fold change compared to vehicle or group means ± standard error of the mean. Data were analyzed by Student's T-test or 2-way ANOVA, and Bonferroni's post-hoc test as denoted in figure legend. Significance was determined by a $p<0.05$.

Results:

Poly-ICLC preconditioned animals have increased expression of IFN-related genes 24 hours following stroke

One hallmark of the neuroprotective phenotype induced by LPS and CpG preconditioning is the increased expression of IFN-related genes following MCAO (Figure 3-1) (Stevens *et al,* 2011). To determine if poly-ICLC preconditioning also exhibited these genetic changes, cortical tissue was collected 72 hours following preconditioning (at the time of MCAO; n=5/group) and 24 hours following MCAO in both vehicle and poly-ICLC preconditioned animals (n=6-7/group) and the expression level of 4 IFN-related genes was measured. ISG15 was significantly upregulated in poly-ICLC-treated animals compared to vehicle-treated controls at the 72-hour time point (3.3 fold change over vehicle; $p<0.001$). IFIT1 was detectable but not expressed differently between treatment groups and Oasl2 and Usp18 were not detectable at the 72-hour time point. Expression of IFIT1, OASL2, USP18 and ISG15 were all significantly increased in poly-ICLC treated animals compared to vehicle treated controls following stroke (Figure 3-2). This indicates that poly-ICLC shares at least part of the genomic signature with other preconditioning stimuli. These data suggest that poly-ICLC preconditioning does reprogram the genomic response to stroke.

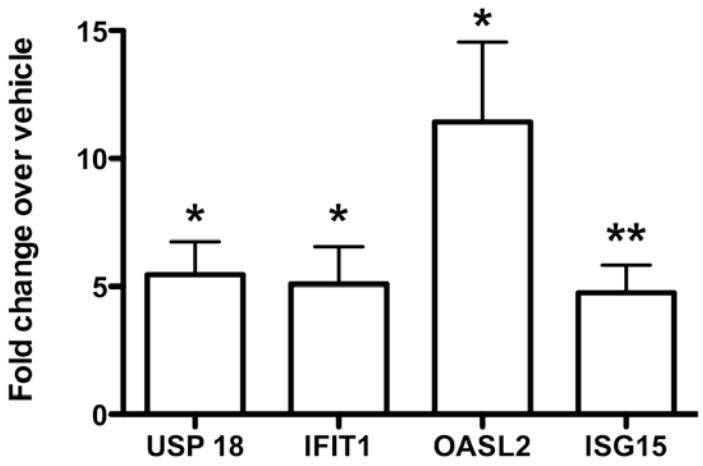

Figure 3-2: Poly-ICLC preconditioned animals have increased expression of interferon-related genes 24 hours following stroke.

Gene expression was evaluated in cerebral cortex samples by qPCR 24 hours following stroke. IFIT1, USP18, OASL2 and ISG15 are significantly upregulated in poly-ICLC preconditioned mice. Fold change compared with vehicle-treated controls are shown. All genes shown are significantly increased compared to vehicle-treated controls using dCT values (t-test; *$p<0.05$, **$p<0.01$) (n=7/group).

IRF7 is required for poly-ICLC-mediated neuroprotection

The transcription factor IRF7 has previously been identified as a critical component of LPS and CpG preconditioning (Stevens *et al,* 2011). Using mice deficient in IRF7 we sought to determine whether IRF7 played a role in poly-ICLC-mediated neuroprotection. WT and IRF7$^{-/-}$ mice were treated with poly-ICLC or vehicle 72 hours prior to MCAO (n=9-10/group) and infarct volume was determined after 24 hours of reperfusion. WT mice were significantly protected from stroke damage by poly-ICLC preconditioning, however IRF7$^{-/-}$ mice were not protected (WT vehicle 26.2 ± 3.3 v poly-ICLC 9.8 ± 2.0 $p<0.05$; IRF7$^{-/-}$ vehicle 28.0 ± 4.9 v poly-ICLC 27.0 ± 4.4 $p>0.05$; Figure 3-3). This indicates that IRF7 is required for poly-ICLC-mediated neuroprotection.

Poly-ICLC preconditioning requires type-1 IFN signaling

The transcription factor IRF7 is known to induce both type-1 IFNs, IFNα and IFNβ, which both signal through the same receptor, IFNAR (Honda *et al,* 2005). Poly-ICLC treatment is also associated with robust production of both IFNα and IFNβ (Bever *et al,* 1985; Bever *et al,* 1988; Levy *et al,* 1975; Levy and Levine, 1981; Longhi *et al,* 2009). To determine if type-1 IFN signaling was involved in poly-ICLC neuroprotection, WT and IFNAR$^{-/-}$ mice were treated with poly-ICLC or vehicle 72 hours prior to MCAO (n=7-10/group). Infarct volume measured at 24 hours following MCAO revealed that while WT mice were significantly protected

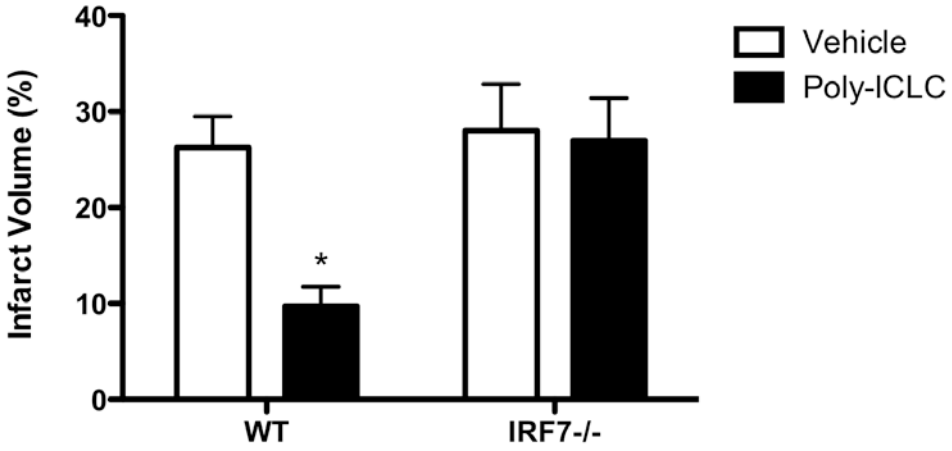

Figure 3-3: IRF7 is required for poly-ICLC-mediated neuroprotection.

WT and IRF7$^{-/-}$ mice were preconditioned with poly-ICLC or vehicle 72 hours prior to MCAO (n=9-10/group). Infarct size was determined 24 hours following MCAO by TTC staining. WT mice were significantly protected by poly-ICLC preconditioning while IRF7$^{-/-}$ mice were not. Values are group means ± SEM; *p<0.05, versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

by poly-ICLC preconditioning treatment IFNAR$^{-/-}$ mice were not protected (WT vehicle 34.7 ± 3.1 v poly-ICLC 13.8 ± 2.2 $p<0.01$; IFNAR$^{-/-}$ 31.5 ± 6.9 v poly-ICLC 35.4 ± 4.9 $p>0.05$; Figure 3-4). Therefore these results demonstrate that type-1 IFN signaling is required for poly-ICLC mediated neuroprotection.

CpG and LPS preconditioning do not utilize type-1 IFN signaling

Having learned that IFNAR$^{-/-}$ is critical for poly-ICLC preconditioning we then asked whether this was also a feature shared with CpG and LPS preconditioning. WT and IFNAR$^{-/-}$ mice were treated with LPS, CpG or vehicle 72 hours prior to MCAO and infarct volume was measured after 24 hours of reperfusion (n=7-18/group). As with WT mice, IFNAR$^{-/-}$ mice were protected from stroke damage by LPS and CpG preconditioning (WT vehicle 37.4 ± 2.2 v CpG 22.1 ± 2.4 $p<0.001$ v LPS 30.0 ± 3.0 $p<0.05$; IFNAR$^{-/-}$ vehicle 42.6 ± 2.2 v CpG 29.7 ± 2.1 $p<0.001$ v LPS 26.9 ± 3.9 $p<0.001$; Figure 3-5). These results reveal the requirement for IFNAR is unique to the mechanism of protection established by poly-ICLC preconditioning and not associated with LPS or CpG preconditioning.

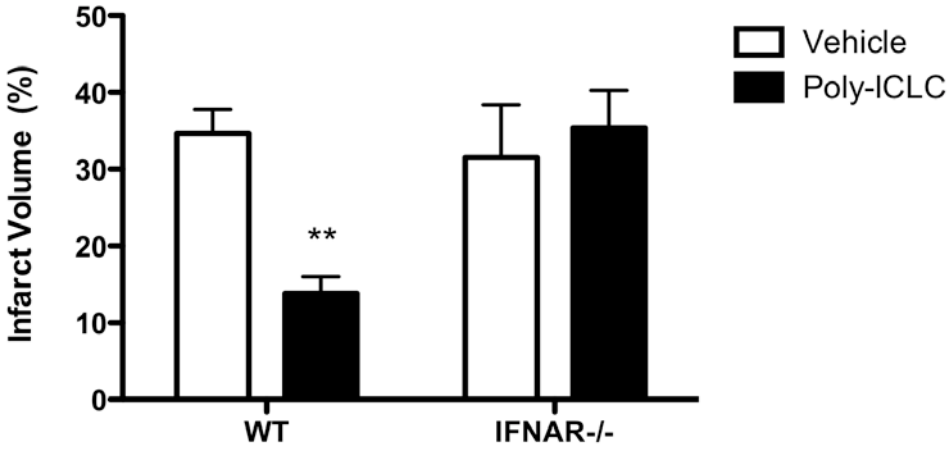

Figure 3-4: Poly-ICLC preconditioning requires type-1 interferon signaling. WT and IFNAR$^{-/-}$ mice were preconditioned with poly-ICLC or vehicle 72 hours prior to MCAO (n=7-10/group). Infarct size was determined 24 hours following MCAO by TTC staining. WT mice were significantly protected by poly-ICLC preconditioning while IFNAR$^{-/-}$ mice were not. Values are group means ± SEM; **p<0.01, versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

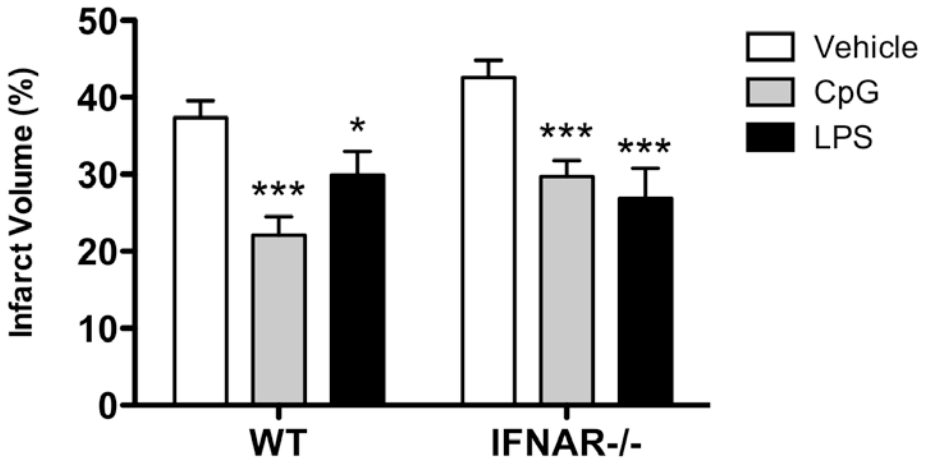

Figure 3-5: CpG and LPS preconditioning do not utilize type-1 interferon signaling

WT and IFNAR$^{-/-}$ mice were preconditioned with CpG, LPS or vehicle 72 hours prior to MCAO (n=7-18/group). Infarct size was determined 24 hours following MCAO by TTC staining. WT and IFNAR$^{-/-}$ mice were both significantly protected by CpG and LPS. Values are group means ± SEM; *p<0.05,***p<0.001 versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

Poly-ICLC neuroprotection does not require TLR3

To assess if TLR3, a known receptor for poly-ICLC, is required for poly-ICLC neuroprotection we utilized $TLR3^{-/-}$ mice. WT and $TLR3^{-/-}$ mice were treated with poly-ICLC or vehicle 72 hours prior to MCAO (n=6-8/group) and infarct volume was determined after 24 hours of reperfusion. Both WT and $TLR3^{-/-}$ mice were significantly protected from MCAO damage by poly-ICLC preconditioning (WT vehicle 25.9 ± 11.2 v poly-ICLC 8.2 ± 5.8 $p<0.01$; $TLR3^{-/-}$ 37.6 ± 10.6 v poly-ICLC 21.5 ± 10.9 $p<0.01$; Figure 3-6). This indicates that TLR3 is not required for poly-ICLC neuroprotection.

