THE ROLE OF INFLAMMATION IN THE NEUROPROTECTIVE EFFECTS OF LPS PRECONDITIONING AGAINST STROKE

By

Holly Lallman Rosenzweig

A Doctoral Dissertation

Presented to the Department of Molecular Microbiology and Immunology

and the Oregon Health & Science University

School of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

December 2005

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Holly L. Rosenzweig

has been approved

[redacted]
Mentor/Advisor

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

TABLE OF CONTENTS

List of Tables

Chapter 2

Chapter 3

Appendix

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Abbreviations

AP-1	activator protein-1
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma *Xenopus laevis*
CINC	cytokine-induced neutrophil chemoattractant
COX-2	cyclooxygenase-2
CNS	central nervous system
DPL	diphosphoryl lipid A
FADD	fas-associated death domain
IAP	inhibitor of apoptosis protein
ICAM	intracellular adhesion molecule
IFN	interferon
IκB	inhibitor of I-kappa B
IKK	IκB-kinase
IL	interleukin
IRAK	IL-1 receptor associated kinases
IRF3	interferon regulatory factor 3
JNK	c-Jun NH2-terminase kinase
KC	keratinocyte chemoattractant
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein
MMP	matrix metalloprotease
MnSOD	manganese-superoxide dismutase
MyD88	myeloid differentiation protein 88
NADPH	nicotinamide adenine dinucleotide phosphate dehydrogenase
NF-κB	nuclear factor kappa-B
NO	nitric oxide
NOS	nitric oxide synthase
OGD	oxygen-glucose-deprivation
RIP	receptor-interacting protein
ROS	reactive oxygen species
s-TNFR1	soluble-TNFR1
SHIP1	SH2-containing inositol-5'-phosphatase 1
SOCS-1	suppressor of cytokine signaling
SOD	superoxide dismutase activity
TACE	TNFα converting enzyme

TAK-1	transforming growth factor-B-activated kinase-1
TBK1	TRAF-family member associated NF-κB activator-binding kinase 1
TGF-β	tumor growth factor-beta
TIA	transient ischemic attack
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNFα	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll receptor-associated activator of interferon
VCAM	vascular cell adhesion molecule
WBC	white blood cells

Acknowledgements

I would first like to thank my mentor Mary Stenzel-Poore, who taught me to continually strive to improve my work and to become a better scientist. Without her excellent training, I would not be the scientist that I am today. Likewise, I would like to thank Roger Simon as my "mentor in-law," who challenged me to think more critically about my own work. Thank you also to my committee members, James Rosenbaum, Jay Nelson and Magdalene So for their helpful comments throughout my graduate work.

I have come to know so many wonderful people in the Stenzel-Poore and Simon laboratories over the past several years. I appreciate getting to know all of them and am thankful for their support. I would especially like to thank Susan Murray who is an inspiration and role model for me both in and out of lab. I cannot express enough gratitude toward Sarah Coste and Susan Stevens who in their own special ways have taught me to become a better person and scientist.

Last, but certainly not least, I would especially like to thank my husband, Jesse, for his unfaltering love and support. I always knew he would be there for me, no matter what happened. He has taught me to see the big picture and to never lose my humor about life. I would also like to thank my entire family for their continual love and support. I am so fortunate to be surrounded by such wonderful and amazing people.

Preface

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

Chapter 2 is a manuscript as it appears in the original paper published in the journal *Stroke*.[1] Chapter 3 is a manuscript as it has been prepared for publication and submission to *The Journal of Neuroscience*. Chapter 4 pertains to data in preparation for a paper for *Brain Research*. The appendix contains additional data regarding the neuroprotective effects of LPS preconditioning in mice, including the effects of LPS preconditioning on physiological parameters and on peripheral cellular changes.

Abstract

Treatment with a small dose of lipopolysaccharide (LPS) prior to stroke, termed LPS preconditioning, induces marked neuroprotection against damage caused by subsequent stroke. The cellular and molecular events involved in the neuroprotective effects of LPS preconditioning remain poorly understood. Activation of inflammatory cascades and production of cytokines such as tumor necrosis factor (TNFα) appear to be important in the establishment of LPS-induced neuroprotection, although their roles have not been well defined. Regulation of inflammatory responses during stroke could be a crucial component of neuroprotection, as it has long been appreciated that excessive inflammation triggered by stroke exacerbates ischemic brain damage. I hypothesized that LPS preconditioning induces neuroprotection against stroke by suppression of injurious inflammatory responses to ischemic injury.

To investigate this hypothesis, the optimal neuroprotective conditions of LPS preconditioning were established in a mouse model of stroke. Neuroprotection in mice that have been preconditioned by LPS occurred within one day of systemic LPS administration and lasted for at least one week. LPS preconditioning resulted in diminished peripheral cellular infiltration in the brain and decreased accumulation in the blood following stroke. Furthermore, LPS preconditioning suppressed the cellular activation of microglia in the brain and monocytes in the blood in response to stroke. These findings indicate that attenuation of cellular inflammatory responses that would

otherwise exacerbate ischemic brain damage could be involved in the neuroprotective effects of LPS preconditioning.

TNFα, which exacerbates neuronal damage, is a prominent inflammatory cytokine induced during stroke. Paradoxically, in the setting of preconditioning, TNFα plays a neuroprotective role. This apparent discrepancy has hindered the formulation of a clear understanding of the role of TNFα in the neuroprotective effects of LPS preconditioning. Thus, the role for TNFα in LPS-induced neuroprotection was further defined using TNFα knock-out mice. TNFα knock-out mice preconditioned with LPS did not show neuroprotection against stroke, which indicates that TNFα plays an essential role in the neuroprotective effects of LPS preconditioning. In contrast to the protective role for TNFα in the establishment of neuroprotection by LPS preconditioning, *following* stroke the cytotoxic effects of TNFα were reduced—a feature that could contribute to LPS-induced neuroprotection. LPS preconditioning suppressed the production of TNFα and neuronal expression of TNF-receptor (TNFR1) and the signaling mediator TNFR-associated death domain (TRADD) following stroke. Moreover, LPS preconditioned mice showed increased levels of soluble-TNFR1 following stroke, which could further neutralize TNFα-mediated injury during ischemic injury. Finally, mice that were preconditioned by LPS showed marked resistance to brain damage caused by intracranial administration of exogenous TNFα following stroke. The capacity of LPS preconditioning to diminish the neuronal sensitivity to TNFα-induced injury was also evinced in studies *in vitro*, as cortical neuronal cultures preconditioned by LPS were resistant to increasing cytotoxic doses of TNFα in the setting of ischemia. These studies

reveal dual functions for TNFα in the neuroprotective process of LPS preconditioning. That is, TNFα mediates the establishment of LPS-induced neuroprotection, while also mitigating TNFα-induced damage initiated by stroke. These studies advance the hypothesis that LPS preconditioning confers neuroprotection against stroke by suppression of injurious inflammatory responses that would otherwise exacerbate ischemic brain damage.

CHAPTER 1

INTRODUCTION

1. Significance and Rationale

Stroke is the third leading cause of death in developed countries and is our nation's leading cause of long-term disability. Of the 700,000 people who are afflicted by stroke each year, 163,000 die annually. The actual number of strokes is predicted to increase to over one million people per year by 2050. Moreover, 4.5 million survivors of stroke are alive today and as many as 30% are permanently disabled and 20% require institutional care. As such, health care costs for this disease exceed 57 billion dollars annually.[2-5]

There is an obvious need for therapeutics for stroke, including neuroprotective therapy prior to the ischemic event. For example, more than a third of patients who experience a transient ischemic attack (TIA), which is a mini-stroke that does not produce any lasting damage, will have a stroke within one year. This patient population comprises 30% of the stroke incidence each year.[2] In addition, of the 336,000 patients that undergo coronary artery bypass surgery each year 50% suffer permanent cognitive decline due to intraoperative emboli that cause strokes.[6] Treating such high-risk patient populations could reduce brain damage caused by stroke and is the motivation of this research.

The current therapeutic strategies for acute stroke offer little promise for the majority of stroke victims. The only effective treatment is recombinant tissue plasminogen activator or t-PA, also referred to as 'clot buster'[7]which is a thrombolytic therapy that essentially dissolves the clot and restores blood flow to the brain. However, t-PA treatment is only effective when administered within three hours of the occurrence of stroke.

Unfortunately, most stroke victims do not receive medical attention until much too late and miss the window of therapeutic efficacy of t-PA. As such, this treatment is limited to ~3% of all stroke patients.

Many of the stroke therapies that have failed in clinical trials are known to target early biochemical events in neuronal damage. These treatments include interventions that modify calcium ion channel function, glutamate-mediated excitotoxicity or oxygen radical effects to name a few.[8, 9] The logic that underlies these interventions has been questioned recently because patient treatment is most often initiated well after the activation of these biochemical events that cause neuronal injury. There is a greater emphasis placed on strategies that target biological events that occur later or have an effective time window that spans greater periods of time.

Preconditioning the brain against ischemic injury is a strategy that offers new promise in the prevention of damage from stroke. In the setting of preconditioning, the brain's own protective mechanisms are employed to limit ischemic injury and a state referred to as tolerance is established. Tolerance to ischemia in the brain can be induced by various preconditioning stimuli including brief ischemia, brief episodes of seizure, glutamate excitotoxicity, exposure to anesthetic inhalants and endotoxin (lipopolysaccharide, LPS).[10] The common theme of preconditioning is that a small dose of an otherwise harmful stimulus induces neuroprotection against subsequent injurious ischemic challenge. Understanding how preconditioning provides protection against ischemia may lead to the identification of therapeutic targets. In addition, preconditioning as a means

of therapy offers potential treatment for those individuals in whom brain ischemia is anticipated, such as during surgery of heart and brain or high-risk patient populations.

Preconditioning with LPS is a powerful means of protecting the brain against ischemic injury and is the focus of this thesis research. LPS is a component of gram-negative bacteria and is a potent immuno-modulator. Although inflammatory mediators such as TNFα have been implicated in the induction of ischemic tolerance against stroke, their roles are not defined. In my interest to understand how LPS preconditioning reduces cerebral ischemic injury, I considered that a related model of LPS preconditioning exists wherein tolerance to the otherwise toxic effects of endotoxin challenge can be induced by a prior low dose treatment with LPS. This process, referred to as endotoxin tolerance, is a protective state that is manifested by suppressed macrophage activation and a shift in the balance between inflammatory and anti-inflammatory cytokine production.[11, 12] Thus, I reasoned that certain protective features of endotoxin tolerance might also contribute to neuroprotection against stroke as a result of LPS preconditioning. In particular, I chose to focus on the idea that LPS preconditioning has the capacity to suppress subsequent injurious inflammatory responses to injury. Such an effect may be critical to neuroprotection against stroke, as cerebral ischemia triggers an inflammatory response that contributes to ischemic brain damage.

The hypothesis that underlies this thesis is that **LPS preconditioning induces neuroprotection against stroke by suppression of injurious inflammatory responses to ischemic injury.**

Based on this hypothesis, I postulated that LPS preconditioning might suppress two key aspects of the inflammatory response during stroke that are known to be deleterious to neuronal survival, as described below.

1) **LPS preconditioning suppresses the cellular inflammatory response to stroke.** Resident brain macrophages, referred to as microglia, are the primary immune cells of the brain and are rapidly activated by stroke. In addition, recruitment of peripheral leukocytes such as neutrophils and monocytes in the ischemic brain occurs following stroke. Such cellular inflammatory responses are thought to contribute to ischemic brain damage due to their excessive production of inflammatory mediators that are neurotoxic. Thus, LPS preconditioning may limit cellular activation and/or diminish the composition of the cellular response in the brain and peripheral blood following stroke, which would be expected to be neuroprotective.

2) **LPS preconditioning suppresses the potential of TNFα, a cytokine known to mediate neuronal injury, to cause damage following stroke.** TNFα has been referred to as a double-edged sword. TNFα is known to be an essential component of LPS preconditioning *prior* to MCAO for ischemic tolerance. However, numerous studies

have shown that TNFα *during* ischemic injury contributes to neuronal death. Thus, I reasoned that the reduction in brain damage conferred by LPS preconditioning could involve attenuation of the cytotoxic effects of TNFα during stroke. I postulated that LPS preconditioning might suppress the potential of TNFα to induce injury by producing a state wherein neuronal cells are protected against TNFα-induced injury and/or by impairing activation of the TNFα signaling pathway through modulation of proximal signaling effectors, thereby providing neuroprotection in the setting of ischemia.

2. Cerebral Ischemic Injury

During stroke, cerebral ischemic injury occurs when local cerebral blood flow is obstructed, in most cases as a result of cerebral artery occlusion by an embolus or focal thrombosis. Restricted blood supply limits the delivery of oxygen and glucose and leads to profound impairment of energy production that is essential for cellular homeostasis and survival. With deficits in vital energy substrates, cells are no longer able to maintain ionic gradients across the cell membrane and membrane depolarization ensues, which leads to excitotoxic glutamate release and intracellular calcium overload. The accumulation of intracellular calcium triggers calcium-activated proteases and pro-oxidant enzymes that are cytotoxic. Additionally, loss of energy results in mitochondrial dysfunction, release of free radicals, protein and DNA damage, generation of inflammatory mediators and activation of caspase cascades that mediate cell death (refer to more detailed reviews of the many mechanisms investigated in stroke[13-17]).

Two distinct regions of injury within the ischemic territory of an occluded blood vessel are easily distinguished. In the center or core of the infarct, blood flow is severely reduced to less than 20% of normal. Here, energy depletion and ionic disruption are followed by cell death due to necrosis within hours. Between this terminally damaged core and normal brain tissue is the penumbra, an area in which cells suffer milder ischemic injury due to residual blood flow from collateral vessels. In this region, energy production is partially preserved and cell death progresses over several days.[13, 18] The delayed cell damage results from endogenous processes that are triggered in response to

the ischemic injury, such as inflammation and apoptosis. Thus, it is believed that targeting such injurious events that transpire within this later time window could salvage potentially viable cells within the penumbra.

3. The Cellular Inflammatory Response to Stroke

In general, inflammatory responses are designed to defend against toxic organisms and are also important for tissue regeneration, repair, and restoration of homeostasis and elimination of damaged cells following tissue damage. However, in the setting of stroke the brain is especially vulnerable to damage that might be caused by the very means that the immune system uses to promote tissue regeneration. The pronounced inflammatory response in the brain that ensues following stroke is considered detrimental to neurological function and viability. Thus, it is currently believed that targeting the cascade of inflammatory events initiated by cerebral ischemia could be a new strategy to spare ischemic brain damage caused by stroke.

Inflammatory cells and their production of mediators are important contributing factors to ischemic brain injury. Peripheral cellular infiltration in the brain is a well-documented inflammatory response to stroke. However, it is now become apparent that many inflammatory responses induced rapidly *within* the brain also contribute to the neuropathology of stroke. These include the activation of resident macrophages in the

brain, called microglia, and the expression and release of inflammatory mediators such as acute-phase proteins, eicosanoids, complement factors and cytokines (refer to more detailed reviews of inflammatory events involved in stroke[19-22]). The role of cytokines in particular is a rapidly growing area of stroke research.[23] Cytokines such as TNFα and IL-1β are produced by activated microglia and stimulate expression of chemokines and adhesion molecules that promote peripheral leukocyte infiltration. As such, alterations in the activation state and/or composition of inflammatory cells in the brain and their production of cytokines following stroke could influence the extent of ischemic brain damage. For the purpose of testing my hypothesis, I chose to focus on the effects of LPS preconditioning on microglia activation, peripheral cellular infiltration and the cytotoxic effects of TNFα following stroke.

3-1. The Role of Microglia in Stroke

Microglia, the resident macrophages of the brain, comprise approximately 20% of the total number of cells in healthy brain tissue.[24] Microglia are the principle immune effector cells of the central nervous system (CNS), where it is believed that they have both neuroprotective and neurotoxic effects. In healthy brain tissue microglia reside in a resting state, but in response to cerebral ischemic injury microglia become activated, proliferate and migrate to the site of neuronal damage.[24 25, 26]

The response of microglia to cerebral ischemia has been particularly well-studied in experimental models of stroke in rodents induced by middle cerebral artery occlusion (MCAO), wherein the microglial activation process has been characterized by morphological changes (amoeboid shape, hypertrophy), upregulation of the myeloid cell surface markers (CD11b, CD45, MHC II) and acquisition of a phagocytic phenotype at later phases.[27, 28] This activation process is initiated rapidly by ischemia, prior to any detectable neuronal death, and peaks between 18 and 24 hours following MCAO.[25, 27-30] Although the process of microglial activation is well documented, the mechanisms responsible for the initiation of microglial activation are still largely unknown. It has been suggested that early events initiated by cerebral ischemia that cause neuronal damage such as glutamate, cortical spreading depression (wave of membrane depolarization that spreads across the cortex) and TNFα could all be involved in microglial activation.[31-33]

As the predominant resident immune cell, microglia are viewed as key modulators of inflammation within the CNS. Activated microglia possess the ability to produce increased amounts of cytokines (TNFα, IL-1β, IL-6), chemokines (MCP-1, MIP-1α, MIP-2, MIP-1β), reactive oxygen and nitrogen species, enzymes (COX-2) and proteases.[28, 34-36] Thus, microglia are thought to contribute to ischemic damage through their excessive production of neurotoxic inflammatory mediators. As such, activated microglia play an important role in the immune responses during stroke that impact the pathological events leading to neuronal death.

Several lines of evidence support a pathological role for activated microglia in brain damage following stroke. Studies in rodent models of stroke have shown that inhibition of microglia activation by treatment with tetracyclines at the time of MCAO improves stroke outcome.[37] This is consistent with studies *in vitro* that also demonstrated inhibition of microglia activation by treatment with tetracyclines reduces neuronal damage in the setting of ischemia or glutamate toxicity.[38-40] Other studies *in vivo* have demonstrated the correlative relationship between reduced activation of microglia and improved ischemic outcome, as rodents made hypothermic showed decreased microglia activation and reduced ischemic injury.[41] In addition, stroke-prone spontaneously hypertensive rats (SHR), which have a genetically determined increased sensitivity to cerebral ischemic damage, show a significant increase in activated microglia in response to MCAO compared to normal rat strains.[42] Of note, naïve SHR rats also show an increase in the number of microglia compared to normal rats. Such an increase in microglia *prior* to stroke also supports a contributory link between microglia and ischemic damage. Recent studies *in vitro* have further established the capacity for activated microglia to directly mediate neuronal death, as medium from activated microglia is neurotoxic.[43, 44] The upregulation of FasL and release of TNFα are known to be mediators of microglial-induced neuronal death.[44-47] Furthermore, activated microglia have recently been implicated in the exacerbation of other types of neuronal insults such as glutamate-mediated neurotoxicity.[40, 48]

Although the deleterious role of activated microglia in stroke has attracted much attention, some functions of activated microglia have been proposed to be beneficial to

neuronal survival following stroke. Microglia can produce anti-inflammatory cytokines such as IL-10 and TGF-β that are known to be neuroprotective.[49-52] Moreover, their capacity for phagocytosis and removal of neuronal debris may promote the recovery process following stroke. In addition to these more classical roles for microglia, it was recently suggested that microglia might also function as glutamate scavengers.[53, 54] Our knowledge about the neuroprotective roles for microglia in ischemia and the factors that may determine the nature of such microglial responses is still limited. However, this dichotomy in microglial actions suggests that their strict regulation could be crucial to stroke outcome.

3-2. The Role of Peripheral Leukocytes in Stroke

In addition to microglia, peripheral leukocytes are also an component of the post-ischemic cellular inflammatory response. Peripheral leukocytes are not typically present in the healthy brain but infiltrate the ischemically compromised brain tissue following stroke, where they are thought to exacerbate neuronal damage. Leukocyte rolling, adherence and transendothelial migration into brain tissue requires specific receptor-ligand interactions between adhesion molecules expressed on the surfaces of leukocytes and endothelial cells.[20, 21] Once activated by stroke, leukocytes bind to adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) that are upregulated on endothelial cells, through CD11/CD18 integrin interactions. Within ischemically compromised brain tissue, leukocytes such as neutrophils and macrophages are thought to

directly participate in the ongoing neuronal pathogenesis through their production of neurotoxic molecules, including reactive oxygen and nitrogen species, proteases, cytokines and lipid-derived mediators, that are destructive to the compromised brain tissue.[55-59] In addition, leukocyte-endothelial interactions cause cerebral vasculature damage and thereby contribute indirectly to the extent of ischemic brain damage. Following stroke, activated neutrophils and monocytes in the blood form aggregates with circulating platelets. These aggregates adhere to the cerebral vasculature and cause capillary plugging, thrombosis and damage to the cerebral vasculature. This process results in reduced post-ischemic blood flow, termed hypoperfusion, and thereby prolonged ischemic injury of potentially viable tissue.[58, 60 61]

Many laboratories, including ours, have evaluated the temporal pattern of the peripheral cellular response in the brain following MCAO in rodents. The cellular response involves a relatively early presence of neutrophils in the ischemic brain hemisphere, which peaks between 24 and 48 hours following MCAO.[30, 55, 62, 63] The appearance of neutrophils in the brain parenchyma is preceded by adherence of neutrophils to the cerebral vasculature as early as 1 hour following cerebral ischemia.[60, 61, 63] Peripheral monocytes also infiltrate into the ischemic brain tissue and appear to peak between two and three days following MCAO.[55, 56] In the brain, the peripheral macrophage infiltrate is indistinguishable from resident activated microglia and together this collective "microglia-macrophage" population dominates the infarct region for weeks following MCAO.[64] T cells have also been shown to infiltrate the brain following stroke in a delayed fashion beginning at 3 to 4 days following MCAO.[30, 64] However, T cells are

considered relatively minor constituents of the cellular inflammatory response to stroke compared to the more well established role for neutrophils and monocytes.

Several lines of evidence support a deleterious role for peripheral leukocytes in ischemic brain damage. For example, increased leukocyte infiltration correlates with greater ischemic brain damage. Furthermore, depletion of leukocytes such as neutrophils at the time of MCAO reduces infarct size in rodents.[65, 66] More recent studies have shown that inhibition of cellular infiltration at the time of MCAO improves stroke outcome. These studies were performed by targeting chemokines (IL-8, CINC, MCP-1, MIP-1, MIP-2)[67-71] or adhesion molecules (ICAM-1, CD11b, P-selectin, E-selectin)[72-79] by pharmacological treatment with neutralizing antibodies or by the generation of knock-out mice. Inhibition of such chemokines and adhesion molecules blocked cellular infiltration in the brain following MCAO and reduced ischemic brain damage.

4. Cerebral Ischemic Tolerance

Ischemic tolerance has emerged as a powerful means of conferring neuroprotection against stroke. Experimental studies have demonstrated that a brief insult to the brain, that is not harmful by itself, results in an endogenous protective response in the brain against the subsequent damaging effects of stroke. Thus far, ischemic tolerance has been investigated in experimental models of stroke, however recent retrospective studies in

humans suggest that an analogous process occurs, wherein a previous transient ischemic attack confers a more favorable prognosis on subsequent stroke.[80, 81] Thus, it is believed that elucidating the molecular mechanisms responsible for neuroprotection in ischemic tolerance could lead to future discovery of therapeutic targets for acute treatment of stroke as well as preventative therapy when ischemia to the brain is anticipated.

Tolerance to cerebral ischemia can be induced by exposure to a variety of preconditioning stimuli, for example a brief episode of ischemia or epilepsy, cortical spreading depression, excitotoxic glutamate, inhaled anesthetics and inflammation induced by endotoxin.[82-87] Ischemic tolerance induced by such diverse preconditioning stimuli all share common underlying features. That is, ischemic tolerance induced by various preconditioning stimuli develops over time (greater than 1 day) and involves *de novo* synthesis of proteins thought to ultimately mediate the brain's increased resistance to the subsequent damaging effects of stroke. Although the mechanisms behind the formation of tolerance induced by these different preconditioning stimuli are largely unknown, a common theme is that a small dose of an otherwise harmful stimulus is protective against cerebral ischemic injury (as reviewed[10]). The research focus of this thesis was specifically on the neuroprotective effects induced by LPS preconditioning, which are discussed in more detail below.

5. Neuroprotection Induced by Endotoxin Preconditioning

LPS is a surface component of gram-negative bacteria and is a potent immuno-modulator. LPS induces its effects through the receptor, Toll-like receptor 4 (TLR4), which is expressed predominantly on immune cells throughout the periphery and CNS (refer to Section 6 in Chapter 1 for further detail on LPS and TLR4). Treatment with moderate to high doses of LPS stimulates a robust inflammatory response that can lead to lethal septic shock. However, administration of a low dose of LPS prior to stroke confers robust neuroprotection against ischemic brain damage.[88]

The underlying mechanisms of the tolerant effects of LPS preconditioning are poorly understood, although *de novo* protein synthesis and inflammatory responses appear to play beneficial roles. Using an experimental model of stroke, MCAO, it was shown that a prior systemic treatment with LPS confers neuroprotection against MCAO-induced injury in rats. LPS preconditioned rats showed a ~35% reduction in infarct compared to saline pretreated controls.[88, 89] These studies also demonstrated that LPS-induced ischemic tolerance develops over several days and requires *de novo* protein synthesis, as co-treatment with cyclohexamide (an inhibitor of translation) reversed LPS-induced neuroprotection against MCAO.[89] Activation of inflammatory cascades *prior* to stroke appear to play a central role in the establishment of tolerance. Treatment with anti-inflammatory drugs (dexamethasone or indomethacin) at the time of LPS administration blocks neuroprotection against MCAO in rats.[89] These findings support a protective role for LPS-induced inflammatory responses *prior* to stroke.

TNFα in particular has been identified as an important mediator of the protective response initiated by LPS preconditioning. This is supported by the finding that systemic administration of TNFα binding protein (chimeric form of the soluble TNFR1) given concurrently with LPS treatment blocked the neuroprotective effects of LPS preconditioning.[88] In addition, TNFα or ceramide, its downstream signaling mediator, can substitute for LPS preconditioning to induce tolerance to cerebral ischemia.[90, 91] Thus, proximal members of the TNFα pathway, such as TNFα and its receptors TNFR1 (p55) and TNFR2 (p75), as well as sphingomyelin-based second messengers such as ceramide, are likely mediators of the protective effects of LPS preconditioning. Beneficial roles provided by NO and superoxide dismutase (SOD) further support the critical involvement of inflammatory pathways in LPS preconditioning.[89, 92, 93]

Despite the identification of certain inflammatory mediators that play a role in priming the ischemic tolerant effect of LPS preconditioning, little is known about the cellular and molecular events that mediate the neuroprotective state during ischemic injury, or *following* stroke. The evidence to date suggests LPS preconditioning results in cerebrovascular protective effects following MCAO, as ischemic-induced hypoperfusion deficits as well as endothelial and smooth muscle dysfunction are diminished in LPS preconditioned rats in a delayed manner following stroke.[92, 94] Improved vascular function following stroke could contribute indirectly to the neuroprotective effects of LPS preconditioning by restoring blood flow following stroke. Alternatively, preconditioning induced by treatment with diphosphoryl lipid A (DPL), a derivative of the lipid A moiety of LPS, increases SOD activity *within the brain* and decreased

myeloperoxidase activity (marker for neutrophils and/or activated microglia) out to 24h following MCAO in rats—a critical time window for the evolution of cell death following stroke.[95] This finding suggests that enhanced endogenous anti-oxidant defense mechanisms and diminished inflammatory responses within the brain could be involved in the neuroprotective effects of DPL preconditioning. Thus, it is reasonable to speculate that the neuroprotective state induced by LPS preconditioning, like DPL preconditioning, could involve modulation of the inflammatory response following stroke.

6. LPS-Induced Inflammatory Responses and Endotoxin Tolerance

In my interest to understand the mechanisms by which LPS preconditioning induces tolerance to stroke, I considered that LPS preconditioning is even better known for its ability to protect against the otherwise toxic effects of large dose LPS—a phenomenon termed endotoxin tolerance. Thus, I reasoned that we might be able to improve our comprehension of the mechanisms of LPS-induced neuroprotection by a closer examination of how LPS modulates the immune system through activation of TLR4 and the underlying mechanisms involved in endotoxin tolerance.

6-1. Immune Recognition of Lipopolysaccharide

Lipopolysaccharide is an integral component of the outer membrane of gram-negative bacteria. LPS is a highly complex molecule with three main structural regions: a lipid (called lipid A), a core oligosaccharide and O-polysaccharide side chains of variable lengths **(Figure 1-1A)**. It is now known that the Lipid A portion of LPS **(Figure 1-1B)** is responsible for the biological actions of endotoxin through activation of TLR4.[96-98]

Immune recognition of pathogenic organisms represents an essential feature of host defense and is mediated primarily through the pattern recognition receptors **Toll-like receptors** (as reviewed[99]). Currently, eleven and thirteen TLR family members have been identified in humans and mice, respectively. The TLRs share an extracellular leucine rich repeat region and a common cytoplasmic Toll/interleukin-1 receptor (TIR) domain.[100] The TLRs themselves have evolved with the capacity to distinguish different structural motifs referred to as pathogen-associated molecular patterns, (PAMPs) from bacterial, viral and fungal organisms. Recently, TLRs have also been implicated in recognizing endogenous molecules related to host tissue injury such as heat shock proteins and extracellular matrix molecules (as reviewed[101, 102]). Activation of TLRs triggers the initial innate immune response that ultimately leads to inflammatory gene expression, elimination of the infectious agent and tissue repair.

TLR4 specifically responds to the LPS PAMP by triggering signaling pathways that lead to activation of the transcription factor NF-κB and expression of numerous inflammatory

genes. Although TLR4 is vital for the immune response to LPS, hyperactivation of TLR4 triggers excessive inflammation that causes adverse effects, such as septic shock. Thus, it is important that inflammation triggered by LPS be kept under tight control.

6-2. Toll-like Receptor 4

Cellular expression of TLR4

TLR4 mediates the cellular response to LPS in a wide variety of cell types throughout the periphery and brain. TLR4 is expressed predominantly on monocytes and macrophages in the periphery, which are considered the primary cellular mediator of inflammatory responses to systemically administered LPS. However, TLR4 is expressed on other immune cells such as neutrophils and lymphocytes,[103, 104] as well as non-classical immune cells such as endothelial, smooth muscle and epithelial cells.[105-108] It has recently become apparent that TLR4 is also expressed in the brain, where it localizes primarily to the resident immune cells such as microglia and astrocytes.[109-111 112, 113] Microglia and astrocytes differ in endogenous TLR4 expression levels and functional response to LPS. Microglia appear to have the highest levels of TLR4 and are more responsive to LPS than astrocytes.[109, 110] This is consistent with other studies that implicate microglia as the major LPS-responsive cell within the CNS.[109, 110, 112, 113]

TLR4-induced signaling response

The signaling response initiated by TLR4 has been studied in great detail (please refer to comprehensive reviews of the signaling pathways of TLR4[114-116]). Complete TLR4 activation in response to LPS involves the association of LPS and LPS-binding protein in the plasma and the subsequent interaction with the cofactor MD-2 and the glycoprotein CD14 which form a "receptor complex" on the cell surface.[117] This association results in TLR4 dimerization and recruitment of several adaptor molecules which couple TLR4 activation to downstream signaling cascades and activates transcription factors (**Figure 1-2**).

