DEFINING A PHYSIOLOGICAL MODEL OF CLASSICAL ACTIVATION IN MICROGLIA

By

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Abstract

Microglia are resident immune cells of the central nervous system that can become activated following injury, disease, or infection. *In vitro*, they can be activated by different stimuli, which determine the inflammatory phenotype they will develop. In this thesis, stimulating microglia with tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) resulted in classical activation, characterized by proliferation, increased transcription of complement receptor 3 and major histocompatibility class II molecules, and elevated production and transcription of interleukin-1β and nitric oxide. Stimulation with TNFα and IFNγ also changed the intensity of phosphorylated (activated) cyclic adenosine monophosphate response element binding protein (pCREB) immunoreactivity in microglia. Specifically cells were differentiated into populations with either high or low pCREB intensity. This was the first example of such a response in microglia. Furthermore, these results were representative of what occurred *in vivo*, after ICH. Thus, the characterization of this model will be useful for future studies of this and other intracellular pathways involved in the classical activation of microglia.

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List of Abbreviations

2% MEM	MEM containing 2% FBS and 0.05 mg/mL gentamycin
Aβ	β Amyloid protein
AP-1	Activator protein-1
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
C1q	Complement 1q
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRE	cAMP response element
CREB	CRE binding protein
CBP	CREB binding protein
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
EAE	Experimental autoimmune encephalitis
eNOS	Endothelial nitric oxide synthase
HAT	Histone acetyltransferase
HIV	Human immunodeficiency virus
Iba1	Ionized calcium-binding adaptor protein 1
ICH	Intracerebral Haemorrhage
IFNγ	Interferon-γ
IL-	Interleukin-
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
Jak	Janus activated kinase
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor
MEM	Minimum essential medium
MHC II	Major histocompatibility class II molecules
MR1	Mannose receptor
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PBS	Potassium buffered saline
PBS-#B	PBS containing #% BSA
TGFβ	Transforming growth factor-β
TNFα	Tumor necrosis factor-α
STAT	Signal transducers and activators of transcription

1. Introduction

Microglia play a central role in inflammation in the brain, following brain injury. Many stimuli are used to activate them *in vitro* and it remains unclear how these induce different inflammatory profiles. Therefore, the goal of this thesis was to carefully describe a model of microglia activation induced by stimuli with physiological importance that represented well an acute brain injury *in vivo*. In this introduction, I will first present an overview of microglia. Second, I will describe some immunomodulators that were relevant to this study. Third, I will review *in vivo* and *in vitro* inflammatory models of microglia activation. Fourth, I will describe a specific intracellular signalling pathway examined to assess whether *in vitro* model results represented *in vivo* model results. Finally, I will present an outline of the main thesis experiments.

1.1. Microglia: Resident CNS Immune Cell

Microglia are resident brain macrophages that become activated following infection, disease, or injury (Ling and Wong, 1993). Their activation has been defined as the "acquisition of competence to execute a complex function" (Adams, 1989). *In vivo*, the role of activated microglia is controversial. Indeed, they have been shown to be cytoprotective by clearing debris and taking up glutamate. They also release chemokines that attract peripheral immune cells and induce acquired immunity, anti-inflammatory cytokines that diminish inflammation, and growth factors that promote neuronal survival (Turrin and Rivest, 2006). However, early after an insult, they are generally considered to be detrimental since they release pro-inflammatory cytokines and reactive oxygen and nitrogen species, which can lead to neurotoxicity (Block and Hong,

2005). Specific stimulators that induce activated microglia to acquire different inflammatory phenotypes remain largely unknown.

In vitro, unstimulated isolated primary microglia are usually somewhat in an activated state (Moller et al., 2000). This is largely because of the nature of *in vitro* microglia isolation from the brain but also because astrocytes produce factors that maintain microglia in a more resting state (Sievers et al., 1994). Thus, the extent to which primary microglia cultures are activated depends partly on culturing conditions (Bohatschek et al., 2001; Giulian and Baker, 1986; Ponomarev et al., 2005). Microglia can be recognized by different markers such as the ionized calcium-binding adaptor protein 1 (Iba1), the cluster of differentiation (CD) 11b, tomato lectin, and isolectin B_4; these are often up-regulated following activation (Acarin et al., 1994; Graeber et al., 1998; Streit and Kreutzberg, 1987). Additionally, CD 63 (or ED1) is a cytoplasmic marker that binds to a glycoprotein in the membrane of lysosomes, it is up-regulated in activated phagocytic macrophages and microglia (Dijkstra et al., 1985; Graeber et al., 1990; Graeber et al., 1998). Thus, these markers can give some indication of the activation state.

Even though *in vitro* microglia may be partly activated, they can be further activated following stimulation (Nakamura, 2002). The specific stimulus used defines the activation state that results. Indeed, depending on the membrane receptors that are activated, different intracellular inflammatory pathways will be induced (Rivest, 2003). These pathways lead to the release of various immunomodulators by microglia, which include reactive oxygen/nitrogen species, cytokines, and chemokines (Rivest, 2003). In this thesis, I used two cytokines to stimulate the microglia and monitored microglial production of a reactive nitrogen species and two cytokines to assess their activation state.

1.2. Immunomodulators

1.2.1. Nitric oxide (NO)

Nitric oxide is a reactive nitrogen species that is created from the oxidation of L-arginine by one of three nitric oxide synthases (NOS). NOS is constitutively expressed in neurons (nNOS) and in endothelial cells (eNOS); it can also be induced in astrocytes and microglia (iNOS) (Lipton, 1999b). Because nitric oxide is unstable, it can interact with another reactive species, superoxide, which arises from the reduction of one electron from oxygen (Ortega Mateo and Amaya Aleixandre de, 2000). The extremely cytotoxic molecule generated from this irreversible reaction is peroxynitrite (Ortega Mateo and Amaya Aleixandre de, 2000). Peroxynitrite can oxidize and nitrate DNA, protein, and lipids; the alteration of which can lead to cell death (Krantic et al., 2005). Following inflammation, microglia are thought to play an important role in mediating neuronal injury through their induction of iNOS and consequently, their release of NO (Boje and Arora, 1992; Chao et al., 1992; Zielasek et al., 1992). Indeed, *in vitro*, microglia release NO after stimulation with lipopolysaccharide (LPS), prion protein fragments, HIV nuclear proteins, IL-12, and ATP (Fabrizi et al., 2001; Nakamura et al., 1999; Ohtani et al., 2000; Pahan et al., 2001; Polazzi et al., 1999). However, *in vivo*, microglia may not be the primary source of NO (Matsumoto et al., 2007).