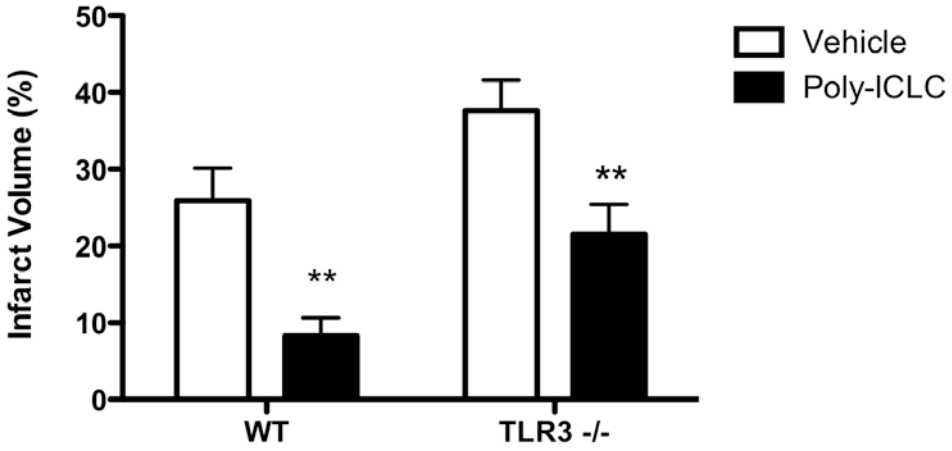

Figure 3-6: Poly-ICLC neuroprotection does not require TLR3

WT and TLR3$^{-/-}$ mice were preconditioned with poly-ICLC or vehicle 72 hours prior to MCAO (n=6-8/group) and infarct volume was determined after 24 hours of reperfusion. Both WT and TLR3$^{-/-}$ mice were significantly protected from MCAO damage by poly-ICLC preconditioning. Values are group means ± SEM; **p<0.01 versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

Discussion:

Several lines of evidence suggest a major role for type-1 IFN in the neuroprotective mechanism of preconditioning: IRF3 and IRF7 are both required for LPS and CpG preconditioning, and there is a genomic fingerprint of IFN-related genes with preconditioning following stroke which correlates with increased activity of IRF3 and increased expression of IRF7. Here we show that poly-ICLC preconditioning also results in a reprogrammed response to the injury demonstrated by induction of IFN-related genes following stroke that are not present at the time of stroke. Further poly-ICLC preconditioning requires IRF7 and type-1 IFN signaling via the IFNAR. The requirement of IFNAR may be unique to poly-ICLC, as CpG and LPS preconditioning induce ischemic tolerance in mice deficient in IFNAR while poly-ICLC preconditioning does not. This suggests that although all 3 preconditioning paradigms induce an IFN genomic signature following stroke, the establishment of neuroprotection is dependent on the preconditioning stimulus.

IRF7 is the master regulator of IFNα and IFNβ gene induction (Honda *et al,* 2005). In contrast to IRF3, which contributes little to IFN induction in the absence of IRF7, IRF7 plays a major role and functions even in the absence of IRF3. IRF7 is activated downstream of the pattern recognition receptors TLR3, TLR7, TLR9, RIG-I and mda-5. Following poly-IC administration IRF7 expression is increased nearly 18-fold together with an increase in IRF7 protein in the mouse

thymus (Demoulins *et al,* 2009). A robust type-1 IFN response follows poly-ICLC administration in primates and rodents (Bever *et al,* 1985; Bever *et al,* 1988; Levy *et al,* 1975; Levy and Levine, 1981; Longhi *et al,* 2009). Following poly-IC administration, primarily IRF7 mediates induction of type-1 IFN and a positive feedback loop of type-1 IFN production ensues as type-1 IFN activates it's receptor IFNAR leading to the production of more IFN and IFN-related genes (Ablasser and Hornung, 2011; Zust *et al,* 2011). IFNAR is required for poly-ICLC neuroprotection; therefore, in addition to IRF activity, the establishment of protection may also require this positive feedback loop of IFN.

Increased type-1 IFN following stroke is neuroprotective. Damage can be decreased by IFNβ administration directly into the brain following stroke (Marsh *et al,* 2009a). IFNβ administered systemically at the time of or after stroke also reduces infarct volume and improves blood brain barrier integrity (Liu *et al,* 2002; Veldhuis *et al,* 2003a; Veldhuis *et al,* 2003b). Therefore, if poly-ICLC preconditioning increases type-1 IFN production following stroke, this may be an affecter of neuroprotection by IFNAR signaling. Additionally, by an indirect route, type-1 IFN generated at the time of poly-ICLC administration may contribute to neuroprotection by reprogramming the genomic response. The mechanism by which IFNAR-mediated signaling contributes to genomic reprogramming in the brain to stroke is unknown; however, there is evidence from other models that IFN can regulate and redirect the immune response. In macrophages, pretreatment with IFNα is able to shift the inflammatory response to TLR ligands

towards an IFN dominated response (Siren *et al,* 2005). Likewise, in the setting of stroke pre-exposure to IFN in the brain with poly-ICLC preconditioning may be able to shift the subsequent TLR immune response following stroke from detrimental inflammation towards a protective IFN-dominated response. Systemic treatment with IFNα is able to alter gene expression in the brain and induce IFN-stimulated genes; therefore, peripheral IFN generated by poly-ICLC can stimulate changes in the brain (Wang *et al,* 2008). This evidence suggests type-1 IFN may contribute to poly-ICLC neuroprotection in two ways, by reprogramming the immune response to stroke towards neuroprotective factors (inhibiting NFκB and enhancing IFN-related genes) and by increasing IFN following stroke which may reduce damage and protect the neurovascular unit.

Poly-ICLC shares the requirement for IRF7 with both CpG and LPS; however, a role for IFNAR is unique to poly-ICLC. If the induction of IFNα and IFNβ by IRF7 is not a critical component of LPS or CpG-mediated preconditioning, it remains to be tested which genes induced by IRF7 are important in the case of CpG and LPS. It may be those IFN-associated genes expressed following stroke, potentially induced by IRF7, are critical to preconditioning and the reduction of stroke injury. With poly-ICLC we have identified a novel component of preconditioning neuroprotection, IFNAR, which itself may also serve as a potential target for direct therapeutic intervention.

Our finding that poly-ICLC preconditioning does not require TLR3 suggests that we have discovered a novel pharmacological target for inducing ischemic tolerance. Another poly-ICLC receptor mda-5 is a member of the RLR family and a cytosolic RNA receptor and may mediate poly-ICLC neuroprotection. As with TLRs, activation of mda-5 also leads to the activation of NFκB and IRF families of transcription factors. Poly-ICLC treatment leads to the robust production of type-1 IFN and previous work has found that this type-1 IFN response is dependent on the mda-5 receptor and not TLR3 (Gitlin *et al,* 2006; Longhi *et al,* 2009; McCartney *et al,* 2009). Therefore, in the case of poly-ICLC neuroprotection where we have shown that type-1 IFN activity is essential, mda-5 is likely the required receptor for poly-ICLC for neuroprotection. However, it should be noted that protection in TLR3 deficient animals is not as robust as WT animals preconditioned with poly-ICLC. Therefore, TLR3 signaling may contribute to poly-ICLC neuroprotection and potentially both mda-5 and TLR3 could be essential.

Our current findings suggest a potential model for poly-ICLC preconditioning induced neuroprotection. Poly-ICLC induces neuroprotection via a TLR3-independent route, potentially through mda-5-mediated activation of IRF7 and the induction of type-1 IFN and IFN-associated genes. Critically, the IFN produced may activate the IFNAR whose signaling further enhances the positive feedback loop of IFN and IFN-related genes. We can posit that this process sets up the genomic reprogramming event whereby following stroke IFN-related

genes, such as IFIT1, USP18, OASL2 and ISG15, are induced to a greater extent. This IFN response shifts the balance of the immune response to stroke from detrimental inflammation to protective IFN-related signaling thus resulting in neuroprotection.

Chapter 4:

Poly-ICLC preconditioning

Summary and Perspectives

1. Poly-ICLC preconditioning is a promising therapeutic approach

We have tested poly-ICLC as a candidate preconditioning stimulus to induce tolerance to ischemia. Poly-ICLC preconditioning protects primary cortical cells from cell death by modeled ischemia *in vitro* in a dose-dependent manner. Similarly in a mouse model of cerebral ischemia, poly-ICLC preconditioning significantly reduces infarct volume. Preconditioning with poly-ICLC also improves functional outcomes measured by neurological scoring and performance in a corner test. Importantly preconditioning with poly-IC provides protection out 3 days following reperfusion; therefore, this protection appears to be lasting and not transient. Additionally, poly-IC preconditioning provides protection not only against cerebral ischemia but also against renal ischemia. Previous studies have shown that LPS preconditioning induces ischemic tolerance in multiple organ systems however we believe poly-ICLC preconditioning may be a superior therapeutic candidate. Induction of proinflammatory cytokines is significantly reduced with Poly-ICLC preconditioning compared to LPS preconditioning. Poly-ICLC treatment also has a long record of safe usage in human patient populations even with chronic treatment regimens (discussed in detail below). We believe poly-ICLC preconditioning is a promising treatment option to develop for antecedent use in patients at-risk of ischemic damage.

Guidelines for development of stroke treatments

As we plan future studies to develop poly-ICLC preconditioning for potential clinical use, the STAIR criteria provide guidelines for development of stroke therapeutics. We must also be mindful of recent drug development efforts to learn from their examples. One drug that has been developed since the establishment of these criteria is NXY-059. The recent failure of NXY-059 in phase III trials was a disappointment for the field of stroke neuroprotection as this drug appeared to meet the STAIR criteria (Macleod *et al,* 2008). NXY-059 is a free radical trapping agent that was shown to decrease infarct volume and improve functional outcome in a number of animal models when the drug was given out to 4 hours after ischemia. These studies even included a non-human primate model, the marmoset, that was administered NXY-059 4 hours after ischemia onset and had a 28% reduction of infarct volume and improved arm function and attenuated spacial neglect (Marshall *et al,* 2003). However, there was significant reduction in damage for the putamen only and the marmoset may not be an appropriate model as it has a lissencephalic brain, not a gyrencephalic brain like humans. The first Stroke-Acute Ischemic NXY Treatment (SAINT) trial of 1699 patients also had positive results with significantly reduced disability at 90 days following NXY-059 treatment (Lees *et al,* 2006) as measured by the modified Rankin Scale (van Swieten *et al,* 1988). The success of the SAINT I trial could not be repeated in the larger subsequent SAINT II trial where NXY-059 gave no improvement to modified Rankin Scale measures or secondary outcome

measures (Shuaib *et al,* 2007) such as the National Institutes of Health Stroke Scale (Brott *et al,* 1989).

There are many possible explanations for the failure of this promising drug in phase III trials. Collectively the pre-clinical studies met the STAIR criteria but retrospective analysis raised concerns about the quality of these studies on an individual basis for issues such as blinding, randomization, concealment of treatment allocation, hypertensive animal subjects, blood flow monitoring, statistics, behavior evaluation, long-term efficacy and conflict of interest bias (Macleod *et al,* 2008). Individual studies with a greater adherence to the STAIR criteria gave significantly lower estimates of efficacy than those that did not (Macleod *et al,* 2008). In addition unpublished data do exist that show negative results and some studies did not show sustained improvement (Macleod *et al,* 2008; Savitz, 2007). Other concerns have been raised about the lack of information about how the drug worked including specific identification of the drug target, understanding the drug-target interaction and the amount of drug that actually made it into the brain (Feuerstein *et al,* 2008; Savitz, 2007). Another concern not often mentioned in the literature is the effect of age, the most important risk factor for stroke, as the mean age for patients in the SAINT trial was 69 yet many of the animal studies used young adult male subjects including the marmoset report. Additionally there were concerns about the design for the clinical trial's choice of patients and drug time window. As the drug was only found to be protective of grey matter the participants could have been limited to

those with grey matter injury or middle cerebral artery occlusions as was used in pre-clinical testing (Savitz, 2007; Savitz and Schabitz, 2008). The subjects could also have been selected on the basis of diffusion/perfusion magnetic resonance imaging to find subjects with salvageable tissue, though this idea has not been validated (Feuerstein *et al,* 2008). The greatest concern may be that NXY-059 was given at an inappropriate time, up to 6 hours following ischemia in the SAINT trials, whereas animal studies showed that it was only effective when given at most 4 hours following ischemia (Savitz, 2007). Though this drug proved to be a failure there is much to be learned from this example for future work with neuroprotectant drugs. Keeping in mind the STAIR criteria and the lessons we have learned from NXY-059 and the SAINT trials we hope to rigorously test preconditioning treatments as neuroprotectant therapies and understand the mechanisms behind preconditioning-mediated protection.