Myeloid differentiation protein 88 (MyD88) is a crucial adaptor molecule which mediates the most well established TLR4-induced signaling response that leads to activation of the transcription factor NF-κB. Upon TLR4 activation, MyD88 is recruited to, and interacts with, the TIR domain of TLR4. This interaction between MyD88 and TLR4 then allows the subsequent association and activation of the downstream family of kinases called IL-1 receptor associated kinase (IRAK) 1, 2 and 4. The IRAK kinases interact with MyD88 through death domains common to both proteins and initiate a phosphorylation cascade which involves the downstream kinases TNF-receptor associated factor (TRAF) 6, transforming growth factor-B activated kinase (TAK-1) and IκB-kinase (IKK). The latter phosphorylates IκB leading to its ubiquitination and proteolytic degradation which leads to translocation of NF-κB into the nucleus, as reviewed.[118] A well-described consequence of NF-κB activation is the induction of numerous inflammatory genes, including inflammatory cytokines (TNFα, IL-1β, IL-6), chemokines (IL-8, MCP-1, MIP-1α),

adhesion molecules (E-selectin, ICAM-1) and oxidative enzymes (COX-2, iNOS). Moreover, the MyD88-mediated signaling pathway has also been reported to involve mitogen-activated protein kinase (MAPK) kinases, including c-Jun NH2-terminase kinase (JNK) and p38 as well as activation of the transcription factor activator protein-1 (AP-1).

In addition to the core pathway for LPS signaling, which is mediated by MyD88, other adaptor molecules also play a role in the initiation of TLR4 signal transduction pathways (as reviewed[119]). These include the adaptor molecule Mal, which appears to contribute to the MyD88-mediated signaling pathway and activation of NF-κB. Other adaptor molecules include Toll receptor-associated activator of interferon (TRIF) and TRIF-related adaptor molecule (TRAM). TLR4 recruits and activates the adaptor molecules TRIF and TRAM independently of MyD88/Mal in order to initiate a signaling pathway leading to activation of the kinase, TRAF-family member associated NF-κB activator-binding kinase 1 (TBK1) and the transcription factor interferon regulator factor 3 (IRF3). IRF3 is known to participate in the transcriptional induction of IFNα and IFNβ. Thus, recruitment of diverse adaptors molecules and activation of different signaling pathways could mediate the array of inflammatory responses induced by activation of TLR4 in response to LPS.

6-3. Endotoxin Tolerance

Although activation of inflammatory responses through TLR4 is critical for host defense, excessive production of inflammatory mediators can itself be maladaptive as in the case of septic shock. A protective regulatory property of the immune response to LPS is the induction of endotoxin tolerance, wherein a prior exposure to non-toxic, low dose of LPS protects against re-exposure to an otherwise septic dose of LPS. The endotoxin tolerant state protects against LPS-induced mortality and excessive production of inflammatory cytokines (refer to more detailed reviews of endotoxin tolerance[12, 120]). Thus, it is believed that endotoxin tolerance represents an evolutionarily conserved protective mechanism against the deleterious effects of sepsis or persistent bacterial infection.

The classic endotoxin tolerance paradigm is "homologous tolerance," wherein LPS is both the trigger of tolerance and the challenge. However, endotoxin tolerance can also be induced by ligand-mediated activation of other TLRs by PAMPs distinct from LPS or by treatment with cytokines (TNFα, IL-1β, TGF-β, IL-10 and NO).[12] Tolerance induced through this latter process has been termed "cross-tolerance," as the priming stimulus differs from a subsequent challenge with LPS.[121]

Investigation of endotoxin tolerance modeled in macrophages *in vitro* has provided insight into the cellular and molecular mechanisms involved in endotoxin tolerance.[122, 123] These studies demonstrate that diminished macrophage activation is likely protective because, unlike naïve macrophages, macrophages previously primed by a low dose of

LPS *do not* produce inflammatory cytokines that would typically promote tissue damage during sepsis (TNFα, IL-6, IL-1β and IL-12) in response to subsequent LPS challenge. The inhibition of inflammatory cytokine production in endotoxin tolerant macrophages occurs at the transcriptional level and extends to other inflammatory genes such as chemokines (IL-8, IP-10, MIP-1α, MCP-1, MIP-2α, KC) and enzymes (COX-2).[11, 123, 124]

Importantly, endotoxin tolerance is not merely a global suppression of macrophage responsiveness, but rather a complex regulated response. This is supported by studies that show in response to LPS challenge, macrophages rendered tolerant by a prior exposure to a low dose of LPS produce increased amounts of anti-inflammatory mediators that are protective against sepsis (IL-10, IL-1R-antagonist), s-TNFR1 and suppressor of cytokine signaling 1 (SOCS-1)[11, 123, 125] As such, priming with a low dose of LPS changes the macrophage response to re-exposure to LPS with the outcome being a shift in the balance of inflammatory and anti-inflammatory mediators. This specific and adaptive protective response observed in endotoxin tolerance is called "reprogramming."[11]

The change in the macrophage response characteristic of endotoxin tolerance is thought to arise secondary to alterations in the TLR4 signal transduction pathway. It has been shown that diminished TLR4 signaling during endotoxin tolerance is achieved by disruption of NF-κB and AP-1 activation and increased expression of negative regulators of the TLR4 signaling pathway.[123] Attenuation of NF-κB and AP-1 is considered a contributing factor to the inability of endotoxin tolerant macrophages to produce

inflammatory cytokines. Several negative regulators of TLR4 have been identified that inhibit proximal signaling events induced by LPS in a negative feedback fashion (refer to detailed review of negative regulators of TLR4 signaling[126]). To date, the negative regulators identified as *necessary* effectors of endotoxin tolerance include ST2 (also called T1 or DER4), IL-1R-associated kinase-M (IRAK-M), SOCS-1 and SH2-containing inositol-5'-phosphatase 1 (SHIP1).[127-130] A crucial role for these negative feedback inhibitors in endotoxin tolerance is supported by studies in null mutant mice, which exhibit hyper-responsiveness to LPS as evidenced by elevated inflammatory cytokine production and increased mortality. Importantly, mice deficient in these crucial negative regulators are not protected against sepsis by a prior treatment with low dose LPS.

7. The Dual Role of TNFα in Stroke

In light of the pathological role of TNFα in ischemic brain damage, I postulated that LPS-induced neuroprotection could involve a reduction in the cytotoxic effects of TNFα *following* stroke. Intriguingly, TNFα has also been shown to play an essential role in the *induction* of tolerance to stroke by LPS preconditioning,[88] which supports a neuroprotective role for TNFα. Thus, two contrasting roles have been proposed for TNFα in cerebral ischemia. The effects of TNFα in both of these different scenarios will be discussed.

7-1. TNFα and its Receptors

The dual role of TNFα in stroke is poorly understood perhaps due to the complexity of the TNFα-signaling network that leads to diverse biological functions including inflammatory processes, cell survival and induction of apoptosis. Thus, I first describe the basic signaling events induced by TNFα.

TNFα is a pleiotropic inflammatory cytokine that occurs as both a soluble protein, representing the classical form of the cytokine, and as a type II transmembrane protein (mTNFα). TNFα is initially synthesized as a transmembrane protein that can bind directly to both receptors through cell-to-cell contact or it can be proteolytically cleaved by the metalloproteinase TACE to act in its soluble form.[131, 132] Although the membrane form of TNFα exhibits biological activity, its functional role in TNFα-mediated responses has not been clearly established. Indeed, the majority of the known biological effects of TNFα are attributed to the soluble form of TNFα.

TNFα exerts its biological effects through the interaction with two distinct cell surface receptors, **TNFR1 (p55)** and **TNFR2 (p75)**. TNFR1 is constitutively expressed on all cell types, including those in the brain and is considered the predominant mediator of the cellular response to soluble TNFα. In contrast, TNFR2 expression is limited principally to immune cells and endothelial cells and is only fully activated by the transmembrane form of TNFα.[131, 133]

A common feature of both TNFα receptors is that their extracellular domains are proteolytically cleaved from the cell membrane, giving rise to soluble forms of the receptor.[134, 135] Shedding of the soluble forms of the receptors, s-TNFR1 and s-TNFR2, is increased in response to TNFα and is thought to function as an important regulatory mechanism to limit the signaling capacity of TNFα, as the s-TNFRs bind and neutralize TNFα.[134, 136-138] Thus, cleavage of the TNF-receptors could serve to desensitize the cell to the action of TNFα by both decreasing the number of receptor molecules on the cell surface and by increasing the number of soluble TNFRs that function as physiological inhibitors of TNFα activity.

7-2. TNFα-Induced Signal Transduction

Signal pathways initiated by TNFR1 lead to inflammatory and apoptotic cellular responses and have been studied in great detail (refer to more detailed reviews of the signaling pathways initiated by TNFα[133, 139, 140]). As shown in **Figure 1-3**, recruitment of the adaptor molecule TNFR-associated death domain (TRADD) is essential for signal transduction initiated by TNFR1 activation. The TNFR1-TRADD interaction forms a complex that serves as a central platform for the subsequent recruitment of other signaling proteins that mediate the various downstream actions of TNFα.[141] Recruitment of Fas-associated death domain (FADD) leads to activation of caspase-8, which initiates downstream caspase cascades that results in apoptosis. Alternatively, recruitment of receptor-interacting protein (RIP) and the TNFR-associating factor (TRAF) proteins 2, 4

and 6 lead to NF-κB transcriptional response via the classical ubiquitin-mediated degradation of its inhibitor IκB (as reviewed[142]). Other pathways initiated by TNFR1 involve activation of MAPKs, involving JNK and p38, the lipid mediator ceramide[143] and reactive oxygen species.[143, 144]

NF-κB is one of the most conserved components of TNFα signaling pathway and its activation is thought to mediate many of the diverse effects of TNFα that have been implicated in stroke. For example, TNFα-stimulation increases NF-κB-regulated genes such as inflammatory cytokines (TNFα, IL-1β, IL-6, IL-12), chemokines (IL-8, MIP-1α, MCP-1), adhesion molecules (ICAM-1, VCAM-1, E-selectin), reactive enzymes (COX-2, iNOS, eNOS, NADPH) and apoptotic mediators, such as Fas. TNFα-induced expression of such inflammatory mediators is thought to exacerbate ischemic brain damage. However, TNFα induction of NF-κB has also been reported to promote cell survival through the upregulation of anti-apoptotic and neuroprotective proteins (Bcl-2, Bcl-x, MnSOD, A20) which could be beneficial in the setting of ischemic tolerance.[145]

In contrast to TNFR1, there is much less information regarding the molecular mechanisms of the signal pathways and cellular responses solely initiated by TNFR2. TNFR2 is only fully activated by the membrane form of TNFα[131] and it is thought to function in a localized signaling role during cell-cell interactions. TNFR2 lacks a cytoplasmic death domain and is not thought to directly signal apoptosis. As such, it has been postulated that TNFR2 antagonizes the apoptotic function of TNFR1.[146] Indeed, neuroprotective effects have been attributed to TNFR2.[147] However, it has also been

suggested that the two receptors act cooperatively to enhance the inflammatory and apoptotic functions of TNFα.[148, 149]

7-3. TNFα is Damaging During Cerebral Ischemia

TNFα is a prominent inflammatory cytokine that is increased in the brain following cerebral ischemia. The temporal and cellular regulation of TNFα following stroke has been particularly well documented in experimental models of stroke in rodents. TNFα is induced in the brain rapidly following ischemia, well before neuronal death and remains elevated for several days following stroke. [150-153] There are several likely cellular sources of TNFα in the brain following stroke including neurons and endothelial cells. However, activated microglia-macrophages are considered the major pathophysiological source of TNFα during ischemia. This has been demonstrated by studies that show TNFα mRNA and TNFα immunoreactivity occur predominately in microglia-macrophage cells.[34, 154]

The information regarding the regulation of the TNF-receptors in the brain during cerebral ischemia is limited to a few studies performed in rat and human brain tissue.[155, 156] It has been shown that cerebral ischemic injury results in increased expression of both TNF-receptors in the brain, where they are found upregulated on a variety of cell types including neurons, activated microglia-macrophages and endothelial cells. Thus, it seems possible that upregulation of TNF-receptors during ischemia may act to further sensitize cells to the injurious actions of TNFα following stroke.

Several lines of evidence support a deleterious role for TNFα in ischemic brain injury. Studies in rodent models of stroke have shown that inhibition of TNFα activity during MCAO improves stroke outcome. These studies were performed by treatment with neutralizing antibodies or TNFα-binding protein (chimeric form of s-TNFR1) either systemically or intracranially at the time of MCAO. Blockade of TNFα in these cases resulted in reduced ischemic infarct size, enhanced cerebral blood flow and improved neurological outcome.[157-162] Conversely, exogenous administration of TNFα intracranially in SHR rats prior to MCAO worsens ischemic injury.[161] Likewise, direct treatment of organotypic brain slices with TNFα following ischemia *in vitro* augments neuronal cell death.[161, 163] These pharmacological studies are supported by the finding that TNFα knock-out mice show increased resistance to ischemic brain damage.[164] TNFα knock-out mice showed reduced infarct size, improved locomotor performance and reduced peripheral cellular infiltration following MCAO. Furthermore, cortical neuronal cultures prepared from TNFα knock-out mice also show reduced cell death following ischemic injury *in vitro*. Less clear are the studies performed in TNF-receptor double knock-out mice that showed enhanced ischemic injury after MCAO,[165] an effect that is mediated solely through the loss of TNFR1.[166] This finding could suggest a protective role for TNFR1 signaling against ischemic injury. An alternative interpretation could be that lack of endogenous TNFR1-induced cell survival signals creates a disrupted state of homeostasis and decreased expression of neuroprotective proteins such as MnSOD.[145] This may actually render TNF-receptor knockout mice more susceptible to cerebral injury in the setting of ischemia.

Due to its pleiotropic effects, TNFα is thought to play a role in several aspects of cerebral ischemic injury. TNFα activates numerous inflammatory responses, as it stimulates microglia-macrophages and the cerebrovasculature to produce inflammatory cytokines, reactive oxygen and nitrogen species, chemokines, adhesion molecules and coagulation factors (as described in section 7-2). As a result, TNFα exacerbates inflammation and promotes cellular infiltration, thereby increasing brain damage (as reviewed[145]). Moreover, TNFR1-mediated signaling has the capacity to directly trigger neuronal apoptosis (as described in section 7-2). In addition to such classical roles for TNFα in mediating inflammatory responses, recent studies suggest that TNFα is a contributing factor to early biochemical events initiated by stroke such as glutamate-toxicity and oxidative stress.[44, 163, 167] Thus, TNFα may contribute to the initiation and progression of ischemic brain damage following stroke.

7-4 TNFα is Neuroprotective the Setting of Preconditioning Prior to Stroke.

In contrast to the deleterious role of TNFα in the progression of cerebral ischemic injury, TNFα *prior* to stroke has been shown to have a neuroprotective role. Low dose treatment with TNFα, or its downstream signaling mediator, ceramide, prior to ischemia confers neuroprotection against MCAO in rodents[90, 91] or against ischemic injury modeled *in vitro* in cortical neuronal cultures.[168-171] Moreover, TNFα has been shown to play a beneficial role in the establishment of LPS-induced neuroprotection against stroke.[88]

The mechanisms by which TNFα pretreatment confers neuroprotection against ischemic brain damage are largely unknown, however suppression of the cellular inflammatory response appears to be involved. This is supported by a study wherein TNFα pretreated mice, which are protected against ischemic brain damage caused by MCAO, showed reduced microglia activation and neutrophil infiltration in the ischemic brain tissue.[90] Other studies performed in neuronal cultures *in vitro* have shown that the neuroprotective effects of TNFα are mediated by activation of the transcription factor NF-κB and increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-x.[172] TNFα pretreatment has also been shown to directly increase expression of other NF-κB regulated genes such as the anti-oxidant enzyme MnSOD and the calcium-binding protein calbindin, which are known to be neuroprotective against cerebral ischemic injury and suppress elevation of intracellular calcium.[163, 171] TNFα pretreatment could also provide neuroprotection by diminishing early events triggered by ischemia that cause

neuronal damage, as cultured brain cells preconditioned by TNFα are protected against exitotoxic-glutamate,[171, 173] calcium ionophore toxicity and acidosis,[174, 175] as well as TNFα itself.[169, 176]

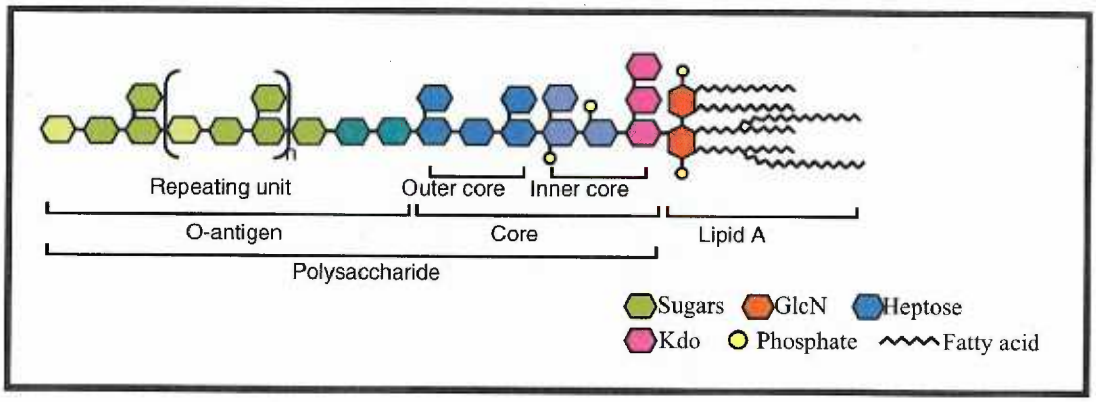

Figure 1-1A) Schematic structure of LPS

Kdo: 3-deoxy-d-manno-octulosonic acid GlcN: galactosamine

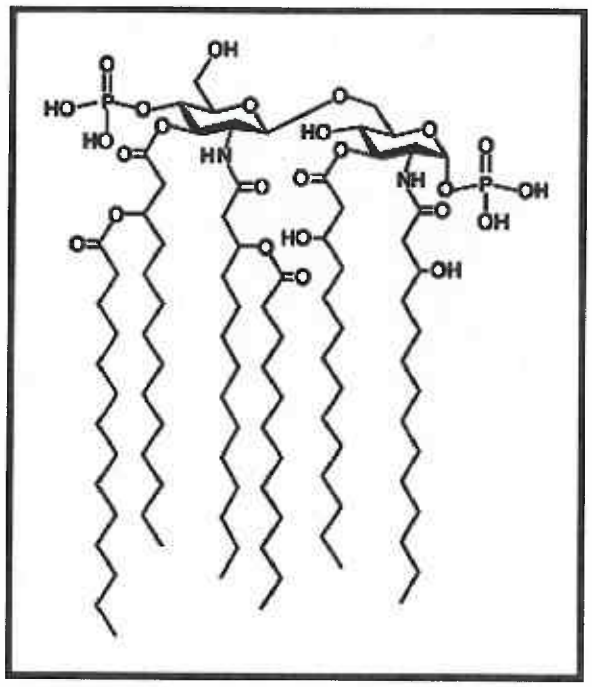

Figure 1-1B) Chemical structure of Lipid A

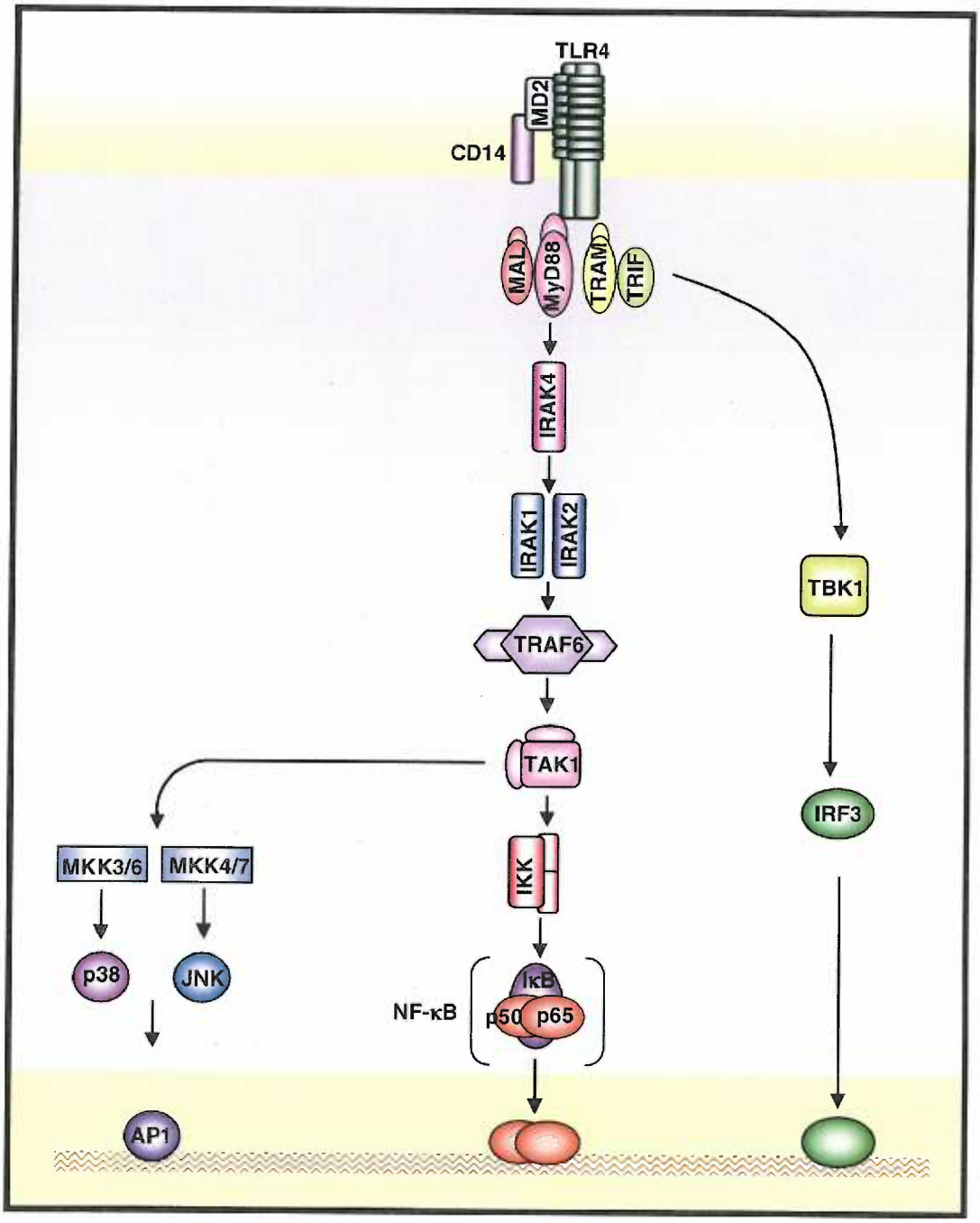

Figure 1-2. Summary of LPS-induced signaling pathways

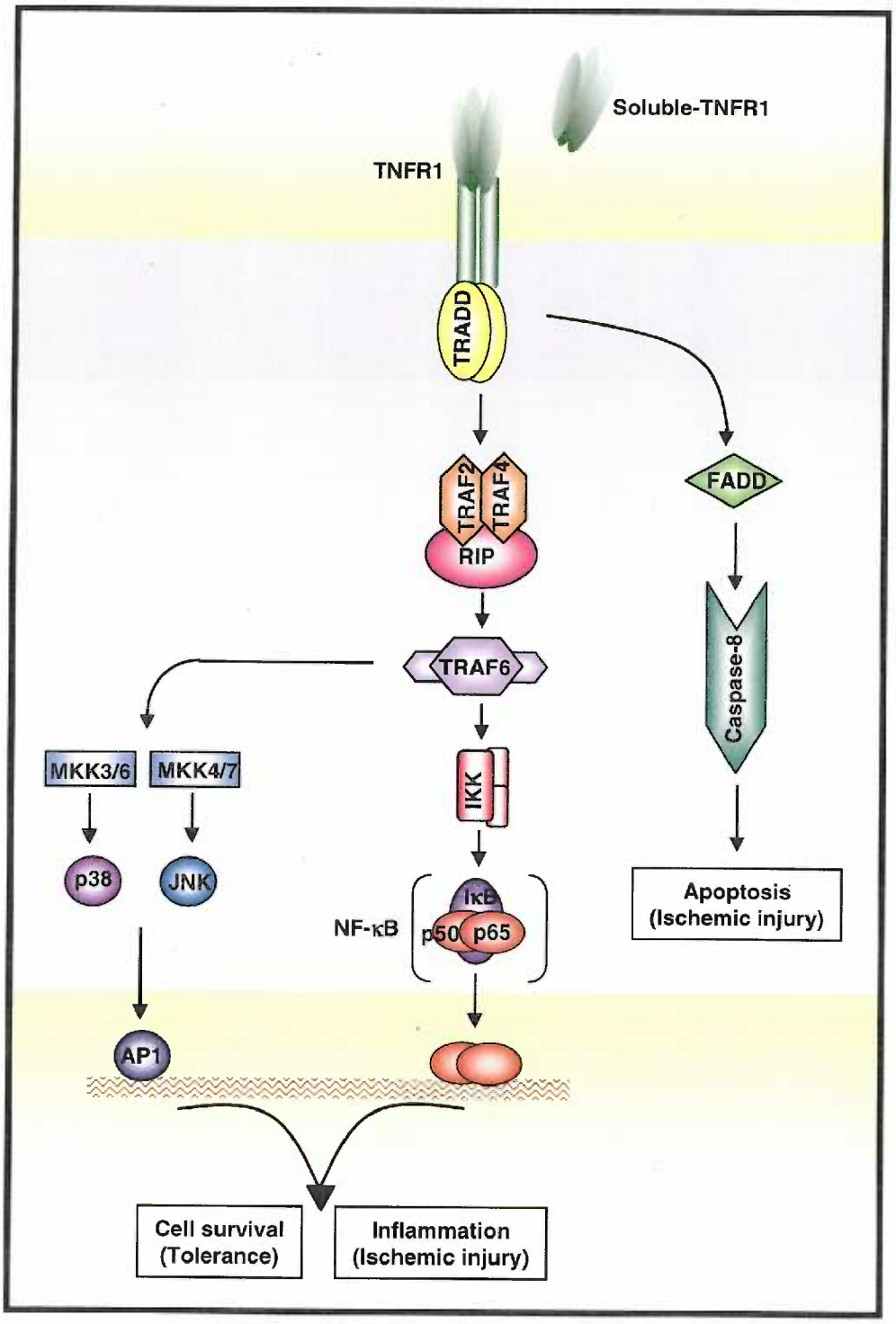

Figure 1-3. Summary of TNFα-induced signaling pathways

RESEARCH DESIGN

The goal of my research was to test the hypothesis that **LPS preconditioning induces neuroprotection against stroke by suppression of injurious inflammatory responses to ischemic injury.** In order to test this hypothesis the neuroprotective conditions of LPS preconditioning were first established in a mouse model system of stroke and in an *in vitro* system of primary cortical neuronal cultures. I then proposed to test the two postulates as described below.

Establish experimental models systems of LPS preconditioning and define the role of TNFα in the induction of ischemic tolerance: In order to test my hypothesis, the neuroprotective conditions of LPS preconditioning were first established in a mouse model of stroke, MCAO (refer to Chapter 3, appendix). The basic properties of LPS preconditioning were also defined in a novel model of LPS preconditioning *in vitro* in primary cortical neuronal cultures exposed to oxygen-glucose-deprivation (OGD) (refer to Chapters 3 and 4).

The development of these two different models systems of LPS preconditioning then enabled me to define the role of TNFα in the induction of ischemic tolerance by LPS preconditioning. TNFα production in response to LPS preconditioning was measured by ELISA over time following LPS or saline treatment in mice. Induction of TNFα activity following LPS preconditioning in primary cortical cultures *in vitro* was measured by WEHI assay. I then tested whether TNFα is necessary for the neuroprotective effects of

LPS preconditioning using TNFα knock-out mice. Stroke outcome was assessed in TNFα knock-out mice that had been preconditioned with LPS or saline prior to MCAO. The role for TNFα in LPS-induced neuronal resistance to ischemic injury *in vitro* was also examined. The activity of soluble form of TNFα was inhibited at the time of LPS preconditioning and the amount of cell death was the measured following exposure to OGD.

Test postulate #1: LPS preconditioning suppresses the cellular inflammatory response to stroke (refer to Chapter 2).

1) The establishment of a mouse model of LPS preconditioning allowed me to then determine whether LPS preconditioning alters activation of microglia and monocyte populations in response to MCAO. The effect of LPS preconditioning on microglia and monocyte responsiveness to stroke was quantified by flow cytometry and immunoflorescence in the brain and blood. Differences were compared to saline pretreated controls.

2) I also determined whether LPS preconditioning alters peripheral leukocyte infiltration in the brain and blood following MCAO. The effect of LPS preconditioning on peripheral cellular infiltration in the brain and blood was quantified by flow cytometry following stroke in mice that had received a prior LPS or saline treatment.

Test postulate #2: LPS preconditioning diminishes the cytotoxic effects of TNFα following ischemia (refer to Chapter 3 and Chapter 4).

1) I determined whether LPS preconditioning attenuates the cytotoxic effects of TNFα during ischemia through modulation of proximal mediators of the TNFα-signaling pathway following MCAO in mice. The effects of LPS preconditioning on the production of TNFα, TNFR1, TNFR2, TRADD and s-TNFR1 following MCAO in mice was measured over time by ELISA, immunoflourescent staining and western blotting techniques.

2) I determined whether LPS preconditioning alters the potential of TNFα to induce injury following cerebral ischemia. Mice that were preconditioned with LPS or saline were administered an i.c.v. injection of TNFα (or artificial cerebral spinal fluid) following MCAO and stroke outcome was assessed. The capacity of LPS preconditioning to alter the neuronal response to TNFα-induced injury was also tested in the setting of ischemia *in vitro* in cortical neuronal cultures. Following OGD, increasing concentrations of TNFα were added to cortical cultures that had been preconditioned by LPS or media. The effect of TNFα on OGD-induced death was then compared between LPS preconditioned and non-preconditioned cultures.

CHAPTER 2-Manuscript #1

Endotoxin Preconditioning Prevents the Cellular Inflammatory Response During Ischemic Neuroprotection in Mice

Holly L. Rosenzweig[1], Nikola S. Lessov[2], David C. Henshall[3], Manabu Minami[3], Roger P. Simon[3], Mary P. Stenzel-Poore[1]

[1] Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon, USA

[2] Oregon Stroke Center, Oregon Health & Science University, Portland, Oregon, USA

[3] Robert S. Dow Neurobiology Labs, Legacy Research, Portland, Oregon, USA

Chapter 2 is a manuscript as it appears in the original paper published in the journal *Stroke.*[1]

Abstract

Background and Purpose—Tolerance to ischemic brain injury is induced by several preconditioning stimuli, including lipopolysaccharide (LPS). A small dose of LPS given systemically confers ischemic protection in the brain—a process that appears to involve activation of an inflammatory response prior to ischemia. We postulated that LPS preconditioning modulates the cellular inflammatory response following cerebral ischemia resulting in neuroprotection.