In addition, NO has beneficial effects in the healthy brain (Ortega Mateo and Amaya Aleixandre de, 2000). Even during inflammation, there is evidence that it inhibits caspases that are activated following injury and that would otherwise induce apoptosis (Lipton, 1999b). On the other hand, it is equally possible that inhibiting these caspases is actually detrimental by causing cell death to

be necrotic instead of apoptotic (Lipton, 1999b). Taken together, it is more likely that up-regulation of iNOS and release of NO in activated microglia leads to cytotoxicity (Chao et al., 1992). Because iNOS and NO are important in mediating cytotoxicity *in vivo* and *in vitro* and because microglia have been shown to up-regulate iNOS and release NO, I examined *iNos* transcription and NO production in activated microglia.

1.2.2. Cytokines

Cytokines are small proteins or glycoproteins that regulate normal function in tissues (Hopkins, 2003). Most importantly, they play a crucial role in inflammation and immunity. In the brain, cytokines are one of the main modulators of, and products of activated microglia (John et al., 2003). Following injuries and infections, cytokines regulate different aspects of inflammation *in vivo* (Morganti-Kossmann et al., 2002; Stoll et al., 2002). Four cytokines are studied in this thesis and will be described in this section. Tumor necrosis factor (TNFα) and interleukin-1β (IL-1β) are two pro-inflammatory cytokines released, in the brain early following infection, Alzheimer's Disease, or ischemia (Barone and Feuerstein, 1999; Dickson et al., 1993; Merrill and Chen, 1991). Interferon-γ (IFNγ) is a regulatory pro-inflammatory cytokine that is of interest when examining early events following infections and autoimmune disease (Gendelman et al., 1994; Suzuki, 1999). Finally, interleukin-10 (IL-10) is an anti-inflammatory cytokine that suppresses the activation of immune cells (Morganti-Kossmann et al., 2002). These cytokines were chosen because TNFα and IL-1β are the two orchestrators of the acute response, IFNγ is the main mediator of acquired immunity, and IL-10 is a well-studied anti-inflammatory cytokine.

1.2.2.a Tumor Necrosis Factor-α (TNFα)

TNFα is produced initially as a 26 kDa precursor that is cleaved by the TNFα converting enzyme into a 17 kDa mature form (Hallenbeck, 2002). TNFα can bind to two different receptors, the TNFα receptor Type I (TNFR1; or p55 or CD 120b) or Type II (TNFR2; or p75 or CD120a). Activated macrophages are the major source of TNFα although TNFα is also produced by neurons, astrocytes, and endothelial cells (Buttini et al., 1996; Gregersen et al., 2000; Hofman et al., 1989; Lee et al., 1993; Liu et al., 1994; Medana et al., 1997; Sawada et al., 1990a; Seilhean et al., 1997; Uno et al., 1997). TNFα is produced specifically in microglia in response to LPS, β-amyloid peptide (Aβ), IFNγ, and ATP *in vitro* and *in vivo* (Hide et al., 2000; Meda et al., 1995; Sawada et al., 1989; Si et al., 2000; Yates et al., 1999). Its release after injury is known to occur early and orchestrate the ensuing inflammatory response (Shohami et al., 1999).

The cytotoxic actions of TNFα are thought to be mediated through binding to the higher affinity TNFR1 (Fontaine et al., 2002; Tartaglia et al., 1991). Specifically, the TNFR1 contains an intracellular sequence, a death domain, that binds adaptor proteins, which are recruited following ligand-receptor binding, and cause the activation of two transcription factors: nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) that mediate the transcription of inflammation-related genes (Chen and Goeddel, 2002; Hallenbeck, 2002). As a consequence of the actions of TNFα acting on TNFR1, TNFα can have deleterious effects. *In vitro*, some groups have shown that TNFα can induce cytotoxicity in oligodendrocytes, Schwann cells, and non-primary neurons (Boyle et al., 2005; Selmaj and Raine, 1988; Westmoreland et al., 1996). *In vivo*, several studies have found a beneficial impact of deleting TNFR1 or injecting a TNFα antibody (Bermpohl et al., 2007; Grau et al., 1987; Iosif et al., 2006; Martin-Villalba et al., 2001).

TNFα can also bind to TNFR2, which has a lower affinity, and is generally associated with cytoprotective functions (Barna et al., 1990; Cheng et al., 1994; Davidson et al., 1996; Liu et al., 1998; Shen et al., 1997). Interestingly, Dopp *et al.* (1997) found that microglia are the only cell type in the brain to constitutively express TNFR2 mRNA and that TNFR2 mRNA is induced by either TNFα or IFNγ stimulation. Taken together, these results suggest a dual role of TNFα that is dependent on its concentration (and thus, on the receptor type activated); the timing and duration of TNFα activation may also affect the response (Shohami et al., 1999). Because of its dual-role and its role as early orchestrator of the immune response, and because TNFα is released from dying neurons after injury, I chose it as a stimulus of microglia activation (Lipton, 1999a).

1.2.2.b Interleukin-1β (IL-1β)

IL-1β is a 17 kDa protein initially formed as a precursor, pro-IL-1β, which is cleaved by caspase-1 (also known as IL-1β cleaving enzyme, ICE). Caspase-1 only becomes active when pro-caspase-1 binds an adaptor protein, ASC, to mediate the assembly of an 'inflammasome' complex, which cleaves pro-IL-1β (Martinon and Tschopp, 2007; Srinivasula et al., 2002; Stehlik et al., 2003). Once mature, IL-1β is released and binds to the receptor IL-1RI, which causes activation of signalling pathways leading to transcription via NF-κB (O'Neill and Greene, 1998). IL-1β can also bind to IL-1RII, a decoy receptor, which is not linked to any intracellular signalling pathway (O'Neill and Greene, 1998). The interleukin-1 receptor antagonist (IL-1ra) is an endogenous competitive inhibitor of IL-1β actions; the soluble isoform of IL-1ra binds IL-1RI but is not associated with intracellular signalling (Allan et al., 2005).