Poly-ICLC preconditioning and the STAIR criteria

Development of poly-ICLC preconditioning as a therapeutic has only just begun but we have already met some of the STAIR criteria. We have performed a dose-response curve and determined the most efficacious dose to be 1.6 mg/kg in mice. We have also found that a low dose (0.4 mg/kg) or a high dose (4 mg/kg) does not confer significant neuroprotection nor do they exacerbate damage. We have performed the animal experiments in a blinded manner with predefined inclusion/exclusion criteria (age, gender, cerebral blood flow measurements,

temperature) using an adequate sample size of young male animals based on power analysis. We began our studies looking first at acute outcome measures out to 72 hours following reperfusion; however, future studies will need to examine long-term outcomes. Future experiments with poly-ICLC will need to address additional STAIR criteria. These include determining a therapeutic time window, performing experiments in female animals, aged animals and animals with co-morbid conditions, examining other biomarker endpoints, performing interaction studies with commonly used medications, determining drug-target interactions and performing studies in gyrencephalic species.

Poly-ICLC treatment in non-human primates

An important criteria for preclinical stroke therapeutic testing is utilizing a gyroencephalic animal model that replicates clinical phenotypes observed in human stroke. Non-human primates (NHPs) are more closely related to humans in several aspects critical to stroke pathology. Many NHPs have a gyroencephalic brain with similar neuroanatomy and vasculature and NHPs exhibit complex behaviors more analogous to humans. Poly-ICLC is a superior option to poly-IC in primates in that poly-ICLC is protected from degradation by nucleases, as it is complexed with poly-L-lysine and carboxymethylcellulose (Levy *et al,* 1976; Nordlund *et al,* 1970). Poly-ICLC treatment is a potent inducer of IFN and has been safely and effectively used in NHPs (Sammons *et al,* 1977). Poly-ICLC has been used as an antiviral compound, as an adjuvant to improve

vaccine responses and protects against malaria (Flynn *et al,* 2011; Levy *et al,* 1976; Puri *et al,* 1996; Sariol *et al,* 2011; Stahl-Hennig *et al,* 2009; Stephen *et al,* 1977; Stephen *et al,* 1979; Tewari *et al,* 2010). Collectively these studies suggest that poly-ICLC preconditioning might also be successful at neuroprotection in a NHP model of stroke as this compound has been effectively used in monkeys.

Our laboratory has recently developed a NHP model of cortical stroke in Rhesus Macaques (West *et al,* 2009) and used this model to test the efficacy of CpG preconditioning. CpG preconditioning administered 72 hours prior to stroke significantly reduced infarct volume measured at 48 hours following reperfusion by MRI and TTC staining (Bahjat *et al,* 2011). Further CpG preconditioned animals also had improved neurological function at this timepoint demonstrated by improved neurological scores assessed by a modified Spetzler neurologic scale (Bahjat *et al,* 2011). This work is the first demonstration of efficacy for pharmacological preconditioning in NHPs. The success of this project suggests that this NHP model will be an appropriate approach to further evaluate the clinical potential of poly-ICLC preconditioning.

Poly-ICLC treatment in human patients

More importantly, poly-ICLC treatment has been used in human subjects over the past few decades and is generally well tolerated with no major side effects at reasonable doses even with repeated administration (Caskey *et al,* 2011;

Champney *et al,* 1979). Treatment with poly-ICLC activates innate immune system pathways including IFN, inflammasomes, and complement in people similar to those described in animal models (Caskey *et al,* 2011). Poly-ICLC has been tested as a treatment for various indications in clinical trials; these include multiple myeloma, leukemia, neuroblastoma, metastatic carcinoma, malignant glioma, multiple sclerosis and vaccine adjuvants (Bever *et al,* 1986; Butowski *et al,* 2009a; Butowski *et al,* 2009b; Durie *et al,* 1985; Krown *et al,* 1985; Lampkin *et al,* 1985; Markosian and Hyde, 2005; McFarlin *et al,* 1985; Okada *et al,* 2011; Rosenfeld *et al,* 2010; Salazar *et al,* 1996; Stevenson *et al,* 1985). Due to the extended history of using poly-ICLC in clinical populations, the use of poly-ICLC as a prophylactic treatment for patients at risk of ischemic damage holds true clinical potential.

Poly-ICLC preconditioning to protect multiple organs simultaneously

Here we have shown that poly-ICLC preconditioning is able to protect not only the brain from ischemic damage but also the kidneys. This suggests that, like LPS preconditioning which protects numerous organs including the brain, liver, kidneys, retina and lungs (Colletti *et al,* 1994; Franco *et al,* 2008; Heemann *et al,* 2000; Merry *et al,* 2010; Tasaki *et al,* 1997), poly-ICLC preconditioning may have the ability to protect multiple organs simultaneously. This usage could be particularly helpful for prophylactic treatment of major surgery patients who risk ischemic damage to multiple organs or potential preconditioning of organs prior

to transplant. The proof-of-concept studies that preconditioning can protect multiple organs simultaneously has been tested in humans using remote ischemic preconditioning (RIPC) (Kharbanda *et al,* 2009). For RIPC, non-target tissue is subjected to brief periods of ischemia (for instance by inflation of a blood pressure cuff on the arm), which then protects other organs from ischemic damage. From early clinical trials it appears RIPC can reduce myocardial injury in children and adults undergoing surgery and this protection may also extend to the lungs, kidneys and liver (Ali *et al,* 2007; Cheung *et al,* 2006; Hausenloy *et al,* 2007; Hoole *et al,* 2009; Venugopal *et al,* 2009). These studies demonstrate preconditioning can be a successful approach to protect multiple organs from ischemic injury in humans. Poly-ICLC offers a pharmacological alternative to RIPC treatment that could also potentially provide widespread protection from ischemia. A pharmacological approach offers the advantage of carefully titrating dose and site of administration to improve treatment outcomes.

2. Potential mechanisms of poly-ICLC-induced neuroprotection

We have also begun to study the mechanisms by which poly-ICLC preconditioning induces neuroprotection. This investigation will help to inform therapeutic development of poly-ICLC preconditioning by understanding key drug targets and drug actions. Additionally, learning about the mechanisms of preconditioning will also help to appreciate endogenous methods of neuroprotection and even find potential new targets for acute therapies.

Poly-ICLC preconditioning requires IRF7 and type-1 IFN signaling

Our recent work has shown that poly-ICLC preconditioning shares common IFN-related attributes with other preconditioning stimuli, specifically LPS and CpG. Poly-ICLC preconditioning leads to the upregulation of IFN-related genes following stroke in protected brain tissue including IFIT1, USP18, ISG15 and OASL2 (Figure 3-2). As with CpG and LPS preconditioning, neuroprotection by poly-ICLC requires the presence of IRF7. This was demonstrated using IRF7-deficient mice that were not protected by these preconditioning stimuli (Figure 3-3). These findings suggest that poly-ICLC preconditioning may cause a similar reprogramming event that can be characterized by an IFN fingerprint that is driven by the activity of IRFs. However, we have also learned that poly-ICLC preconditioning is not identical to LPS and CpG preconditioning, as poly-ICLC requires the activity of type-1 IFN while LPS and CpG do not. Mice deficient in the IFNAR are protected by LPS and CpG but they are not protected by poly-ICLC (Figures 3-4 & 3-5). This may mean that while poly-ICLC shares aspects of the IFN fingerprint, it acts through a novel route to induce reprogramming. LPS and CpG treatment lead to the production of TNFα, which may be a critical factor in initiating the preconditioning process for these stimuli. On the other hand, Poly-ICLC treatment leads to the robust production of type-1 IFN. It may be the action of IFN on their IFNAR that is a critical factor prior to stroke for poly-ICLC preconditioning. IFN actions are essential for many poly-ICLC functions such as humoral immunity, isotype switching and CD8 Tcell expansion, and blocking the

IFNAR significantly dampens the genomic response to poly-IC (Caskey *et al,* 2011; Stahl-Hennig *et al,* 2009) and poly-ICLC neuroprotection is no exception.

Just as poly-IC treatment can provide heterotolerance to LPS challenge, poly-ICLC can also provide tolerance to ischemia (Biswas *et al,* 2007; Jiang *et al,* 2005). Therefore, we believe that the mechanism of protection afforded by poly-ICLC preconditioning may be similar to the reprogramming model (Figure 1-2) proposed for LPS and CPG but, the initiation of that protection is not identical. Poly-ICLC is sensed by dsRNA receptors in the cell. These receptors are activated and downstream signaling events lead to the activation of IRFs and the production of type-1 IFN and IFN-related genes. This productivity is further enhanced by a positive feedback loop as IFN acts on its own receptor leading to further production of IFN-related genes (Figure 4-1). This initiation step leads to currently unknown reprogramming processes in the cell whereby, upon challenge with ischemia, signaling is redirected from a proinflammatory response to an immune response dominated by IFN-related neuroprotective effectors akin to the model proposed for CpG and LPS (Figure 1-2).

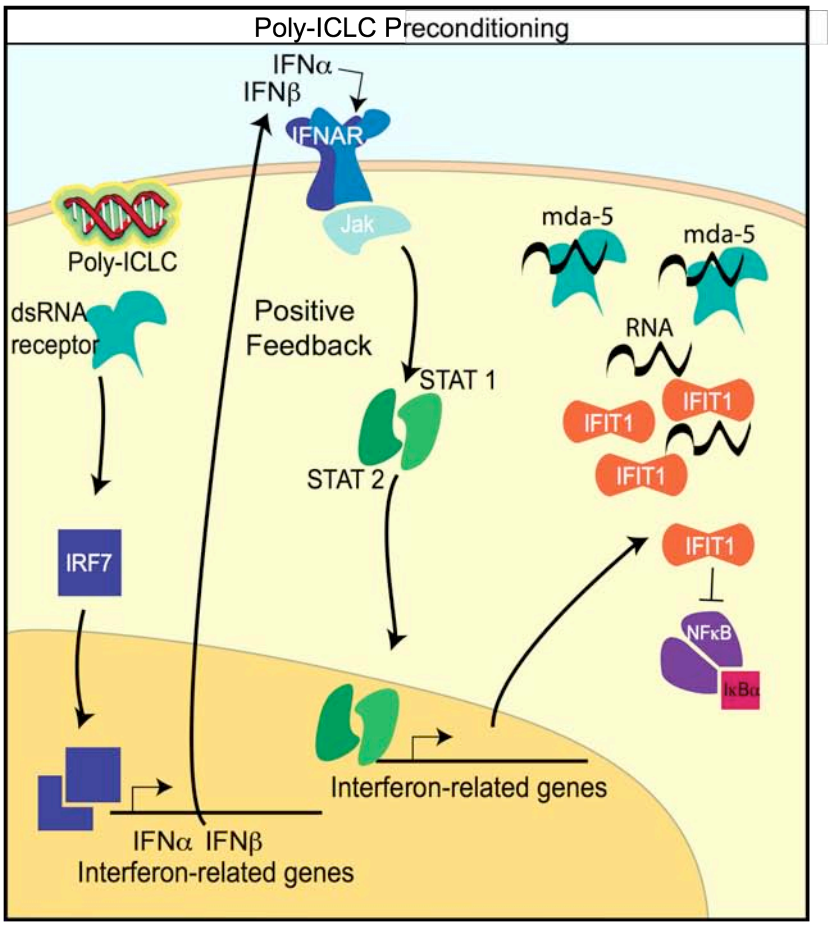

Figure 4-1: Poly-ICLC preconditioning model

Poly-ICLC induces neuroprotection via a TLR3-independent route, potentially through mda-5-mediated activation of IRF7 and the induction of type-1 IFN and IFN-associated genes. Critically the IFN produced may activate the IFNAR whose signaling drives a positive feedback loop of expression of IFN and IFN-related genes. We posit that poly-ICLC preconditioning causes a genomic reprogramming event whereby following stroke the immune response is redirected from detrimental inflammation to protective IFN-related signaling thus resulting in neuroprotection. Increased expression of IFN-related genes following stroke, such as IFIT1 or mda-5, may act as effectors of neuroprotection by inhibiting NFκB activity and suppressing translation or inflammation.