Methods—Mice were treated with LPS (0.2 mg/kg) 48h prior to ischemia induced by transient middle cerebral artery occlusion (MCAO). The infarct was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Microglia-macrophage responses following MCAO were assessed by immunofluorescence and flow cytometry. The effect of MCAO on white blood cells in the brain and peripheral circulation was measured by flow cytometry 48h following MCAO.

Results—LPS preconditioning induced significant neuroprotection against MCAO. Administration of low dose LPS prior to MCAO prevented the cellular inflammatory response in the brain and blood. Specifically, LPS preconditioning suppressed neutrophil infiltration into the brain and microglia-macrophage activation in the ischemic hemisphere, which was paralleled by suppressed monocyte activation in the peripheral blood.

Conclusions— LPS preconditioning induces neuroprotection against ischemic brain injury in a mouse model of stroke. LPS preconditioning suppresses the cellular inflammatory response to ischemia in the brain and circulation. Diminished activation of cellular inflammatory responses that ordinarily exacerbate ischemic injury may contribute to neuroprotection induced by LPS preconditioning.

Introduction

Tolerance to ischemic brain injury is induced by several distinct preconditioning stimuli that confer neuroprotection including brief periods of ischemia, cortical spreading depression, brief episodes of seizure, and exposure to anesthetic inhalants.[82, 85-87] Although the mechanisms that underlie the various forms of preconditioning are not well understood they share a common link—small doses of an otherwise harmful stimulus induce protection against subsequent injurious challenge.

Preconditioning with low doses of endotoxin (lipopolysaccharide, LPS) in the rat provides protection against subsequent challenge with injurious focal ischemia in the brain.[88] The mechanisms involved in LPS preconditioning are incompletely understood, however activation of inflammatory pathways appear to play a role. In particular, LPS-induced activation of tumor necrosis factor-α (TNFα) and its downstream signaling mediator, ceramide are important for neuroprotection against ischemic injury.[90, 91] Beneficial roles provided by superoxide dismutase (SOD) and endothelial nitric oxide synthase have also been postulated which supports the critical involvement of inflammatory pathways in LPS preconditioning.[89, 93, 95]

Some evidence suggests that LPS preconditioning reduces ischemic injury without a corresponding decrease in inflammatory cell infiltration. Pretreatment of rats with low doses of LPS decreased ischemic infarct size despite increased numbers of inflammatory cells in the ischemic hemisphere.[177] This is paradoxical, as it is generally accepted that

the degree of inflammatory infiltration corresponds with the extent of ischemic injury.[55, 178] The observed reduction in ischemic injury, despite increased numbers of inflammatory cells may reflect a condition in which the inflammatory cells exist in an altered state of activation at the time of ischemia. Such altered activity occurs in macrophages primed with a low dose of LPS, wherein they show reduced cytokine activity upon subsequent challenge with LPS.[11] We hypothesized that LPS preconditioning prior to ischemia renders peripheral macrophages and microglia hypo-responsive to activation by ischemia.

We investigated the effect of LPS preconditioning on the cellular inflammatory response following cerebral focal ischemia in mice. We report that LPS preconditioning induces significant neuroprotection against focal ischemic injury. LPS preconditioning modulates the cellular inflammatory response to ischemia in the brain and peripheral circulation and leads to decreased cellular infiltration and suppressed microglia and monocyte activation, which may contribute to neuroprotection.

Materials and Methods

Mice. Male C57Bl/6 mice (8-10 weeks, NCI) were housed in an AALAC approved facility. Procedures were conducted according to Oregon Health & Science University, IACUC and NIH guidelines. Mice were given free access to food and water.

LPS Treatment. Mice were given an intraperitoneal (i.p.) injection of saline or LPS (0.2 mg/kg, *E coli* serotype *055:B5*, Sigma) 48h before MCAO. Mice administered LPS

showed no differences in body weights and glucose levels. Mean body temperature fluctuate within a normal physiological range <37°C.

Ischemia Reperfusion Model. Mice were anesthetized with 4% halothane and subjected to 60min of MCAO using the monofilament suture method described previously.[30] Cerebral blood flow was monitored throughout surgery by laser Doppler flowmetry. Body temperature was maintained at 37°C with a thermostat-controlled heating pad following surgery.

Infarct Evaluation. A coronal brain section (2mm) was removed at bregma and placed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline at 37°C, 30min.[179] Stained sections were scanned and %area infarct was measured by a blind observer (NIH Image1.62). We have documented that infarct area of this coronal section correlates highly ($r^2 = 0.96$) with infarct volume in this model.[180]

Preparation of WBCs from Brain Tissue. 48h following MCAO mice were transcardially perfused with heparinized saline (2 U/ml heparin). A section was excised for infarct analysis as above, and cells were isolated from the ischemic and non-ischemic hemispheres (excluding the olfactory bulb and cerebellum) as described.[30] Tissue was dissociated (20 U/ml collagenase II and 25 U/ml Dnase I) and cells were purified on Percoll gradients and counted using trypan blue exclusion.

Preparation of WBCs from Blood. Mice were anesthetized 48h post MCAO with isoflurane inhalant. For flow cytometry, blood was collected via the retro-orbital sinus into tubes containing heparin (1,000 U/mL). Red blood cells were lysed in buffer (0.15 mol/L NH_4Cl, 1.0 mmol/L KHO_3, 0.1 mmol/L Na_2EDTA) and resuspended in PBS containing 3% FBS. For total WBC and platelet counts, truncal blood was collected into EDTA-coated microtainer tubes. Cell counts were measured using an automated Cell Dyne 3500R counter (Antech Labs).

Flow Cytometric Analysis. Antibody staining and flow cytometry were performed as previously described.[30] Samples were treated with Fc Block (anti-CD16/CD32) and primary antibodies: anti-CD45 CyChrome-conjugated (WBCs), anti-CD11b R-phycoerythrin (PE)-conjugated (monocytes, macrophages, microglia, neutrophils), anti-CD45R/B220 fluorescein isothiocynate (FITC)-conjugated (B lymphocytes), anti-CD3 PE-conjugated (T lymphocytes), and anti-Ly6G (neutrophils). A FITC-conjugated antibody was used to detect Ly6G. Antibodies were obtained from BD PharMingen, except anti-Ly6G (gift from T. Malek).[181] Flow cytometry was performed with a FACScan (Becton Dickinson); data were analyzed using Cell Quest software on equal numbers of $CD45^+$ cells.

CD11b-Immunofluorescence on Brain Tissue. Brain tissue was prepared for immunofluorescence as described.[182] Brain sections were treated with anti-CD11b (BD PharMingen) and detected with an anti-FITC-conjugated antibody (Jackson Immuno Research). Microglia were quantified from 10 randomly selected 40X fields of view

within the cortex for each individual mouse. Images were collected using a Leica microscope with an Optronics DEI-750 three-chip camera equipped with a BQ 8000 sVGA frame grabber and analyzed using Bioquant (Nashville, TN).

Statistical Analysis. Data are represented as mean ± SEM and were analyzed using two-factor (treatment and hemisphere) analysis of variance (ANOVA). Post hoc analyses were performed using one-way ANOVA or student's t test. Differences were considered statistically significant when $p<0.05$.

Results

LPS Preconditioning is Neuroprotective Against Cerebral Ischemia in Mice. We examined the effects of low doses of LPS on ischemic outcome in pilot studies to determine the optimal dose of LPS that provides the most neuroprotection against MCAO.[183] Based on these findings mice were pretreated systemically with 0.2 mg/kg LPS 48h prior to MCAO. The extent of ischemic injury was assessed 48h following MCAO. LPS preconditioning showed a 31%infact compared to saline controls (52%, **Figure 2-1**). Thus LPS preconditioning provides a 40% reduction in infarct size induced by MCAO.

LPS Preconditioning Attenuates Activation of Microglia Following MCAO. Microglia are activated following ischemia and release inflammatory mediators that exacerbate injury. We quantified microglia in the ischemic brains of LPS preconditioned

mice using fluorescent immunocytochemistry to detect CD11b, a microglial/macrophage marker. Following MCAO (24h) the number of microglia in the ischemic hemisphere was reduced by prior LPS treatment (6.4 $\pm$ 1.0), compared to that of control, saline-treated mice (21.8 $\pm$ 4.1, $p<0.05$, **Table 2-1**). LPS preconditioning alone did not alter the number of microglia. Immunocytochemical staining of CD11b-positive microglia detects activated microglia due to their high level of expression of CD11b, but does not easily detect resting microglia. That LPS preconditioning reduces activation of microglia is analogous to the effects of LPS preconditioning on macrophage activation, which results in lack of macrophage responsiveness to subsequent LPS stimulation.[11]

We have previously demonstrated maximal activation of microglia at 48h following MCAO by flow cytometry.[30] As flow cytometry is a more sensitive means of assessing CD11b expression, we tested whether LPS preconditioning altered microglia activation following MCAO. Microglia populations were quantified as activated (CD11b-hi) or resting (CD11b-low). LPS preconditioning did not alter the total microglia population (resting + activated) in the ischemic hemisphere following MCAO, **Table 2-2**. However, LPS-treated mice showed a marked reduction in an activated microglia population that ordinarily increases in response to MCAO (**Figure 2-2A**). The mean percentage of activated microglia of the total microglia population in the ischemic hemisphere increased significantly (57.2% $\pm$ 5.3, $p<0.05$) following MCAO compared to the non-ischemic hemisphere (25.2% $\pm$ 3.0, dashed line, **Figure 2-2B**). In contrast, LPS preconditioned mice showed no increase in the percentage of activated microglia in the ischemic hemisphere (29.9% $\pm$ 5.4) following MCAO compared to the non-ischemic

hemisphere (29.0% ± 4.6, dashed line). This indicates that a neuroprotective dose of LPS reduces microglial activation following MCAO.

LPS Preconditioning Reduces Neutrophil Infiltration in the Brain following MCAO. Inflammatory events can exacerbate injury following ischemia via recruitment of activated microglia and circulating WBCs. Neutrophils and lymphocytes present in the brain were quantified by flow cytometry 48h following MCAO to determine whether LPS pretreatment alters infiltration of circulating WBCs. Comparisons of cell populations were made between ischemic and non-ischemic hemispheres of each individual mouse following MCAO as previously described.[30] Neutrophils in the brain were assessed using expression of CD45 and CD11b, and distinguished from microglia by expression of Ly6G, a neutrophil-specific marker. Equal numbers of $CD45^+$ cells were gated from each hemisphere of the brain and expressed as percentage of $CD45^+$ cells. Neutrophils were significantly increased (14.7% ± 2.1) in the ischemic hemisphere compared to the non-ischemic hemisphere (8.3% ± 1.4, $p<0.05$) in saline-pretreated controls (**Table 2-2**). In contrast, LPS preconditioned mice exhibited no increase in neutrophils in the ischemic hemisphere following MCAO compared to the control, non-ischemic hemisphere (9.6% ± 1.6 and 9.4% ± 2.3, respectively). Thus neuroprotection is associated with reduced neutrophil recruitment following MCAO. B and T lymphocytes were assessed by expression of CD45 and either CD45R/B220 or CD3 expression, respectively and were not altered by prior LPS treatment.

LPS Preconditioning Alters WBCs in the Blood Following MCAO. Based on altered peripheral cellular infiltration in ischemic brain in LPS preconditioned mice, we postulated that LPS preconditioning modulates the response of circulating WBCs to MCAO. MCAO resulted in a decrease in total WBCs and a modest increase in platelets—effects that were not altered in LPS preconditioned mice **(Table 2-3A)**. However, LPS preconditioning did significantly alter the number of lymphocytes and monocytes following MCAO. We further quantified the percentage of specific cell populations by flow cytometry **(Table 2-3B)**. LPS preconditioning reduced neutrophil composition in the blood following MCAO compared to non-preconditioned controls (13.3% $\pm$ 2.2 versus control 21.3% $\pm$ 2.9; $p<0.05$) which correlates with reduced neutrophil numbers. The reduction of circulating neutrophils may contribute to reduced infiltration in the ischemic brain following MCAO.

LPS Preconditioning Attenuates Monocyte Activation in Blood Following Ischemia. As the aforementioned studies do not distinguish resident microglia in the brain from peripheral macrophages infiltrating from the circulation (although both cell types would be expected to respond similarly to MCAO), we tested whether LPS preconditioning modulates monocyte activation in the blood following MCAO by flow cytometry. We found an activated population of monocytes identified as those $CD45^+$ cells with high levels of CD11b expression present following MCAO (58 $\pm$ 6.3%, **Figure 2-3),** but reduced in LPS preconditioned mice (21.3% $\pm$ 1.7%, $p<0.05$). This indicates that LPS modulates cellular activation of peripheral monocytes in parallel to that of microglia activation in the brain following cerebral ischemia.

Discussion

We investigated the effect of LPS preconditioning on infarct size and the cellular inflammatory response to ischemia. Administration of a low dose of LPS prior to MCAO conferred marked neuroprotection against subsequent cerebral focal ischemia (40% reduction in infarct size). LPS preconditioning prior to MCAO reduced neutrophils in the blood and caused a corresponding reduction of neutrophil infiltration into the brain. In addition, LPS preconditioning attenuated cellular activation of monocyte/macrophage and microglial populations in the peripheral circulation and ischemic hemisphere.

Neutrophils are considered pathogenic in ischemic injury, due to their ability to release inflammatory cytokines and free radicals that exacerbate tissue damage within the brain parenchyma as well as the microvasculature.[55, 178] Here, LPS-induced neuroprotection is associated with a significant reduction in neutrophil infiltration in the brain. Reduced neutrophils in the ischemic hemisphere of LPS preconditioned mice may be due to a corresponding decrease in neutrophils in the blood following MCAO. LPS preconditioning may also suppress neutrophil activity and adherence following ischemia, which would be consistent with the finding that LPS preconditioning promotes preservation of microvascular perfusion in MCAO.[94] Alternately, reduced infiltrate may simply reflect reduced ischemic injury. It is difficult to distinguish between these possibilities, particularly *in vivo*. Investigation of molecular mediators involved in cellular recruitment induced prior to infarct development may indicate whether the immune response is affected early, independent of neuronal injury.

We show that LPS pretreatment caused a marked attenuation in microglial activation in response to MCAO. This is noteworthy, as microglial activation may exacerbate inflammatory injury in ischemia due to their participation in the inflammatory response.[184] Mice preconditioned with LPS showed ~ 30% of the microglia are activated in the ischemic hemisphere following MCAO, which is comparable to the non-ischemic hemisphere. In contrast, saline controls showed robust microglial activation (60% of microglia) in response to MCAO. Thus, the microglial response to LPS preconditioning results in a state that is refractory to activation by ischemia.

Inhibition of microglial activation may be associated with improved ischemic outcome. Rodents made hypothermic or preconditioned with TNFα show increased neuroprotection that corresponded to decreased microglial activation in response to MCAO.[41] In addition, inhibition of microglia activation with minocyline reduces ischemic injury.[37, 90] Thus, a mechanism of LPS-induced neuroprotection may be suppression of microglial activation during ischemia. Interestingly, monocytes in the peripheral circulation of LPS preconditioned mice also showed reduced activation following MCAO. This contrasts with larger doses of LPS that activate microglia and have a negative impact on neuronal injury. High doses of LPS increase the severity of neurodegeneration and attenuation of long-term potentiation associated with the pathology of ALS and Alzheimer's disease.[185, 186] Thus, both dose and timing of LPS administration influence neurological outcome.

Hypo-responsiveness in macrophages occurs in endotoxin tolerance in which small doses of LPS provide protection against greater doses of LPS. Endotoxin tolerance is manifested by suppressed production of many inflammatory cytokines, e.g., TNFα, and IL-12 however, other anti-inflammatory mediators, e.g. IL-10 and IL-1R antagonist are not inhibited.[11] Thus, endotoxin tolerance is not due solely to unresponsiveness of macrophages but reflects a reprogramming of the cellular response to LPS signals.

Mechanisms that underlie endotoxin tolerance in macrophages may be similar to LPS-induced ischemic neuroprotection. Pretreatment with low dose LPS may induce a similar reprogramming in microglia that alters their responsiveness to a subsequent ischemic insult. That LPS preconditioning renders microglia refractory to activation by ischemia supports this notion. Similar to LPS-tolerant macrophages, microglia exposed to a low dose of LPS may shift the balance between pro- and anti-inflammatory mediators following ischemia. This scenario may also be envisioned for neutrophils given that endotoxin-tolerance results in hypo-responsive neutrophil activity.[187] Such reduced activity could extend to their ability to infiltrate the brain following MCAO in LPS preconditioned mice. In addition to suppression of inflammatory responses, beneficial antioxidant responses may be enhanced with endotoxin preconditioning. For example, ischemic protection due to diphosphoryl lipid A pretreatment reduced neutrophil infiltration and this reduction was accompanied by enhanced SOD activity.[95] This implicates both responses as potential mechanisms involved in the neuroprotective process.

It is tempting to speculate that LPS preconditioning reprograms the cellular response to ischemia via genomic changes that render the brain refractory to ischemic injury. This is supported by our studies that suggest preconditioning by brief periods of non-injurious ischemia reprograms the genomic response to subsequent injurious ischemia.[179] Similar studies to decipher the genomic response to LPS preconditioning are currently underway in our laboratory and should prove informative regarding the cellular and molecular events responsible for LPS-induced ischemic neuroprotection.

Table 2-1. Effect of LPS preconditioning on number of $CD11b^+$ cells in ischemic brain tissue

	SALINE	*LPS*
Pre-MCAO	0.5±0.2	0.8±0.4
24 h Post-MCAO	21.8±4.1	6.4±1.0*

Values are mean number of CD11b microglia per 40X field of view within the cortex (mean sum of ten different fields of view for each mouse) ± SEM; *$p<0.05$ versus saline controls; n=6 mice/group.

Table 2-2. Effect of LPS preconditioning on WBC infiltration in the brain following MCAO in mice

	SALINE		LPS	
	Non-Ischemic	**Ischemic**	**Non-Ischemic**	**Ischemic**
Total Microglia	55.2±3.8	60.8±3.9	56.5±8.8	68.9±4.1
Neutrophils	8.3±1.4	14.7±2.1*	9.4±2.3	9.6±1.6
T lymphocytes	4.5±0.9	4.0±1.2	2.5±0.6	4.8±2.0
B lymphocytes	3.5±1.0	4.5±0.6	2.6±0.6	4.9±1.1

WBCs were quantified by flow cytometry 48 h following MCAO. Equal numbers of $CD45^+$ cells per brain hemisphere were gated, and values are mean percent of $CD45^+$ cells ± SEM; * $p<0.05$ versus non-ischemic hemisphere within a treatment; n = 8-12 mice/group.

Table 2-3. Effect of LPS preconditioning on circulating WBCs following MCAO

	Saline control	*Saline/MCAO*	*LPS control*	*LPS/MCAO*
A. CELL NUMBER				
WBC ($x10^6$/ml)	3.5±0.31	2.2±0.45*	4.1±0.45	2.7±0.27*
Platelets ($x10^6$/ml)	992.6±66.20	1,204.57±87.72	851.2±69.44	1,051.88±83.95
Monocytes ($x10^3$/ml)	104.5±18.8	96±23.4	124.3±34.2	45.9±6*†
Neutrophils ($x10^3$/ml)	877.6±138.7	1117±355.2	626.4±105.4	517.3±58.4
Lymphocytes ($x10^3$/ml)	2427±175	835.9±145.9*	3321±482	1824.1±320.4*†
B. % DIFFERNTIAL WBC POPULATIONS (Flow Cytometry)				
Total Monocytes	8.0±0.8	7.5±0.5	14.7±0.9*	8.6±1.0*
Neutrophils	4.2±0.6	21.3±2.9*	4.0±0.5	13.3±2.2*†
T cells	24.0±1.9	15.5±1.3*	22.8±0.8	15.5±1.1*
B cells	53.4±1.6	33.2±4.1*	52.6±1.4	42.2±4.0*

Blood was collected 48 h following MCAO or 4 d following control injections for quantification of WBC and platelet numbers **(A)**. Quantification of distinct %WBC populations was performed by flow cytometry **(B)**. Values are mean ± SEM; * $p < 0.05$ versus saline or LPS control; † $p < 0.05$ versus saline/MCAO; n = 8-12 mice/group.

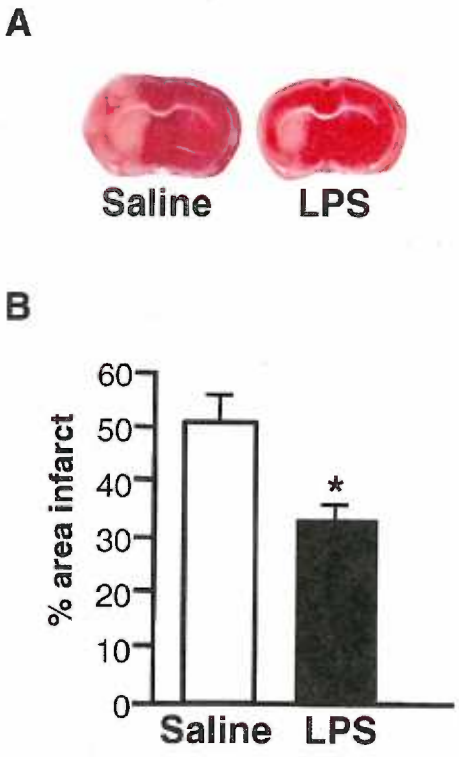

Figure 2-1. Effect of LPS preconditioning on infarct size. Mice were pretreated with LPS or saline 48h before MCAO. Infarcts were assessed 48h after MCAO and quantified as percentage area of ischemic hemisphere. Values are mean $\pm$ SEM, *$p<0.05$ vs saline treatment, n = 12 mice/treatment group.

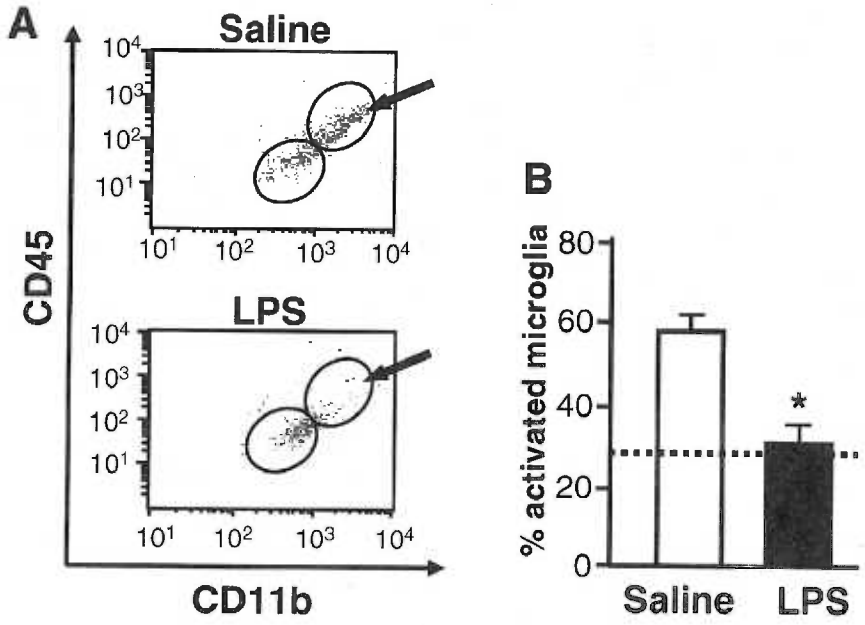

Figure 2-2. Effect of LPS preconditioning on microglia activation in the brain after MCAO. Activated microglia populations were quantified by flow cytometry in the ischemic and non-ischemic brain hemispheres (dashed line) 48h after MCAO in LPS preconditioned mice. A) Representative flow cytometric plot of the ischemic hemisphere of an individual mouse preconditioned by LPS or saline. Arrow indicates activated microglia (CD45-hi and CD11b-hi) present after MCAO but diminished in LPS preconditioned mice. B) Values are mean percentage of activated microglia of total population $\pm$ SEM, *$p<0.05$ vs saline treated mice, n = 8-12 mice/treatment group.

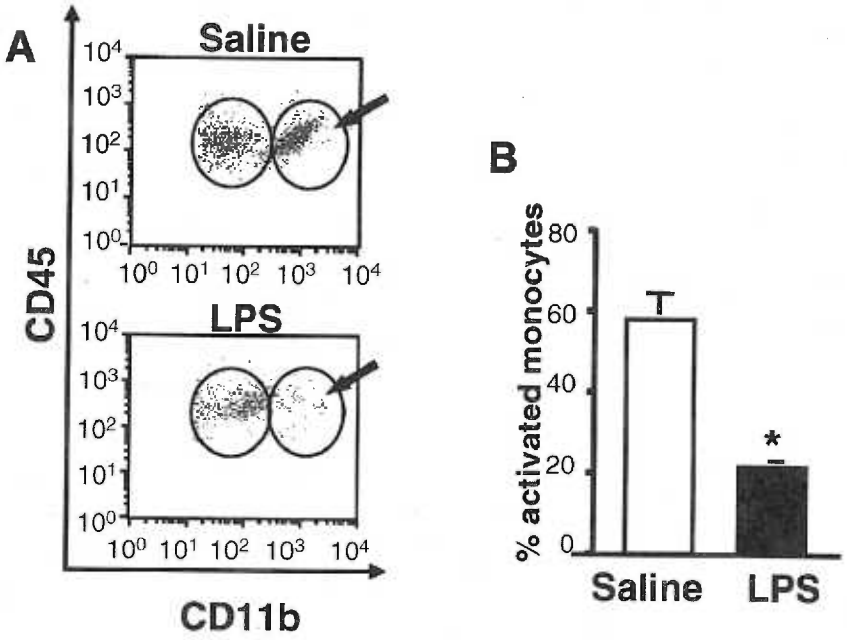

Figure 2-3. Effect of LPS preconditioning on monocyte activation in the blood after MCAO. Activated monocytes in the blood were quantified by flow cytometry 48h after MCAO in LPS preconditioned mice. A) Representative flow cytometric plots of blood sample from an individual mouse preconditioned by LPS or saline. Arrow indicates activated monocytes present after MCAO (CD11b-hi) but diminished in LPS preconditioned mice. B) Values are mean percentage of activated monocytes of total monocytes ± SEM. *$p<0.05$ vs saline treated mice, n = 8-12 mice/treatment group. Controls: saline injection (39.3 ± 4.3%) and LPS injection (25.3 ± 4.3%) vs saline/MCAO $p<0.05$.

CHAPTER 3—Manuscript #2

Endotoxin Preconditioning Protects Against the Cytotoxic Effects of TNFα Following Stroke: A Novel Role for TNFα in LPS-Ischemic Tolerance

Holly L. Rosenzweig[1], Manabu Minami[2], Nikola S. Lessov[1], Sarah C. Coste,[1], Susan Stevens[1], David C. Henshall,[2] Robert Meller[2], Roger P. Simon[2], Mary P. Stenzel-Poore[1]

[1] Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon, USA

[2] Robert S. Dow Neurobiology Labs, Legacy Research, Portland Oregon, USA

[3] Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland

Chapter 3 is a manuscript as it has been prepared for publication and submission to *The Journal of Neuroscience*.

Abstract

Lipopolysaccharide (LPS) preconditioning provides neuroprotection against subsequent cerebral ischemic injury. Tumor necrosis factor (TNFα) has been assigned a pivotal neuroprotective role in LPS-induced preconditioning. Paradoxically, TNFα also exacerbates neuronal injury in the setting of ischemia. Here, we defined the role of TNFα in the development of LPS-induced ischemic tolerance in a murine model of stroke and test whether LPS preconditioning attenuates the cytotoxic effects of TNFα following ischemia. We show that LPS preconditioning increases TNFα in mice prior to stroke induced by (middle cerebral artery occlusion, MCAO). Further, we demonstrate that TNFα is required to establish subsequent neuroprotection against ischemia, as mice lacking TNFα fail to be protected from ischemic injury by low dose LPS pretreatment. Interestingly, following stroke, mice preconditioned with LPS have reduced levels of TNFα and proximal TNFα signaling molecules, neuronal TNFR1 and TRADD. In addition, levels of soluble-TNFR1 were increased following stroke in mice preconditioned with LPS. Increased soluble-TNFR1 may neutralize the effect of TNFα and reduce TNFα-mediated injury in ischemia. Finally, mice preconditioned with LPS showed marked resistance to brain damage caused by intracranial administration of exogenous TNFα following stroke. Thus, our studies suggest that TNFα is a twin-edged sword in the setting of stroke: TNFα upregulation is needed to establish LPS-induced tolerance prior to ischemia while suppression of TNFα signaling during ischemia seems to be an important component of the neuroprotection process following LPS preconditioning.

Introduction

Endotoxin (lipopolysaccharide, LPS), a surface component of gram-negative bacteria, is a potent modulator of the immune system that acts through activation of Toll-like receptor 4 (TLR4). Administration of high doses of LPS stimulates a robust inflammatory response that can lead to lethal septic shock, whereas administration of low doses of LPS induces a protective or tolerant state to subsequent doses injurious of LPS that would ordinarily cause serious injury.[12] Low dose exposure to LPS also induces cross-tolerance wherein protection occurs against heterologous injury unrelated to LPS, such as ischemia. This protective state known also as LPS preconditioning, is not well understood although some evidence suggests that modulation of inflammatory responses and release of cytokines, particularly tumor necrosis factor (TNFα), play an important role. [1, 88, 89, 93, 95]

The role of TNFα is particularly intriguing because it is protective in the setting of preconditioning,[90, 169] yet deleterious in ischemic brain damage following stroke.[161] Support for a beneficial effect of TNFα in preconditioning is underscored by the finding that neutralization of TNFα in the systemic circulation at the time of LPS preconditioning blocks neuroprotection against ischemic injury in rats[88] and pretreatment with either TNFα or its downstream signaling mediator ceramide, is neuroprotective against ischemia *in vivo*[90, 91] and *in vitro*.[169, 170] In contrast to the aforementioned beneficial effects of TNFα *prior* to stroke injury, there is substantial evidence that TNFα is induced in stroke and worsens ischemic damage. TNFα is increased very early after

stroke [151, 152] and affects numerous inflammatory responses, including microglia and vascular endothelial activation, coagulation cascades and up-regulation of enzymes such as COX-2, all of which contribute to the pathogenesis of brain damage.[145] In addition, TNFα causes cell death directly by activating apoptotic signaling pathways mediated by FADD and caspase-8.[140] A cytotoxic role for TNFα in ischemic damage is evidenced by studies demonstrating that systemic or intracranial inhibition of TNFα at the time of cerebral ischemia reduces infarct size in rodent models of stroke.[157-161]

The fact that TNFα plays a protective role in preconditioning by LPS and a damaging role in ischemic injury led us to speculate that LPS primes the neuroprotective process via TNFα production whose effect ultimately suppresses TNFα pathway activation following ischemic insult. We reasoned that the deleterious effects of TNFα in ischemia may be reduced in LPS preconditioning by dampened TNFα production and/or impaired ability to signal following stroke.