In general, increases in IL-1β signalling are thought to be detrimental (Allan et al., 2005). For example, deletion of the IL-1R1 in mice affected by a mild hypoxic ischemic insult decreased the infarct size, the pro-inflammatory cytokine transcription (*Il-1*, *Tnfα*, *M-csf*), and the release of cyclooxygenase-2 (COX-2), and the number of phagocytic microglia (Lazovic et al., 2005). Consistent with these findings, IL-1ra deletion increases the infarct size and oedema after ischemia, *in vivo* (Pinteaux et al., 2006). Furthermore, inhibition of caspase-1 reduced infarct size and improved neurological score after ischemia *in vivo*. I*n vitro*, it delayed motor neuron death caused by growth factor deprivation (Hara et al., 1997; Milligan et al., 1995). Also, blocking the actions of IL-1β by adding IL-1ra diminished neurotoxicity induced by LPS+IFNγ-stimulated microglia in *in vitro* (Ma et al., 2002).

Nevertheless, IL-1β might not directly cause injury but cause neurotoxicity by increasing the permeability of the blood brain barrier (Ferrari et al., 2004; Hailer et al., 2005; Rothwell, 2003). Indeed, some evidence suggests that chronic up-regulation of IL-1β expression renders the blood brain barrier more permeable to leukocyte such as neutrophils (Shaftel et al., 2007). Other stimuli (possibly from these infiltrating neutrophils) may be required to cause neurodegeneration. Consistent with this view, Depino *et al.* (2005) found that neuron death due to an injection of IL-1β caused death only around the needle track and, therefore, may be caused by the synergy between IL-1β and substances released from neurons dying because of the injection.

Taken together, all these studies suggest that IL-1β might have a direct neurotoxic effect or is a participant in the events leading to neuron death. Because TNFα and IL-1β are both early cytokines involved in propagating inflammation, and because their signalling converges on NF-

κB, their synergistic effect may be highly detrimental to surrounding cells (Mercurio and Manning, 1999). Microglia release both of these cytokines. For reasons described in the previous section, I used TNFα as a stimulus. Additionally, I chose to monitor the microglial production of IL-1β as marker of their activation response to TNFα.

1.2.2.c Interleukin-10 (IL-10)

Interleukin-10 is an important anti-inflammatory cytokine that plays a role in reducing the levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, and their receptors (Sawada et al., 1999). It binds to its receptor (IL-10R) to activate intracellular pathways leading to Janus activated kinase (Jak) and signal transducer and activators of transcription (STAT)-mediated transcription. Although the mechanisms for IL-10-induced immunosuppression remain unclear, changes in pro-inflammatory cytokine transcription and post-transcriptional degradation have been suggested (Grutz, 2005).

In contrast to TNFα and IL-1β, increased IL-10 is believed to reduce damage after brain injury and to improve the prognosis. Indeed, IL-10 knockout mice have larger necrotic areas after ischemic stroke (Grilli et al., 2000). Moreover, injections of IL-10 have been shown to reduce the size of excitotoxic brain lesions (Mesples et al., 2003). Conversely, there are suggestions that IL-10 might impair wound healing (Werner and Grose, 2003). Explicitly, by preventing neutrophil and macrophage infiltration and reducing pro-inflammatory cytokines and chemokines, IL-10 might reduce both wound clearance and production of important growth factors involved in repair (Werner and Grose, 2003). Microglia are likely involved in IL-10 regulation since this cytokine can be released from LPS-activated primary rat microglia *in vitro* and is present *in vivo* in microglia after LPS challenge (Ledeboer et al., 2002; Park et al., 2007).

Therefore, because IL-10 is released by microglia *in vivo* and *in vitro* and plays a prominent role in mediating immunosuppression, I monitored IL-10 production as an indicator of microglia deactivation.

1.2.2.d Interferon-γ (IFNγ)

IFNγ is a type II interferon that can bind the IFNγ receptor type I (IFNγ R1) to cause signalling via the Jak/STAT pathway (Schroder et al., 2004). The genes up-regulated by Jak-STAT signalling contain an interferon regulatory factor (IRF)-element site and are controlled by IRF-1 (Schroder et al., 2004). These sites can also be bound by IRF-2, which represses transcription (Schroder et al., 2004). Although IFNγ was originally exclusively associated with T lymphocyte Type I helper cells, it is now accepted that other types of immune cells, including B cells, natural killer cells, and antigen-presenting cells (such as macrophages and microglia) can produce IFNγ (Schroder et al., 2004). IFNγ is known to have diverse effects on microglia. It can be anti-proliferative, and can induce antigen presentation, apoptosis, production of reactive oxygen and nitrogen species, and leukocyte attraction (Schroder et al., 2004).

In vivo, IFNγ is associated with the infiltration of T lymphocytes into the CNS, cells that are widely studied in infection and multiple sclerosis models (Gaddi and Yap, 2007; Harari et al., 2006; Weiss et al., 2007). Interestingly, IFNγ has been shown to be intrinsically linked to the production and levels of TNFα in monocytes and macrophages (Cox et al., 1992; Sanceau et al., 1992). Increased levels of IFNγ are associated with the onset of multiple sclerosis (MS) in

humans, and of experimental allergic encephalitis (EAE) in animals (Beck et al., 1988; Issazadeh et al., 1995).

IFNγ studies are largely restricted to infection and auto-immune diseases, with very few studies of acute brain injury. In fact, IFNγ changes following haemorrhagic stroke have never been examined. Fewer than 10 studies have examined IFNγ after ischemic stroke (Baird, 2006; Beck et al., 1988; Gaddi and Yap, 2007; Harari et al., 2006; Hurn et al., 2007; Issazadeh et al., 1995; Lambertsen et al., 2004; Lau and Yu, 2001; Li et al., 2001; Raghavendra Rao et al., 2002; Theodorou et al., 2008; Weiss et al., 2007; Yilmaz et al., 2006). In some of them, resident brain cells have been implicated in the production of IFNγ, including astrocytes, macrophages, and microglia (Hurn et al., 2007; Lambertsen et al., 2004; Lau and Yu, 2001; Yilmaz et al., 2006). Knockout mice studies suggest that IFNγ is associated with exacerbation of ischemic injury (Lambertsen et al., 2004; Yilmaz et al., 2006). For this thesis, I wanted to examine whether IFNγ was changed following haemorrhagic stroke. In addition, since IFNγ is crucial for the participation of macrophages in acquired immunity, and because it acts through a different signalling pathway than TNFα, I decided to use IFNγ as a co-stimulus with TNFα *in vitro*.