Poly-ICLC and mda-5

Poly-ICLC mainly activates two receptors, TLR3 and mda-5. TLR3 is not required for poly-ICLC preconditioning as TLR3 deficient mice are protected from stroke by poly-ICLC (Figure 3-6). This finding is surprising given that other TLR ligands that induce neuroprotection require their related TLR receptor. Our finding with poly-ICLC suggests that another receptor is primarily accountable for initiating poly-ICLC preconditioning. Mda-5 is responsible for type-1 IFN production following poly-IC administration. As type-1 IFN activity is required for poly-ICLC neuroprotection (Gitlin *et al,* 2006; Longhi *et al,* 2009), this makes mda-5 the likely receptor target in poly-ICLC preconditioning. The prospective identification of mda-5 as a target widens the potential preconditioning targets to not only include TLRs but also RLRs and other pattern recognition receptors as well.

Previous studies have shown that TLR3 does not play a major role in response to cerebral ischemia (Hua *et al,* 2009; Hyakkoku *et al,* 2010) despite the fact that TLR3 has been identified as a sensor of tissue damage (Bsibsi *et al,* 2010; Cavassani *et al,* 2008; Kariko *et al,* 2004a). A role for mda-5 in response to stroke injury has not yet been tested. Mda-5 also recognizes DAMPS such as self-RNA (Malathi *et al,* 2007); therefore, it is possible that mda-5 could play a role in sensing tissue damage following stroke. Mda-5 signaling leads to the robust production of IFN and IFN-related genes; therefore, it is possible that

Table 4-1: Expression of mda-5 following stroke

PRECONDITIONING STIMULUS	FOLD CHANGE MDA-5	P VALUE
Vehicle 1	1.04	0.8106
LPS	1.39	0.0046 *
CpG	1.36	0.0082 *
Vehicle 2	1.08	NA
Poly-ICLC	2.06	0.0183 *
Vehicle 1, LPS and CpG results by microarray vs. unhandled control Vehicle 2 and Poly-ICLC results by qPCR vs. vehicle control		

mda-5 activity in the context of ischemia would be neuroprotective. In fact, mda-5 is significantly upregulated in the brains of animals preconditioned with LPS, CpG or poly-ICLC 24 hours following stroke challenge (Table 4-1). Enhancement of the mda-5 pathway may be neuroprotective and a critical part of the IFN-related response following stroke however this possibility remains to be tested (Figure 4-1).

Potential role for IFIT1 in poly-ICLC preconditioning and neuroprotection

The gene IFIT1 is significantly upregulated following stroke in the brains of mice that were preconditioned with LPS, CpG, brief ischemia and poly-ICLC (Stevens *et al,* 2011) (Figure 3-2). This gene is one of a number of IFN-related genes that is a hallmark of preconditioning protection. However we do not know if IFIT1 and other IFN-related genes have a protective function in the response to ischemia or if these genes are merely bystanders, a result or indication of reprogramming but not neuroprotective. Recent publications on the antiviral functions of IFIT1 suggest potential functions for IFIT1 in response to ischemia.

IFIT1 (also known as ISG56), stands for IFN-induced protein with tetratricopeptide repeat motifs. IFIT1 is a member of a small family of evolutionarily conserved IFIT proteins (Fensterl and Sen, 2010). Expression of the IFIT1 gene is triggered by STAT1/2 and IRF activity induced by IFN, virus infections, and PAMPs such as dsRNA and LPS (Fensterl and Sen, 2010). One

function of IFIT1 that has been proposed is to block translation by binding to the eIF3 complex that initiates protein synthesis (Hui *et al,* 2005). IFIT1 also functions as a negative feedback regulator to dampen the innate immune response by inhibiting NFκB activity (Li *et al,* 2009). As a cytoplasmic protein IFIT1, like TLR3 and mda-5, is a sensor for RNA (5'-triphophate RNA) and it blocks translation of viruses such as Hepatitis C and Human Papillomavirus (Fensterl and Sen, 2010; Pichlmair *et al,* 2011). In the setting of ischemia IFIT1 may function to modulate translation or dampen the innate immune response.

How might preconditioning with poly-ICLC enhance the expression of IFIT1 following stroke? Poly-ICLC preconditioning induces IFN which leads to histone3 variant H3.3 to be deposited on IFIT1 and this enrichment is in place for at least 48 hours (Tamura *et al,* 2009). Therefore, histone deposition may serve as an epigenetic mark that enhances transcription of IFIT1 following stroke. Increased IFIT1 protein following stroke could potentially be activated by aberrant RNA released from damaged cells and then act as a negative regulator of immune responses thereby dampening the damaging immune response to stroke (Figure 4-1). Though currently not investigated, a neuroprotective role for IFIT1 is a possibility.

Potential role for NK cell responses in poly-ICLC preconditioning

Genomic methods have also been applied to investigate the response in blood cells to CpG preconditioning and stroke. Microarray analyses revealed 422 genes that were differentially regulated in the blood of CpG preconditioned animals relative to vehicle at 24 hours following stroke (Marsh *et al,* 2009b). Subsequent analysis identified overrepresented transcriptional regulatory elements based on the promoter sequences of these genes and discovered just one, GATA-3, which was linked to 53% of the genes (Marsh *et al,* 2009b). GATA-3 plays a critical role in natural killer (NK) cells and a number of the upregulated genes in the blood of CpG preconditioned mice are NK cell-associated. This suggests CpG preconditioning activates an NK cell response following stroke. NK cells may play a protective role by limiting neuroinflammation as NK cells provided neuroprotection in a model of autoimmune disease (Hammarberg *et al,* 2000). While we have not tested this directly in the setting of stroke, indirect evidence suggests that poly-ICLC preconditioning may also enhance the activity of NK cells, perhaps an additional means of providing neuroprotection by preconditioning.

Poly-IC activates NK cells indirectly through stimulatory cytokines IFN and IL-12 and leads to the production of IFNγ by NK cells (Girart *et al,* 2007; Longhi *et al,* 2009; McCartney *et al,* 2009; Miyake *et al,* 2009; Perrot *et al,* 2010; Salem *et al,* 2006). Poly-IC-induced activation of NK cells is primarily mediated by mda-5,

which is consistent with our suggestion that neuroprotection by poly-ICLC is mda-5 but not TLR3 mediated (McCartney *et al,* 2009). NK cells appear to be a critical mediator of poly-IC functions as NK cell activity is required for some poly-IC adjuvant effects (Salem *et al,* 2006). Perhaps most important to the setting of preconditioning, poly-IC treatment not only activates NK cells but also promotes NK cell resistance to suppression by stressors like surgery (Rosenne *et al,* 2007). Therefore poly-ICLC preconditioning may not only activate NK cells but also help to boost their activity following a stressor like stroke.

Critical site of action of poly-ICLC preconditioning

One of the STAIR criteria is to "document the drug accesses the target organ, the brain" (Fisher *et al,* 2009). However with the preconditioning paradigm we do not know if we necessarily need the drug to access the brain to confer protection. The site of action of preconditioning is a critical question we need to answer in order to make significant progress in understanding the mechanism of action. We are able to administer preconditioning agents systemically to later protect the brain from ischemic insult. It is possible that the critical drug target in the case of preconditioning may be peripheral cells, perhaps circulating immune cells, endothelial cells or stromal cells. These initial cellular targets may then produce secondary messengers that help to confer ischemic tolerance in the brain. On the other hand, it is also possible that poly-ICLC does cross into the brain or reach brain cells via circumventricular organs, to have direct effects on cells of the

CNS. Systemic poly-IC treatment does affect gene regulation in the brain suggesting it may be acting in the brain directly (Konat *et al,* 2009). Poly-IC treatment causes disruption of the blood brain barrier integrity; therefore, this disruption may improve poly-IC access to the brain (Wang *et al,* 2004). We have also shown that direct pretreatment of primary mouse cortical cells with poly-ICLC protects against ischemic damage modeled *in vitro*, suggesting that the peripheral responses are not required for neuroprotection (Packard *et al,* 2011). However in a complex *in vivo* environment where multiple systems are contributing to damage following ischemia, treating solely cortical cells may not be sufficient. Studies that have investigated the adjuvant activity of poly-IC to an HIV vaccine have found that both hematopoietic and non-hematopoietic cells must sense poly-IC to have the desired effect (Longhi *et al,* 2009). This adjuvant activity of poly-IC also requires a type-1 IFN response and the source of type-1 IFN was via mda-5 on non-hematopoietic cells (Longhi *et al,* 2009; Wang *et al,* 2010). This suggests that for complex immune system responses multiple cell types from multiple compartments must respond to poly-IC. Therefore it may be that poly-IC preconditioning has to act on multiple cell types in multiple compartments to result in neuroprotection as well.

The following chapter begins to tackle this important question: what is the critical site of action of pharmacological preconditioning agents? Rather than utilizing poly-ICLC that we have just begun to study, the following project utilizes CpG, a TLR9 agonist. CpG preconditioning is also initiated through systemic

administration, thus determining the site of action for this compound may be similar to identifying the site of action for poly-IC. CpG preconditioning has been studied in detail in our mouse stroke model and also in the NHP stroke model, therefore we have already learned many important aspects of CpG preconditioning (Bahjat *et al,* 2011; Stevens *et al,* 2008; Stevens *et al,* 2011). With CpG, we have tested multiple routes of administration, we have performed extensive genomic analyses and we have identified several critical mediators of protection TNFα, IRF3, and IRF7. From this background information we have chosen to focus on the site of initiation, TLR9 expressing cells, and the production of TNFα, a cytokine we believe is critical to the initiation of preconditioning. We hope to apply what we learn from this CpG project to gain knowledge as to the site of action of poly-ICLC and to develop future experiments that will identify critical cellular targets of poly-ICLC which induce ischemic tolerance.

Chapter 5:

TLR9 expression on hematopoietic cells is required but not sufficient for central TNFα expression and ischemic neuroprotection by CpG preconditioning

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Introduction:

Systemic administration of a low dose of the Toll-like receptor 9 (TLR9) agonist unmethylated cytosine-guanine-rich DNA oligonucleotides (CpG) prior to stroke induces robust neuroprotection against ischemic damage demonstrated by the significant reduction of infarct size in both mouse and primate stroke models (Bahjat *et al* 2011; Stevens *et al* 2008). The neuroprotective outcome induced by CpG requires TNFα, as TNFα deficient mice are not protected against stroke by preconditioning with CpG (Stevens *et al,* 2008). Classically, TNFα is believed to exacerbate damage following cerebral ischemia (Nawashiro *et al,* 1997a; Rosenzweig *et al,* 2007) however, the dependence of CpG preconditioning on TNFα suggests that it plays pleiotropic roles in ischemic injury. Importantly, several preconditioning stimuli also require TNFα to exert protection, including preconditioning with the TLR4 agonist lipopolysaccharide (LPS) to protect against stroke (Rosenzweig *et al,* 2007) and preconditioning with brief ischemia to protect against myocardial infarction (Ren *et al,* 2004). These data suggest that TNFα may be a common mediator through which ischemic tolerance may be achieved. Interestingly, direct intracisternal, but not systemic, injection of TNFα into the brain prior to stroke results in reduced infarct size in response to cerebral ischemia (Nawashiro *et al,* 1997c). Further direct TNFα preconditioning protects neuronal or astrocyte cultures from ischemic damage (Ginis *et al,* 2002; Saha *et al,* 2009). However the systemic addition of a TNFα blocking protein abolishes the LPS-induced neuroprotective effects against stroke (Tasaki *et al,* 1997)

suggesting that systemic TNFα is required for LPS preconditioning, but not sufficient to induce protection. We have previously shown that CpG induces systemic TNFα prior to stroke (Stevens *et al,* 2008), but whether TNFα is also induced in the brain is unknown. Thus, although TNFα is critical for CpG-induced neuroprotection, the source and site of action of TNFα in response to CpG is unclear.

In mice, TLR9 is expressed on a wide variety of cell types both in the brain and the periphery including dendritic cells, monocytes/macrophages, B cells, pulmonary tissue (Knuefermann *et al,* 2007), endothelium (El Kebir *et al,* 2009; Erridge *et al,* 2008; Li *et al,* 2004; Martin-Armas *et al,* 2006), cerebrovascular endothelium (Constantin *et al,* 2004), microglia (Jack *et al,* 2005) and neurons (Qi *et al,* 2011; Tang *et al,* 2007). Following systemic administration it is unclear whether CpG crosses the blood brain barrier to directly act upon the CNS or whether CpG acts through systemic mediators that induce neuroprotection. Even though CpG is not generally thought to cross the barrier (Ho and Hartig, 1999), some anti-sense oligonucleotides administered intravenously at large doses have the ability to cross by the oligonucleotide transport system-1 (OTS-1) (Banks *et al,* 2001). Moreover, despite the fact that circulating hematopoietic cells express TLR9 and are readily exposed to systemically administered CpG, ample experimental evidence suggests that hematopoietic cells may not be the primary site of CpG action in preconditioning for stroke. We have shown previously that mouse mixed cortical cells pretreated with CpG have significantly decreased cell

death in an *in vitro* model of ischemia – oxygen glucose deprivation (OGD). These data indicate that, in the absence of hematopoetic cells, cortical cells respond to CpG and are protected against ischemic insult (Stevens *et al,* 2008). Further, a study utilizing TLR4 knockout bone marrow chimeric mice showed that TLR4 expression on hematopoietic cells was not required to elicit an LPS-mediated inflammatory response in the CNS by systemically administered LPS (Chakravarty and Herkenham, 2005), diminishing the potential role of hematopoietic cells in the observed CNS inflammatory response. Thus, we sought to define whether systemic or central responses to CpG are required to produce TNFα and ultimately to promote neuroprotection against stroke.