Here, we examined the effects of LPS preconditioning on systemic TNFα production over time in a mouse model of stroke, middle cerebral artery occlusion (MCAO) and assessed whether LPS preconditioning influences the proximal mediators involved in the initiation of TNFα signaling. Specifically, we examined the type one receptor (TNFR1, p55) and its intracellular adaptor molecule TRADD, which mediates the majority of the biological effects attributed to TNFα.[139, 141] We also examined the cleaved and soluble form of TNFR1, as it binds TNFα and inhibits its signaling capacity. We went on to test whether LPS preconditioning diminishes the deleterious effects of centrally administered

TNFα on ischemic brain damage following MCAO. Our findings suggest that LPS preconditioning changes the neuronal response to TNFα following ischemia and attenuates ischemic brain damage through suppressed ligand production and decreased expression of proximal signaling molecules.

Materials and Methods

Mice. Age-matched male (8-10 wk) C57Bl/6 mice, TNFα knock-out mice (B6129SF-tnf) and the control strain (B6129F2/J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Procedures were conducted according to National Institute of Health guidelines and Oregon Health and Sciences University Institutional Animal Care and Use Committee.

LPS Preconditioning and Ischemia in mice. Mice were preconditioned with phenol-extracted LPS from *E. coli* (Sigma, L-2880, L-2630) by an intraperitoneal (i.p.) injection of 200ul volume 3d prior to challenge with MCAO. Control mice received an i.p. injection of sterile saline. Due to differences in LPS purity and EU activity that exist between batches of LPS, the optimal preconditioning dose was determined for each batch of LPS. Mice were treated doses of LPS that ranged between 7,500 and 25,000 EU. For surgery, mice were anesthetized with 4% halothane and subjected to MCAO using the monofilament suture method described previously.[30] Briefly, a silicone-coated 8-0 monofilament nylon surgical suture was threaded through the external carotid artery into

the internal carotid artery in order to block the middle cerebral artery, and maintained intraluminally for 60min (except for Figure 3-5, where mice received a shorter period of MCAO to test the effect of TNFα). The suture was then removed to restore blood flow. Regional cerebral blood flow was monitored throughout surgery by laser doppler flowmetry throughout surgery. Body temperature was maintained at 35°C with a thermostat-controlled heating pad.

Intracerebral Ventricular Injection of TNFα following MCAO. The effect of LPS preconditioning on central administration of recombinant mouse TNFα (Chemicon) was studied in mice following 33min MCAO. At 25min post termination of MCAO, TNFα (1.5ul volume/30ng) was injected into the right lateral ventricle as previously described.[188] A control group of animals received an injection of the same volume of sterile, artificial cerebral spinal fluid (aCSF). Infarct volume was measured 24h following stroke.

Infarct Measurement. Infarct measurements were assessed as described.[188] Briefly, dissected mouse brains (minus the olfactory bulb and cerebellum) were sliced into 7-1mm coronal sections from the rostral end (Stoelting tissue slicer, Wood Dale, IL). Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO) in saline (37°C, 15min) and fixed in 10% formalin. An observer blind to treatment groups measured the area of infarct and ischemic hemisphere of each scanned section using NIH Image1.62. Infarct size (% volume) was determined by calculating the area of each section by the section thickness (1mm) and summed over the entire brain

and calculated as: (infarct volume)/(ischemic hemisphere volume) x 100. In one experiment (Figure 1B), % area infarct was calculated so that the remaining brain tissue could be examined for immunohistochemistry and microglia activation as reported previously.[1] We have previously documented that % area infarct of this coronal section correlates highly ($r^2 = 0.96$) with infarct volume in our MCAO model[180]

Physiological measurements. Mean arterial blood pressure and arterial blood gases were measured via a femoral catheter made of PE-50 and micro-renathane tubing in anesthetized mice (1.5% halothane). Blood pressure values were collected using a Statham P23ID pressure transducer (Gould Inc., Oxnard, CA) in line with a Grass Model 7 polygraph (Grass Instruments, Quincy, MA) and expressed as an average across 30min of sampling (sampling rate of 100 Hz). Blood gases were measured using an Instrument Laboratory Synthesis 10 (Barcelona, Spain). Body temperature was measured by a rectal probe.

LPS Preconditioning and Ischemia in vitro. Preparation of primary rat cortical neuronal cultures and oxygen-glucose-deprivation (OGD) was performed according to our previously published method.[179, 188] Cultures were prepared from 1 to 2d old Sprague-Dawley rat pups (Harlan). Cortices were dissected and dissociated with papain (Worthington Biochemicals) and plated at a density of 1×10^6 cells/ml onto coverslips coated with poly-D-lysine. Cells were cultured in Neurobasal-A media (supplemented with Glutamax and B27, Invitrogen) for 7 days prior to each experiment. Cultures consisted of $76.8 \pm 2.4\%$ neurons as determined by staining for NeuN (n=8 separate

cultures, 200 cells/culture examined). The remaining ~20% of the cortical cells was comprised of glia (astrocytes, $GFAP^+$) and (microglia, $CD11b^+$). Cortical neuronal cultures were pretreated with LPS (1ug/ml, Sigma L-2880) for a 24h duration prior to OGD. OGD was performed by removal of the culture medium and replacement with PBS (supplemented with 0.5mM CaCl2, 1.0mM MgCl2, pH7.4), followed by incubation in an anaerobic atmosphere of 85% N2, 5% H2, 10% CO2 for 2h at 35°C. The anaerobic conditions within the chamber were confirmed by the use of Gaspack anaerobic indicator strips (Forma Scientific). OGD was terminated by replacing the exposure medium with Neurobasal-A, medium (supplemented with Glutamax) and the cells were returned to a normoxic incubator.

Acidosis exposure. Acidosis was induced according to a previously published method.[189] Cortical neuronal cultures were exposed to extracellular pH 6.0 for 1.5h during a 2h-exposure of OGD. Exposure to acidosis and OGD was terminated by replacing the medium with Neurobasal-A medium, pH 7.2 (supplemented with Glutamax) and returning of the cells to a normoxic incubator.

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

GMBH) and analyzed using Bioquant software. The number of PI and DAPI stained cells were counted in two random fields of view on each coverslip, and percent cell death was calculated as mean (PI)/(DAPI) x 100 per field of view. Each treatment was performed in duplicate coverslips within an experiment and the entire experiment was repeated three or more times.

Western Blotting. Protein extraction was performed as described previously[188] with some modifications. Briefly, tissue samples were dissected from the cortex or striatum of each hemisphere and lysed in a buffer containing a protease inhibitor cocktail (Roche). Protein concentrations were determined using a BCA kit (Pierce-Endogen). Protein samples (50ug) were denatured in a gel-loading buffer (Bio-Rad, Labs) at 100°C for 5min and then loaded onto 12% Bis-Tris polyacrylamide gels (Bio-Rad Labs). Following electrophoresis, proteins were transferred to polyvinylodene difluoride membranes (Bio-Rad Labs) and incubated with anti-TNFR1 or TNFR2 antibodies (Santa Cruz Biotechnology) at 4°C overnight. Membranes were then incubated with anti-mouse IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and detected by chemiluminescence (NEN Life Science Products) and exposure to Kodak film (Biomax). Images were captured using an Epson scanner and the densitometry of the gel bands, including α-tubulin as a loading control, was analyzed using scanning integrated optical density software (ImageJ).

Immunofluorescence. Brain tissue was prepared for immunofluorescence as previously described.[1] Brain sections were treated with anti-TNFR1 or anti-TRADD antibodies

(Santa Cruz Biotechnology), which were detected with a Cy3-conjugated antibody (Jackson ImmunoResearch, PA, USA). TNFR1 and TRADD immunofluorescence was quantified from 10 randomly selected fields of view at 20X within the cortex or striatum of each individual mouse, and scored by a blinded observer on a scale of 0 to 3 (0 = no staining (i.e. equivalent to background of negative control), 1 = light staining, 2 = moderate staining and 3 = heavy staining). Cell phenotype was determined by counterstaining sections with a neuronal specific antibody (anti-NSE antibody, Chemicon) and detected by an anti-fluorescein isothiocyanate-conjugated antibody (Jackson ImmunoResearch). Co-localization of TNFR1 and TRADD with NSE was quantified from 5 different fields of view at 40X and the mean count obtained. Images were collected using a Leica microscope with an Optonics DEI-750 3-chip camera equipped with a BQ 8000sVGA frame grabber and analyzed using Bioquant.

Soluble-TNFR1 measurement. Protein extraction and quantification was performed as described above on tissue dissected from the cortex of each mouse brain hemisphere. s-TNFR1 was measured with a commercial mouse s-TNFR1 ELISA kit (R&D Systems). Equal amounts of protein (267ug) for each sample were added in duplicate wells, and measured according to a standard curve.

TNFα measurements. Plasma TNFα levels in mice were measured by a commercial mouse TNFα ELISA kit (BD Pharmingen) from blood samples obtained via the retro-orbital plexus. TNFα bioactivity in supernatants of cortical neuronal cultures *in vitro* was determined using a cytotoxic bioassay with the TNFα-sensitive indicator cell line

WEHI-164/clone-20[190] derived from WEHI 164 (CRL-1751, American Type Culture Collection, Manassas, VA). WEHI-164 cells were cultured in RPMI-1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 50uM 2-mecaptoethanol, 2% penicillin/streptomycin/glutamine). For assessment of TNFα activity, WEHI cells were plated at a density of 40,000 cells/well in 96-well plates and then sensitized with LiCl2 and Actinomycin D (25mM and 2ug/ml respectively, Sigma) prior to adding samples. WEHI cells were then incubated overnight at 37°C and cell death was assessed by reduction of Alamar Blue dye (BioSource) based on the absorbance at 570nm (for reduced) and 600nm (for oxidized). TNFα levels were determined in duplicate compared to a standard curve of known amounts of recombinant rat TNFα (Chemicon).

Reagents. Recombinant mouse or rat TNFα was purchased from Chemicon. Rabbit anti-TNFα neutralizing antibody (3ug/ml) was purchased from Pierce-Endogen, TAPI-1 (8uM) was purchased from Calbiochem and cyclohexamide was purchased from Sigma.

Statistical Analysis. Mean differences were analyzed using a one-way or two-way ANOVA with Bonferroni's post hoc test or student's t test analysis. Data are represented as mean ± SEM and differences were considered statistically significant when $p<0.05$.

Results

TNFα plays a necessary role in LPS preconditioning in mice. We investigated whether TNFα played an essential role in LPS preconditioning in a mouse model of stroke. Previous work in a rat model showed that neutralization of TNFα at the time of LPS administration blocked neuroprotection against subsequent stroke.[88] Here we examined whether TNFα knock-out mice could be given a low dose of LPS to induce neuroprotection against subsequent stroke injury. We first established the optimal neuroprotective conditions (dose and time) of LPS preconditioning in the mouse model of MCAO. Mice were administered increasing doses of LPS systemically 72h prior to MCAO and stroke outcome was assessed 24h later (**Figure 3-1A**). We found that mice treated with doses of LPS between 0.05 and 0.2mg/kg showed significant protection compared to saline-treated controls. To determine the duration of neuroprotection induced by LPS treatment, mice were preconditioned with LPS for different time intervals *prior* to MCAO (**Figure 3-1B**) and stroke outcome assessed. We found that LPS-induced neuroprotection developed within one day following administration and extended through day 7. Protection was no longer evident 14 days post treatment with LPS. Importantly, such neuroprotection by LPS preconditioning was not associated with physiological differences between treatment groups in mean arterial blood pressure, arterial blood gases, cerebral blood flow or body temperature at the time of MCAO (refer to **Appendix Table 1**). These data define the specific dose response and time window of LPS preconditioning in mice and allowed us to determine the effect of LPS preconditioning in stroke outcome in genetically engineered mice that lacked TNFα. We

administered LPS to TNFα knock-out and wild-type (WT) control mice 72h prior to MCAO and assessed stroke outcome. WT mice pretreated with LPS showed a significant reduction in ischemic injury, however, $TNF\alpha^{-/-}$ mice were not protected by LPS preconditioning (**Figure 3-1C**). This finding supports a critical role for TNFα in mediating the neuroprotective effects of LPS preconditioning against ischemic injury.

TNFα production is suppressed in LPS-preconditioned mice following MCAO. We next tested whether production of TNFα in response to stroke was altered in LPS preconditioned mice. LPS preconditioned mice showed increased systemic TNFα levels within 1h following LPS administration, which returned to baseline within 24h. Following MCAO, systemic TNFα levels increased in LPS treated and untreated mice to similar levels at 1.5h and 3h. However, by 24h post MCAO. TNFα levels were 3-fold lower in mice preconditioned with LPS compared to those not so treated (**Table 3-1**).

LPS preconditioning modulates proximal mediators of the TNFα signaling pathway following MCAO. We went on to assess whether LPS preconditioning alters proximal mediators of TNFα signaling in addition to TNFα (**Figure 3-2**). We first tested whether TNFR1 expression in the brain was altered by MCAO in mice and found a marked increase in TNFR1 as early as 1.5h post MCAO, which remained elevated 24h following MCAO in the ischemic hemisphere of saline-treated control mice (**Figure 3-2A**). In contrast, mice preconditioned with LPS showed very low induction of TNFR1 following MCAO. Importantly, LPS-induced suppression of TNFR1 occurred in the cortex (region

of protection) and striatum (core of infarct, region of damage) following MCAO (**Figure 3-2B**) which indicates that diminished TNFR1 expression was not due simply to less injury in the cortex, but is specifically associated with LPS treatment. Next we examined the effect of LPS preconditioning on the soluble form of TNFR1 (s-TNFR1), which binds and inhibits the actions of TNFα. Following MCAO, LPS preconditioned mice showed significantly greater levels of s-TNFR1 in ischemic brain hemispheres compared to saline-treated control mice (**Figure 3-2C**). This difference was evident early and sustained out to 24 hr post MCAO.

We also used immunofluorescence to examine TNFR1 and its adaptor molecule, TRADD. This approach allowed us to determine the cellular localization of the TNFR1-complex (**Figure 3-2D**). We found that expression of TNFR1 and TRADD was increased following MCAO in the ischemic hemisphere, however LPS preconditioning suppressed expression of both molecules equally in the cortex and the striatum. Co-staining for neuronal cells with NSE revealed that TNFR1 and TRADD expression were generally co-localized with neurons (95 ± 1%). The neuronal phenotype indicated by staining was also consistent with neuronal morphology.

Blockade of TNFα abrogates LPS preconditioning in modeled ischemia *in vitro*. Our results indicate that LPS preconditioning may attenuate TNFα signaling in ischemia. We postulated that LPS preconditioning reduces neuronal sensitivity to the injurious effects of TNFα in the setting of ischemia. To test this directly, we developed an *in vitro* model of LPS preconditioning where LPS treatment of cortical neurons for 24h confers

protection against injury induced by exposure to oxygen-glucose-deprivation (OGD) (**Figure 3-3A**). The neuroprotective effect of LPS was dependent on *de novo* protein synthesis as the addition of cyclohexamide, an inhibitor of protein translation, reversed neuroprotection against OGD (as described in Chapter 4, Figure 4-4). This is consistent with previous reports regarding the effect of LPS preconditioning *in vivo*.[89] To assess the involvement of TNFα in our *in vitro* system of LPS preconditioning, we measured TNFα levels following LPS treatment of neuronal cultures and found a marked increase in TNFα levels prior to OGD (**Figure 3-3B**). To test whether TNFα activity was important in the neuroprotective effect of LPS, neuronal cultures were treated with an anti-TNFα neutralizing antibody to block the effect of TNFα at the time of LPS preconditioning. Neutralization of TNFα reversed the neuroprotective effects of LPS preconditioning (**Figure 3-3C**). Treatment with anti-TNFα antibody or control IgG 24h prior to OGD had no affect on cell viability or OGD-induced cell death (data not shown).

TNFα is a type II transmembrane protein (mTNFα) that can bind directly to its receptors through cell-to-cell contact. mTNFα can also undergo cleavage (via the protease TACE) and subsequently bind its receptors as a soluble protein, TNFα.[191] To establish whether the neuroprotective effect of TNFα was mediated through a soluble form of the molecule we inhibited cleavage of mTNFα by treatment with TAPI, an inhibitor of TACE, at the time of LPS preconditioning and found that LPS-induced neuroprotection against OGD-induced injury was lost completely (**Figure 3-3D**). There was a modest reduction in cell death in control TAPI-treated cells following OGD which may result from residual TAPI that remained after washing prior to OGD. This is consistent with the fact that TACE is

upregulated following OGD and contributes to ischemic injury.[192, 193] Treatment with TAPI alone in control cultures not exposed to OGD had no affect on cell viability (data not shown). Taken together, these data reveal that the soluble form of TNFα mediates the protective actions of TNFα during LPS preconditioning.

LPS preconditioning ameliorated TNFα-exacerbated neuronal injury following ischemia. We next assessed whether TNFα exacerbated ischemic injury to neurons in the setting of prior LPS preconditioning. Not unexpectedly, we found that endogenous release of TNFα during OGD is cytotoxic as evinced by the fact that treatment with anti-TNFα neutralizing antibody following OGD limits cell death (**Figure 3-4A**). To assess whether LPS preconditioning alters the susceptibility of ischemia-exposed neuronal cells to TNFα-induced injury, TNFα was added to LPS preconditioned cortical neuronal cultures after exposure to OGD (**Figure 3-4B**). We found that exogenous TNFα enhanced OGD-induced cell death in control, non-preconditioned cortical neuronal cultures. However, LPS preconditioned cortical neuronal cultures were completely protected against TNFα-induced injury following OGD. In control cultures not exposed to OGD, TNFα treatment alone did not affect cell viability (data not shown), which supports the deleterious role of TNFα in the setting of ischemia. These data demonstrate that LPS preconditioning changes the neuronal response to the cytotoxic actions of TNFα in the setting of ischemia—an effect that may contribute to the neuroprotective process of LPS preconditioning. To assess whether LPS preconditioning changed the neuronal response to injurious stimuli other than TNFα in the setting of ischemia, we examined the effect of acidosis on OGD-induced cell injury. Acidosis occurs following ischemia

which, in turn causes neuronal damage via membrane acid-sensiong ion channel, or ASIC.[189] We tested whether LPS protected against acidosis-induced injury (exposure to extracellular pH 6.0) in the setting of OGD. Figure **4-4C** showes that lowering extracellular pH to 6.0 for 1.5h during exposure to OGD induces marked cell death in the presence or absence of LPS treatment. Thus, LPS preconditioning protects against TNFα but not acidosis-induced injury in the setting of ischemia, which suggests that acidosis-mediated damage is independent of TNFα.

We went on to assess whether LPS preconditioning protects against TNFα cytotoxicity during ischemia *in vivo.* We reasoned that in the absence of LPS preconditioning the addition of TNFα would exacerbate stroke injury. The duration of MCAO was reduced to 33min (from 60min) to induce less damage and thereby allow detection of increased damage by exogenous TNFα. TNFα (30ng) or artificial cerebral spinal fluid (aCSF) was injected into the right lateral ventricle 25min following termination of MCAO in LPS preconditioned mice or saline controls and infarct size was evaluated 24h later (**Figure 3-5**). TNFα administration failed to worsen stroke damage in mice preconditioned with LPS whereas mice not preconditioned suffered significantly larger stroke injury with the administration of TNFα. We did not observe any brain injury due to TNFα treatment following sham surgery (data not show). These findings suggest that neuroprotective effects of LPS preconditioning are mediated partially through diminished sensitivity of the brain to the injurious effects of TNFα at the time of stroke.

Discussion

Here we report the novel finding that LPS preconditioning suppresses the TNFα response to cerebral ischemic injury. We show that LPS preconditioning alters proximal mediators of the TNFα signaling pathway following stroke. That is, TNFα, TNFR1 and TRADD were reduced and s-TNFR1 was increased in LPS preconditioned mice following stroke. We demonstrated that cortical neuronal cultures preconditioned by LPS were less susceptible to TNFα-induced injury following ischemia *in vitro*. Moreover, the capacity of LPS preconditioning to protect against TNFα was also evident *in vivo*, as TNFα treatment failed to exacerbate stroke injury in LPS preconditioned mice. These findings suggest that LPS preconditioning may provide neuroprotection against ischemic injury by diminishing the deleterious actions of TNFα induced after stroke.

Somewhat paradoxically, we also show that TNFα plays an essential beneficial role as an initiator of LPS preconditioning against ischemic injury in mice. This is indicated by the fact that LPS preconditioning in TNFα knock-out mice does not protect against injurious MCAO. In addition, our studies suggest that the soluble form of TNFα mediates the neuroprotective effects of LPS preconditioning *in vitro* because cortical cultures treated with a TACE inhibitor are not protected by LPS preconditioning. This latter finding implicates TNFR1 as the mediator of protective signaling because the soluble form of TNFα primarily signals through this receptor subtype rather than TNFR2.[131]

LPS preconditioning, which increased TNFα levels in the circulation prior to ischemia and which returned to baseline by the time of stroke (72h). Early induction of TNFα may be essential in the emergence of neuroprotection as inhibition of TNFα at the time of LPS preconditioning by systemic administration of TNFα-binding protein reversed neuroprotection against MCAO in rats.[88] These data suggest that TNFα may be an early signal that primes the brain against subsequent ischemic injury. The mechanisms by which TNFα mediates LPS-ischemic tolerance are not known, although studies *in vitro* have shown that TNFα pretreatment alone is protective against ischemic injury and that activation of the transcription factor NF-κB plays an essential role in the induction of tolerance by TNFα. NF-κB activation by TNFα has been shown to increase expression of cell survival and neuroprotective proteins such as bcl-2 and MnSOD,[163, 172] which could coutermand the damaging effects of cerebral ischemia. Strong evidence also points to a protective role for TNFα-induced signaling events and activation of NF-κB in the induction of ischemic tolerance by other preconditioning stimuli, such as sub-injurious ischemia.[194] Thus, it is reasonable that similar TNFα-induced signaling events may be involved in LPS-ischemic tolerance.

TNFα may also mediate LPS-induced ischemic tolerance by suppression of subsequent TNFα-signaling response in the setting of ischemia. Studies show that TNFα pretreatment in cortical brain cells suppresses subsequent TNFα-induced signaling events, as NF-κB activity was reduced and ICAM-1 expression was inhibited upon re-exposure to TNFα.[169, 176] The negative autocrine regulation induced by prior TNFα treatment is thought to occur through increased expression of negative feedback

inhibitors such as MnSOD, A20, c-IAP and c-FLIP that inhibit TNFα-signaling events.[140] Such features of TNFα tolerance could be protective against the cytotoxic effects of TNFα during ischemia. Indeed, it has been shown that preconditioning with LPS or diphosphoryl lipid A increased SOD activity during MCAO in rats.[89, 95] This finding is consistent with TNFα tolerance wherein MnSOD expression is increased during re-exposure to TNFα [176] and that MnSOD inhibits TNFα-signaling responses and apoptosis.[195-198]

Our data suggest the possibility that neuroprotection induced by LPS preconditioning depends on TNFα production which ultimately causes suppression of proximal mediators of the TNFα signaling pathway *following* stroke. LPS-induced suppression of effectors of the TNFα signaling pathway following MCAO is evinced by our data that show reductions in systemic TNFα production and neuronal TNFR1 and TRADD expression. TNFR1 expression was suppressed at early times following MCAO and sustained out to 24h—a critical time window in the development of brain injury. Suppression of TNFR1 was coincident with enhanced s-TNFR1 and together these changes would be expected to decrease the effect of soluble TNFα. This observation underscores further the importance of the modulating TNFR1 for neuroprotection by LPS preconditioning. In addition, it has been shown previously that improved stroke outcome results from exogenous treatment with s-TNFR1 at the time of ischemia.[199] Such improvement occurs presumably by neutralization of the actions of TNFα. Our data support a similar protective role for enhanced levels of *endogenous* s-TNFR1 after stroke and suggest one way that TNFR1-mediated signaling may be dampened in the acute response to stroke injury. TNFR2

expression was not induced substantially until later times following stroke (24h post MCAO)—an upregulation that was suppressed by prior LPS preconditioning. Although others have suggested that TNFR2 may play a beneficial role in neuronal survival,[200] our data suggests that TNFR2 is not a major mediator of neuroprotection in LPS preconditioning. Collectively, our findings showing increased levels of s-TNFR1 in association with decreased expression of neuronal TNFR1 and TRADD indicate that LPS preconditioning may limit TNFα signaling and thereby enhance neuronal survival in the setting of ischemia.

That LPS preconditioning may alter the neuronal responses to the injurious effects of TNFα is bolstered further by our finding that LPS preconditioning decreases the vulnerability of neurons to TNFα-mediated injury following ischemia *in vitro*. Mitigation of the effect of TNFα would be expected to be beneficial in ischemia as indicated by our studies *in vitro* showing that inhibition of TNFα at the time of OGD is protective. This is consistent with a deleterious role for endogenous TNFα in neuronal survival in this model system as suggested by studies done previously showing that the absence of TNFα at the time of OGD is neuroprotective in cortical cultures.[164] Robust protection against TNFα-mediated injury may be a specific outcome of LPS preconditioning, as there was no protection against the deleterious effects of acidosis during ischemia in LPS preconditioned cortical cultures. That protection is not present in the setting of acidosis may suggest that LPS preconditioning specifically alters TNFα associated effects but does not alter ASIC induced changes in intracellular calcium flux that lead to neuronal death.[189] Thus, LPS preconditioning may protect against TNFα

induced injury via inhibition of TNFα-induced cell death directly, as TNFα triggers apoptosis through FADD and the caspase-8 signaling pathway, or by possibly diminishing neuronal sensitivity to other insults such as glutamate-toxicity or oxidative stress.[163, 171, 192, 198] Furthermore, our findings *in vivo* demonstrate that TNFα administered into the brain at the time of stroke does not exacerbate ischemic brain damage in mice that had been preconditioned, which suggests that neuroprotection by LPS may also involve diminished inflammatory responses. In the absence of LPS-preconditioning, TNFα exacerbates reperfusion injury by initiating inflammatory responses such as microglial and endothelial activation, increasing vascular permeability and peripheral cellular infiltration. In support of attenuated inflammation as an effect of LPS-induced neuroprotection we have demonstrated previously that LPS preconditioning reduces neutrophil infiltration and activation of microglia and monocytes following MCAO.[1] In addition, others have shown that LPS preconditioning results in preservation of microvascular perfusion and enhanced endothelial cell function after MCAO in rats.[92, 94]

Our finding that LPS preconditioning decreases the ratio of TNFα to s-TNFR1 resembles effects seen in endotoxin tolerance, wherein a low dose of LPS is protective against a greater, lethal dose of LPS. Pretreatment with low dose LPS suppresses production of pro-inflammatory mediators such as TNFα, IL-1β and IL-6 following subsequent challenge with a large dose of endotoxin. Furthermore, LPS preconditioning leads to protection against cell death via a process referred to as "reprogramming" whereby priming by exposure to low dose LPS alters the subsequent response to LPS. Such

reprogramming leads to sustained or enhanced production of anti-inflammatory mediators such as s-TNFR1 and IL-10.[11, 123, 201] It is possible that LPS preconditioning also *reprograms* the response to ischemic injury and leads to increased cell survival. Genomic expression patterns observed in LPS preconditioned animals provide supportive evidence of genomic reprogramming[202] and indicate that protection may result, in part, from marked suppression of deleterious inflammatory pathways such as TNFα and induction of beneficial anti-inflammatory and neuroprotective pathways that enhance cell survival. Such findings have important implications for therapeutic treatment of patients at risk of stroke, as LPS preconditioning offers the potential to minimize the deleterious effects of TNFα while enhancing beneficial neuroprotective mediators following stroke.

Table 3-1. Effect of LPS preconditioning on systemic TNFα production over time

Time relative to MCAO							
	-71h	-69h	-66h	-48h	1.5h	3.0 h	24h
Saline	85.5 ± 3.8	67.2 ± 5.4	76.8 ± 5	65.9 ± 7	142.4 ± 45	120 ± 36	1066 ± 66
LPS	2065.9 ± 299*	236.8 ± 20*	165 ± 30*	94.7 ± 22	168 ± 53	298 ± 60*	347 ± 55*

Mice were treated with LPS (0.2mg/kg) 72h prior to MCAO and blood was collected at the indicated times to measure plasma levels of TNFα by ELISA. Values are mean pg/ml ± SEM, *$p < 0.05$ compared to saline-controls for each time point, n = 8 mice/treatment group.

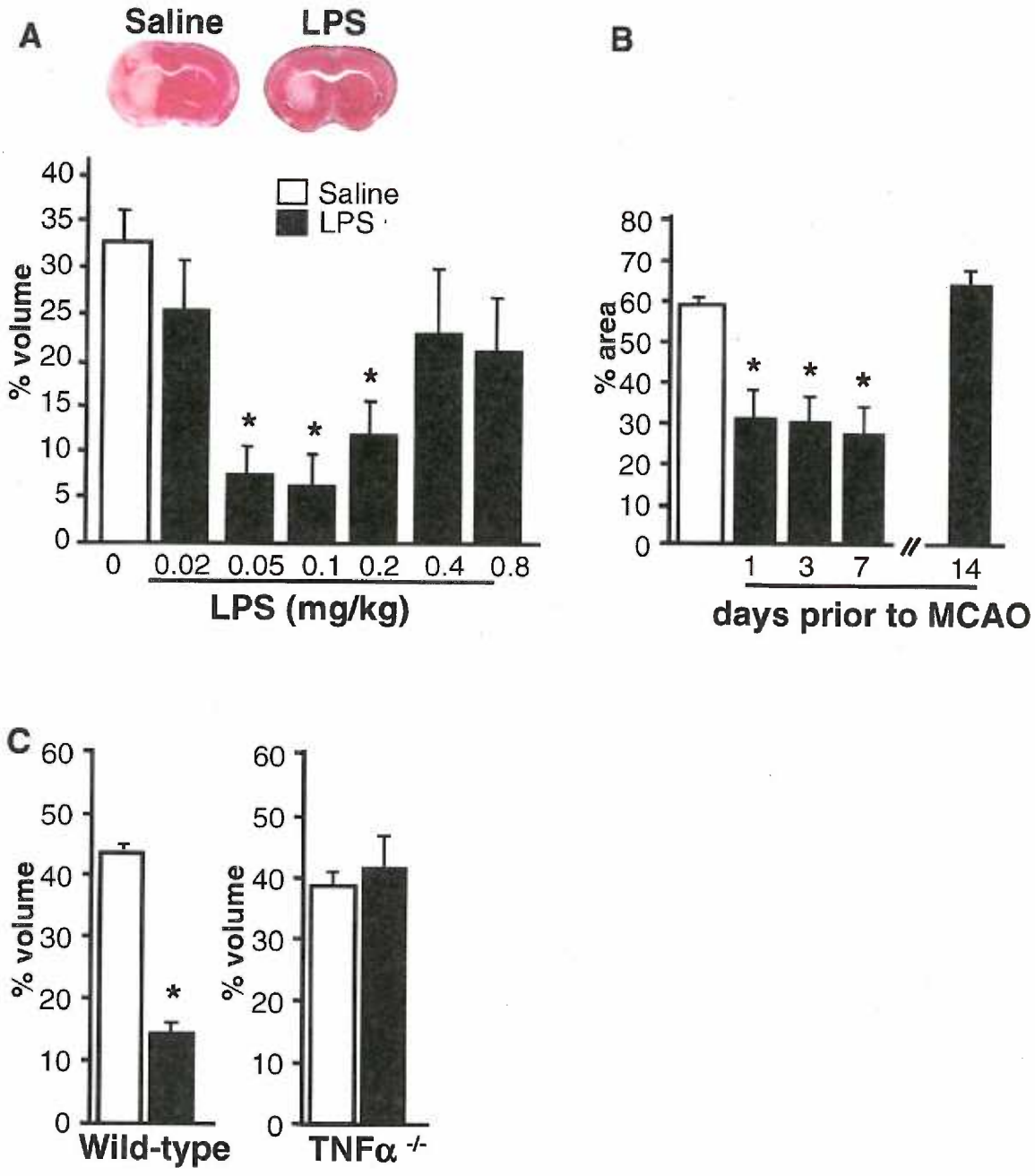

Figure 3-1. TNFα plays a necessary role in LPS preconditioning in mice. A) C57Bl/6 mice were treated with different doses of LPS 72h prior to MCAO and infarcts were assessed 24h following MCAO by TTC staining. A) Representative brain sections stained with TTC and the mean % infarct volume is plotted as a function of LPS dose. B) C57Bl/6 mice were treated with 0.2mg/kg LPS at different times *prior* to MCAO and infarcts were assessed 24h following MCAO. C) Control, wild-type strain or TNFα knock-out mice ($TNF\alpha^{-/-}$) were treated with 0.2mg/kg LPS 72h prior to MCAO and infarcts were assessed 24h post MCAO. Values are mean ± SEM, *$p<0.05$ vs saline treatment, n = 6-8 mice/treatment group.