1.3. Inflammation Models

1.3.1. Choosing an *In Vivo* Model to Study Brain Inflammation

Stroke occurs following a change in blood flow in the brain. This can be caused by haemorrhage or ischemia. In ischemia, blood flow is restricted from reaching an area of the brain; whereas in

haemorrhage, a blood vessel breaks and blood leaks into the parenchyma. There are two animal models of intracerebral haemorrhage (ICH): the autologous blood model and the collagenase-induced model (Wang and Dore, 2007). In the latter, collagenase, a proteolytic enzyme, is injected into the brain where it breaks down the blood brain barrier causing a haemorrhage (Rosenberg et al., 1990). This model causes a well-delineated lesion with a core where cells die and macrophages/microglia and neutrophils enter to clean up the debris (Wasserman and Schlichter, 2007b). Around the lesion, a wall of activated macrophages/microglia contains the injury (Wasserman et al., 2007). In this model, activated microglia and astrocytes are shown to release both pro- and anti-inflammatory cytokines (Wasserman and Schlichter, 2007a; Wasserman et al., 2007). The time-course of cytokine induction has been described (Wasserman et al., 2007). To better understand the role of microglia in inflammation *in vivo* after acute brain injury, I used the collagenase-induced ICH model because it is highly reproducible and is well-characterized in my laboratory.

1.3.2. Choosing an *In Vitro* Model of Microglia Activation

There are many ways to study microglia-mediated inflammation *in vitro*. Based on many studies, five types of macrophage activation have been differentiated by Gordon (2003). By analogy, different stimuli will likely induce different types of microglia activation. The importance of determining the specific activation type lies in the increased ability to predict a response from the stimulated microglia. In this thesis, I activated microglia using two cytokines, TNFα and IFNγ, and described outcomes that allowed me to pinpoint the type of activation induced. The different types of activation described for macrophages include innate activation,

classical activation, humoral activation, alternative activation, and innate/acquired deactivation (Table 1). Each of these activation types will be briefly described in this section.

The main stimulus currently used to induce microglia activation is LPS. LPS is an endotoxin from the outer wall of Gram negative bacteria. *In vivo*, it is rather unlikely for microglia to encounter bacterial cell walls; thus, LPS is used, not because of its relevance *in vivo*, but because of its ability to readily activate microglia (Nakamura et al., 1999). Indeed, LPS causes the release of NO, TNFα, IL-1β, IL-6, IL-10, and COX-2 from microglia, consistent with a state of innate activation (Table 1) (Gordon, 2003; Nakamura et al., 1999). Innate activation is generally associated with phagocytosis of pathogens through macrophage receptors following their opsonisation by complement proteins (Fig. 1A) (Bonifati and Kishore, 2007). If LPS also activates an acquired immune response, antibodies produced by lymphocytes can coat the pathogen and cause phagocytosis via Fc receptors (Fig. 1B) (Orr et al., 2005).

LPS is often combined to IFNγ to increase microglia activation. The combination of LPS+IFNγ also causes phagocytosis via Fc receptors but then antigens can be presented at the microglia surface by major histocompatibility complex class II molecules (MHC II) (Fig. 1C) (Gordon, 2003). This entails both production of the pro-inflammatory molecules mentioned above (note that IL-10 and COX-2 are not induced) and up-regulation of MHC II (Table 1) (De Simoni et al., 1997; Matsuoka et al., 1999; Minghetti et al., 1996; Roach et al., 1995).

Microglia can also be stimulated in a fashion that does not cause them to become pro-inflammatory. Following stimulation with IL-4 or IL-13, microglia enter a state that was first described in macrophages as alternatively activated (Table 1) (Gordon, 2003). Mannose receptors on the microglia surface are up-regulated and phagocytosis of mannosylated residues

can occur (Fig. 1D). This leads to the down-regulation of LPS-induced inflammatory modulators, including pro-inflammatory cytokines such as TNFα, IL-1β, and IL-12, but also anti-inflammatory cytokines such as IL-10 (Hart et al., 1999). This type of activation does not lead to suppression of inflammation but leads to immunoregulation (Gordon, 2003).

Finally, by analogy with macrophages, microglia can become deactivated by anti-inflammatory stimuli or by apoptotic cells (Fig. 1E) (Table 1) (Gordon, 2003). This leads to down-regulation of pro-inflammatory mediators such as TNFα, IL-1β, IL-12, iNOS, and up-regulation of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGFβ) (Chang and Liu, 2000; Nakamura et al., 1999; Prinz et al., 2001; Tan et al., 1999).