We hypothesized that hematopoietic cells are not required for the neuroprotective effect of CpG. In order to test our hypothesis we first utilized intranasal administration of CpG to directly target the CNS to determine if the mice would be protected against ischemic injury. The results demonstrated robust protection against stroke, illustrating the protective effects of CpG on CNS cells *in vivo*. Next we validated that TLR9 deficient mice (TLR9KO) were not protected by CpG. To further investigate the site of action of CpG we used TLR9KO mice to generate TLR9-deficient bone marrow chimeric mice whereby TLR9 is expressed only on parenchymal cells, including CNS cells, but not on the systemic hematopoietic cells (TLR9KO→WT). We found that preconditioning TLR9KO→WT with CpG did not protect them from subsequent stroke, suggesting that the hematopoietic TLR9 expressing cells were required for

neuroprotection. Additionally, we tested the reciprocal chimeric mice whereby TLR9 was expressed on systemic hematopoietic cells, but not parenchymal cells (WT→TLR9KO), and found that they were also not protected against stroke by CpG preconditioning. This indicates that the response of hematopoietic TLR9 expressing cells is not sufficient to induce neuroprotection. Importantly, we found that both reciprocal chimeric mice had suppressed CpG-induced TNFα mRNA response in the brain. Thus, the absence of TNFα mRNA in the brain correlates with the loss of CpG preconditioning-induced neuroprotection. Taken together, these data suggest that the mechanism governing CpG-induced protection is derived from the responses of both the hematopoietic and parenchymal compartments and requires a TNFα response in the brain to induce neuroprotection against stroke.

Materials and Methods:

Animal Care and Criteria: C57Bl/6J mice and the congenic mouse strain B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ were obtained from Jackson Laboratories (West Sacramento, CA). TLR9KO mice were purchased from OrientalBioService (Osaka, Japan) and were backcrossed 8 generations onto C57Bl/6. All stroke studies were performed with male mice between 10 – 14 weeks of age. All mice were given free access to food and water and housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal protocols were approved by the Oregon Health & Science

University Institutional Animal Care and Use Committee (OWLAW# A3304-01) and met the guidelines set forth by the National Institutes of Health.

Generating Chimeric Mice: The TLR9KO mice back-crossed onto C57Bl/6 have the CD45.2 allelic version of the CD45 leukocyte common antigen expressed on all leukocytes. In order to distinguish between recipient and donor cells in the chimeric animals we used the congenic strain B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ, which have the CD45.1 allelic version of the CD45 leukocyte common antigen, as our TLR9 replete strain (WT). Recipient mice were irradiated with a split dose of 12 grays (6 grays each dose, separated by 4 hours). Bone marrow was flushed from the femurs and the tibias of donor mice, and $1x10^{6}$ cells were injected intravenously into irradiated recipients. Chimeras were given 250 mg/L ciprofloxacin antibiotic added to drinking water for the first 2 weeks. Three bone marrow chimera groups were produced (donor→recipient): 1) WT recipients receiving bone marrow from TLR9KO mice (TLR9KO→WT) 2) TLR9KO recipients receiving bone marrow from WT mice (WT→TLR9KO) and 3) WT recipients receiving bone marrow from WT mice (WT→WT).

Determination of reconstitution efficiency: After 4–6 weeks of reconstitution, chimerism was verified by flow cytometry via testing for the percent blood leukocytes positive for CD45.1 (TLR9 replete) versus CD45.2 (TLR9KO). Mice were deeply anesthetized with isoflurane, and blood was collected via cardiac puncture in sodium heparin and red blood cells were lysed using 2 volumes of 1X

RBC lysis buffer (Biolegend, San Diego, CA). Samples were washed using cell staining buffer (Biolegend) and were incubated with Fc-Block (BD Biosciences, Franklin Lakes, NJ) for 10 minutes. Cells were stained with mouse anti-mouse CD45.1 (eBiosicences, San Diego, CA) and CD45.2 antibodies (BD Biosciences) conjugated with PeCy7 and FITC, respectively, for 20 minutes at 4°C. Results were acquired using an LSRII cytometer (BD Biosciences) with no fewer than 10,000 total events collected per sample. Post acquisition analysis was performed using FlowJo 9.1 (Tree Star Inc., San Carlos, CA).

Drug treatments: Mice were given a subcutaneous injection of CpG ODN 1826 (CpG; Invivogen, San Diego CA), LPS [*Escherichia coli* serotype *0111:B4*; phenol extraction purified, protein content 3% (Sigma Aldrich, St.Louis MO)], or saline in a total volume of 100µl. For intranasal administration, mice were given CpG or artificial cerebral spinal fluid (aCSF) vehicle in a total volume of 20µl. For ischemia experiments, mice were treated 72 hours prior to the induction of ischemia. For cytokine and quantitative PCR studies, tissues were collected at 1 and 3 hours following injection.

Ischemia-reperfusion model: Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described previously (Stevens *et al,* 2008). In brief, MCAO was performed in anesthetized mice (1.5-2% isoflurane) by threading a 7-0 silicon-coated nylon surgical filament (Docol, Redlands CA) through the external right carotid artery to the internal carotid artery, blocking

blood flow at the bifurcation into the MCA and anterior cerebral artery. Following 45 or 60 minutes of occlusion, the monofilament was removed and blood flow was restored. The duration of MCAO was optimized to obtain consistent baseline infarct sizes across studies between surgeons. Cerebral blood flow was monitored throughout the procedure by laser Doppler flowmetry (Transonic System Inc., Ithaca NY) and animals were excluded if blood flow was not reduced by 80% or greater during occlusion. Body temperature was maintained at 37°C during the surgery.

Evaluation of infarct size: Twenty-four hours following MCAO, mice were deeply anesthetized with isoflurane then perfused with ice-cold saline containing 2 U/ml sodium heparin. Brains with olfactory bulbs removed were sectioned into 1mm slices beginning from the rostral end, for a total of 7 slices. The infarct area was visualized by incubating the sections in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, St. Louis MO) in phosphate buffered saline (PBS) for 10 min at 37°C before transferring to 10% formalin (Sigma Aldrich). Sections were then imaged and the infarct area was measured using ImageJ software (NIH Image, Bethesda MD). Infarct volume was calculated using the indirect method [(contralateral live – ipsilateral live) / contralateral live * 100] to account for the effects of edema, and the final damage data are given as % damage of the contralateral hemisphere normalized to genotype-matched vehicle-treated controls.

Analysis of plasma cytokine levels: Mice were deeply anesthetized with isoflurane and blood was collected via cardiac puncture in sodium heparin. The blood was then centrifuged (2000 rpm for 20 minutes) and the plasma was removed and stored at -80°C. ELISA was used to quantify plasma TNFα (R&D Systems, Minneapolis MN). Samples were run in duplicate.

Tissue processing and quantitative real time PCR: Total RNA was isolated from the brain cortex using the Qiagen Rneasy Lipid Mini Kit (Qiagen, Valencia CA). RNA was reverse transcribed using an Omniscript Reverse Transcription kit (Qiagen). Quantitative PCR (qPCR) was performed using the TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad CA) on an ABI-prism 7700. Results were normalized to β-Actin expression and are represented as dCT values.

Statistical Analysis: Statistical testing was performed using Prism 5 software (GraphPad, LaJolla, CA). Values are given as group means ± standard error of the mean. Data were analyzed by Student's T-test, 1-way ANOVA or 2-way ANOVA, and Bonferroni's post-hoc test as denoted in figure legend. Significance was determined by a $p<0.05$.

Results:

Central CpG preconditioning is neuroprotective.

Our previous studies showed that CpG preconditioning provides direct protection to CNS cells in culture from an *in vitro* model of stroke and also when given systemically by either intraperitoneal or subcutaneous injection in an *in vivo* model of stroke (Stevens *et al,* 2008; Stevens *et al,* 2011). In this study we expanded on this finding by determining if directly targeting CNS cells via intranasal administration of CpG could lead to neuroprotection from stroke *in vivo*. Mice (C57Bl/6J) were preconditioned by either the intranasal route with CpG (3.2 mg/kg; n=8) or aCSF (n=3) or the subcutaneous route as a positive control with CpG (0.8 mg/kg; n=8) or saline (n=6) 72 hours prior to MCAO (60 minute). Infarct volume was significantly decreased in mice treated with CpG regardless of route of administration when compared to vehicle-treated controls (aCSF and saline groups had comparable infarcts and were combined for analysis; Figure 5-1). Therefore, similar to systemic administration of CpG, targeting the CNS directly via intranasal administration is protective in a mouse model of stroke.

Following intranasal administration some CpG may also reach cells outside of the CNS, therefore we investigated the peripheral response. TNFα is critical for CpG-induced neuroprotection; therefore, we determined whether intranasal

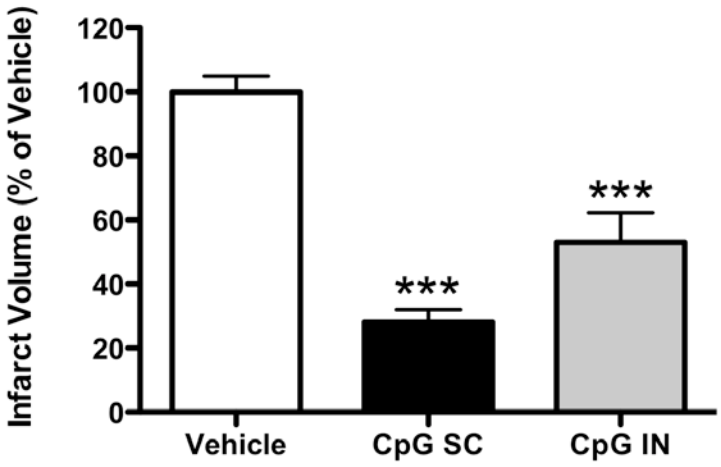


Figure 5-1: Central CpG preconditioning is neuroprotective.

Mice were preconditioned by either intranasal route with CpG (3.2 mg/kg; n=8) or aCSF (n=3) or subcutaneous route with CpG (0.8 mg/kg; n=8) or saline (n=6) 72 hours prior to 60 minute MCAO. Infarct volume was determined by TTC staining 24 hours following MCAO. The aCSF and saline groups had comparable infarcts and were combined for analysis. Preconditioning with intranasal administration of CpG reduced infarct damage following MCAO. Values are group means ± SEM; ***$p<0.001$ versus vehicle control by one-way ANOVA and Bonferroni post-hoc test.

administration of CpG, which is neuroprotective, induced systemic TNFα production. TNFα is detected in the plasma of mice administered with CpG subcutaneously. Plasma was collected 1, 3 or 6 hours following intranasal administration of CpG or vehicle. TNFα was not detected in the plasma of mice treated with CpG by intranasal administration at any time point (TNFα for all groups is less than the limit of detection for the assay, 23.4 pg/ml). This suggests that systemic TNFα is not critical for CpG-induced neuroprotection.

CpG preconditioning is TLR9-dependent.

While CpG is a known ligand for TLR9 and we have previously shown in culture that antagonizing TLR9 with the specific TLR9 antagonist ODN 2088, prevents CpG-mediated neuroprotection of cells from OGD-induced death (Stevens *et al,* 2008), a definitive role for TLR9 in CpG preconditioning *in vivo* had not been established. To define the role of TLR9 in CpG-induced protection, TLR9KO mice were preconditioned with CpG (1.6 mg/kg; s.c.) or saline 72 hours prior to MCAO (45 minute). Saline-treated TLR9KO mice had similarly sized infarcts (99.95 ± 14.47%; n=10) as CpG-treated TLR9KO mice (85.01 ± 13.42% of saline; n=9-10) (Figure 5-2; $p>0.05$). As expected, this indicates that neuroprotection from CpG preconditioning is dependent on TLR9 in the mouse MCAO model of stroke.