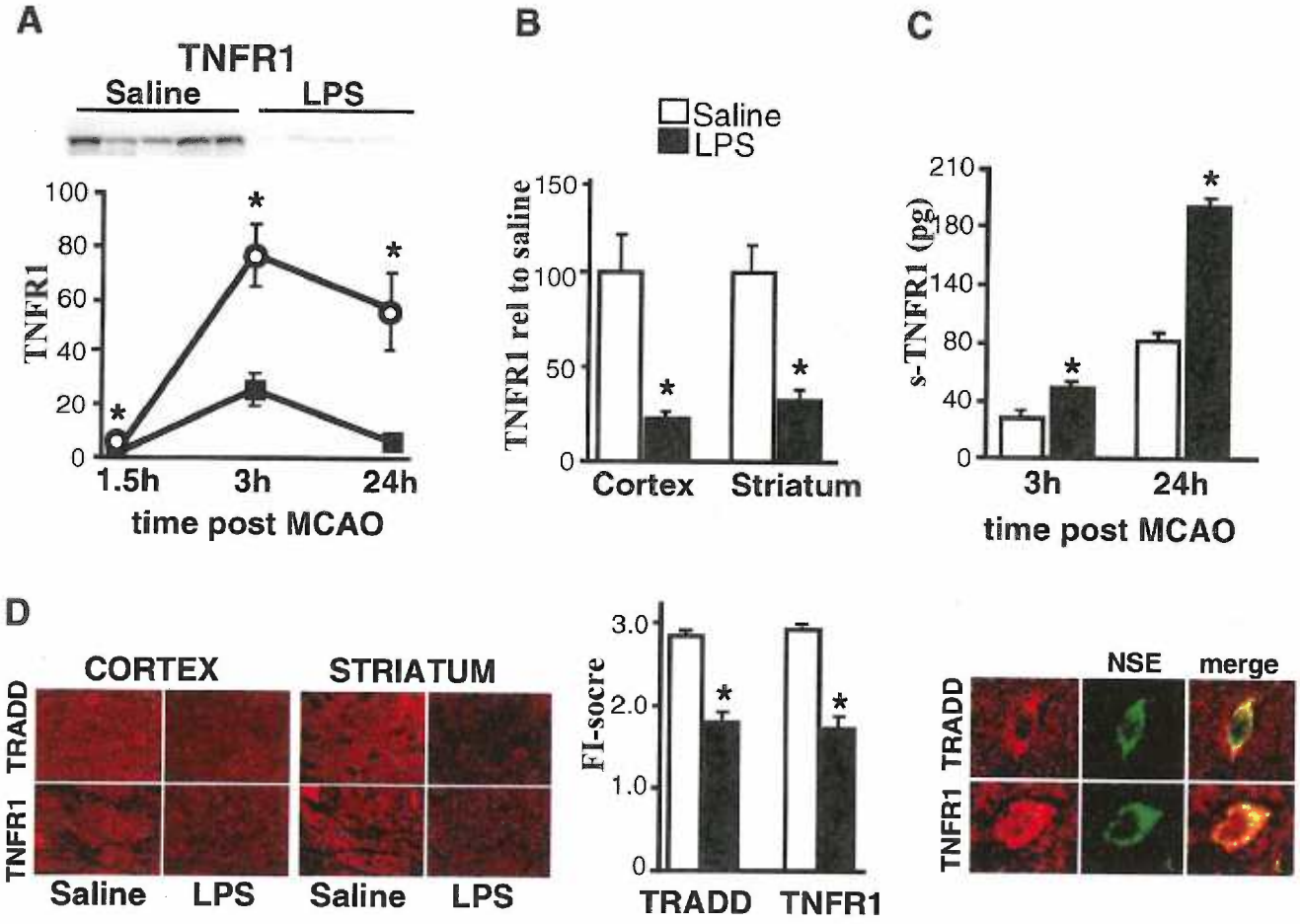

Figure 3-2. LPS preconditioning modulates proximal mediators of the TNFα signaling pathway following MCAO. C57Bl/6 mice were preconditioned with 0.2mg/k LPS 72h prior to MCAO. A) TNFR1 expression in the cortex of the ischemic hemisphere was measured by western blot analysis at the indicated times following MCAO. The densitometric values of TNFR1 are graphically expressed relative to baseline controls as a function of time following MCAO, n = 8 mice/treatment. Open circles: saline pretreated mice, black squares: LPS preconditioned mice B) TNFR1 expression was examined by western blot analysis in the cortex or striatum of the ischemic hemisphere at 24h following MCAO. Mean densitometric values are expressed relative to ischemic hemisphere of saline controls, n = 8 mice/treatment. C) s-TNFR1 in the cortex of the ischemic hemisphere was examined by ELISA at the indicated times following MCAO, n = 5 mice/treatment. D) TNFR1 and TRADD expression was examined by immunofluorescent staining at 24h following MCAO in both the cortex and striatum of the ischemic hemisphere. Fluorescence intensity (FI) was measured on a scoring system (0 to 3) within 10 random fields of view at 20X within each region of the cortex (as shown) in a mouse brain. There was no difference in scoring between the cortex and striatum within each treatment group. Cell phenotype was determined by counterstaining for NSE, and co-localization was quantified from 5 different fields of view at 40X and the mean count obtained, n = 6 mice/treatment group. For all experiments, values are mean ± SEM, *$p<0.05$ vs saline controls.

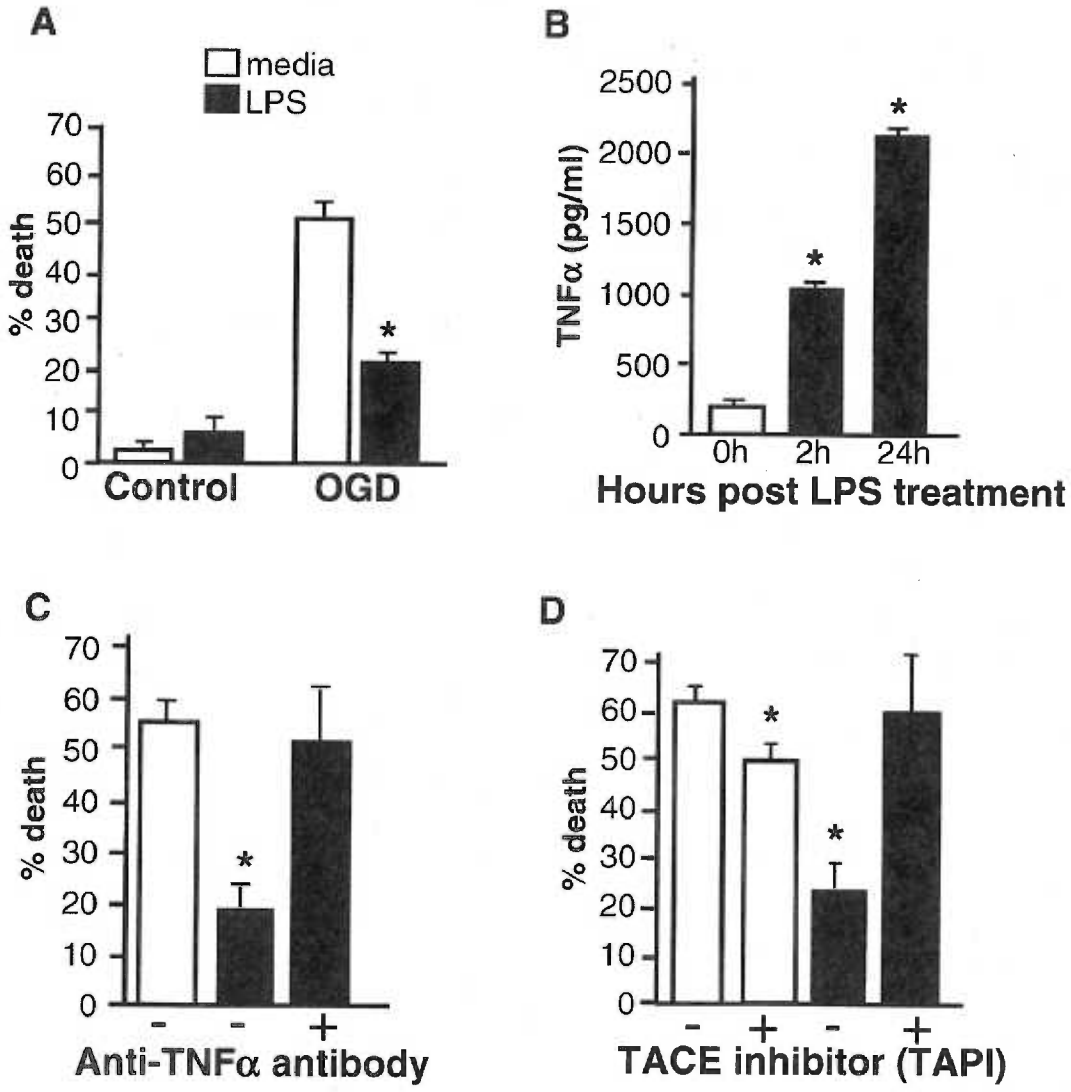

Figure 3-3. Soluble TNFα is essential for LPS preconditioning in an *in vitro* model of ischemia. A) Primary cortical neuronal cultures were pretreated with 1ug/ml LPS for a 24h duration prior to exposure to oxygen-glucose-deprivation (OGD). Cell death % was determined 24h following OGD by PI staining. B) TNFα activity present in the supernatant following LPS treatment alone was measured by WEHI assay. C) Cortical neuronal cultures were co-treated with anti-TNFα neutralizing antibody and LPS for 24h prior to OGD, and % cell death was examined 24h following OGD by PI staining. D) Cortical neuronal cultures were co-treated with TAPI (TACE inhibitor) and LPS for 24h prior to OGD, and % cell death was determined 24h later by PI staining. For all experiments, values are mean ± SEM, *$p<0.05$ vs media-treated OGD controls, n = 4-6 individually repeated experiments.

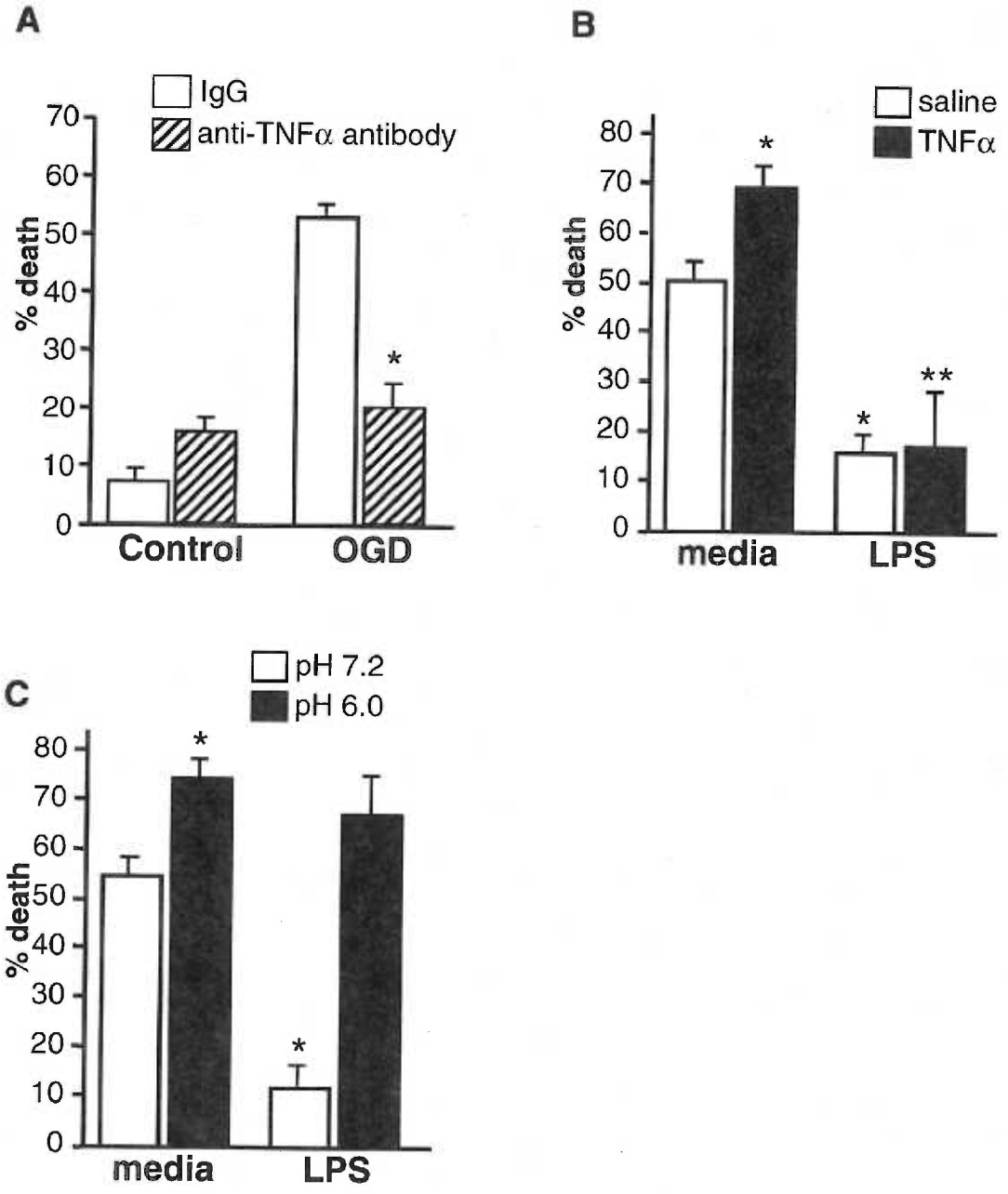

Figure 3-4. LPS preconditioning protects neurons against TNFα-mediated injury in the setting of ischemia *in vitro*. A) Cortical neuronal cultures were treated with anti-TNFα neutralizing antibody at the time of OGD and for the 24h duration following OGD until assessment of cell death by PI staining. B) Cortical neuronal cultures were pretreated with LPS for 24h prior to OGD. Exogenous TNFα (10ng/ml) was added to the medium at the termination of OGD, and % cell death was examined 24h later by PI staining. C) Cortical neuronal cultures were pretreated with LPS for 24h prior to OGD. Cultures were then subjected to acidosis by extracellular by pH 6.0 for 1.5h during OGD, and cell death was determined 24h later by PI staining (n = 3 repeated experiments). Values are mean ± SEM, *$p<0.05$ vs media-treated OGD controls, **$p < 0.05$ vs TNFα-treated OGD, n = 4-5 individually repeated experiments.

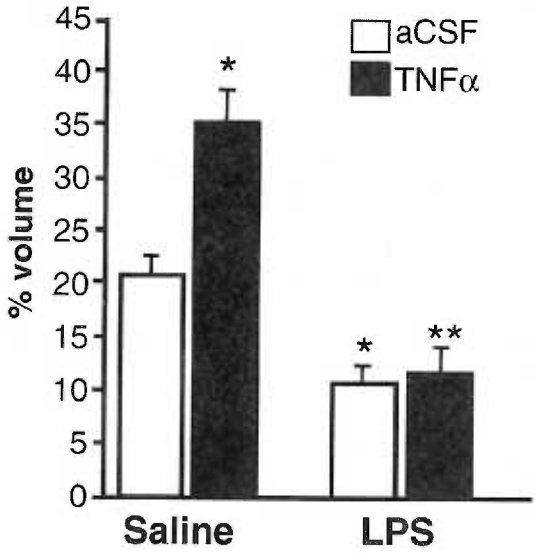

Figure 3-5. LPS preconditioning protects against TNFα-induced ischemic brain damage following cerebral ischemia *in vivo*. Mice that were preconditioned with LPS 72h prior to 33min MCAO were then administered an i.c.v injection of TNFα (30ng) or artificial cerebral spinal fluid (aCSF) after stroke. Infarct volume was measured 24h following MCAO by TTC staining. Values are mean ± SEM, *$p<0.05$ vs saline pretreated-aCSF injected controls, **$p<0.05$ vs saline pretreated-TNFα injected mice, n = 8 mice/treatment group.

CHAPTER 4-Manuscript #3

LPS Preconditioning Increases Neuronal Resistance to TNFα-Induced Injury During Ischemia

Holly L. Rosenzweig[1], Roger P. Simon[2], Mary P. Stenzel-Poore[1]

[1] Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon, USA

[2] Robert S. Dow Neurobiology Labs, Legacy Research, Portland Oregon, USA

Chapter 4 contains data for a paper in preparation for *Brain Research*

Preconditioning with lipopolysaccharide (LPS) reduces ischemic brain damage caused by subsequent stroke in rodents.[1, 88] Our lab and others have demonstrated that modulation of inflammatory responses and release of cytokines, particularly TNFα, are thought to play beneficial roles in the neuroprotective effects of LPS preconditioning (refer to Chapter 3).[88, 89] Our prior studies (as described in Chapter 3) revealed that TNFα mediates dual functions in the neuroprotective process of LPS preconditioning. That is, TNFα induced by LPS preconditioning *prior* to stroke plays an essential role in the establishment of tolerance against ischemia, while TNFα suppression *following* stroke appears to be an important component of the neuroprotective state. In light of the substantial evidence that TNFα is deleterious during stroke, *diminished sensitivity to TNFα* in the setting of ischemia could be an important component of the neuroprotective mechanism of LPS preconditioning.

Here we further explored the capacity of LPS preconditioning to alter the neuronal sensitivity to the injurious effects of TNFα during ischemia. We sought to determine whether LPS preconditioning could directly alter neuronal responses to TNFα in the setting of ischemia, but in the absence of peripheral effectors. We evaluated the effects of LPS preconditioning on the potential of TNFα to directly cause neuronal damage in an *in vitro* model of ischemia, oxygen-glucose-deprivation (OGD). Such a study required that I establish an *in vitro* model system of LPS preconditioning using primary neuronal cultures—a model system lacking heretofore. I first defined the basic features of neuroprotection induced by LPS preconditioning in primary cortical neuronal cultures. The initial parameters included establishment of the dose response and time window of

LPS pretreatment and the requirement of protein synthesis to obtain neuroprotection. The establishment of optimal conditions for LPS preconditioning *in vitro* then afforded an opportunity to examine the question of whether LPS preconditioning alters the neuronal sensitivity to the cytotoxic effects of TNFα during ischemia.

Methods.

Preparation of primary cortical neuronal cultures. Primary rat cortical neuronal cultures were prepared as previously published.[179, 188] Briefly, cultures were prepared from 1 to 2d old Sprague-Dawley rat pups (Harlan). The cortical brain region was dissected and dissociated with papain (Worthington Biochemicals) and plated at a density of 1 x 10^6 cells/ml onto coverslips that had been previously coated with poly-D-lysine. Cortical cells were cultured in Neurobasal-A media (supplemented with Glutamax and B27, Invitrogen) for 7 days prior to use in each experiment. The cellular composition of cortical cultures was assessed by immunofluorescent staining for the cell specific markers on neurons (NeuN), astrocytes (GFAP) and microglia (CD11b).

Oxygen-glucose-deprivation (OGD). OGD was performed according to our previously published method (Lancet paper), wherein the culture medium is removed and replaced with PBS (supplemented with 0.5mM CaCl2, 1.0mM MgCl2, pH7.4). Cultures are then placed in an anaerobic chamber containing atmospheric gases of 85% N2, 5% H2, 10% CO2 for 2h at 35°C. The anaerobic conditions within the chamber were confirmed by the use of Gaspack oxygen-indicator strips (Forma Scientific). OGD was terminated by

replacing the exposure medium with Neurobasal-A medium (supplemented with Glutamax) and the cells were returned to a normoxic incubator.

Measurement of cell death. The amount of cell death caused by OGD in cortical cultures was measured 24h following the termination of OGD by means of fluorescent, cell-permeable, DNA-binding dyes: propidium iodide (PI), as an indicator of cell death, and 4',6-diamidino-2-phenylindole (DAPI), as an indicator of the total number of cells. Coverslips were incubated with PI (1.5ug/ml, Sigma) for 2min, washed with PBS and fixed with Vectashield mounting medium containing DAPI (Vector labs). Stained cells were visualized with a fluorescent microscope (Leica GMBH) and analyzed using Bioquant software. The number of PI and DAPI stained cells were counted in two random fields of view on each coverslip, and percent cell death was calculated as mean (PI)/(DAPI) x 100 per field of view. Each treatment was performed in duplicate coverslips within an experiment and the entire experiment was repeated three or more times.

Reagents. Phenol-purified LPS from *E. coli*, serotype 055-B5 (L-2880) and cyclohexamide were purchased from Sigma. Rat recombinant TNFα was obtained from Chemicon.

Statistical Analysis. Mean differences in % cell death were analyzed using a two-way or one-way ANOVA with student's t-test hoc analysis. Data are represented as mean ± SEM and differences were considered statistically significant when $p<0.05$.

Results.

The neuroprotective effects of LPS preconditioning *in vitro* are dose-dependent. To test whether LPS preconditioning alters the neuronal sensitivity to TNFα during ischemia, the optimal neuroprotective conditions of LPS preconditioning were established with primary rat cortical neuronal cultures using an *in vitro* experimental model of ischemia, oxygen-glucose-deprivation (OGD).[179] The primary cortical neuronal cultures are comprised of 76.8 ± 2.4% neurons, and ~20% astrocytes and microglia (n = 8 separate cultures, 200 cells/culture examined). Cortical cultures were pretreated with increasing doses of LPS for 24h prior to exposure to OGD. The amount of cell death caused by OGD exposure was determined 24h later by propidium iodide (PI) staining (**Figure 4-1**). We found that cortical cultures pretreated with LPS (1 to 10ug/ml) showed significant protection against OGD-induced damage compared to non-preconditioned cultures. Cell viability was not altered in control cortical cultures treated with LPS alone, in the absence of OGD (data not shown). These data demonstrate that LPS pretreatment renders cortical brain cells resistant to the damaging effects of ischemia *in vitro* in a dose-dependent fashion.

Transient LPS exposure induces neuroprotection *in vitro*. To test whether transient exposure to LPS induces neuroprotection *in vitro*, cortical cultures were exposed to 1ug/ml LPS for a 2h duration, after which the cultures were washed and replenished with medium that lacked LPS for the remaining 22h time period prior to OGD. Cell death was

then assessed by PI staining 24h following challenge with OGD (**Figure 4-2**). We found that cortical cultures exposed transiently to LPS showed significant protection against OGD compared to non-preconditioned cultures, and that the degree of protection was similar to that seen with continual LPS treatment. These studies indicate that the neuroprotective effects of LPS preconditioning *in vitro* do not depend on continual exposure to LPS in cortical cultures, and support a beneficial role for LPS as a preconditioning stimulus.

The time course of LPS-induced neuroprotection *in vitro*. To determine the amount of time required for LPS-induced ischemic tolerance to develop *in vitro*, cortical cultures were preconditioned with 1ug/ml LPS for different time intervals *prior* to OGD, and the amount of cell death caused by OGD was then measured 24h later by PI staining (**Figure 4-3**). We found that LPS produced significant ischemic protection within 8h and that maximal protection was evident with 24h of treatment. In contrast, cortical cultures preconditioned by LPS at earlier times prior to OGD were not protected, as treatment with LPS 1h prior to OGD or during OGD (0h) did not reduce cell death. These findings reveal that the neuroprotective effects of LPS preconditioning develop in a delayed fashion, which requires greater than 8h.

The neuroprotective effects of LPS preconditioning *in vitro* require *de novo* protein synthesis. Previous work in a rat model of MCAO showed that inhibition of protein translation at the time of LPS administration blocked neuroprotection against subsequent stroke.[89] To determine whether *de novo* protein synthesis was necessary for LPS-induced

neuroprotection against OGD *in vitro*, cortical cultures were co-treated with cyclohexamide (CHX), an inhibitor of translation, and 1ug/ml LPS for 24h prior to OGD, and cell death was then assessed 24h later by PI staining. As shown in **Figure 4-4**, the presence of CHX during LPS preconditioning blocked the neuroprotective effects against OGD *in vitro.* Control cultures treated with CHX alone, in the absence of OGD, did not show altered cell viability (data not shown). These findings indicate that *de novo* protein synthesis is required for the induction of ischemic tolerance by LPS in cortical cultures *in vitro*.

LPS preconditioning diminishes the neuronal sensitivity to TNFα-induced injury during ischemia *in vitro*. These studies established a model system of LPS preconditioning *in vitro,* and thereby allowed further testing of the capacity of LPS preconditioning to alter the neuronal sensitivity to TNFα during ischemia. To test the effects of LPS preconditioning on TNFα-induced injury in cortical cultures, increasing doses of exogenous TNFα were added to cortical cultures following OGD. The effect of TNFα on cell death was then assessed 24h following OGD by PI staining (**Figure 4-5**). Treatment with exogenous TNFα treatment in control non-preconditioned cortical cultures exacerbated cell death caused by OGD. Cortical neuronal cultures treated with doses of TNFα between 10ng/ml and 100ng/ml showed a significant increase in the amount of cell death following OGD. In contrast, cortical cultures that had been preconditioned by LPS 24h prior to OGD were resistant to the cytotoxic effects of TNFα following OGD. Control cortical cultures treated with TNFα, in the absence of OGD, did not increase cell death (data not shown), which further supports the pathological role of

TNFα in the setting of ischemia. This finding reveals that LPS preconditioning alters the capacity of TNFα to induced neuronal damage during ischemia.

Discussion.

These findings show that LPS preconditioning renders primary cortical neuronal cultures resistant to subsequent injury caused by ischemia *in vitro*. We found that the neuroprotective effects of LPS preconditioning occur in a dose-dependent fashion and develop over time between 8 and 24h following LPS pretreatment. Furthermore, the establishment of LPS-induced neuroprotection requires *de novo* protein synthesis. Collectively, these features define a new *in vitro* model of LPS preconditioning against ischemia.

Importantly, our findings also demonstrate that LPS induces ischemic tolerance directly and does not require additional effectors conveyed from the periphery. In light of this, we tested whether such tolerance to injury was due to increased TNFα-induced damage. Our results show that LPS preconditioning increases the resistance of neurons to TNFα-mediated damage following OGD. Given the deleterious role for TNFα in the setting of ischemia, the capacity of LPS preconditioning to diminish the neuronal response to TNFα may be an underlying neuroprotective mechanism against stroke.

Here we established the salient features of LPS-induced neuroprotection in cortical neuronal cultures *in vitro* and showed that the dose and timing of LPS pretreatment alters

the amount of neuroprotection against subsequent OGD. Maximal neuroprotection against OGD was observed in cortical cultures that had been preconditioned by LPS 24h prior to OGD, which suggests that synthesis of new proteins induced by LPS could be involved in the development of ischemic tolerance. In support of this postulate, *de novo* protein synthesis is essential for the neuroprotective effects of LPS preconditioning *in vitro*, as inhibition of protein translation blocked LPS-induced ischemic tolerance against OGD. These basic features of LPS preconditioning that we observe *in vitro* are consistent with prior studies in rodents, wherein LPS-induced neuroprotection against subsequent MCAO was influenced by the dose and timing of LPS treatment and required *de novo* protein synthesis.[89] This indicates that the basic underlying features of LPS-induced ischemic tolerance that occur *in vivo* are conserved at the cellular level *in vitro*.

The cellular and molecular mechanisms involved in LPS preconditioning are not well understood. LPS is a potent immunostimulant that exerts its effects through Toll-like receptor 4 (TLR4), which is expressed predominantly on macrophages and other peripheral immune cells. Activation of systemic inflammatory responses and production of cytokines such as TNFα have been implicated in the establishment of LPS-induced ischemic tolerance.[88, 89] It is conceivable that inflammatory responses initiated by LPS preconditioning in the periphery mediate the neuroprotective effects of LPS in the brain. However, TLR4 is also expressed in brain tissue where it localizes primarily to resident immune cells such as microglia and astrocytes.[111, 203, 109] Our studies indicate that activation of resident brain cells directly by LPS could also play an important role in the induction of tolerance against subsequent ischemic injury. Indeed, we have previously

demonstrated that cortical cultures preconditioned by LPS are capable of releasing soluble TNFα, which is essential for neuroprotection by LPS preconditioning (refer to Chapter 3). Thus, inflammatory responses within the brain could shape the neuroprotective response initiated by LPS preconditioning.

We demonstrated that LPS preconditioning increases the neuronal resistance to the cytotoxic effects of TNFα in the setting of ischemia. Attenuation of TNFα-mediated damage is protective in ischemia, as our prior studies demonstrated that inhibition of endogenous TNFα activity during OGD reduced cell death (refer to Figure 3-4). This is consistent with other studies that support a deleterious role for TNFα in stroke damage.[159-161, 199] The capacity of LPS preconditioning to abrogate TNFα-mediated damage during ischemia may be an important mechanism involved in the neuroprotective effects of LPS preconditioning. Our findings further suggest that the injurious actions of TNFα are mitigated by changes within neurons themselves, apart from other systemic inflammatory responses that might be altered *in vivo*.