Figure 1

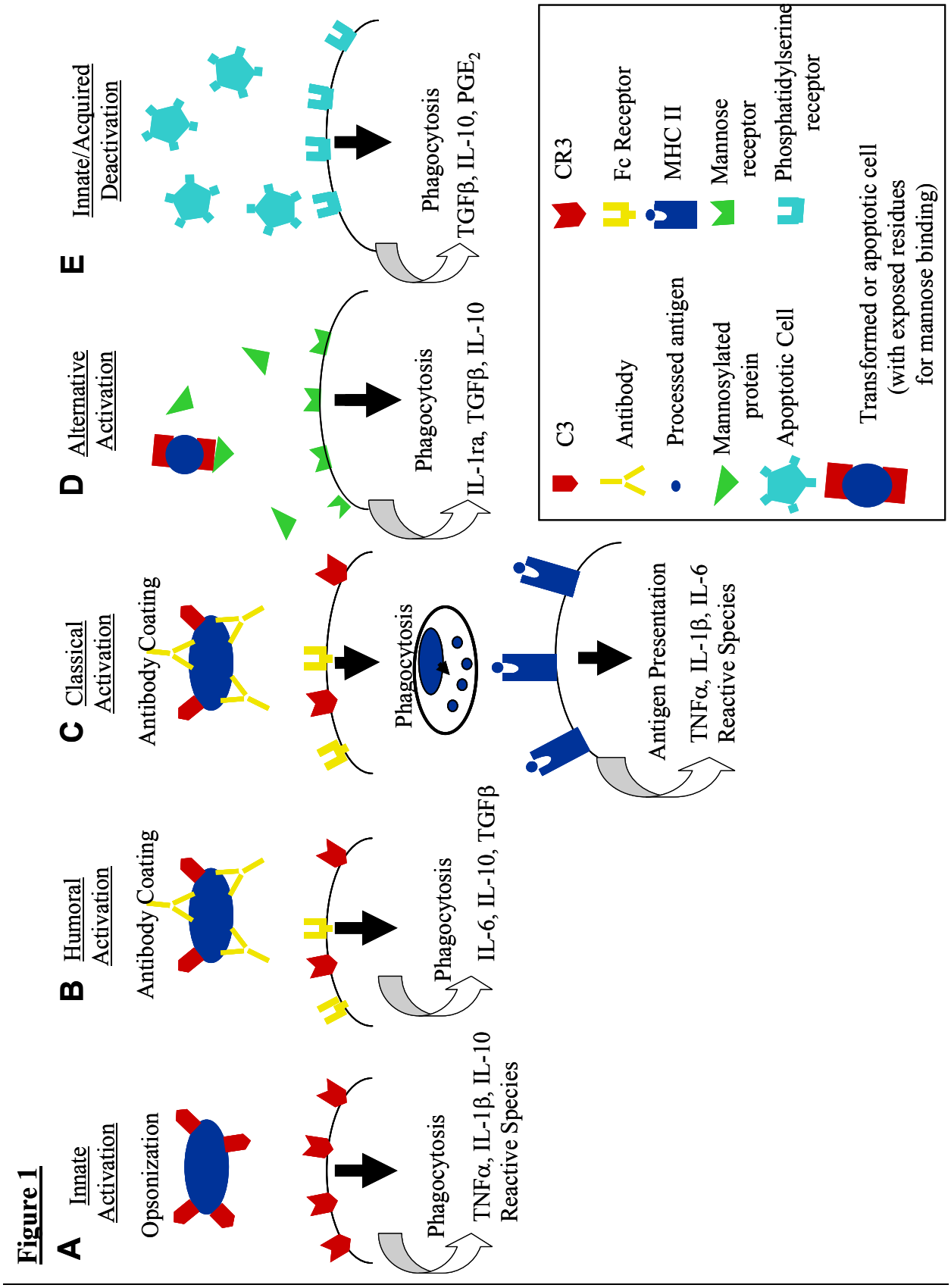
Figure 1
A Innate Activation
Opsonization
Phagocytosis
TNFα, IL-1β, IL-10
Reactive Species
B Humoral Activation
Antibody Coating
Phagocytosis
IL-6, IL-10, TGFβ
C Classical Activation
Antibody Coating
Phagocytosis
Antigen Presentation
TNFα, IL-1β, IL-6
Reactive Species
D Alternative Activation
Phagocytosis
IL-1ra, TGFβ, IL-10
E Innate/Acquired Deactivation
Phagocytosis
TGFβ, IL-10, PGE2
C3
Antibody
Processed antigen
Mannosylated protein
Apoptotic Cell
CR3
Fc Receptor
MHC II
Mannose receptor
Phosphatidylserine receptor
Transformed or apoptotic cell (with exposed residues for mannose binding)

Table 1: Different Types of Microglial Activation

Activation Type	Stimulus	Membrane Receptors involved	Released Molecules	Effect
Innate Activation	Microorganisms	Toll-like receptors	Reactive oxygen/nitrogen species, pro- and anti-inflammatory cytokines	Acute-phase response, opsonization of microbes
Humoral Activation	Complement, antibodies	Complement receptors, Fc receptors	Pro- and anti-inflammatory cytokines	Cytolysis
Classical Activation	IFNγ priming followed by LPS	MHC II	Reactive oxygen/nitrogen species, pro-inflammatory cytokines	Cellular immunity, tissue damage
Alternative Activation	IL-4 or IL-13	MHC II, mannose receptor	Chemokines, IL-1ra	Immunoregulation, allergic responses, repair
Innate/Acquired Deactivation	Anti-inflammatory cytokines, apoptotic cells	Anti-inflammatory cytokine receptors	Anti-inflammatory cytokines, prostaglandins,	Immunosuppression

1.3.3. *In Vitro* Representation of *In Vivo* Models

In vivo, the macrophage (and presumably, microglial) activation types will be much more intertwined. Indeed, following acute brain injuries, the inflammatory profile can be extremely pro-inflammatory at earlier time-points (6 h to 3 d), and then later elevations in anti-inflammatory cytokines can lead to deactivation (Wasserman et al., 2007). These *in vivo* switches in activation type, which likely result from many inflammatory mediators acting in concert, are unlikely to occur *in vitro*. However, both *in vitro* and *in vivo*, microglia can become activated following stimulation, and can proliferate, and release substances that affect

surrounding cells (Garden and Moller, 2006). Thus, although the limitations of activating primary microglia with two cytokines are evident, I think it is important to define how similar (or dissimilar) *in vivo* and *in vitro* responses of microglia are. Therefore, I examined whether the collagenase-induced ICH *in vivo* model resembles the TNFα+IFNγ-stimulation *in vitro* model.

1.4. cAMP Response Element Binding Protein (CREB)

Proliferation of microglia in culture is often examined in response to released stimulators such as colony stimulating factors (Kloss et al., 1997; Sawada et al., 1990b). However, proliferation can also be examined by looking at the effect of changes in transcription factors. One transcription factor that can affect progression through the cell cycle and regulate apoptosis is the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Mayr and Montminy, 2001).

CREB is a transcription factor that is constitutively expressed in the nucleus. It becomes activated following phosphorylation at Serine 133 (Fig. 2) (Brivanlou and Darnell, 2002). Phosphorylated CREB (pCREB) recruits associated proteins, such as CREB binding protein (CBP), or a protein similar to it, p300. CBP/p300 have intrinsic histone acetyltransferase (HAT) function and can also recruit additional HATs (Vo and Goodman, 2001). Because of this, CBP/p300 are instrumental in stimulating transcription of genes containing cAMP response element (CRE) sequences in their promoter (Mayr and Montminy, 2001).