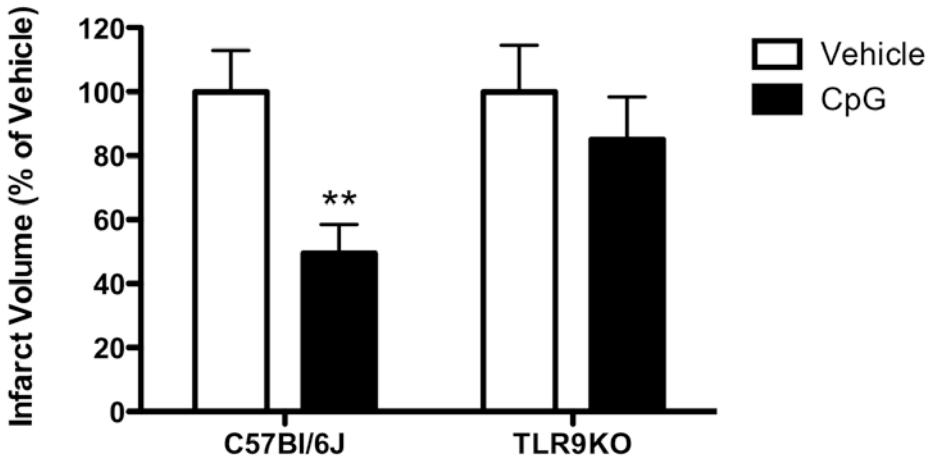


Figure 5-2: CpG preconditioning is TLR9-dependent.

C57Bl/6J or TLR9KO mice were preconditioned by subcutaneous route with CpG (0.8 mg/kg; n=11 C57Bl/6J & n=9 TLR9KO) or saline (n=5 C57Bl/6J & n=10 TLR9KO) 72 hours prior to 45 minute MCAO. Infarct volume was determined by TTC staining 24 hours following MCAO. Preconditioning with CpG reduced infarct damage following MCAO for C57Bl6/J mice but not for TLR9KO mice. Values are group means ± SEM; **p<0.01 versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

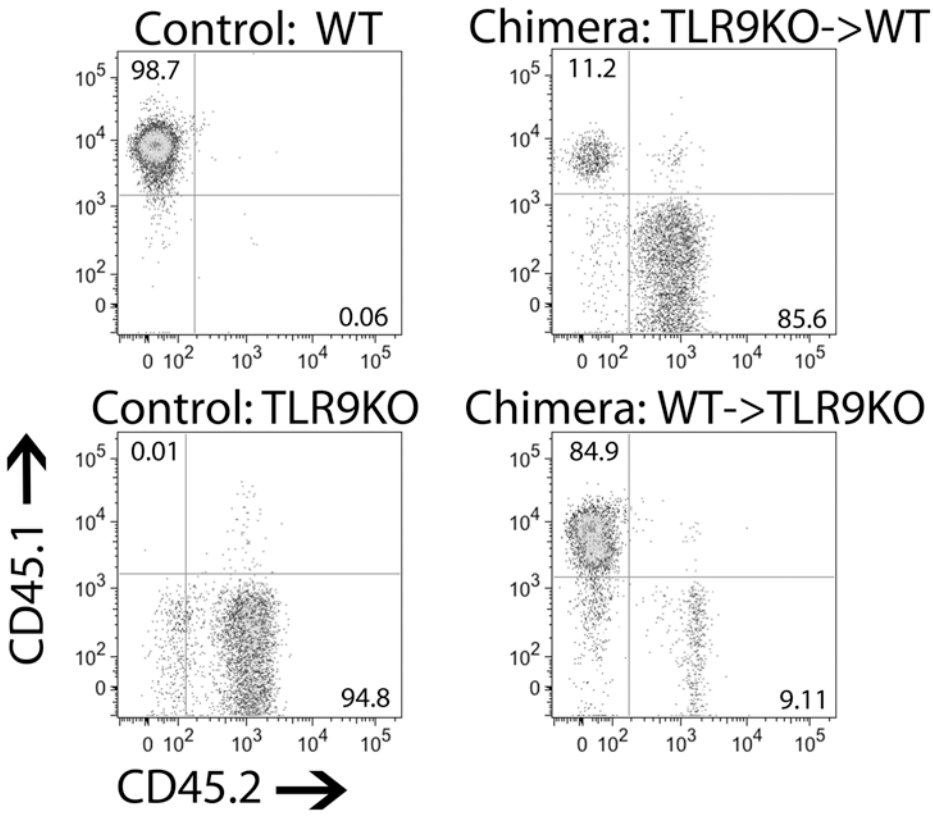


Figure 5-3: Confirmation of TLR9 reciprocal bone marrow chimeric mice generation

Different allelic forms of the CD45 leukocyte common antigen are used to distinguish WT cells (CD45.1 positive; Y-axis) and TLR9KO cells (CD45.2 positive; X-axis). Chimerism was confirmed by flow cytometry. Four to six weeks following bone marrow transfer, more than 80% of the hematopoietic cells were donor-derived in the chimeric mice. Representative plots for each group are shown.

Confirmation of TLR9 reciprocal bone marrow chimeric mice generation.

To test our hypothesis that hematopoietic expression of TLR9 is not required for CpG induced neuroprotection, we generated radiation-induced reciprocal bonemarrow chimeras between WT (WT; CD45.1 haplotype) and TLR9-deficient mice (TLR9KO; CD45.2 haplotype). We generated 3 groups of chimeric mice: 1) mice lacking TLR9 on hematopoietic cells (TLR9KO→WT), 2) mice lacking TLR9 on nonhematopoietic cells (WT→TLR9KO) and 3) control mice replete with TLR9 (WT→WT). To confirm chimerism, we used the CD45.1 (WT) and CD45.2 (TLR9KO) leukocyte common antigen to distinguish between donor and recipient cells by flow cytometry in circulating blood. Four to six weeks following bone marrow transfer, more than 80% of the hematopoietic cells were donor-derived in the chimeric mice (Figure 5-3), indicating successful generation of bone marrow chimeric mice.

Hematopoietic TLR9 is not required to elicit a peripheral TNFα response to CpG.

We have previously shown that CpG fails to protect TNFα-deficient mice against ischemic brain injury, illustrating the requirement for TNFα in this process (Stevens *et al,* 2008). Thus, in order to address the site of TLR9 activation that is required for CpG induced neuroprotection we began by identifying the effect of compartmentalizing TLR9 expression on the induction of TNFα. We first

examined whether TLR9 expression on hematopoietic cells was required for CpG-mediated induction of systemic TNFα using TLR9KO→WT chimeric mice, which lack TLR9 on hematopoietic cells. We found that TNFα was induced in response to CpG (1.6 mg/kg; s.c.) in the TLR9KO→WT mice (442.39 ± 63.8 pg/ml; n=8; Figure 5-4), demonstrating that hematopoietic expression of TLR9 is not required for plasma TNFα induction. However, the response was attenuated compared to data from WT→WT controls (821.44 ± 134.27 pg/ml; n=5; Figure 5-4), suggesting a contributing role for TLR9-expressing hematopoietic cells in the CpG-induced production of serum TNFα. In addition, we examined whether TLR9 expression on hematopoietic cells was sufficient to produce TNFα in response to CpG using WT→TLR9KO chimeric mice, which only express TLR9 on hematopoietic cells. We found that despite the presence of TLR9 on the hematopoietic cells the induction of TNFα was significantly inhibited in response to CpG (80.33 ± 27.08 pg/ml; n=5; Figure 5-4). Together, these data suggests that parenchymal TLR9 is a source for plasma TNFα, however, both hematopoietic and parenchymal TLR9 expression is necessary for optimal induction of plasma TNFα. Of note, all chimeras induced TNFα levels that were equivalent to WT mice in response to the TLR4 ligand, LPS (1 mg/kg), demonstrating that the differences were specific to the CpG-mediated TLR9-driven TNFα response.

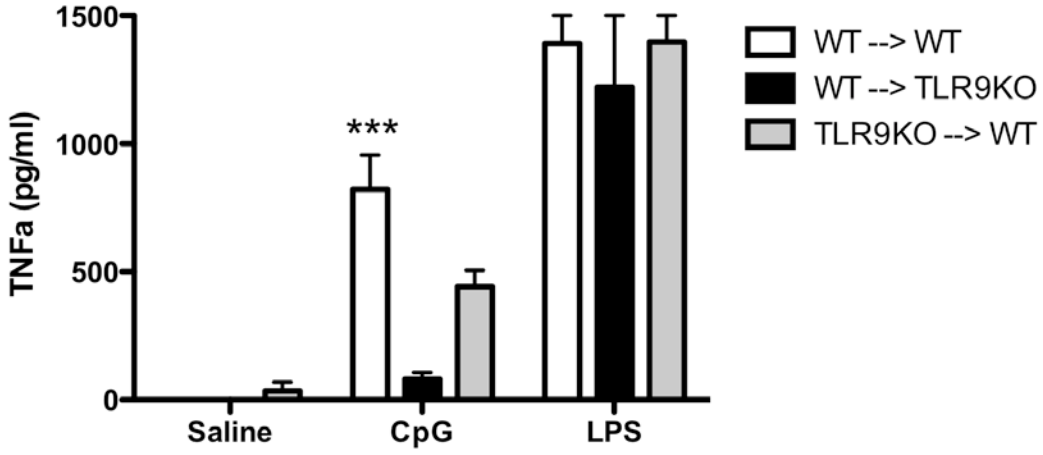


Figure 5-4: Hematopoietic TLR9 is not required to elicit a peripheral TNFα response to CpG

TLR9KO→WT, WT→TLR9KO, and WT→WT mice were injected subcutaneously with either CpG (1.6 mg/kg), LPS (1 mg/kg) or vehicle (n=4-8/group). TNFα was measured by ELISA in plasma samples collected 1 hour following injection. TLR9KO chimeric mice had decreased TNFα response following CpG administration but similar responses to LPS. Values are group means ± SEM; ***p<0.001 WT control versus chimeras by two-way ANOVA and Bonferroni post-hoc test.

CpG induction of brain TNFα requires TLR9 expression in both the hematopoietic and parenchymal compartments.

Pretreatment with TNFα administered directly into the brain is sufficient to induce ischemic neuroprotection (Nawashiro *et al,* 1997c). Further, induction of TNFα expression in the brain following LPS administration is not dependent on systemic TLR4 expression (Chakravarty and Herkenham, 2005). Therefore, we also examined TNFα expression in the brains of mice lacking TLR9 either on hematopoietic cells (TLRKO→WT) or parenchymal cells (WT→TLR9KO), 3 hours following CpG (1.6 mg/kg; s.c.) or saline administration. We found that TNFα mRNA was increased in WT→WT control mice in response to systemically administered CpG (13.9 ± 0.4 dCT; n=4; Table 5-1) while gene induction was abolished in both the TLR9KO→WT and WT→TLR9KO mice (not detected, n=6/group; Table 5-1). There was also no TNFα expression at 1 hour following drug administration in TLR9KO chimeric mice. Of note, TLR9KO chimeras induced TNFα mRNA in response to the TLR4 ligand, LPS (1 mg/ml; s.c.), again demonstrating that the differences observed were specific to the CpG-mediated TLR9-driven response. The fact that both TLR9KO→WT and WT→TLR9KO failed to induce TNFα in response to CpG suggests that TLR9 expression is required on cells in both compartments in order to induce TNFα expression in the brain. These results strongly suggest that interplay between multiple cell types is responsible for CpG-mediated TNFα expression in the brain.

Table 5-1: CpG induction of brain TNFα requires TLR9 expression in both the hematopoietic and parenchymal compartments

CHIMERA	UNHANDLED	CPG	LPS
WT	ND	13.8 ± 0.6	12.6 ± 0.9
WT→WT	ND	14.1 ± 0.2	
WT→TLR9 KO	ND	ND	13.2 ± 0.7
TLR9KO→WT	ND	ND	12.1 ± 0.04
values calculated dCT ND=Not Detected			n=4-6/group

TLR9 is required in both the hematopoietic and parenchymal compartments for CpG-induced neuroprotection against brain ischemia.

To determine if TLR9 expression in the hematopoietic compartment is required for the induction of CpG-induced neuroprotection, chimeric mice were preconditioned with CpG (1.6 mg/kg; s.c.) or saline 72 hours prior to MCAO, and ischemic damage was evaluated 24 hours following occlusion. As expected, WT→WT mice given CpG were significantly protected from brain injury when compared to saline-treated controls (65.66 ± 8.34% of saline; n=11/group; Figure 5-5). However, CpG preconditioning failed to significantly reduce infarct size in either the WT→TLR9KO (90.29 ± 6.82% of saline; n=18 & 16; Figure 5-5) or the TLR9KO→WT mice (91.71 ± 4.63% of saline; n=9 & 9; Figure 5-5). To confirm that the response was specific for TLR9 activation, we preconditioned WT→TLR9KO and the TLR9KO→WT chimeric mice with the TLR4 ligand LPS (1 mg/kg) and achieved significant protection in both cases (70.01 ± 12.96% of saline and 33.92 ± 11.19% of saline respectively; n=6-7/group; $p<0.05$). Thus, a lack of TLR9 in either the parenchymal or the hematopoietic compartments corresponded specifically with the loss of neuroprotection observed following CpG preconditioning. Together, these data suggest that for ischemic neuroprotection to be induced by CpG preconditioning, TLR9 on cells within both compartments is required, and that TLR9 in only one individual compartment is not sufficient.