Although TNFα exerts its effects through two distinct cell-surface receptors, TNFR1 and TNFR2, the majority of the cytotoxic effects of TNFα are mediated by TNFR1.[139, 141, 204, 205] The cellular response to TNFα is thought to result from the balance between distinct intracellular signaling pathways induced by TNFα that promote either cell survival or cell death.[139] Thus, the capacity of LPS preconditioning to diminish the neuronal sensitivity to TNFα during ischemia may be a consequence of impaired injurious signaling events or increased cell survival and protective responses. Our prior studies of

LPS preconditioning and ischemic tolerance *in vivo* have revealed that neuronal expression of TNFR1 and its signaling adaptor protein TRADD are decreased following stroke in LPS preconditioned mice (refer to Chapter 3). This suggests that the signaling potential of TNFα may be dampened within neurons that have been preconditioned by LPS. Thus, further investigation using our *in vitro* model of LPS preconditioning in cortical cultures will enable us to dissect the intracellular changes in signaling events initiated by TNFα within neurons themselves in response to ischemia.

In conclusion, we present a novel model of LPS preconditioning *in vitro* in cortical neuronal cultures. LPS preconditioning rendered cortical neuronal cultures resistant to subsequent ischemic injury. Herein we defined the basic properties of LPS preconditioning *in vitro*. Neuroprotection by LPS preconditioning in cortical neuronal cultures occurs between 8 and 24h of LPS treatment, and requires *de novo* protein synthesis. Importantly, we demonstrated the neuroprotective potential of LPS preconditioning to abrogate TNFα-mediated neuronal damage following ischemia. This finding suggests that attenuation of the TNFα pathway may be a crucial component of the neuroprotective mechanism of LPS preconditioning. The results presented here will further enhance our ability to clarify the cellular and molecular events involved in the neuroprotective effects of LPS preconditioning and to distinguish the involvement of signaling events that occur within brain cells in the absence of confounding systemic effectors *in vivo*.

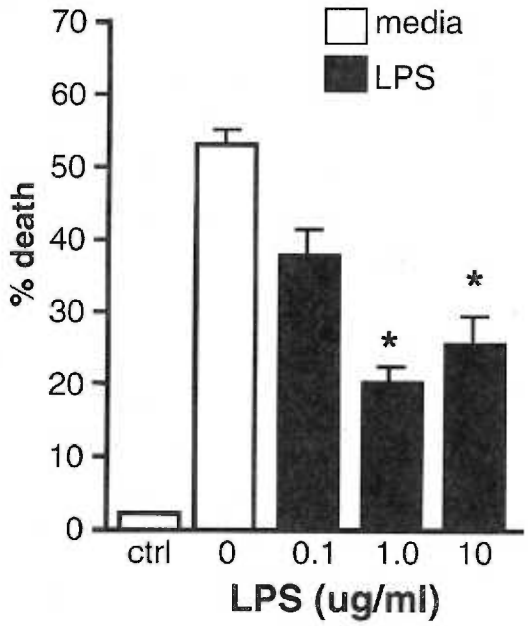

Figure 4-1. The neuroprotective effects of LPS preconditioning *in vitro* are dose-dependent. Primary cortical neuronal cultures were pretreated with different doses of LPS for a 24h duration prior to OGD. Cell death was determined 24h following OGD by propidium iodide staining. Control cultures treated with LPS alone did not show any cell death. Data are mean % death $\pm$ SEM. *$p<0.05$ vs non-preconditioned control cultures exposed to OGD, n = 3 repeated experiments.

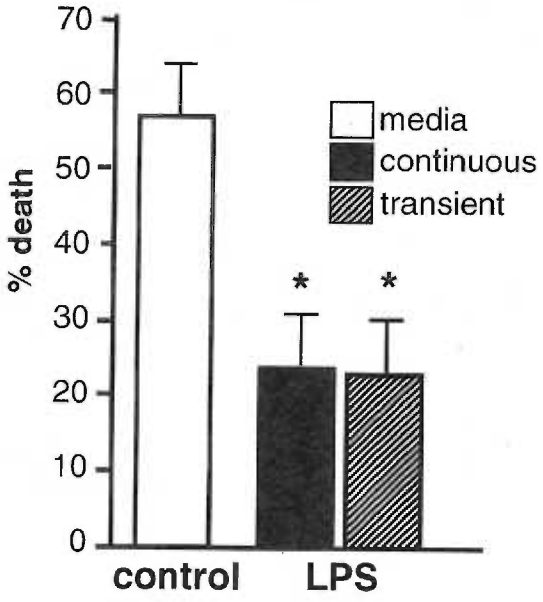

Figure 4-2. Transient LPS exposure induces neuroprotection *in vitro*. Primary cortical neuronal cultures were exposed to 1ug/ml LPS for 24h (continuous) or for 2h only followed by removal of LPS containing media and replenishment with medium that lacked LPS for the remaining 22h duration prior to OGD (transient). Cell death was determined 24h following OGD by propidium iodide staining. Data are mean % death $\pm$ SEM. *$p<0.05$ vs non-preconditioned control cultures exposed to OGD, n = 4 repeated experiments.

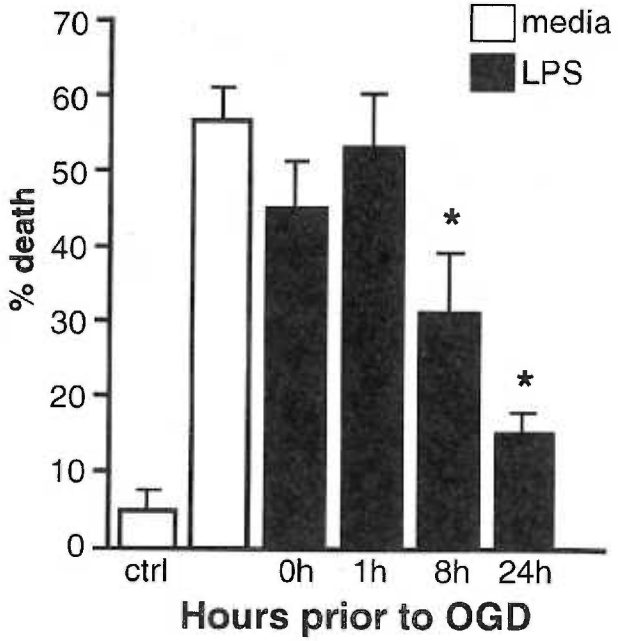

Figure 4-3. The time course of LPS-induced neuroprotection *in vitro*. Primary cortical neuronal cultures were pretreated with 1ug/ml LPS for different durations of time *prior* to OGD. Cell death was then determined 24h following OGD by propidium iodide staining. Data are mean % death $\pm$ SEM. *$p<0.05$ vs non-preconditioned control cultures exposed to OGD, n = 4 repeated experiments.

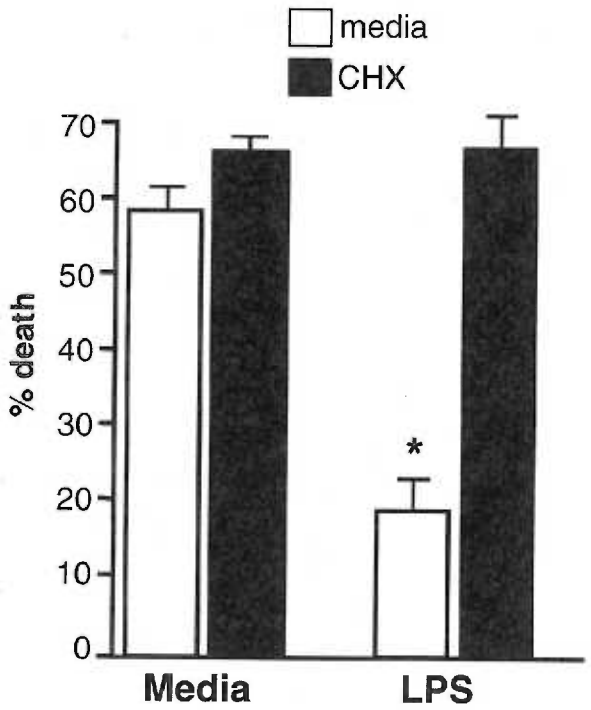

Figure 4-4. Neuroprotection by LPS *in vitro* requires *de novo* protein synthesis. Primary cortical neuronal cultures were pretreated with 1ug/ml LPS concurrently with 1uM cyclohexamide (CHX) for a 24h duration prior to exposure to OGD. Cell death was determined 24h following OGD by propidium iodide staining. Control cultures treated with CHX alone did not show altered cell death or a change in OGD-induced cell death. Data are mean % death $\pm$ SEM. *$p<0.05$ vs non-preconditioned control cultures, n = 3 repeated experiments.

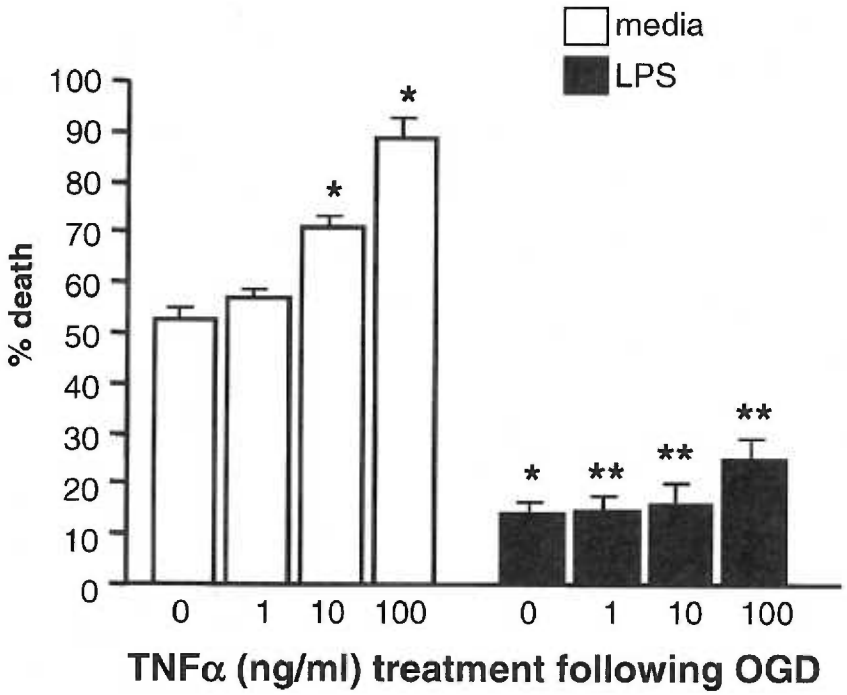

Figure 4-5. LPS preconditioning diminishes the neuronal sensitivity to TNFα-induced injury during ischemia *in vitro*. Primary cortical neuronal cultures were pretreated with 1ug/ml LPS for 24h prior to exposure to OGD. Following the termination of OGD, cultures were exposed to different doses of exogenous TNFα and cell death was assessed 24h later by propidium iodide staining. Data are mean % death ± SEM. *p<0.05 vs OGD-0 treatment group, **p<0.05 vs corresponding OGD control for each dose of TNFα, n = 4-6 repeated experiments for each dose of TNFα.

CHAPTER 5

SUMMARY, CONCLUSIONS AND PERSPECTIVES

The studies presented in this thesis demonstrated that prior exposure to the inflammatory stimulant LPS induces a state of neuroprotection to subsequent stroke in mice and renders neurons resistant to ischemic injury *in vitro*. While the activation of inflammatory cascades and the induction of TNFα *prior* to stroke play crucial roles in the initiation of LPS-induced ischemic tolerance, the findings within this thesis further reveal that suppression of injurious inflammatory responses *following* stroke could be an important component of the neuroprotective state. LPS preconditioning decreased peripheral leukocyte infiltration into the injured ischemic brain and suppressed cellular activation of microglia-macrophages in the brain—features that could contribute to neuroprotection. Moreover, LPS preconditioning suppressed proximal mediators of the TNFα-signaling pathway and attenuated the potential of TNFα to cause neuronal damage following stroke. The results presented in this thesis provide the foundation for future investigation of LPS preconditioning and advance the hypothesis that suppression of injurious inflammatory responses plays an important role in the neuroprotective effects of LPS preconditioning.

TNFα is Essential for the Priming Effects of LPS Preconditioning.

The role of TNFα in cerebral ischemia is particularly intriguing because it has opposing functions on stroke outcome. While the induction of TNFα during stroke is a potent cytotoxic molecule and exacerbates ischemic brain damage,[161] in the setting of preconditioning *prior* to stroke induction of TNFα is neuroprotective.[88, 90] This apparent

discrepancy has hindered a clear understanding of the role of TNFα in the neuroprotective mechanisms of LPS preconditioning. As such, I considered it important to first define the role of TNFα in the neuroprotective effects of LPS preconditioning in mice. Using TNFα knock-out mice, we tested whether LPS-induced neuroprotection requires TNFα. We found that LPS preconditioning in TNFα knock-out mice failed to induce neuroprotection against MCAO, which confirms a fundamental requirement for TNFα in the neuroprotective effects of LPS preconditioning and is consistent with a prior study in a rat model, whereby co-treatment with TNF-binding protein (chimeric s-TNFR1) reversed the neuroprotective effects of LPS preconditioning against MCAO.[88]

I further examined the role of TNFα in the induction of ischemic tolerance by LPS preconditioning in a novel *in vitro* model of LPS preconditioning. We established that cortical neuronal cultures preconditioned by LPS were resistant to the damaging effects of ischemic injury induced by OGD. LPS-induced TNFα activity plays a necessary role in the establishment of LPS-induced neuroprotection, as inhibition of TNFα activity during LPS preconditioning blocked neuroprotection in cortical cultures. Furthermore, the soluble form of TNFα appears to be responsible for mediating the acquisition of LPS-induced ischemic tolerance, as cortical cultures co-treated with LPS and a TACE inhibitor, which prevents cleavage of mTNFα to its soluble form, were no longer protected against ischemic injury. Although the membrane form of TNFα has been implicated in mediating inflammatory responses,[191]these data indicate that ischemic tolerance by LPS preconditioning *in vitro* is mediated solely by the soluble form of TNFα. This latter finding also implicates TNFR1 as the mediator of the protective

signaling as the soluble form of TNFα signals predominantly through this receptor rather than TNFR2.[131]

These studies established that TNFα *prior* to stroke is a prerequisite for LPS-induced neuroprotection against stroke. However, the exact mechanisms by which systemic administration of low dose LPS initiates neuroprotective responses in the brain are not known at this time. This is further confounded by the controversy over whether or not low dose LPS administered peripherally crosses the blood-brain barrier.[206-208]The blood-brain barrier functions to control the exchange of substances between the blood and brain, and is maintained by cerebral capillary endothelial cells and the tight junctions between them.

Due to the increased expression of TLR4 on peripheral innate immune cells such as macrophages, it is conceivable that the systemic release of TNFα in response to LPS preconditioning mediates the protective effects of LPS preconditioning from the periphery to the brain. The presence of transport systems at the blood-brain barrier enables some cytokines in the blood, including TNFα, to reach regions within the brain such as the cortex.[209-211] Microglia and neurons in the brain as well as cells that make up the cerebrovasculature express both TNFR1 and TNFR2[212] and could participate in the induction of neuroprotective responses to TNFα. In addition to TNFα, ceramide generated in the periphery in response to LPS preconditioning could directly contribute to the induction of ischemic tolerance in the brain, as it freely diffuses across the blood-brain barrier.[91, 213] Ceramide is a sphingolipid that is involved in the downstream

signaling events of TNFR1 and is known to mediate the neuroprotective effects of TNFα preconditioning.[169] So in this way, ceramide could bypass the TNF-receptors and mimic many of the biological effects evoked by TNFα within the brain.

The widespread expression of TLR4 throughout the brain on microglia and astrocytes provides the possibility that LPS could directly prime the brain for subsequent ischemia through TLR4 activation. Indeed, our *in vitro* model system of LPS preconditioning demonstrated that cortical brain cells have the intrinsic capacity to respond to LPS preconditioning by becoming tolerant to subsequent ischemic injury. Moreover, as shown in Chapter 3, the ability of cortical cultures to produce TNFα in response to low dose LPS exposure supports the idea that TNFα produced within the brain could then mediate the induction of neuroprotective responses by LPS. Of particular interest, generation of reciprocal bone marrow chimeras between wild-type and TLR4 mutant mice emphasized the critical participation of TLR4 in tissue-resident cells in shaping the inflammatory response in the brain in response to systemic LPS.[214] This study demonstrated that while TLR4 function in hematopoietic peripheral immune cells is required for peripheral inflammation in response to systemic LPS, it is not sufficient for induction of early inflammatory responses in the brain. This indicates that resident cells within the brain that express TLR4 could mediate the priming effects of LPS preconditioning. The relevant TLR4-bearing cells could include endothelial cells and smooth muscle cells that make up the cerebral vasculature, as well cells associated with the cerebral vasculature such as parenchymal microglia and astrocytes.[214] Intriguingly, the presence of TLR4 detected on the vagus nerve[215] suggests that entirely novel

pathways for LPS-induced neural signaling could be involved in transmitting peripheral immune signals directly into the brain. As part of the autonomic nervous system, the vagus nerve is responsible for transducing environmental signals to the brain.[216] In the presence of systemic LPS the vagus nerve signals cytokine production within the brain.[217, 218] This suggests the possibility LPS preconditioning could directly prime the brain for subsequent stroke through vagal nerve activation. Thus, even though there are several possibilities for initiation of LPS preconditioning by TLR4 activation in the periphery and brain, these studies established that TNFα plays a fundamental role in the LPS-induction of neuroprotection against subsequent stroke.

LPS-Induced Neuroprotection Involves Suppression of the Cellular Inflammatory Response to Stroke.

Although LPS-induced inflammatory mediators such as the cytokine TNFα play a beneficial role in the induction of ischemic tolerance to stroke, the effects of LPS preconditioning on the inflammatory events involved in the neuroprotective state *following* stroke were entirely unknown when I began this thesis research. I found that suppression of cellular inflammatory responses following stroke that would ordinarily exacerbate ischemic brain damage may contribute to LPS-induced neuroprotection.

LPS Preconditioning Suppresses the Peripheral Cellular Response Following Stroke.

Reduced peripheral cellular infiltration following stroke may be a neuroprotective effect of LPS preconditioning. Peripheral neutrophils and monocytes produce inflammatory mediators including oxygen radicals and cytokines that may exacerbate neuronal damage in ischemically compromised brain tissue and directly damage the endothelium (as previously discussed in Chapter 1, section 3). Inhibition of cellular infiltration following stroke as observed in LPS preconditioned mice could be due in part to reduced numbers of neutrophils and monocytes in the blood following MCAO in LPS preconditioned mice. In addition, LPS preconditioning could reduce the capacity of circulating leukocytes to adhere to the vasculature following stroke. LPS preconditioning increases the levels of NO in the circulation, eNOS expression in the vasculature and SOD activity in the brain at the time of MCAO in rats,[89, 92, 93] and both endothelial-derived NO and SOD activity are known to exert inhibitory effects on the interaction between neutrophils and the endothelium.[195, 219-224] LPS preconditioning also inhibits neutrophil infiltration into injured ischemic heart tissue through a mechanism involving downregulation of L-selectin on the cell surface of neutrophils.[225] This result is similar to our findings that LPS preconditioning suppressed cell surface expression of CD11b on monocytes in the blood following stroke, and supports a role for diminished leukocyte activation and adherence to the endothelium in the mechanism by which LPS preconditioning reduces cellular infiltration following stroke.

LPS preconditioning could also reduce expression of chemokines and adhesion molecules following stroke as an additional mechanism to diminish the peripheral cellular infiltration and enhance neuroprotection against stroke. In response to stroke, activated microglia in the brain and endothelial cells and astrocytes within the vasculature produce chemokines and adhesion molecules that promote peripheral cellular infiltration. Microarray analysis in our lab indicated that following stroke, expression of chemokines such as IL-8, MIP-1α, MCP-1, MIP-2α/CINC and the adhesion molecules ICAM-1 and VCAM-1 was decreased in LPS preconditioned mice compared to saline controls. Cellular infiltration and ischemic brain damage is reduced in rodents when such chemokines and/or adhesion molecules are blocked at the time of stroke,[67, 69, 70, 72, 77, 79] which suggests the neuroprotective effects of LPS preconditioning involve diminished production of chemokines and adhesion molecules following stroke.

Suppression of the peripheral cellular inflammatory response in the blood could directly contribute to the delayed vascular protective effects of LPS preconditioning. Following stroke in non-preconditioned rodents a progressive impairment in microvascular perfusion normally occurs. Activated neutrophils and monocytes are though to contribute to the vascular damage through excessive production of injurious inflammatory mediators (ROS, cytokines, proteases).[55, 56, 226] Leukocyte adherence to the cerebral vasculature is also thought to directly cause endothelial damage, as the endothelium-dependent vasodilation was preserved following ischemia in mice deficient in the adhesion molecules CD11b and ICAM-1.[227] Moreover, leukocyte and platelet adhesion to the endothelium promotes coagulation and congestive occlusion of the capillary vessel,

thereby further contributing to hypoperfusion.[55, 58, 228] Prior studies showed that LPS preconditioning reduces the development of hypoperfusion deficits at later times following stroke,[92] which is consistent with recent studies that demonstrate LPS preconditioning diminishes the impairment of endothelial vasorelaxation and decrease in K^+ currents in smooth muscle cells that would otherwise occur following cerebral ischemic-reperfusion injury.[94] Taken together with my findings that demonstrated LPS preconditioning suppressed the number of neutrophils and prevented monocyte activation in the blood following stroke, these data suggest that downregulation of systemic cellular inflammatory responses that occur at the blood-endothelial interface could act to reduce vascular damage, and thereby improve cerebrovascular perfusion to otherwise ischemically compromised brain tissue.

LPS Preconditioning Suppresses Microglial Activation in the Brain Following Stroke.

The capacity of LPS preconditioning to prevent cellular activation of microglia in *the brain* in response to stroke could also be a crucial component of the neuroprotective mechanisms induced by LPS preconditioning. As the predominant immune cell in the brain, microglia are considered the primary cellular effector of numerous inflammatory responses following stroke. Once activated, microglia produce inflammatory mediators such as cytokines, chemokines, oxygen radicals and proteases that exacerbate ischemic brain damage. Moreover, activated microglia are capable of directly causing neuronal apoptosis following ischemia through mechanisms involving TNFα and Fas (as previously discussed in detail in Chapter 1, section 3).[44, 45, 47] As such, attenuation of

hyper-responsive microglia during ischemia could limit their production of inflammatory mediators and minimize their ability to cause neuronal death, and thereby contribute to the neuroprotective effects of LPS preconditioning. This explanation would be consistent with my finding that TNFα production was suppressed following MCAO in LPS preconditioned mice. Furthermore, myeloperoxidase activity, which is a feature of activated microglia and mediates release of reactive oxygen species, is also reduced following stroke in rats that have been preconditioned by DPL (diphosphoryl lipid A).[95] Furthermore, microarray studies in our lab indicated that transcription of inflammatory cytokines (IL-1β, IL-6 and IL-12) and chemokines (IL-8, MIP-1α, MCP-1, MIP-2α/CINC) that are typically produced by activated microglia was reduced in the ischemic brain hemisphere of LPS preconditioned mice compared to saline controls. Thus, regulation of microglial responsiveness to stroke during LPS-induced ischemic tolerance could diminish activation of specific inflammatory pathways and limit their capacity to cause neuronal damage.

Although the mechanisms by which LPS preconditioning suppresses microglial activation in response to subsequent stroke are not entirely understood at this time, increased SOD activity in the brain could be involved. SOD plays an important role in immune responses as it inhibits microglial and macrophage activation and prevents their production of inflammatory mediators such as TNFα and ROS,[196, 229] and LPS or DPL preconditioning increases SOD activity in the brain during MCAO[89, 95] Thus, the neuroprotective effects of LPS preconditioning could involve increased expression of

SOD and other negative regulators that suppress microglia activation during stroke (as discussed in more detail later in my proposed model).

LPS-Induced Neuroprotection Involves Attenuation of the Cytotoxic Effects of TNFα Following Stroke.

Our studies showed that LPS preconditioning attenuates TNFα-induced injury following stroke. In light of the evidence that supports a deleterious role for TNFα during stroke, diminished sensitivity of the brain to TNFα could be an important component of the neuroprotective mechanisms involved in LPS preconditioning. The capacity of LPS preconditioning to attenuate the cytotoxic effects of TNFα following stroke appear to be mediated in part by reduction of the amount of available TNFα, as LPS preconditioned mice showed reduced levels of TNFα and increased levels of s-TNFR1 following stroke. The importance of reducing the actions of TNFα for neuroprotection by LPS preconditioning is supported by studies that show exogenous treatment with anti-TNFα antibody or s-TNFR1 at the time of stroke reduces ischemic brain damage in rodents.[157-162, 199] This finding also underscores the importance of modulating TNFR1 for neuroprotection by LPS preconditioning and suggests that increased levels of *endogenous* s-TNFR1 could simultaneously neutralize the cytotoxic effects of TNFα and dampen TNFR1-mediated signaling.

In addition to reduced extracellular actions of TNFα, the capacity of LPS preconditioning to diminish the cytotoxic effects of TNFα appear to involve a novel neuroprotective mechanism whereby the cellular response to TNFα is abrogated. I found that LPS preconditioning reduced neuronal expression of TNFR1 and TRADD following stroke, which supports a role for diminished TNFR1-mediated signaling in LPS-induced neuroprotection. Moreover, the importance of reduced cellular responsiveness to TNFα for neuroprotection by LPS preconditioning is underscored by my finding that i.c.v. administration of TNFα following stroke did *not* exacerbate ischemic brain damage in mice that had been preconditioned by LPS. This was in contrast to non-preconditioned mice, which showed an increase in infarct size in response to TNFα treatment following MCAO. Furthermore, LPS preconditioning increased the neuronal resistance to the cytotoxic effects of TNFα following OGD *in vitro*. These findings indicate that LPS preconditioning abrogates the deleterious effects of TNFα during stroke, which could be a crucial neuroprotective mechanism in LPS-induced ischemic tolerance.

Following stroke, TNFα is a potent cytotoxic molecule and is involved in several facets of cerebral ischemic injury, perhaps due to the fact that TNFα-signaling involves pathways leading to both inflammation and apoptosis (Figure 1-3) (as discussed in detail in Chapter 1, section 7). Thus, LPS preconditioning could diminish TNFα-induced activation of inflammatory responses and/or apoptosis, and thereby provide neuroprotection. In support of attenuated TNFα-induced inflammatory responses as a neuroprotective effect of LPS preconditioning, I showed that peripheral cellular infiltration was suppressed following stroke in LPS preconditioned mice. Blockade of

TNFα during stroke reduces ICAM-1 expression and abrogates peripheral cellular infiltration in the brain following stroke,[158, 164] which suggests that mitigation of TNFα-initiated inflammatory events could be a contributing factor to reduced cellular infiltration during LPS-induced ischemic tolerance. TNFα is also thought to play a critical role as an autocrine-mediator of microglial activation in the brain.[33] As such, in the setting of LPS preconditioning, diminished TNFα signaling in microglia could prevent their prolonged activation following stroke, and would be consistent with our findings that microglial activation was reduced following stroke in LPS preconditioned mice. These data suggest that the neuroprotective effects of LPS preconditioning involve reduced TNFα-mediated inflammatory responses, which could limit the progression of ischemic injury in the brain.

Suppression of TNFα-induced apoptotic signaling events could also be involved in the neuroprotective mechanisms of LPS preconditioning. TNFα is capable of activating apoptosis through signaling events that are mediated by FADD and caspase-8, and could directly initiate neuronal death following stroke (Figure 1-3). Immunofluorescent staining in our lab indicated that neuronal expression of FADD was increased following stroke in non-preconditioned mice. In contrast, mice that were preconditioned by LPS showed reduced neuronal expression of FADD following stroke. Moreover, microarray data analysis performed in our lab indicated that expression of caspase-8 was also reduced following MCAO in LPS preconditioned mice compared to saline controls. Thus, LPS preconditioning could promote neuronal resistance to TNFα during ischemia

by reducing expression of the TNFR1-TRADD complex on the cell surface and by reducing expression of intracellular mediators of apoptosis such as FADD and caspase-8.

An additional mechanism by which the injurious actions of TNFα could be attenuated by LPS preconditioning could be through the upregulation of protective responses such as antioxidant SOD or the anti-apoptotic proteins such as bcl-2 and bcl-x. LPS or DPL preconditioning increases SOD activity in the brain during stroke.[89, 95] SOD activity is considered an important self-defense mechanism against the cytotoxic effects of TNFα, as it functions to inhibit TNFα-mediated NF-κB activation and cell death.[195, 197, 198, 230] In addition, upregulation of cell survival proteins could also be involved in the neuroprotective effects of LPS preconditioning against TNFα-induced injury, as the ischemic tolerant effects of TNFα pretreatment involve increased expression of anti-apoptotic proteins bcl-2 and bcl-x.[172] Such cell survival proteins are neuroprotective against stroke and also protect against TNFα-induced apoptosis.[231, 232] It seems possible that other cell survival proteins that protect against TNFα-induced injury could also be involved in the neuroprotective effects of LPS preconditioning. For example, the zinc finger protein A20, which disrupts recruitment of death domain signaling molecules such as TRADD and FADD to the TNFR1 receptor, was recently shown to have a neuroprotective function in the setting of stroke.[233] Likewise, the inhibitor-of-apoptosis (IAP) family member c-IAP, which contains ubiquitin protein ligase activity, has been implicated in neuroprotection against stroke during ischemic preconditioning.[234] As c-IAP functions to target signaling molecules for proteosomal degradation and blocks activation of caspase-8,[235] it could also ameliorate the cytotoxic effects of TNFα, and

thereby play a role in the neuroprotective effects of LPS preconditioning. Thus, LPS preconditioning could diminish the cellular sensitivity to the cytotoxic effects of TNFα during stroke through both downregulation of injurious signaling pathways and the upregulation of cell survival proteins and negative regulators of TNFα-signaling (SOD, A20, c-IAP) following stroke.

The neuroprotective effects of LPS preconditioning against TNFα-induced injury following stroke could also indirectly involve reduced glutatmate-neurotoxicity, as TNFα and glutamate interact to increase neuronal death. Glutamate is the main excitatory amino acid neurotransmitter that is increased in response to ischemic injury. Glutamate binding to its postsynaptic receptors causes an influx of calcium that promotes neuronal death. Glutamate-excitotoxicity in general is linked to excessive glutamate activation of receptors, particularly the N-methyl-D-aspartate (NMDA) receptor. TNFα directly potentiates glutamate-induced neurotoxicity, which appears to be mediated in part through activation of glutamate-NMDA receptor and activation of NF-κB.[167, 236] TNFα also indirectly contributes to glutamate-neurotoxicity by inhibiting glutamate uptake, which would be expected to further sustain the extracellular levels of glutamate and thereby neurotoxicity.[163, 167, 237-239] Thus, reduced actions of TNFα following stroke could further contribute to the neuroprotective effects of LPS preconditioning by reducing neuronal vulnerability to glutamate excitotoxicity.