Figure 2

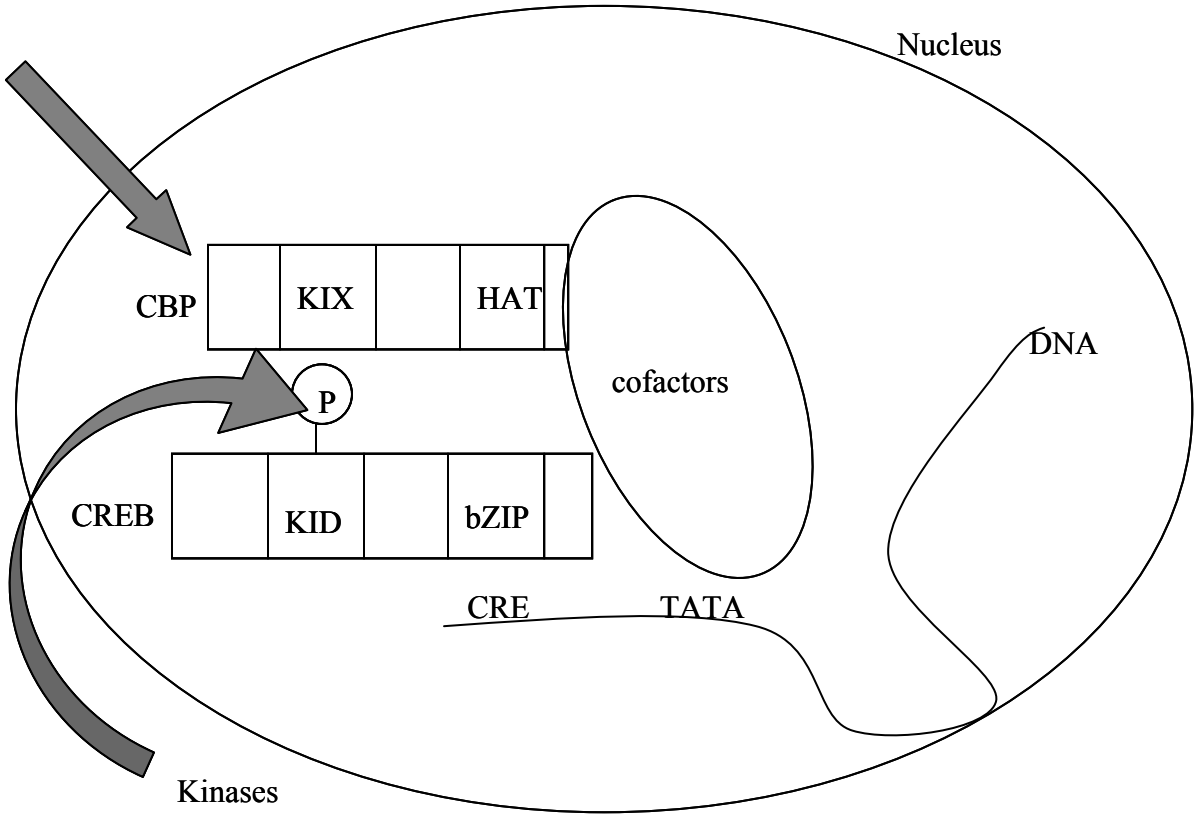

Figure 2: CREB activation of transcription. CREB is a nuclear resident factor that is usually bound to CRE promoter regions. After phosphorylation by kinases on Serine 133, CBP can bind and promote transcription by its own HAT domain and its recruitment of other cofactors. Abbreviations: *CRE*: cAMP response element, *CREB*: CRE binding protein, *CBP*: CREB binding protein, *KID*: Kinase inducible domain, *HAT*: Histone acetyltransferase, *bZIP*: basic region/leucine zipper, *TATA*: A/T rich sequence in the promoter.

pCREB is involved in proliferation and in preventing apoptosis; two processes in microglia that are particularly important in the context of inflammation (Chong et al., 2003; Misra and Pizzo, 2005; Mitrasinovic et al., 2001). Indeed, selectively killing microglia using a transgenic mouse model was shown to increase the lesion size and the number of apoptotic cells after ischemia (Lalancette-Hebert et al., 2007). However, even though CREB and proliferation are linked, and proliferation and inflammation are linked, no one has apparently examined pCREB in microglia

following inflammation *in vivo*. Therefore, to examine the ability of the TNFα+IFNγ-stimulation model to mimic the collagenase-induced ICH model, changes in pCREB were measured in both models.

1.5. Project Description

Many studies have used cytokines to activate microglia *in vitro* (Nakamura, 2002). However, the specific cytokines and their concentrations often vary between studies. Also, no study using cytokine stimulation has systematically defined the type of microglia activation induced. Therefore, the goal of my study was to define a model of microglia activation *in vitro*, using cytokine stimulation, that could reflect a subset of microglia responses that occur following acute brain injury *in vivo*. To do so, I chose two cytokines, TNFα and IFNγ, to stimulate primary microglia in culture. As previously explained, these two cytokines were chosen because they are both elevated relatively early following injury and they activate different signalling pathways. IL-1β was not used as a stimulus because, similarly to TNFα, it causes the activation of NF-κB. Furthermore, TNFα is more interesting as a stimulus because it is released from necrotic neurons (Lipton, 1999a). I hypothesized that these two cytokines would induce microglia to become classically activated. I also hypothesized that the activation of microglia induced could represent events that occurred in microglia following ICH.

Since I wanted this model to be applicable to many different types of CNS insults, I also needed to determine whether IFNγ was a relevant cytokine to use. Although its induction has been demonstrated following infection, auto-immune disease, and ischemia, no one has apparently examined whether it is elevated following haemorrhagic stroke (Baird, 2006; Beck et al., 1988;

Gaddi and Yap, 2007; Harari et al., 2006; Hurn et al., 2007; Issazadeh et al., 1995; Lambertsen et al., 2004; Lau and Yu, 2001; Li et al., 2001; Raghavendra Rao et al., 2002; Theodorou et al., 2008; Weiss et al., 2007; Yilmaz et al., 2006). Therefore, *Ifnγ* mRNA transcription was measured after ICH.

Then, I proceeded to measure different types of variables (including proliferation, pro-and anti-inflammatory mediators, and microglia activation receptors) to define the type of activation induced by TNFα+IFNγ. Finally, once the model was defined, I examined pCREB *in vivo* and *in vitro* to assess whether this *in vitro* model was representative of the responses of microglia *in vivo*.