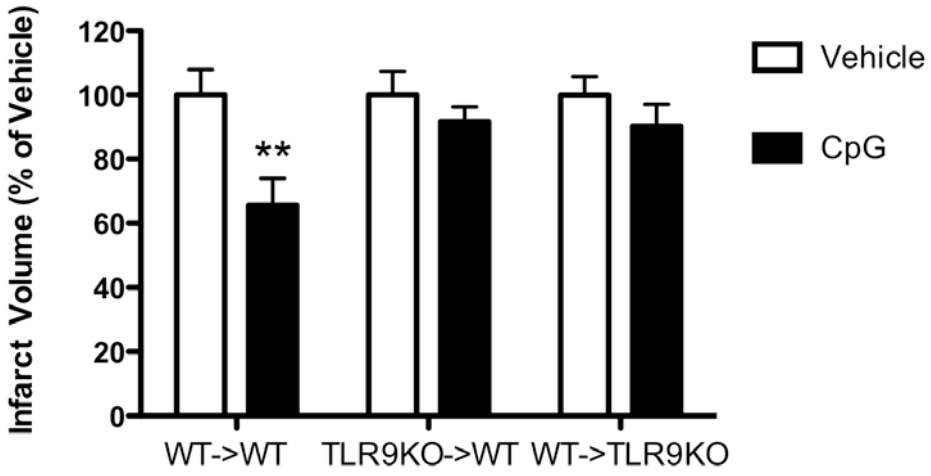


Figure 5-5: TLR9 is required in both the hematopoietic and parenchymal compartments for CpG-induced neuroprotection against brain ischemia. TLR9KO→WT, WT→TLR9KO, and WT→WT mice were injected subcutaneously with CpG (1.6mg/kg; s.c.) or saline 72 hours prior to MCAO (n=9-18/group), and ischemic damage was evaluated by TTC staining 24 hours following occlusion. CpG preconditioning of TLR9KO chimeric mice did not reduce infarct volume following MCAO. Values are group means ± SEM; **p<0.01, versus vehicle control by two-way ANOVA and Bonferroni post-hoc test.

Discussion:

Preconditioning with systemic administration of the TLR9 ligand CpG induces robust neuroprotection against subsequent cerebral ischemia. There are many target cell populations that could potentially initiate CpG-mediated preconditioning as TLR9 is expressed on a wide variety of cell types both in the brain and the periphery. We first confirmed that TLR9 is the major target of CpG whereby TLR9-deficient mice preconditioned with CpG were not protected against stroke. To begin to isolate the potential site of action *in vivo*, we administered CpG intranasally to target the cells of the CNS. We show that intranasal CpG preconditioning significantly reduced infarct volume in response to stroke despite the absence of a systemic TNFα response, demonstrating that neuroprotection can be manifested in mice via the cells of the CNS. This suggests that central stimulation of TLR9 by CpG is sufficient for preconditioning-induced neuroprotection and that activation of the hematopoietic compartment may be unnecessary. This is consistent with our previous work *in vitro* demonstrating that CpG preconditioning of primary cortical cell cultures, in the absence of hematopoietic cells, are protected against ischemic damage induced by oxygen-glucose deprivation (Stevens *et al,* 2008), However, systemic administration has also demonstrated robust neuroprotective action against stroke (Stevens *et al,* 2008). In a system where both hematopoietic and CNS cells have the potential to respond to CpG, the relevant contribution of each cellular compartment is unclear. To address this, we created TLR9 bone marrow

chimeric mice to evaluate the role of TLR9-expressing hematopoietic cells or parenchymal cells on the production of TNFα, a necessary effector of CpG preconditioning, and on the induction of neuroprotection against stroke.

CpG preconditioning does not protect TNFα-deficient mice, indicating that the cytokine TNFα is required for CpG neuroprotection (Stevens *et al,* 2008). Previous research demonstrates that TNFα is secreted into the plasma of mice systemically treated with CpG (Jack *et al,* 2005; Qi *et al,* 2011; Stevens *et al,* 2008). Using TLR9KO chimeric mice, we found that plasma TNFα levels were greatly reduced in WT→TLR9KO mice treated with CpG compared with WT→WT controls, indicating that hematopoietic cells are not the direct source of TNFα in the plasma. This finding is surprising given that multiple hematopoietic cells express TLR9 and are known to produce TNFα, such as macrophages and dendritic cells (Nierkens *et al,* 2011; Talati *et al,* 2008). The attenuated TNFα response in WT→TLR9KO mice implies that parenchymal cells may be the primary source of plasma TNFα induction following CpG treatment. In fact, TLR9KO→WT mice, which have WT parenchymal cells, had increased levels of plasma TNFα in response to CpG administration, further implicating the parenchymal cells as the major source of TNFα. In a similar study Longhi et al. (2009) administered poly-IC to poly-IC receptor chimeric mice and also found that stimulation of parenchymal cells, but not hematopoietic cells, was responsible for the production of type-1 IFN production, again despite the fact that dendritic cells and monocytes produce type-1 IFN in response to poly-IC.

This suggests that there may be a common dominant role for systemic cytokine induction by parenchymal cells. In TLR9KO→WT mice, parenchymal cells that may express TLR9 and could potentially produce TNFα include endothelial, epithelial, stromal, cardiac myocytes, Langerhans type of dendritic cell, neurons and glia (Constantin *et al,* 2004; El Kebir *et al,* 2009; Erridge *et al,* 2008; Jack *et al,* 2005; Knuefermann *et al,* 2007; Li *et al,* 2004; Martin-Armas *et al,* 2006; Merad *et al,* 2002; Qi *et al,* 2011; Tang *et al,* 2007). For example, cardiomyocytes stimulated with CpG produce TNFα in a TLR9-dependent manner (Knuefermann *et al,* 2007). Likewise, primary peripheral neurons express TLR9 and respond to CpG stimulation with the production of inflammatory cytokines IL-1α and IL-1β (Qi *et al,* 2011). CpG has been shown to activate endothelial cells (El Kebir *et al,* 2009) including the activation of NFκB and the induction of intercellular adhesion molecule-1 (ICAM-1) and E-selectin adhesion molecules. Thus, by affecting the state of the endothelium, CpG may indirectly affect the hematopoietic cells and other surrounding parenchymal cells.

Although TLR9KO→WT mice produced TNFα in response to CpG, the plasma TNFα concentration was attenuated compared to that observed in WT→WT mice treated with CpG. This suggests that although the parenchymal compartment is the primary source of TNFα in response to CpG, the hematopoietic compartment may enhance the systemic TNFα response, suggesting a critical interaction between these compartments. Since the endothelium serves as the major interface between these compartments, the necessity for both compartments

further implicates a potential role for the endothelium. El Kebir et al. (2009) demonstrated the importance of this interaction by showing that the number of neutrophils that adhere to the endothelium was significantly increased when both endothelial cells and neutrophils were treated with CpG versus when only the endothelium was treated with CpG. This indicates that TLR9 activation on both the hematopoietic and parenchymal compartments enhanced the inflammatory response following stimulation with CpG.

Previous work has shown that TNFα in the brain plays an important role in preconditioning (Nawashiro *et al,* 1997c; Rosenzweig *et al,* 2007), therefore we investigated whether CpG preconditioning induces TNFα in the brain. We show for the first time that systemic administration of CpG induces TNFα mRNA in the brain of C57Bl/6J and WT→WT mice. It has been previously published that this irradiation model does not compromise the innate immune response in the brain (Turrin *et al,* 2007) and the TNFα induction in the brain of WT→WT controls corroborates that chimeric mice generation does not interfere with the CpG response. We utilized the TLR9 bone marrow chimeric mice to determine the roles of the hematopoietic or parenchymal compartments in the production of TNFα in the brain in response to CpG. Interestingly, following CpG administration, induction of TNFα mRNA in the brain was abolished in both WT→TLR9KO and TLR9KO→WT mice. The lack of TNFα mRNA response in TLR9KO chimeric mice suggests that both hematopoietic and parenchymal cells

are required for systemically administered CpG to induce a TNFα response centrally.

Microglia, the resident immune cells of the brain, are often considered to be a major source of TNFα production in the brain. Microglia have a unique heterogeneous heritage where some microglia are derived from circulating monocytes while others are progeny of proliferating resident microglia (Lawson *et al,* 1992). In chimeric mice, monocyte-derived microglia have been identified in small numbers in the brains at 16-24 weeks of reconstitution but not at 4-12 weeks. In our study, we utilized chimeric mice after a brief 4-6 week reconstitution period; therefore, the microglia would be of recipient origin (Ajami *et al,* 2007; Matsumoto and Fujiwara, 1987; Nakano *et al,* 2001; Priller *et al,* 2001; Ransohoff and Perry, 2009; Vallieres and Sawchenko, 2003). Further, our finding that TNFα is not induced in either TLR9KO chimeric mouse suggests that any unikely extravasation of new microglia from the periphery appears inconsequential for our studies. The loss of brain TNFα induction in TLR9KO→WT mice, where microglia and other cells of the CNS would express TLR9, suggests that the induction of TNFα may not be a direct result of CNS TLR9 activation when CpG is administered systemically. Again, this points to the importance of indirect effects of CpG through other hematopoietic and parenchymal sources acting on the brain. The requirement of both hematopoietic and parenchymal cells for TNFα mRNA in the brain further supports the need for cooperation between these two compartments to respond to CpG.

The neuroprotective effect of systemic CpG preconditioning was eliminated in both forms of TLR9KO chimeric mice. Loss of protection in TLR9KO→WT mice indicates that TLR9-expressing hematopoietic cells are required for neuroprotection. The reciprocal WT→TLR9KO mice also failed to be protected by CpG preconditioning suggesting that TLR9-expressing hematopoietic cells alone are not sufficient for neuroprotection. Therefore, we conclude that TLR9 expression on hematopoietic cells is required but not sufficient for the induction of ischemic neuroprotection by CpG preconditioning. This again indicates that cell types in multiple compartments interact in response to the primary CpG signal to successfully limit ischemic damage in the mouse stroke model. A similar interaction of both hematopoietic and parenchymal cells was also reported in adjuvant studies using the immune modulator poly-IC. Reciprocal bone marrow chimeric mice created with poly-IC receptor knockout did not mount a primary T-cell adjuvant response to poly-IC (Longhi *et al,* 2009). In this case receptor expression was needed in both compartments to produce a poly-IC response equivalent to WT→WT controls. Similar to our findings with neuroprotection, both hematopoietic and parenchymal cells must respond to the stimulus and interact in some fashion to create the desired complex immune response that is responsible for creating the protective state.