LPS-Induced Cerebral Ischemic Tolerance and Endotoxin Tolerance: A Common Reprogramming Mechanism?

My findings in LPS preconditioning reveal that suppression of injurious inflammatory responses to stroke could be an important component of the neuroprotective state. This is similar in nature to endotoxin tolerance, wherein a prior exposure to low dose LPS protects against the otherwise toxic effects of large doses of LPS. The protective state observed in endotoxin tolerance is manifested by suppressed macrophage activation and reduced production of inflammatory cytokines (TNFα, IL-1β and IL-6) in response to challenge with LPS.[12] Furthermore, low dose LPS priming leads to sustained or increased production of anti-inflammatory mediators such as s-TNFR1 and IL-10 that further limit inflammation. This specific change in the cellular response to LPS-induced injury has been referred to as reprogramming.[11] I postulated that the reprogramming capacity observed in endotoxin tolerance could also be involved in LPS-induced neuroprotection against stroke.

In support of this postulate, our findings reveal that neuroprotective features of ischemic tolerance during stroke resemble effects observed in endotoxin tolerance. First, microglia-macrophage and monocyte cell populations are not activated in response to subsequent stroke in LPS preconditioned mice. Secondly, TNFα production is decreased and s-TNFR1 production is increased in response to stroke in LPS preconditioned mice. The altered ratio of TNFα to s-TNFR1 is considered a hallmark of reprogramming in endotoxin tolerance, representing the shift from inflammatory to anti-inflammatory

responses.[12] In view of these parallels between the neuroprotective state induced by LPS preconditioning and endotoxin tolerance, it seems possible that common mechanisms may underlie endotoxin and ischemic tolerance induced by LPS. The neuroprotective effects of LPS preconditioning could involve an analogous reprogramming mechanism in the setting of stroke, thereby limiting injurious inflammatory responses.

The reprogramming capacity of LPS preconditioning would be expected to be neuroprotective in the setting of stroke, as microglia-macrophages may shift the balance between production of inflammatory and anti-inflammatory mediators to ischemic injury. Microarray analysis performed in our lab indicated the genomic response to stroke is altered in LPS preconditioned mice. This unique change in gene expression in response to ischemic injury could represent a neuroprotective response due to reprogramming, as expression of several inflammatory genes such as cytokines (IL-1β, IL-6, IL-12), chemokines (IL-8, MIP-1α, MCP-1, MIP-2α/CINC) and adhesion molecules (ICAM-1 and VCAM-1) was suppressed in response to stroke in mice preconditioned by LPS. Furthermore, uniquely expressed patterns of genes observed following stroke in mice that have been preconditioned by LPS indicated that anti-inflammatory and neuroprotective responses involving TGF-β and type-I interferon were increased following stroke.[202, 240] These findings suggest that the neuroprotective effects of LPS preconditioning may involve reprogramming the brain's response to stroke, wherein injurious inflammatory pathways such as TNFα are decommissioned and beneficial anti-inflammatory and neuroprotective pathways are increased. Such a reprogramming phenotype in the setting of stroke is also consistent with our finding that LPS preconditioning suppressed

neutrophil accumulation in the brain following stroke, as neutrophil activity and infiltration are also decreased in endotoxin tolerance.[187]

In view of this analogy between endotoxin tolerance and LPS-induced neuroprotection, I would further propose that the capacity of LPS preconditioning to reprogram the brain's response to ischemic injury could involve *de novo* synthesis of negative regulators of inflammatory signaling as a consequence of *prior* immune activation by LPS. In endotoxin tolerance, increased expression of feedback inhibitors such as SHIP-1, SOCS-1 and IRAK-M, are crucial for host protection against excessive inflammatory responses initiated by toxic LPS.[127, 128, 241] These negative regulators are induced by *prior* low dose LPS priming and function to extinguish or modulate TLR4 and inflammatory cytokine signaling to subsequent LPS-challenge. Indeed, findings in our lab have revealed that LPS preconditioning increased expression of the negative regulators SHIP-1 and SOCS-1 in the brain prior to stroke, which could function to limit TLR4 and TNFα-induced inflammatory pathways as in endotoxin tolerance. Furthermore, activation of inflammatory pathways and *de novo* protein synthesis are crucial for the ischemic tolerance induced by LPS preconditioning, as the neuroprotective effects of LPS preconditioning were blocked by co-treatment with anti-inflammatory drugs or protein synthesis inhibitors, and common inflammatory mediators such as TNFα and NO appear to be involved in both the ischemic tolerant effects of LPS preconditioning[88, 93] and in the establishment of endotoxin tolerance.[242-245]

Our findings indicate that TNFα in particular, could be a crucial mediator of the reprogramming response to stroke during LPS preconditioning by ultimately diminishing subsequent TNFα-induced inflammatory responses and promoting TNFα-induced neuroprotective responses. The capacity of LPS preconditioning to diminish neuronal vulnerability to subsequent ischemic injury depends on TNFα, which indicates that TNFα is essential for priming the emergence of the neuroprotective state by LPS preconditioning. Indeed, pretreatment solely with TNFα increases expression of the neuroprotective proteins MnSOD, bcl-2, bcl-x and calbindin in cortical neuronal cultures.[163, 171, 172] Importantly, TNFα pretreatment in cultured brain cells reduces cellular activation to subsequent challenge with TNFα, as NF-κB driven gene expression was modified such that ICAM-1 expression is suppressed, while expression of MnSOD is preserved.[169, 176] This capacity of TNFα pretreatment to suppress subsequent inflammatory effects induced by TNFα while preserving neuroprotective responses to TNFα could be a crucial component of the reprogramming response to stroke induced by LPS preconditioning.

In support of this postulate, microarray analysis in our lab indicated that following stroke, expression of ICAM-1 was decreased while expression of MnSOD, bcl-2 and calbindin was increased in LPS preconditioned mice compared to saline controls. This is consistent with a shift in TNFα-induced signaling events observed in "TNFα-tolerance" as mentioned above, suggesting that the inflammatory actions of TNFα are inhibited while neuroprotective actions of TNFα are enhanced during ischemic tolerance induced by LPS preconditioning. That suppression of TNFα-induced injurious inflammatory responses is

a component of the reprogramming response in LPS-induced ischemic tolerance is further supported by our finding that LPS preconditioning suppressed microglial activation and cellular infiltration following stroke, as the ischemic tolerant effects of TNFα preconditioning are sufficient for suppression of microglial activation and cellular infiltration in response to stroke.[90] Finally, neuroprotection against subsequent TNFα-induced neuronal death was a crucial component of LPS-induced ischemic tolerant state.

A Model of LPS-Induced Neuroprotection Against Stroke.

In conclusion, these studies advance our understanding of the neuroprotective effects of LPS preconditioning. The findings in this thesis reveal that suppression of injurious inflammatory responses to stroke may be a crucial component of the neuroprotective mechanism of LPS preconditioning. Intriguingly, the neuroprotective features observed in ischemic tolerance induced by LPS preconditioning parallel the adaptive reprogramming strategy observed in endotoxin tolerance. This suggests that LPS preconditioning could confer neuroprotection by reprogramming the brain's response to subsequent stroke, thereby limiting injurious inflammatory responses.

I present a schematic model of LPS-induced neuroprotection against stroke in **Figure 5**. In response to LPS preconditioning, macrophages and microglia in the periphery and brain initiate inflammatory responses and produce TNFα, thereby inducing *de novo* protein synthesis of negative regulators of inflammatory signaling pathways (SOD, NO,

SHIP-1, SOCS-1, IκB, A20, c-IAP). TNFα *prior* to stroke is a crucial effector of the reprogramming response induced by LPS preconditioning, as it acts in an autocrine manner in microglia and a paracrine fashion in neurons to ultimately uncouple TNFα-induced injurious responses and promote TNFα-induced neuroprotective responses (SOD, bcl-2, calbindin). Thus, in the setting of LPS preconditioning, the host's response to subsequent stroke has been reprogrammed such that activation of injurious inflammatory responses to stroke is suppressed. Activation of monocytes and microglia is suppressed and production of injurious inflammatory mediators such as TNFα is reduced, while production of anti-inflammatory mediators such as s-TNFR1 is increased. This shift in the inflammatory response minimizes cerebral vascular damage and peripheral cellular infiltration, and thereby reduces ischemic brain damage. Moreover, increased resistance within the brain to TNFα-mediated damage is a crucial component of the neuroprotective state, as TNFα-induced neuronal damage is reduced while TNFα-induced neuroprotective actions (SOD, blc-2, calbindin) could be increased.

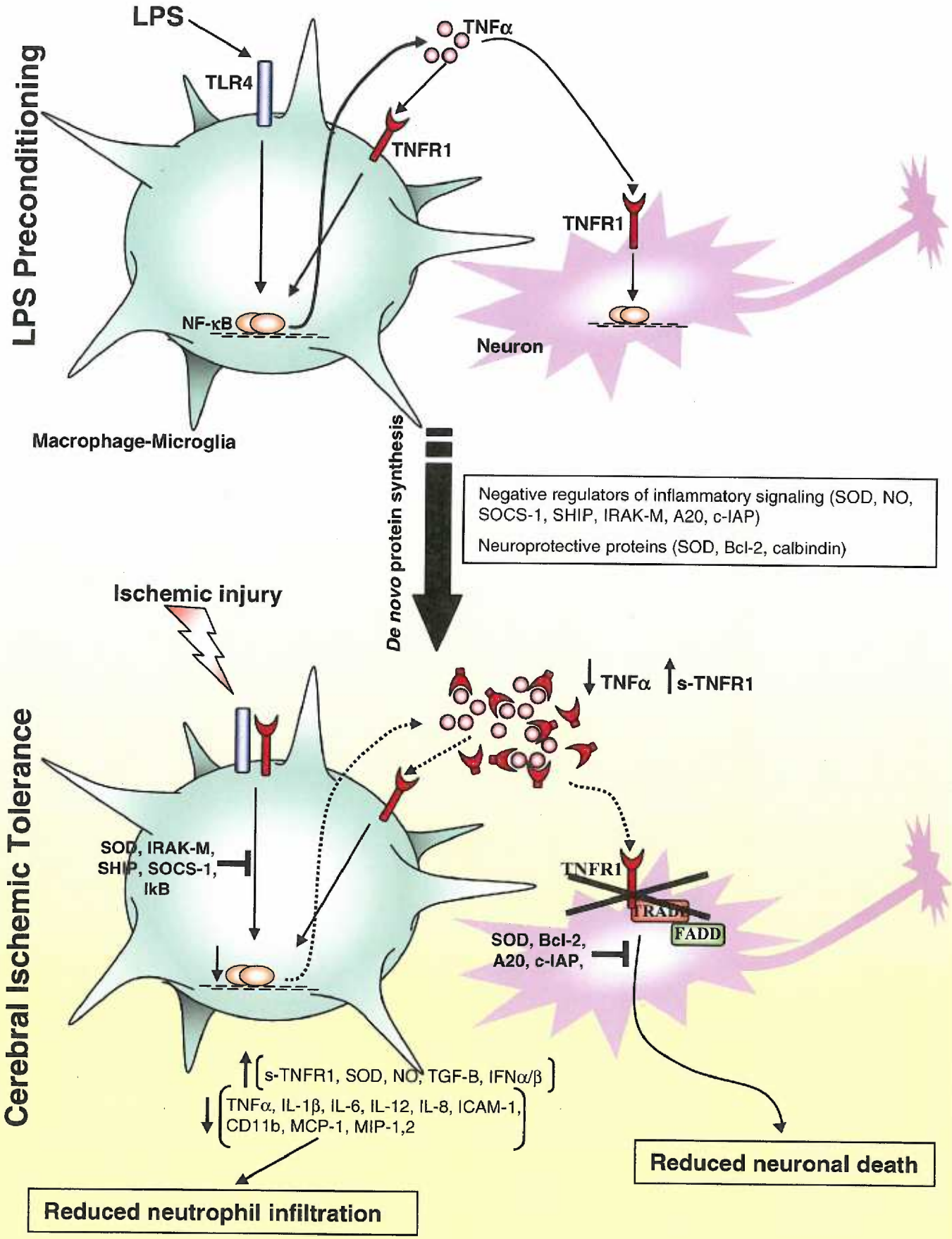

Figure 5. Schematic representation of LPS-induced cerebral ischemic tolerance to stroke.

PERSPECTIVES

The inflammatory response is essential for elimination of invading pathogens, and in the case of tissue injury inflammation plays a beneficial role in tissue repair, regeneration, restoration of homeostasis and elimination of damaged cells. However, it is important that such immune reactions be appropriately regulated, otherwise excessive inflammation leads to tissue damage. Following stroke the intensity of the inflammatory response is deleterious to neuronal function and viability. The finding that LPS preconditioning causes a suppression of injurious inflammatory responses to stroke may reflect a conserved mechanism for regulating the host's response to harmful stimuli. Thus, strategies that have evolved to protect against sepsis or persistent bacterial infections could also to be protective against the injurious effects of ischemia. While the capacity of LPS preconditioning to confer neuroprotection against stroke may not be an evolutionarily conserved mechanism *per se*, common molecular mediators of injury in both sepsis and stroke, such as TNFα or reactive oxygen species, result in cross-tolerance.

Intriguingly, recent studies suggest that endogenous signals (e.g. HSP60, HSP70, fibronectins and hylauronic acid) that originate from injured cells can activate TLR4 in the same way that LPS activates TLR4 (as reviewed[102]). Furthermore, TLR4 and other TLRs are increased in inflamed CNS tissue, and recent reports indicate that TLR4 expression is increased in the brain in response to hypoxia-ischemia in rats.[246] Moreover, TLR4 has been shown to promote injurious inflammatory responses during ischemia in

other organs such as the heart and liver, as TLR4-deficiency reduces ischemic tissue damage.[247-250] Thus, it is plausible that a minor tissue insult and inflammatory response could release endogenous "danger" signals that prime the brain for tolerance via TLR4 to subsequent ischemic injury that would otherwise involve activation of TLR4.[251] As such, TLR4 could be involved in reprogramming the brain's response to subsequent stroke via mechanisms similar to those we observed in LPS-induced ischemic tolerance, wherein deleterious inflammatory responses are suppressed. Such homologous tolerance mediated through TLR4 could be an evolutionarily conserved mechanism to protect against inflammatory-mediated tissue injury, and in the case of the brain, neuroprotection against stroke.

These findings in LPS preconditioning have important implications in terms of neuroprotective mechanisms against stroke and suggest that inhibition of specific inflammatory pathways, such as TNFα could be promising targets for intervention in acute stroke. Importantly, LPS preconditioning offers the therapeutic potential to prevent stroke damage in patients where brain damage is anticipated. Induction of ischemic tolerance by LPS preconditioning could be crucial for patients that undergo coronary artery bypass surgery or brain surgery and for individuals at high risk for stroke. Many stroke survivors are left with severe mental and physical disabilities. Thus, the ability to salvage the ischemic brain by LPS preconditioning could improve recovery, reduce requirements for physical therapy and lessen medical costs. Furthermore, induction of ischemic tolerance by LPS is also known for other organs such as the heart[252] and liver,[253] and common protective mechanisms could be involved. This implies that the therapeutic

potential of LPS preconditioning could be very broad in its applications. In addition, "ischemic-reperfusion injury" is also thought to exacerbate allograft injury during organ transplantation and is thought to activate the innate immune system.[254] Thus, LPS preconditioning has therapeutic potential for protection against other types of injurious inflammatory responses as in the case of tissue transplantation. For therapeutic purpose, it is conceivable that modified forms of Lipid A such as DPL or Lipid A mimetics that might have fewer side effects in humans would be a beneficial alternative to pretreatment with LPS. Moreover, the immune stimulatory effects of other PAMPs such as CpGs, are currently being used for therapeutic treatment of human diseases such as cancer and allergies.[255] It seems reasonable that therapeutic preconditioning with PAMPs other than LPS could provide neuroprotection against stroke in a similar mechanism as LPS preconditioning.

Appendix Table 1. The Physiological Effects of LPS Preconditioning in Mice

	SALINE	LPS
Blood gases (mmHg)		
pH	7.2±0.01	7.2±0.03
pCO_2	49.8±1.3	58.1±5.2
pO_2	345±52.3	369±58.5
Blood flow (PU)	322.2±58.7	328.1±66.4
MABP (mmHg)	84.7±2.4	82.5±0.5
Heart rate (bpm)	442.6±18.4	448.5±14.7
Temperature (°C)	36.14±0.21	36.3±0.17
Weight (g)	24.25±0.34	23.56±0.41
Glucose (mg/dL)	97.11±3.34	100.75±2.67

Physiological effects of LPS were measured at the time of MCAO (72h post treatment with 0.2mg/kg LPS or saline) under anesthesia. Values are mean ± SEM, *$p<0.05$ vs saline controls, n = 8 mice/treatment. MABP: mean arterial blood pressure; PU: perfusion units.

Appendix Table 2. The Effect of LPS Preconditioning on Peripheral Cellular Composition in Mice

	1d	2d	3d	4d
Neutrophils				
Saline	6.1 ± 0.3	4.6 ± 0.5	6.5 ± 0.6	4.3 ± 0.6
LPS	42.6 ± 3.2*	7.3 ± 1.2	4.8 ± 0.5	4.0 ± 0.5
Monocytes				
Saline	8.6 ± 0.6	8.0 ± 0.2	8.1 ± 0.8	8.0 ± 0.8
LPS	13.9 ± 1.3*	10.1 ± 0.8*	18.3 ± 2.0*	14.7 ± 0.9*
T cells				
Saline	22.0 ± 0.6	23.5 ± 2.2	22.2 ±0.6	24.0 ± 1.8
LPS	15.0 ± 1.8*	18.5 ± 1.1	22.5 ± 1.1	22.8 ± 0.8
B cells				
Saline	54.5 ± 0.8	54.8 ± 1.4	49.8 ± 1.7	53.4 ± 1.6
LPS	28.2 ± 2.1*	50.8 ± 1.1*	46.7 ± 2.9	52.6 ± 1.3

Blood was collected over time from mice that were preconditioned with LPS (0.2mg/kg) or saline, and the cell populations were quantified by flow cytometry. Equal numbers of $CD45^+$ cells were gated and the values are mean percentage of $CD45^+$ cells ± SEM, * $p<0.05$ vs saline controls, n = 8 mice/ treatment. Note: Actual cell number was also determined at these same times and was found to directly correlate with the changes in cell percentages shown here.

LITERATURE CITED

1. Rosenzweig HL, Lessov NS, Henshall DC, Minami M, Simon RP, Stenzel-Poore MP. Endotoxin preconditioning prevents the cellular inflammatory response during ischemic neuroprotection in mice. *Stroke*. 2004;11:2576-2581
2. Association AH. Heart and stroke statistical update. 2005
3. Stephenson J. Rising stroke rates spur efforts to identify risks, prevent disease. *JAMA*. 1998;279:1239-1240
4. Fisher M, Bogousslavsky J. Further evolution toward effective therapy for acute ischemic stroke. *JAMA*. 1998;279:1298-1303
5. Plum F. Neuroprotection in acute ischemic stroke. *JAMA*. 2001;285:1760-1761
6. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA. Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. *The New England Journal of Medicine*. 2001;344:395-402
7. Zoppo GJD. Thrombolytic therapy in the treatment of stroke. *Drugs*. 1997;54:90-98
8. Zoppo GJD. Clinical trials in acute stroke: Why have they not been successul? *Neurology*. 1998;51:S59-61
9. Devuyst G, Bogousslavsky J. Recent progress in drug treatment for acute stroke. *J Neurol Neurosurg Psychiatry*. 1999;67:420-425
10. Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends in Neuroscience*. 2003;26:248-254

11. Shnyra A, Brewington R, Alipio A, Amura C, Morrison D. Reprogramming of lipopolysaccharide-primed macrophages is controlled by a counterbalanced production of Il-10 and Il-12. *Journal of Immunology*. 1998;160:3729-3736

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

13. Dirnagl U, Iadecola C, Moskowitz M. Pathobiology of ischaemic stroke: An integrated view. *TINS*. 1999;22:391-397

14. Mergenthaler P, Dirnagl U, Meisel A. Pathophysiology of stroke: Lessons from animal models. *Metabolic Brain Disease*. 2004;19:151-167

15. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. *Nature Reviews Neuroscience*. 2003;4:399-145

16. Zheng Z, Zhao H, Steinberg GK, Yenari MA. Cellular and molecular events underlying ischemia-induced neuronal apoptosis. *Drug News Perspect*. 2003;16:497-503

17. Iadecola C, Ross, M.E. Molecular pathology of cerebral ischemia: Delayed gene expression and strategies for neuroprotection. *Annals New York Academy of Sciences*. 1997;835:203-217

18. Hossmann KA. The hypoxic brain. Insights from ischemia research. *Adv Exp Med Biol*. 1999;474:155-169

19. Zoppo GDJ, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feurstein GZ. Inflammation and stroke: Putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol*. 2000;10:95-112

20. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: New opportunities for novel therapeutics. *J Cereb Blood Flow & Metab*. 1999;19:819-834

21. Stanimirovic D, Satoh K. Inflammatory mediators of cerebral endothelium: A role in ischemic brain inflammation. *Brain Pathol*. 2000;10:113-126

22. Zheng Z, Yenari MA. Post-ischemic inflammation: Molecular mechanisms and therapeutic implications. *Neurol Res*. 2004;26:884-892

23. Wang CX, Shuaib A. Involvement of inflammatory cytokines in central nervous system injury. *Prog Neurobiol*. 2002;67:161-172

24. Lawson LJ. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience*. 1990;39:151-170

25. Abraham H, Lazar G. Early microglia reaction following mild forebrain ischemia induced by common carotid artery occlusion in rats. *Brain Research*. 2000;862:63-73

26. Morioka T, Kalehua AN, Streit WJ. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *Journal of Comparative Neurology*. 1993;327:123-132

27. Kato H, Walz W. The initiation of the microglial response. *Brain Pathol*. 2000;10:137-143

28. Ladeby R, Wirenfeldt M, Garcia-Ovejero D, Fenger C, Dissing-Olesen L, Dalmau I, Finsen B. Microglia cell population dynamics in the injured adult central nervous system. *Brain Res Rev*. 2005;48:196-206

29. Raivich G, Bohatschek M, Kloss C, Werner A, Jones L, Kreutzberg G. Neuroglial activation repertoire in the injured brain: Graded response, molecular mechanisms and cues to physiological function. *Brain Research Reviews*. 1999;30:77-105

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

31. Abraham H, Losonczy A, Czeh G, Lazar G. Rapid activation of microglial cells by hypoxia, kainic acid, and potassium ions in slice preparations of the rat hippocampus. *Brain Res*. 2001;906:115-126

32. Eun SY, Hong YH, Kim EH, Jeon H, Suh YH, Lee LE, Jo C, Jo SA, Kim J. Glutamate receptor-mediated regulation of c-fos expression in cultured microglia. *Biochem Biophys Res Commun*. 2004;325:320-327

33. Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Autocrine activation of microglia by tumor necrosis factor-alpha. *J Neuroimmunol*. 2005;162:89-96

34. Gregersen R, Lambertsen K, Finsen B. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *Journal of Cerebral Blood Flow and Metabolism*. 2000;20:53-65

35. Hanisch UK. Microglia as a source and target of cytokines. *Glia*. 2002;40:140-155

36. Davies CA, Loddick SA, Toulmond S, Stroemer RP, Hunt J, Rothwell NJ. The progression and topographic distribution of interleukin-1 beta expression after

permanent middle cerebral artery occlusion in the rat. *J Cereb Blood Flow & Metab*. 1999;19:87-98

37. Yruanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci*. 1998;95:15769-15774

38. Suk K. Minocycline suppresses hypoxic activation of rodent microglia in culture. *Neurosci Lett*. 2004;366:167-171

39. Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci*. 2001;21:2580-2588

40. Tikka TM, Koistinaho JE. Minocycline provides neuroprotection against n-methyl-d-aspartate neurotoxicity by inhibiting microglia. *J Immunol*. 2001;166:7527-7533

41. Wang G, Deng H, Maier C, Sun G, Yenari M. Mild hypothermia reduces ICAM-1 expression, neutrophil infiltration and microglia/monocyte accumulation following experimental stroke. *Neuroscience*. 2002;114:1081-1090

42. Marks L, Carswell HV, Peters EE, Graham DI, Patterson J, Dominiczak AF, Macrae IM. Characterization of the microglial response to cerebral ischemia in the stroke-prone spontaneously hypertensive rat. *Hypertension*. 2001;38:116-122

43. Kingham PJ, Duzner ML, Pocock JM. Apoptotic pathways mobilized in microglia and neurons as a consequence of chromogranin A-induced microglial activation. *J Neurochem*. 1999;73:538-547

44. Taylor DL, Jones F, Kubota ES, Pocock JM. Stimulation of microglia metabotropic glutamate receptor mglu2 triggers necrosis factor alpha-induced neutrotoxicity in concert with microglia-derived Fas ligand. *J Neurosci.* 2005;25:2952-2964

45. Cacci E, Claasen JH, Kokaia Z. Microglia-derived tumor necrosis factor-alpha exaggerates death of newborn hippocammpal progenitor cells in vitro. *J Neurosci Res.* 2005;80:789-797

46. Vogt M, Bauer MK, Ferrari D, Schulze-Osthoff K. Oxidative stress and hypoxia/reoxygenation trigger CD95 (apo-1/fas) ligand expression in microglial cells. *FEBS lett.* 1998;429:67-72

47. He BP, Wen W, Strong MJ. Activated microglia (bv-2) facilitation of TNF-alpha-mediated motor neuron death in vitro. *J Neuroimmunol.* 2002;128:31-38

48. Zhao W, Xie W, Le W, Beers DR, He Y, Henkel JS, Simpson EP, Yen AA, Xiao Q, Appel SH. Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism. *J Neuropathol Exp Neurol.* 2004;63:694-977

49. Lehrmann E, Keifer R, Christensen T, Toyka KV, Zimmer J, Diemer NH, Hartung HP, Finsen B. Microglia and macrophages are major sources of locally produced transforming growth factor-beta 1 after transient middle cerebray artery occlusion in rats. *Glia.* 1998;24:437-448

50. Lu YZ, Lin CH, Cheng FC, Hseuh CM. Molecular mechanisms responsible for microglia-derived protection of sprague-dawley rat brain cells during in vitro ischemia. *Neurosci Lett.* 2005;373:159-164

51. Simone RD, Ajmone-Cat MA, Tirassa P, Minghetti L. Apoptotic PC12 cells exposing phosphatidylserine promote the production of anti-inflammatory and neuroprotective molecules by microglia cells. *J Neuropathol Exp Neurol.* 2003;62:208-216

52. Ledeboer A, Breve JJ, Wierinckx Z, Jagt Svd, Bristow AF, Leysen JE, Tilders FJ, Dam AMV. Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglia and microglial cells. *Eur J Neurosci.* 2002;16:1175-1185

53. Landeghem FKV, Stover JF, Bechmann I, Bruck W, Unterberg A, Buhrer C, Deimling AV. Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat. *Glia.* 2001;35:167-179

54. Shaked I, Tchoresh D, Gersner R, Meiri G, Mordechai S, Xiao X, Hart RP, Schwartz M. Protective autoimmunity: Interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. *J Neurochem.* 2005;92:997-1099

55. Kochanek P, Hallenbeck J. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. *Stroke.* 1992;23:1367-1379

56. Hallenbeck JM. Cytokines, macrophages, and leukocytes in brain ischemia. *Neurology.* 1997;49:S5-9

57. Dawson D, Ruetzler C, Carlos T, Kochanek P, Hallenbeck J. Polymorphonuclear leukocytes and microcirculatory perfusion in acute stroke in the SHR. *Keio J Med.* 1996;45:248-252

58. Ritter LS, Stempel KM, Coull BM, McDonagh PF. Leukocyte-platelet aggregates in rat peripheral blood after ischemic stroke and reperfusion. *Biol Res Nurs.* 2005;6:281-288

59. Arvin B, Neville LF, Barone FC, Feuerstein GZ. The role of inflammation and cytokines in brain injury. *Neuroscience & Biobehavioral Reviews.* 1996;20:445-452

60. Del-Zoppo GJ, Scmid-Schonbein GW, Mori E, Copeland BR, Chang CM. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke.* 1991;22:1276-1283

61. Hallenbeck JM, Dutka AJ, Tanishima T, Kochanek PM, Kumaroo KK, Thompson CB, Obrenovitch TP, Contreras TJ. Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. *Stroke.* 1986;17:246-253

62. Garcia J, Liu K, Yoshida Y, Lian J, Chen S, Zoppo GD. Influx of leukocytes and platelets in an evolving brain infarct (wistar rat). *American Journal of Pathology.* 1994;144:188-199

63. Barone FC, Hillegass LM, Price WJ, White RF, Lee EV, Feurerstein GZ, Sarau HM, Clark RK, Griswold DE. Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: Myeloperoxidase activity assay and histologic verification. *J Neurosci Res.* 1991;29:336-345

64. Schroeter M, Jander S, Witte OW, Stoll G. Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. *Journal of Neuroimmunology.* 1994;55:195-203

65. Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T, Kogure K. Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat. Effects of neutrophil depletion. *Stroke*. 1994;25:1469-1475

66. Shiga Y, Onodera H, Kogure K, Yamasaki Y, Yashima Y, Syozuhara H, Sendo F. Neutrophil as a mediator of ischemic edema formation in the brain. *Neurosci Lett*. 1991;125:110-112

67. Mukaida N, Matsumoto T, Yokoi K, Harada A, Matsushima K. Inhibition of neutrophil-mediated acute inflammation injury by an antibody against interleukin-8 (IL-8). *J Interferon Cytokine Res*. 1998;18:905-912

68. Beech JS, Reckless J, Mosedale DE, Grainger DJ, Williams SC, Menon DK. Neuroprotection in ischemia-reperfusion injury: An anti-inflammatory approach using a novel broad-spectrum chemokine inhibitor. *J Cereb Blood Flow & Metab*. 2001;21:683-689

69. Hughes PM, Allegrini PR, Rudin M, Perry VH, Mir AK, Wiessner C. Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model. *J Cereb Blood Flow & Metab*. 2002;22:308-317

70. Takami S, Minami M, Nagata I, Namura S, Satoh M. Chemokine receptor antagonist peptide, viral MIP-ii, protects the brain agains focal cerebral ischemia in mice. *J Cereb Blood Flow & Metab*. 2001;21:1430-1435

71. Takami S, Minami M, Katayama T, Nagata I, Namura S, Satoh M. Tak-779, a nonpeptide CC chemokine receptor antagonist, protects the brain against focal cerebral ischemia in mice. *J Cereb Blood Flow & Metab*. 2002;22:780-784

72. Zhang RL, Chopp M, Li Y, Zaloga C, Jiang N, Jones ML, Miyasaka M, Ward PA. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. *Brain Research.* 1994;654:85-95

73. Chen H, Chopp M, Zhang RL, Bodzin G, Chen Q, Rusche JR, Todd RF 3rd. Anti-CD11b monoclonal antibody reduces ischemic cell damage after transient focal cerebral ischemia in rat. *Ann Neurol.* 1994;35:458-463