2. Methods

2.1. Isolation of microglia

All experiments followed the Canadian Council on Animal Care guidelines. Microglia were purified in three steps slightly modified from our previous culturing method (Kaushal and Schlichter, 2008). First, the cerebellum and the meninges of 1day-old Sprague-Dawley rat pup brains (Charles River, St-Constant, Quebec, Canada) were removed in Minimal Essential Medium (MEM; University Health Network, Toronto, Ontario, Canada) and passed through a 100-mesh screen. Second, the minced brains were centrifuged (1000 g for 15 min) and resuspended in MEM (Invitrogen, San Diego, CA) containing 10% of heat-inactivated fetal bovine serum (FBS) (Wisent, St-Bruno, QC) and 0.05 mg/ml gentamycin (Invitrogen). Third, the dissociated cerebral cells were seeded onto 75 cm^2 flasks at a density of 1 brain for 5 flasks. The medium was changed on day 2 to remove cellular debris and non-adherent cells, and microglia were isolated on day 10-12 by shaking for 3-4 h on an orbital shaker (Barnstead International, Dubuque, IA) at 70 rpm. Floating cells were harvested and plated in 96-well microtiter plates or onto 12 mm coverslips (Fisher Scientific, Ottawa, ON) in 24-well plates (VWR, Mississauga, Ontario, Canada) at 50 000 cells/coverslip in MEM containing 2% FBS and 0.05 mg/mL gentamycin (2% MEM) for 1 h, washed, and then cultured for 48 h in 100 µL 2% MEM/well for 96- and 500 µL/well for 24-well plates. This procedure yields highly pure microglia cultures (>99%), confirmed by labeling with CD11b (OX-42) with very few contaminating oligodendrocytes. The microglia were slightly ramified, often either unipolar or bipolar, and had a minimal amount of vesiculation (Fig. 3). If the microglia did not have this type of morphology, generally because they contained too many vesicles, they were rejected from experiments.

Figure 3

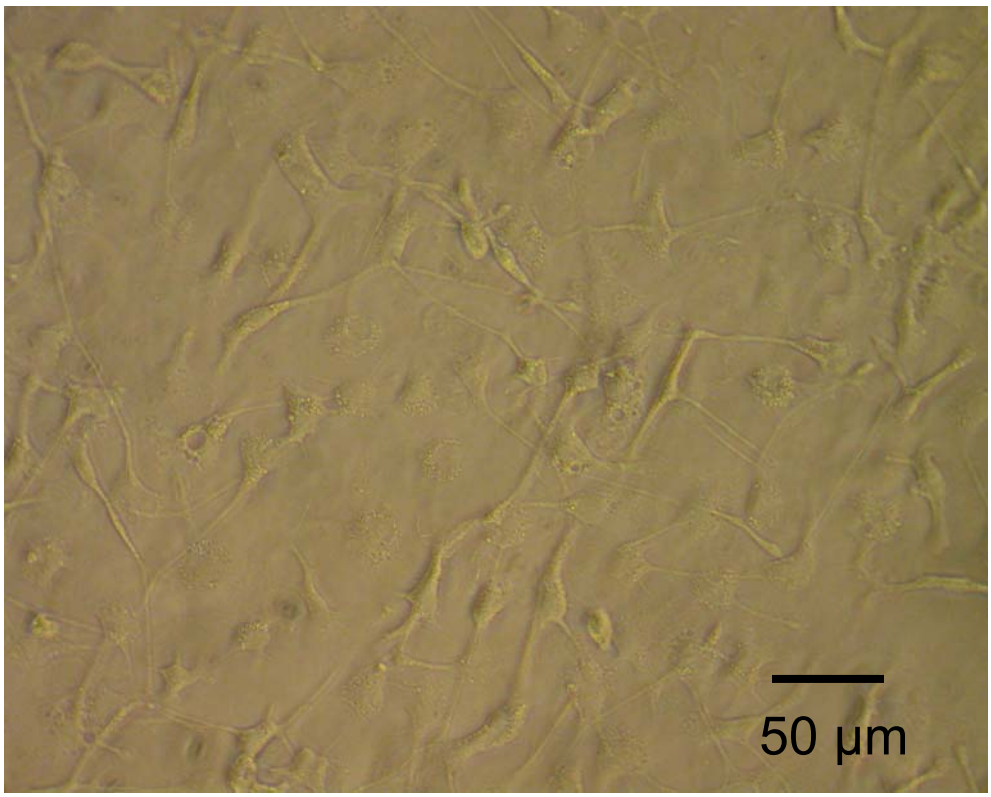

Figure 3: Descriptive picture of isolated primary microglia. Microglia were visualized using an Olympus CK2 microscope. They were slightly ramified, either unipolar or bipolar, and had a minimal amount of vesiculation.

2.2. Microglia stimulation

Recombinant rat TNFα (R&D Systems, Minneapolis, MN) and IFNγ (Sigma) were used as cytokine activators of microglia. TNFα was reconstituted in PBS containing 0.1% BSA to make a stock of 10 µg/mL, which was kept in aliquots at -20°C and diluted to a final concentration of 50 ng/mL. IFNγ was reconstituted in PBS containing 0.1% BSA and 1 mM dithiothreitol, according to manufacturer recommendations, to make a stock of 100 µg/mL, which was kept in aliquots at -80°C. Within three weeks of use, aliquots of 10 µg/mL IFNγ were made from the

100 μg/mL stock, kept at -20°C, and diluted to a final concentration of 20 ng/mL on the day of use. Cells on coverslips in the 24-well plate were activated using 200 μL of solution while those in 96-well microtiter plates were activated using 100 μL of solution. These specific concentrations of TNFα and IFNγ were chosen because preliminary studies I did have shown them to cause the greatest release of IL-1β release.

2.3. Fluorescent staining

2.3.1. *In vitro*

For *in vitro* staining, cells were fixed onto coverslips with 4% paraformaldehyde (PFA) in PBS for 30 min and then rinsed thoroughly 3x with PBS. For immunostaining, non-specific antigens were blocked using 5 % immunoglobulin (IgG)-free bovine serum albumin (BSA) in PBS (PBS-5B) for 60 min. Then, the cells were incubated with specific primary antibodies overnight at 4°C in PBS containing 2.5% IgG free BSA (PBS-2.5B): rabbit anti-rat pCREB (1:1000) (Millipore, Temecula, CA), mouse anti-rat CD63 (1:500) (AbD Serotec, Raleigh, NC), or goat anti-rat IL-10 (1:50) (R&D Systems). On day 2, all the coverslips were washed 3x for 5 min each with PBS-2.5B. In the case of IL-10 staining, amplification was needed. Therefore, a donkey anti-goat biotin antibody was applied for 85 min at 1:500 and washed off (3x, 5 min each with PBS-2.5B) prior to application of streptavidin-bound Cy3 (both from Jackson ImmunoResearch, West Grove, PA). For other stainings, the coverslips were incubated at 1:500 with one of the following secondary antibodies: donkey anti-rabbit Cy3, goat anti-rabbit Cy3, or goat anti-mouse FITC (all from Jackson ImmunoResearch). After 85 min, the cell nuclei were stained with a DNA-specific stain: 4',6-diamidino-2-phenylindole (DAPI) (Sigma) which was added to the coverslips in a

1:3000 dilution in PBS for 5 min and then washed 3x 5 min with PBS. Finally, the coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, Burlington, ON) and then fixed into place using clear nail polish.