We found that expression of TLR9 in both the hematopoietic and parenchymal compartments was required for the induction of TNFα in the brain following CpG treatment and for the induction of neuroprotection against stroke. Therefore, the

loss of TNFα in the brain following CpG administration corresponds to the loss of neuroprotection. Systemic levels of TNFα did not demonstrate the same correlation with neuroprotection, as we observed detectable levels of systemic TNFα in TLR9KO→WT mice, yet these mice were not protected. Additionally we did not detect TNFα systemically in mice administered CpG intranasally, however, those mice were protected. This finding is consistent with earlier work on TNFα preconditioning, which concluded that central, but not peripheral, TNFα preconditioning induced neuroprotection (Nawashiro *et al,* 1997c). Additionally, in the context of LPS-mediated preconditioning in cortical cells, the TNFα neutralizing antibody blocked the neuroprotective effects of LPS against oxygen glucose deprivation (Rosenzweig *et al,* 2007). Mechanistically, TNFα may potentially play a role in neuroprotection through initiation of TLR tolerance – a process by which TLR signaling is reprogrammed to produce protective effects against a damaging stimulus. We have previously shown that CpG induces TLR signaling in the brain following preconditioning and that the response to subsequent stroke is reprogrammed to produce an IRF driven response (Stevens *et al,* 2011). In TLR tolerance, TNFα induction following preconditioning is required to produce the protective response that is also characterized by significant IRF activation (Biswas and Lopez-Collazo, 2009). Thus, while TNFα may be induced systemically, the primary role of TNFα may be in the brain to initiate TLR tolerance in response to stroke. This is especially important since TLRs are activated following stroke by endogenous ligands in a manner that

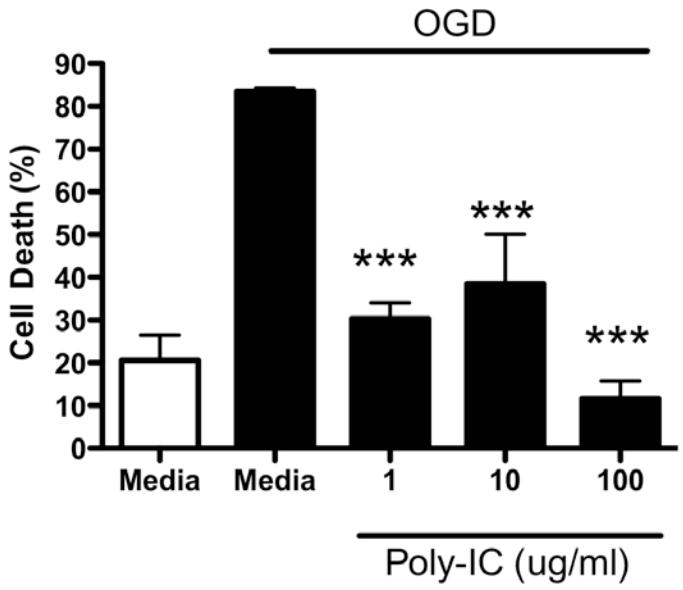
exacerbates damage. Thus, reprogramming the response of TLRs would be a critical factor in reducing stroke-induced damage.

Our current results indicate that the neuroprotective effects of CpG stem from cellular responses in hematopoietic and parenchymal compartments. While the hematopoietic response is required to produce the neuroprotective effects, activation of the hematopoietic cells alone is not sufficient to produce neuroprotection against stroke. Further, our results suggest a cooperative role for hematopoietic and parenchymal cells to induce TNFα in the brain. The loss of TNFα induction in the brain correlated with the loss of neuroprotection, emphasizing that TNFα in the brain may be critical to the production of neuroprotection in the setting of CpG-mediated preconditioning. Taken together, these data strongly implicate synergistic activity of TLR9-expressing cells from both hematopoietic and parenchymal compartments following CpG stimulation in order to induce TNFα-mediated neuroprotection against stroke.

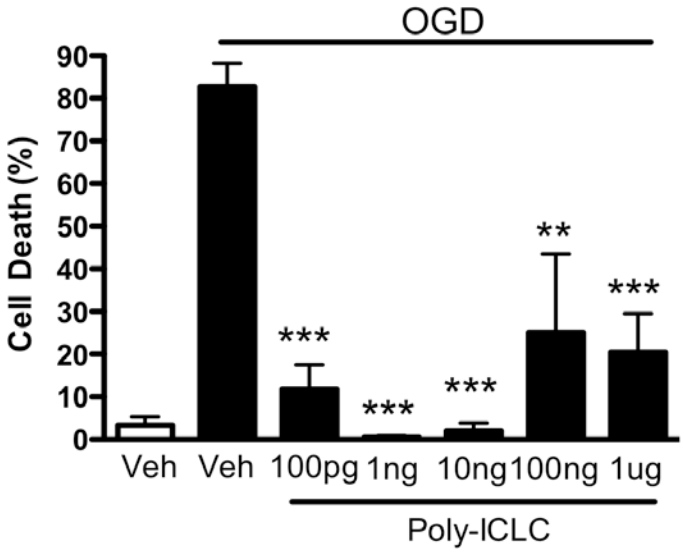
Appendix:

Preconditioning is a powerful method to reduce ischemic damage that may provide prophylactic protection for patients at risk of stroke. However, acute neuroprotective treatment that could be delivered for an extended time window following stroke would potentially benefit an enormous number of stroke patients. Therefore, we sought to apply what we have learned from preconditioning studies to find an acute neuroprotective treatment. Poly-ICLC treatment induces a robust type-1 IFN response and promotes the expression of IFN-related genes. These IFN-related genes include those genes of the IFN fingerprint that was identified as a common genetic signature of LPS, CpG and brief ischemia preconditioning. We hypothesized that acute poly-ICLC treatment following stroke would be neuroprotective due to the ability of poly-ICLC to induce a protective IFN related response. We first tested this hypothesis *in vitro* and found that acute treatment with poly-IC or poly-ICLC of primary mixed cortical cultures following OGD was neuroprotective (Appendix 1 & 2). However, we have not been able to replicate this neuroprotective effect in our MCAO model. Both poly-IC and poly-ICLC given by subcutaneous administration following MCAO do not confer significant neuroprotection against ischemic damage (Appendix 3 & 4). In order to better mimic our *in vitro* model of neuroprotection we also delivered poly-ICLC to the brain directly using intracerebral ventricular injection or intranasal administration following MCAO. Nevertheless, we did not observe neuroprotection with either of these routes of administration (Appendix 5

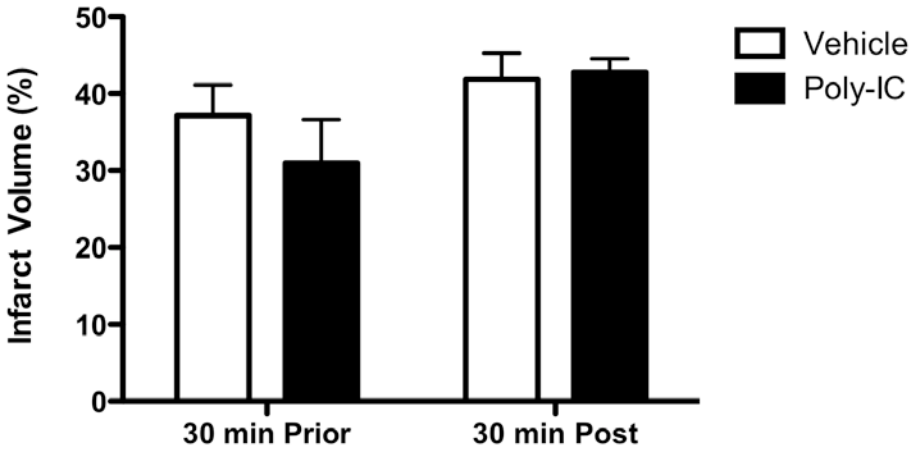
& 6). Future studies may be able to vary the timing of acute administration of poly-ICLC or broaden the range of doses poly-ICLC in order to further test our hypothesis that acute poly-ICLC treatment is neuroprotective.



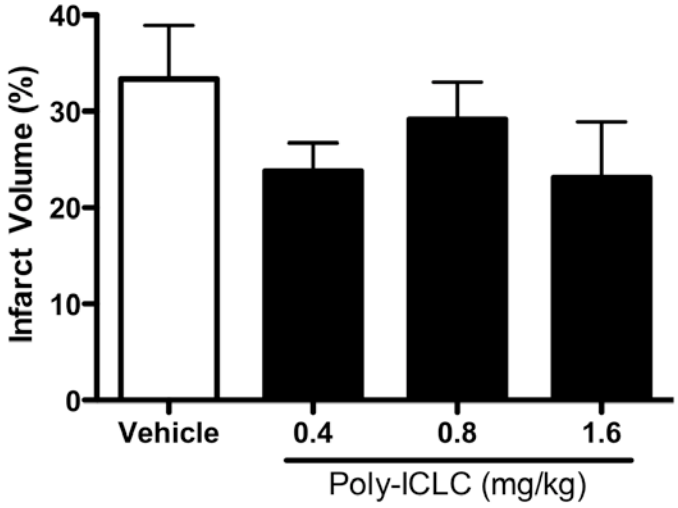
Appendix 1: Poly-IC acute treatment is neuroprotective in modeled ischemia *in vitro*. Mixed cortical cultures were treated with poly-IC (1-100 ug/ml) following 3 hour OGD. Cell death was determined by PI staining 24 hours following OGD. A representative example of 3 independent experiments is shown. Values are group means ± SEM; ***p<0.001 versus media control by ANOVA and Bonferroni post-hoc test. This figure was published previously (Marsh *et al* 2009a).



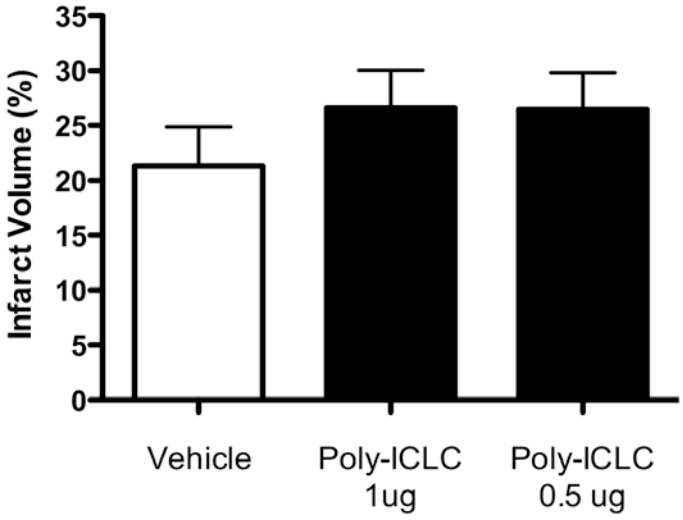
Appendix 2: Poly-ICLC acute treatment is neuroprotective in modeled ischemia *in vitro*. Mixed cortical cultures were treated with poly-ICLC (100 pg-1ug/ml) or vehicle following 3 hour OGD. Cell death was determined by PI staining 24 hours following OGD. A representative example of 2 independent experiments is shown. Values are group means ± SEM; **p<0.01, ***p<0.001 versus vehicle control by ANOVA and Bonferroni post-hoc test.



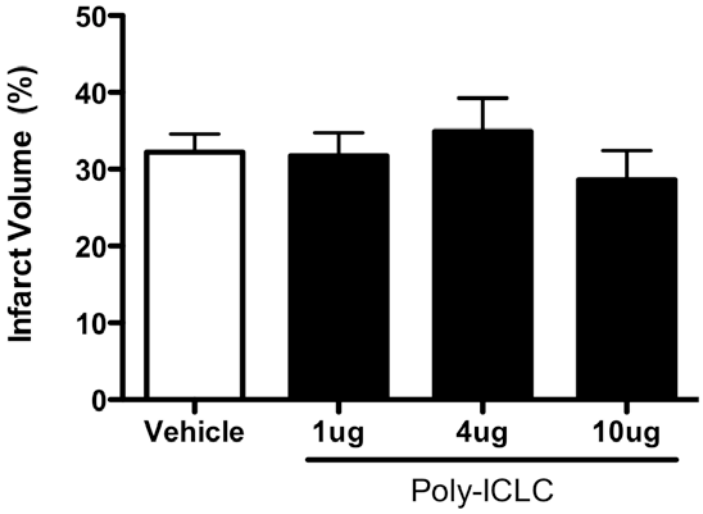
Appendix 3: C57Bl/6J mice were treated by subcutaneous route with poly-IC (1 mg/kg) or saline 30 minutes prior to 45 minute MCAO or 30 minutes post 45 minute MCAO (n=9-10/group). Infarct volume was determined by TTC staining 24 hours following MCAO. Acute treatment with poly-IC did not change infarct damage following MCAO. Values are group means ± SEM; $p>0.05$ versus vehicle control by two-way ANOVA.



Appendix 4: C57Bl/6J mice were treated by subcutaneous route with poly-ICLC (0.4-1.6 mg/kg) or vehicle 30 minutes following 45 minute MCAO (n=5-10/group). Infarct volume was determined by TTC staining 24 hours following MCAO. Acute treatment with poly-ICLC did not change infarct damage following MCAO. Values are group means ± SEM; $p>0.05$ versus vehicle control by one-way ANOVA.



Appendix 5: C57Bl/6J mice were treated by intracerebral ventricular injection with poly-ICLC (0.5-1ug in 2ul total volume) or vehicle approximately 20-30 minutes following 45 minute MCAO (n=11-12/group). Infarct volume was determined by TTC staining 24 hours following MCAO. Acute treatment with poly-ICLC did not change infarct damage following MCAO. Values are group means ± SEM; $p>0.05$ versus vehicle control by one-way ANOVA.



Appendix 6: C57Bl/6J mice were treated by intranasal administration with poly-ICLC (1-10ug in 5 ul total volume) or vehicle approximately 20-30 minutes following 45 minute MCAO (n=11-12/group). Infarct volume was determined by TTC staining 24 hours following MCAO. Acute treatment with poly-ICLC did not change infarct damage following MCAO. Values are group means ± SEM; $p>0.05$ versus vehicle control by one-way ANOVA.

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