74. Zhang ZG, Chopp M, Tang WX, Jiang N, Zhang RL. Postischemic treatment (2-4 h) with anti-CD11b and anti-CD18 monoclonal antibodies are neuroprotective after transient (2 h) focal cerebral ischemia in the rat. *Brain Res.* 1995;698:79-85

75. Huang J, Choudhri TF, Winfree CJ, McTaggart RA, Kiss S, Mocco M, Kim LJ, Protopsaltis TS, Zhang Y, Pinsky DJ, Connolly ES. Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. *Stroke.* 2000;31:3047-3053

76. Suzuki H, Hayashi T, Tojo SJ, Kitagawa H, Kimura K, Mizugaki M, Itoyama Y, Abe K. Anti-P-selectin antibody attenuates rat brain ischemic injury. *Neurosci Lett.* 1999;265:163-166

77. Connolly ES, Jr., Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *Journal of Clinical Investigation.* 1996;97:209-216

78. Soriano S, Coxon A, Wang Y, Frosch M, Lipton S, Hickey P, Mayadas T. Mice deficient in mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. *Stroke*. 1999;30:134-139

79. Soriano SG, Lipton SA, Wang YF, Xiao M, Springer TA, Gutierrez-Ramos JC, Hickey PR. Intercellular adhesion molecule-1-deficient mice are less susceptible to cerebral ischemia-reperfusion injury. *Annals of Neurology*. 1996;39:618-624

80. Moncayo J, deFreitas GR, Bogousslavsky J, Altieri M, vanMelle G. Do transient ischemic attacks have a neuroprotective effect? *Neurology*. 2000;54:2089-2094

81. Wegener S, Gottschalk B, Jovanovic V, Knab R, Fiebach JB, Schellinger PD, Kucinski T, Jungehulsing GJ, Brunecker P, Muller B, Banasik A, Amberger N, Wernecke KD, Siebler M, Rother J, Villringer A, Weih M. Transient ischemic attacks before ischemic stroke: Preconditioning the human brain? *Stroke*. 2004;35:616-621

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

83. Chen J, Simon R. Ischemic tolerance in the brain. *Neurology*. 1997;48:306-311

84. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M. Ischemic tolerance phenomenon found in the brain. *Brain Research*. 1990;528:21-24

85. Kobayashi S, Harris V, Welsh F. Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. *J Cereb Blood Flow & Metab*. 1995;15:721-727

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

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

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

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

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

91. Furuya K, Ginis I, Takeda H, Chen Y, Hallenbeck J. Cell permeable exogenous ceramide reduces infarct size in spontaneously hypertensive rats supporting *in vitro* studies that have implicated ceramide in induction of tolerance to ischemia. *J Cereb Blood Flow & Metab*. 2001;21:226-232

92. Dawson DA, Furuya K, Gotoh J, Nakao Y, Hallenbeck JM. Cerebrovascular hemodynamics and ischemic tolerance: Lipopolysaccharide-induced resistance to

focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. *Journal of Cerebral Blood Flow and Metabolism*. 1999;19:616-623

93. Puisieux F, Deplanque D, Pu Q, Souil E, Bastide M, Bordet R. Differential role of nitric oxide pathway and heat shock protein in preconditioning and lipopolyscaccharide-induced brain ischemic tolerance. *European Journal of Pharmacology*. 2000;389:71-78

94. Bastide M, Gele P, Petrault O, Pu Q, Caliz A, Robin E, Deplanque D, Duriez P, Bordet R. Delayed cerebrovascular protective effect of lipopolysaccharide in parallel to brain ischemic tolerance. *J Cereb Blood Flow & Metab*. 2003;23:399-405

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

96. Galanos C, Freudenberg MA, jay F, Nerkar D, Veleva K, Brade H, Strittmatter W. Immunogenic properties of lipid A. *Rev Infect Dis*. 1984;6:546-552

97. Galanos C, Luderitz O, Rietschel ET, Westphal O, Brade H, Brade L, Freudenberg M, Schade U, Imoto M, Yoshimura H. Synthetic and natural escherichia coli free lipid a express identical endotoxic activites. *Eur J Biochem*. 1985;148:1-5

98. Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR, Golenbock DT. Toll-

like receptor 4 imparts ligand-specific recognition of bacterial lipopolysacchairde. *J Clin Invest*. 2000;105:497-504

99. Janeway C, Jr, Medzhitov R. Innate immune recognition. *Annual Review of Immunology*. 2002;20:197-216

100. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the drosophila toll protein signals activation of adaptive immunity. *Nature*. 1997;388:394-397

101. Beg AA. Endogenous ligands of toll-like receptors: Implications for regulating inflammatory and immune responses. *Trends Immunol*. 2002;23:509-519

102. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev*. 2005;204:27-42

103. Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (tlr)2 and tlr4 in human peripheral blood granulocytes: A critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol*. 2002;168:4701-4710

104. Muzio M, Bosisio D, Polentaurutti N, D'amico G, Stoppacciaro A, Manicinelli R, Veer Cvt, Penton-Rol R, Ruco LP, Allavena P, Mantovani A. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: Selective expression of TLR3 in dendritic cells. *J Immunol*. 2000;164:5998-6004

105. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of toll-like receptors in human monocytes and dendritic cells. *J Immunol*. 2001;166:249-255

106. Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, Polentarutti N, Muzio M, Arditi M. Bacterial lipopolysaccharide activated NF-kappaB through toll-like receptor (TLR4) in cultured human dermal endothelial cells. Differential expression of TLR4 and TLR2 in endothelial cells. *J Biol Chem.* 2000;275:11058-11063

107. Stoll LL, Denning GM, Li WG, Rice JB, Harrelson AL, Roming SA, Gunnlaugsson ST, Miller FJ, Weintraub NL. Regulation of endotoxin-induced proinflammatory activation in human coronary artery cells: Expression of function membrane-bound CD14 by human coronary artery smooth muscle cells. *J Immunol.* 2004;173:1336-13343

108. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expession of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun.* 2000;68:7010-7017

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

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

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

112. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *Journal of Neuroscience*. 2002;22:2478-2486

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

114. Kawai T, Akira S. Toll-like receptor downstream signaling. *Arthritis Res Ther*. 2005;7:12-19

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

116. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, toll-like receptor-4. *Immunology*. 2004;113:153-162

117. Correia JDS, Soldau K, Christian U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. *J Biol Chem*. 2001;276:21129-21135

118. Li Q, Verma IM. Nf-kappab regulation in the immune system. *Nat Rev Immunol*. 2002;2:725-734

119. Dunne A, O'Neill LA. Adaptor usage and toll-like receptor signaling specificity. *FEBS lett*. 2005;579:3330-3335

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

121. Lehner MD, Morath S, Michelsen KS, Schumann RR, Hartung T. Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different toll-like receptors independent of paracrine mediators. *J Immunol.* 2001;166:5161-5167

122. Virca GD, Kim SY, Glaser KB, Ulevitch RJ. Lipopolysaccharide induced hyporesponsiveness to its own action in RAW 264.7 cells. *J Biol Chem.* 1989;264:21951-21956

123. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: Dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *Journal of Immunology.* 2000;164:5564-5574

124. Learn CA, Mizel SB, McCall CE. mRNA and protein stability regulate the differential expression of pro- and anti-inflammatory genes in endotoxin-tolerant THP-1 cells. *J Biol Chem.* 2000;275:12185-12193

125. Frankenberger M, Pechumer H, Ziegler-Heitbrock HW. Interleukin-10 is upregulated in LPS tolerance. *J Inflamm.* 1995;45:56-63

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

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

128. Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, Kishimoto

T. SOCS-1 participates in negative regulation of LPS responses. *Immunity*. 2002;17:677-687

129. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, Yoshida H, Kubo M, Yoshimura A. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity*. 2002;17:583-591

130. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, Liew FY. ST2 is an inhibitor of interleukin 1 receptor and toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol*. 2004;5:373-379

131. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell*. 1995;83:793-802

132. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP. A metalloproteinase disintegrin that releases tumor-necrosis factor-alpha from cells. *Nature*. 1997;385:729-733

133. Aggarwal BB. Signalling pathways of the TNF superfamily: A double-edged sword. *Nat Rev Immunol*. 2003;3:745-756

134. Kohno T, Brewer MT, Baker SL, Schwartz PE, King MW, Hale KK, Squires CH, Thompson RC, Vannice JL. A second tumor necrosis factor receptor gene product can shed a naturally occuring tumor necrosis factor inhibitor. *Proc Natl Acad Sci*. 1990;87:8331-8335

135. Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D. Soluble forms of tumor necrosis factor receptor (s-TNFR). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO*. 1990;9:3269-3278

136. Lantz M, Malik S, Slevin ML, Olsson I. Infusion of tumor necrosis factor (TNF) causes an increase in circulating TNF-binding protein in humans. *Cytokine*. 1990;2:402-406

137. Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D. Soluble forms of tumor necrosis factor receptors (s-TNFR). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO Journal*. 1990;9:3269-3278

138. Zee KJV, Kohno T, Fischer E, Rock CS, Moldawer LL, Lowry SF. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. *Proc Natl Acad Sci*. 1992;89:4845-4849

139. Chen G, Goeddel DV. TNF-R1 signaling: A beautiful pathway. *Science*. 2002;296:1634-1635

140. Muppidi JR, Tschopp J, Siegel RM. Life and death decisions: Secondary complexes and lipid rafts in TNF receptor family signal transduction. *Immunity*. 2004;21:461-465

141. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*. 1995;81:495-504

142. Li Q, Verma IM. NF-kB regulation in the immune system. *Nature Reviews Immunology*. 2002;2:725-734

143. Kolesnick RN, Kronke M. Regulation of ceramide production and apoptosis. *Annu Rev Physiol*. 1998;60:643-665

144. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: Role of p47phox phosphorylation and binding to TRAF4. *Mol Cell Biol*. 2005;25:2320-2330

145. Hallenbeck JM. The many faces of tumor necrosis factor in stroke. *Nature Medicine*. 2002;8:1363

146. Li X, Yang Y, Ashwell JD. TNF-R2 and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature*. 2002;41:345-347

147. Shen Y, Li R, Shiosaki K. Inhibition of p75 tumor necrosis factor receptor by antisense oligonucleotides increases hypoxic injury and beta-amyloid toxicity in human neuronal cell line. *J Biol Chem*. 1997;272:3550-3553

148. Mukhopadhyay A, Suttles J, Stout RD, Aggarwal BB. Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. *J Biol Chem*. 2001;276:31906-30912

149. Sipe KJ, Dantzer R, Kelley KW, Wehenmeyer JA. Expression of the 75 kda TNF-receptor and its role in contact-mediated neuronal cell death. *Brain Res Mol Brain Res*. 1998;62:111-121

150. Buttini M, Appel K, Sauter A, Gebicke-Haerter PJ, Boddeke HW. Expression of tumor necrosis factor alpha after focal ischemia in the rat. *Neuroscience*. 1996;7:1-16

151. Liu T, Clark RK, McDonnell PC. Tumor necrosis factor alpha expression in ischemia neurons. *Stroke*. 1994;25:1481-1488

152. Gong C, Qin Z, Betz AL, Liu XH, Yang GY. Cellular localization of tumor necrosis factor alpha following focal cerebral ischemia in mice. *Brain Research*. 1998;801:1-8

153. Wang X, Yue TL, Barone FC, White RF, Gagnon RC, Feuerstein GZ. Concomitant cortical expression of tnf-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia. *Mol Chem Neuropath*. 1994;23:103-114

154. Lambertsen KL, Meldgaard M, Ladeby R, Finsen B. A quantitative study of microglial-macropahge synthesis of tumor necrosis factor during acute and late focal cerebral ischemia in mice. *J Cereb Blood Flow & Metab*. 2005;25:119-135

155. Botchkina GI, Meistrell MEr, Botchkina IL, Tracey KJ. Expression of TNF and TNF receptors (p55 and p75) in the rat brain after focal cerebral ischemia. *Molecular Medicine*. 1997;3:765-781

156. Dziewulska D, Mossakowski MJ. Cellular expression of tumor necrosis factor alpha and its receptors in human ischemic stroke. *Clin Neuropathol.* 2003;22:35-40

157. Lavine SD, Hofman FM, Zlokovic BV. Circulating antibody against TNF-alpha protects rat brain from reperfusion injury. *Journal of Cereberal Blood Flow and Metabolism.* 1998;18:52-58

158. Yang GY, Gong C, Qin Z, Ye W, Mao Y, Bertz AL. Inhibition of TNF-alpha attenuates infarct volume and ICAM-1 expression in ischemic mouse brain. *Neuroreport.* 1998;9:2131-2134

159. Dawson DA, Martin D, Hallenbeck JM. Inhibition of tumor necrosis factor-alpha reduces focal cerebral ischemic injury in the spontaneously hypertensive rat. *Neurosci Lett.* 1996;218:41-44

160. Nawashiro H, Martin D, Hallenbeck JM. Inhibition of tumor necrosis factor and amelioration of brain infarction in mice. *Journal of Cerebral Blood Flow & Metabolism.* 1997;17:229-232

161. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, Lysko PG, Feuerstein GZ. Tumer necrosis factor-alpha: A mediator of focal ischemic brain injury. *Stroke.* 1997;28:1233-1244

162. Meistrell ME, Botchkina GI, Wang H, Di Santo E, Cockroft KM, Bloom O, Vishnubhakat JM, Ghezzi P, Tracey KJ. Tumor necrosis factor is a brain damaging cytokine in cerebral ischemia. *Shock.* 1997;8:341-348

163. Wilde GJC, Pringle AK, Sundstrom LE, Mann DA, Lannotti F. Attenuation and augmentation of ischemia-related neuronal death by tumor necrosis factor-alpha in vitro. *European Journal of Neuroscience*. 2000;12:3863-3870

164. Martin-Villalba A, Hahne M, Kleber S, Vogel J, Falk W, Schenkel J, Krammer PH. Therapeutic neutralization of CD95-ligand and tnf attenuates brain damage in stroke. *Cell Death Differ*. 2001;7:679-686

165. Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nature Medicine*. 1996;2:788-794

166. Gary DS, Bruce-Keller AJ, Kindy MS, Mattson MP. Ischemic and excitotoxic brain injury is enhanced in mice lacking the p55 tumor necrosis factor-receptor. *J of Cerebral Blood Flow and Metab*. 1998;18:1283-1287

167. Zou JY, Crews FT. TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: Neuroprotection by NF kappa B inhibition. *Brain Res*. 2005;1034:11-24

168. Liu J, Ginis I, Spatz M, J.M. H. Hypoxic preconditioning protects cultured neurons against hypoxic stress via tnfa and ceramide. *American Journal of Physiology*. 2000;278:C144-C153

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

170. Chen Y, Ginis I, Hallenbeck JM. The protective effect of ceramide in immature rat brain hypoxia-ischemia involves up-regulation of bcl-2 and reduction of tunel-positive cells. *J Cereb Blood Flow & Metab.* 2001;21:34-40

171. Cheng B, Christakos S, Mattson M. Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. *Neuron.* 1994;12:139-153

172. Tamatani M, Che Y, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M. Tumor necrosis factor induces bcl-2 and bcl-x expression through NF-kappaB activation in primary hippocampal neurons. *Journal of Biological Chemistry.* 1999;274:8531-8538

173. Furukawa K, Mattson MP. The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. *J Neurochem.* 1998;70:1876-1886

174. Wernyj RP, Mattson MP, Christakos S. Expression of calbindin in C6 glial cells stabilizes intracellular calcium levels and protects against apoptosis induced by calcium ionophore and amyloid beta-peptide. *Brain Res Dev Brain Res.* 1999;64:69-79

175. Barger SW, Horster D, Furukawa K, Goodman Y, Krieglstein J, Mattson MP. Tumor necrosis factors alph and beta protect neurons against amyloid beta-peptide toxicity: Evidence for involvement of a kappa B-binding factor and attenuation of peroxide and CA^{2+} accumulation. *Proc Natl Acad Sci.* 1995;92:9328-9332

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

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

178. Akopov SE, Simonian NA, Grigorian GS. Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. *Stroke*. 1996;10:1739-1743

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

180. Hill J, Gunion-Rinker L, Kulhanek D, Lessov N, Kim S, Clark W, Nishi R, Stenzel-Poore M, Eckenstein F. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. *Brain Research*. 1999;820:45-54

181. Fleming TJ, Fleming ML, Malek TR. Selective expression of LY-6G on myeloid lineage cells in mouse bone marrow. *J Immunol*. 1993;151:2399-2408

182. Araki T, Simon RP, Taki W, Lan JQ, Henshall DC. Characterization of neuronal death induced by focally evoked limbic seizures in the C7Bl/6 mouse. *Journal of Neuroscience Research*. 2002;69:614-621

183. Minami M, Stevens S, Andrews D, Simon R, Stenzel-Poore M. LPS-induced neuroprotection from ischemic injury is associated with marked mRNA downregulation of TNFRp55, TRADD and FADD in mice. *Abstract 20819 Society for Neuroscience Meeting, San Diego, CA*. 2001

184. Mabuchi T, Kitagawa K, Ohtsuki T, Kuwabara K, Yagita Y, Yanagihara T, Hori M, Matsumoto M. Contribution of microglia/macrophages to expansion of infarction and response of oligodendrocytes after focal cerebral ischemia in rats. *Stroke*. 2000;31:1735-1743

185. Nguyen M, D'Aigle T, Gowing G, Julien J, Rivest S. Exacerbation of motor neuron disease by chronic stimulation of innate immunity in a mouse model of amyotrophic lateral sclerosis. *Journal of Neuroscience*. 2004;24:1340-1349

186. Hauss-Wegrzyniak B, Lynch M, Vraniak P, Wenk G. Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. *Experimental Neurology*. 2002;176:336-341

187. Yao Z, Foster P, Gross G. Monophosphoryl lipid A protects against endotoxic shock via inhibiting neutrophil infiltration and preventing disseminated intravascular coagulation. *Circ Shock*. 1994;43:107-114

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

189. Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, Price MP, Welsh MJ, Simon RP. Neuroprotection in ischemia: Blocking calcium-permeable acid-sensing ion channels. *Cell*. 2004;118:687-698

190. Gold MC, Munks MW, Wagner M, Koszinowski UH, Hill AB, Fling SP. The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by m45-specific ctl but does not alter the immunodominance of the m45-specific CD8 t cell response in vivo. *Journal of Immunology*. 2002;169:359-365

191. Eissner G, Kolch W, Scheurich P. Ligands working as receptors: Reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine Growth Factor Review*. 2004;15:353-366

192. Hurtado O, Lizasoain I, Fernandez-Tome P, Alvarez-Barrientos A, Leza JC, Lorenzo P, Moro MA. Tace/adam17-TNF-alpha pathway in rat cortical cultures after exposure to oxygen-glucose deprivation or glutamate. *J Cereb Blood Flow & Metab*. 2002;22:576-585

193. Wang X, Feuerstein GZ, Xu L, Wang H, Schumacher WA, Ogletree ML, Traub R, Duan JW, Decicco CP, Liu RQ. Inhibition of tumor necrosis factor-alpha converting enzyme by a selective antagonist protects brain from focal ischemic injury in rats. *Molecular Pharmacology*. 2004;65:890-896

194. Pradillo JM, Romera C, Hurado O, Cardenas A, Moro MA, Leza JC, Davalos A, Castillo J, Lorenzo P, Lizasoain I. TNFR1 upregulation mediates tolerance after brain ischemic preconditioning. *J Cereb Blood Flow & Metab*. 2005;25:193-203

195. Lin SJ, Shyue SK, Hung YY, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. Superoxide dismutase inhibits the expession of vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1 induced by tumor necrosis factor-alpha

in human endothelial cells through the jnk/p38 pathways. *Arterioscler Thromb Vasc Biol.* 2005;25:334-340

196. Chang SC, Kao MC, Fu MT, Lin CT. Modulation of NO and cytokines in microglial cells by Cu/Zn-superoxide dismutase. *Free Radical Biology and Medicine.* 2001;31:1084-1089

197. Li JJ, Oberley LW, Fan M, Colburn NH. Inhibition of AP-1 and NF-kappaB by manganse-containing superoxide dismutase in human breast cancer cells. *FASEB.* 1998;12:1713-1723

198. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *Journal of Biological Chemistry.* 1998;273:13245-13254

199. Nawashiro H, Martin D, Hallenbeck JM. Neuroprotective effects of TNF binding protein in focal cerebral ischemia. *Brain Research.* 1997;778:265-271

200. Marchetti L, Klein M, Schlett K, Pfizenmaier K, Eisel UL. Tumor necrosis factor-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by n-methyl-d-aspartate receptor activation. Essential role of TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *Journal of Biological Chemistry.* 2004;279:32869-32881

201. Wang HM, Meng X, Tasai B, Wang JF, Turrentine M, Brown JW, Meldrum DR. Preconditioning up-regulates the soluble TNF receptor 1 response to endotoxin. *Journal of Surgical Research.* 2004;121:20-24

202. Stenzel-Poore MP, Simon RP. Lipopolysaccharide (LPS) preconditioning in stroke: A look into the neuroprotective signature. In: Krieglstein JK, S., ed. *Pharmacology of cerebral ischemia*. MedPharm, Stuttgart; 2004:379-384.

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

204. Haviv R, Stein R. The intracellular domain of p55 tumor necrosis factor receptor induces apoptosis which requires different caspases in naive and neuronal PC12 cells. *J Neurosci Res*. 1998;52:380-389

205. Condorelli F, Sortino MA, Stella AM, Canonico PL. Relative contribution of different receptor subtypes in the response of neuroblastoma cells to tumor necrosis factor-alpha. *J Neurochem*. 2000;75:1172-1179

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

207. Tunkel AR, Rosser SW, Hansen EJ, Scheld WM. Blood-brain barrier alterations in bacterial meningitis: Development of an in vitro model and observations on the effects of lipopolysaccharide. *In Vitro Cell Dev Biol*. 1991;27:113-120

208. Yan E, Castillo-Melendex M, Nicholls T, Hirst J, Walker D. Cerebrovascular responses in the fetal sheep brain to low-dose endotoxin. *Pediatr Res*. 2004;55:855-863

209. Gutierrez EG, Banks WA, Kastin AJ. Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. *J Neuroimmunol*. 1993;47:169-176

210. Pan W, Kastin AJ. Tnfalpha transport across the blood-brain barrier is abolished in receptor knockout mice. *Exp Neurol*. 2002;174:193-200

211. Banks WA, Kastin AJ, Broadwell RD. Passage of cytokines across the blood-brain barrier. *Neuroimmunomodulation.* 1995;2:241-248

212. Armitage RJ. Tumor necrosis factor receptor superfamily members and their ligands. *Curr Opin Immunol.* 1994;6:407-413

213. Zimmermann C, Ginis I, Furuya K, Klimanis D, Ruetzler C, Spatz M, Hallenbeck J. Lipopolysaccharide-induced ischemic tolerance is associated with increased levels of ceramide in brain and in plasma. *Brain Research.* 2001;895:59-65

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

215. Hosoi T, Okuma Y, Matsuda T, Nomura Y. Novel pathway for LPS-induced afferent vagus nerve activation: Possible role of nodose ganglion. *Auton Neurosci.* 2005;120:104-107

216. Czura CJ, Tracey KJ. Autonomic neural regulation of immunity. *J Intern Med.* 2005;257:156-166

217. Laye S, Bluthe RM, Kent S, Combe C, Medina C, Parnet P, Kelley K, Dantzer R. Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. *Am J Physiol.* 1995;268:R1327-1331

218. Laye S, Parnet P, Goujon E, Danzer R. Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice. *Brain Rese Mol Brain Res.* 1994;27:157-162

219. Becker M, Menger MD, Lehr HA. Heparin-released superoxide dismutase inhibits postischemic leukocyte adhesion to venular endothelium. *Am J Physiol.* 1994;267:H925-930

220. Bowler RP, Nicks M, Tran K, Tanner G, Chang LY, Young SK, Worthen GS. Extracellular superoxide dismutase attenuates lipopolysaccharide-induced neutrophilic inflammation. *Am J Respir Cell Mol Biol.* 2004;31:432-439

221. Fullerton DA, Eisenach JH, McIntyre RC, Friese RS, Sheridan BC, Roe GB, Agarfojo J, Banerjee A, Harken AH. Inhaled nitric oxide prevents pulmonary endothelial dysfunction after mesenteric ischemia-reperfusion. *Am J Physio.* 1996;271:L326-331

222. Biffl WL, Moore EE, Moore FA, Barnett C. Nitric oxide reduces endothelial expression of intracellular adhesion molecule (ICAM)-1. *J Surg Res.* 1996;63:328-332

223. Kanwar S, Kubes P. Nitric oxide is an antiadhesive molecule for leukocytes. *New Horiz.* 1995;3:93-104

224. Batteur-Parmentier S, Margaill I, Plotkine M. Modulation of nitric oxide of cerebral neutrophil accumulation after transient focal ischemic in rats. *J Cereb Blood Flow & Metab.* 2000;20:812-819

225. Lipton BP, Bautista AP, Delcarpio JB, McDonough KH. Effects of endotoxin on neutrophil-mediated I/R injury in isolated perfused rat hearts. *Am J Physiol Heart Circ Physiol.* 2001;280:H802-811

226. Del Zoppo G. Microvasculature responses to cerebral ischemia/inflammation. *Ann NY Acad Sci.* 1997;823:132-147

227. Banda MA, Lefer DJ, Granger DN. Postischemic endothelium-dependent vascular reactivity is preserved in adhesion molecule-deficient mice. *Am J Physiol.* 1997;273:H2721-2725

228. Zoppo GJD, Schmid-Schonbein GW, Mori E, Copeland BR, Chang CM. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke.* 1991;22:1276-1283

229. Kang J, Park EJ, Jour I, Kim JH, Joe EH. Reactive oxygen species mediate a beta (25-35)-induced activation of bv-2 microglia. *Neuroreport.* 2001;12:1449-1452

230. Delhalle S, Deregowski V, Benoit V, Merville MP, Boursq V. NF-kappa B-dependent MnSOD expression protects adenocarcinoma cells from TNF-alpha-induced apoptosis. *Oncogene.* 2002;21:3917-3924

231. Talley AK, Dewhurst S, Perry SW, Dollar SC, Gummuluru S, Fine SM, New D, Epstein LG, Gendelman HE, Gelbard HA. Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: Protection by the antioxidant n-acetylcysteine and the genes bcl-2 and crma. *Mol Cell Biol.* 1995;15:2359-2366

232. Jaattela M, Benedict M, Tewari M, Shayman JA, Dixit VM. Bcl-x and bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene.* 1995;10:2297-2305

233. Miao HS, Yu LY, Hui GZ, Guo LH. Antiapoptotic effet both in vivo and in vitro of A20 gene when transfected into rat hippocampal neurons. *Acta Pharmacol Sin.* 2005;26:33-38

234. Tanaka H, Tokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS. Ischemic preconditioning: Neuronal survival in the face of caspase-3 activation. *J Neurosci.* 2004;24:2750-2759

235. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. NF-kappa B antiapoptosis: Induction of TRAF1 and TRAF2 and c-IPA1 and c-IPA2 to suppress caspase-8 activation. *Science.* 1998;281:1680-1683

236. Goffi F, Boroni F, Benarese M, Sarnico I, Bennetti A, Spano PF, Pizzi M. The inhibitor of I kappa B alpha phosphorylation BAY 11-7082 prevents NMDA neurotoxicity in mouse hippocampal slices. *Neurosci Lett.* 2005;377:147-151

237. Chao CC, Hu S. Tumor necrosis factor-alpha potentiates glutamate neurotoxicity in human fetal brain cell cultures. *Dev Neurosci.* 1994;16:172-179

238. Ogoshi F, Yin HZ, Kuppumbatti Y, Song B, Amindari S, Weiss JH. Tumor necrosis-factor-alpha (TNF-alpha) induces rapid insertion of CA^{2+}-permeable alpha-amino-3-hydorxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate (CA-a/k) channels in a subset of hippocampal pyramidal neurons. *Exp Neurol.* 2005;193:384-393

239. Hermann GE, Rogers RC, Bresnahan JC, Beattie MS. Tumor necrosis factor-alpha induces c-fos and strongly potentiates glutamate-mediated cell death in the rat spinal cord. *Neurobiol Dis.* 2001;8:590-599

240. Stevens SL, Rosenzweig HL, Gopalan B, Harrington CA, Cannon W, Simon RP, Stenzel-Poore MP. LPS-induced ischemic tolerance: TNF primes the emergence of neuroprotection by reprogramming the ischemic response from cell death to cell survival. *Abstract, Society for Neuroscience Meeting, Washington, DC.* 2005

241. Sly LM, Rauh MJ, Kalesnikoff J, Song CH, Krystal G. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity*. 2004;21:227-239

242. Ogle CK, Guo X, Chance WT, Ogle JD. Induction of endotoxin tolerance in rat bone marrow cells by in vivo infusion of tumor necrosis factor. *Crit Care Med*. 1997;25:827-833

243. Zhou BR, Gumenscheimer M, Freudenberg MA, Galanos C. Lethal effect and apoptotic DNA fragmentation in response of D-Galn-treated mice to bacterial LPS can be suppressed by pre-exposure to minute amount of bacterial LPS: Dual role of TNF receptor 1. *World J Gastroenterol*. 2005;11:3398-3404

244. Zingarelli B, Halushka PV, Caputi AP, Cook JA. Increased nitric oxide synthesis during the development of endotoxin tolerance. *Shock*. 1995;3:102-108

245. Fresno Cd, Gomez-Garcia L, Caveda L, Escoll P, Arnalich F, Zamora R, Lopez-Collazo E. Nitric oxide activates the expression of IRAK-M via the release of TNF-alpha in human monocytes. *Nitric Oxide*. 2004;10:213-220

246. Maslinska D, Laure-Kamionowska M, Maslinski S. Toll-like receptor in rat brains injured by hypoxia-ischemia or exposed to staphylococcal alpha-toxin. *Folia Neuropathol*. 2004;42:125-132

247. Oyama J, Blais C, Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T. Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. *Circulation*. 2004;109:784-789

248. Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem*. 2001;276:5197-5203

249. Zhai Y, Shen XD, O'Connell R, Gao F, Lassman C, Busuttil RW, Cheng G, Kupiec-Weglinski JW. Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IFN regulatory factor 3-dependent Myd88-independent pathway. *J Immunol.* 2004;173:7115-7119

250. Wu HS, Wang L, Rotstein O. Toll-like receptor 4 involvement in hepatic ischemia/reperfusion injury in mice. *Hepatobiliary Pancreat Dis Int.* 2003;3:250-235

251. Kariko K, Weissman D, Welsh FA. Inhibition of toll-like receptor and cytokine signaling--a unifying theme in ischemic tolerance. *J Cereb Blood Flow & Metab.* 2004;24:1288-1304

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

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

254. Land WG. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. *Transplantation.* 2005;79:504-514

255. Vollmer J. Progress in drug development of immunostimulatory CPG oligodeoxynucleotide ligands for TLR9. *Expert Opin Biol Ther.* 2005;5:673-682