2.3.2. *In vivo*

For *in vivo* staining, the animals were sacrificed by Jason Wasserman by an overdose of isoflurane 3 d after the onset of ICH and then perfused through the heart with 100 mL PBS followed by 60 mL of fixative (4% paraformaldehyde, 2% sucrose in PBS; pH 7.5). Dissected brains were stored in the same fixative at 4°C overnight, and transferred to 10% sucrose for 24 h, and 30% sucrose for 48 h. Fixed brains were cut coronally through the needle entry site (easily identified on the brain surface), and at 4 mm anterior and 4 mm posterior to that plane. Frozen brain sections (16 μm thick) were made by Helen Yang using a cryostat (JungCM 3000, Leica, Richmond Hill, ON) and were stored at − 40°C until used. Immunohistochemistry was done with the help of Jason Wasserman. Slices were incubated at 4°C overnight with primary antibodies directed against pCREB (1:1000) (Millipore) and CD11b (1:100) (AbD Serotec) in PBS containing 3% normal goat serum and 0.3% Triton-X-100. For secondary antibody labeling, the sections were washed in PBS (3x, 10 min each) and incubated (2 h, RT) in PBS containing 3% normal goat serum, 0.3% Triton X-100 and goat anti-mouse Cy3 and goat anti-rabbit FITC (1:500; Jackson Laboratories). DAPI was applied at 1:3000 for 5 min before washing (3x, 10 min each, in PBS) and coverslips were applied to the slides using a 1:1 solution of PBS:glycerol and fixed into place using clear nail polish.

All images for pCREB analysis were taken using an upright wide-field Zeiss Axioplan 2 with a high resolution Zeiss Axiocam (Fig. 4) (Carl Zeiss Canada Ltd, Toronto, ON). IL-10 (Fig. 11) and descriptive pCREB images (Fig. 13 and 16) were obtained with a Zeiss LSM 510 META (Carl Zeiss Canada) with the help of Jason Wasserman.

2.4. Image Analysis

The intensity of pCREB was measured specifically in the nucleus using different functions and PlugIns of Image J, version 1.38. This was done by double-staining for DAPI and pCREB. A mask of the DAPI-positive staining was created by first adjusting the threshold to only view cell nuclei. This created an image of either saturated pixels or empty pixels. Second, this image was turned into a binary image whereby all locations where there was DAPI staining were assigned an intensity value of 1 and all non-DAPI stained areas had a value of 0 by dividing by the saturation value (Fig. 4A). Third, this image was multiplied by the pCREB image (Fig. 4B) so that all the pCREB in the nucleus maintained its original intensity and any pCREB staining that did not colocalize with DAPI staining had an intensity of 0 (Fig. 4C). Fourth, the threshold was set between 1 and the saturation value to measure all pCREB intensities in the nucleus. Finally, mean, minimum and maximum intensity values, as well as size for each particle were generated using the Image J Particle Analysis PlugIn. The background staining was measured from negative controls for each experiment and was subtracted from the intensity value of each cell. The mean values per treatment were averages of the intensity minus background of all the cells (~70 cells/field) in 3 different fields for 3 different coverslips per *n*. There was a size-limiting

factor to exclude the smaller nuclei of dead cells. Mean pCREB intensity is expressed as the % intensity compared to naive cells that were merely fixed with no change in medium.

Figure 4

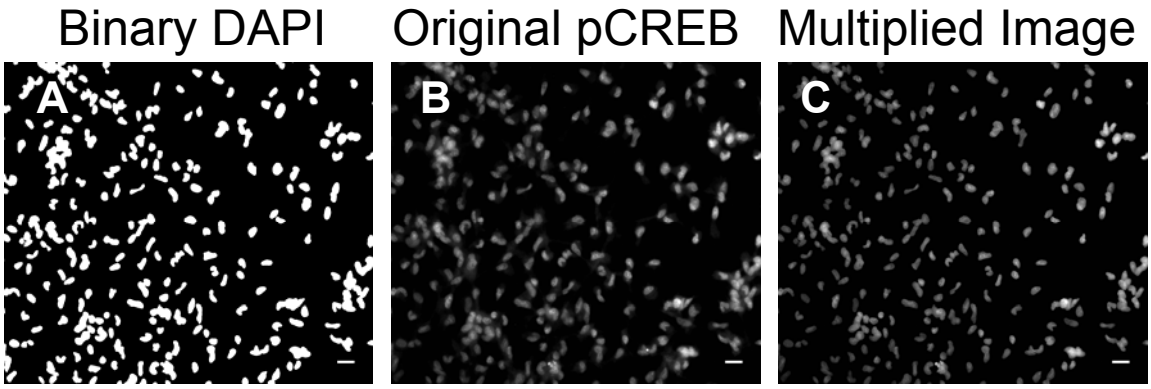

Figure 4: Image Analysis to measure pCREB intensity includes making a DAPI mask where the intensity value in the nuclei is set to 1 and the background set to 0 (A), and multiplying it by the pCREB image (B), to get a final image where the intensity values of pCREB are observed only in the nuclei (C). Scale bars = 20 μm.

2.5. Real-time quantitative reverse transcriptase-PCR

Real-time quantitative reverse transcription (qRT)-PCR was performed by Xiao-Ping Zhu and used to monitor gene transcript levels, using primers designed with the "Primer3Output" program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RNeasy mini kits (Qiagen, Mississauga, Ontario, Canada) were used to isolate RNA after degrading any contaminating DNA with DNaseI (0.1 U/ml, 15 min, 37°C; Amersham Biosciences, Baie d'Urfe, Quebec, Canada). A two-step reaction was performed according to the instructions of the manufacturer (Invitrogen): total RNA (2 μg) was reverse transcribed using 200 U of SuperScriptII RNase H-reverse transcriptase, with 0.5 mM dNTPs (Invitrogen) and 0.5 μM oligo-dT (Sigma). Amplification was performed on an ABI PRISM 7700 Sequence Detection System (PE

Biosystems, Foster City, CA) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. "No-template" and "no-amplification" controls (Bustin and Nolan, 2004) were included for each gene. For *in vitro* results, the threshold cycle (C_T) for each gene was determined, and normalized against the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT1) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Cells were stimulated with TNFα and IFNγ alone or in combination for 24 h; results are expressed as fold changes relative to 2% MEM control. For ICH results, the changes in mRNA expression of *Ifnγ* were compared to time-matched sham-operated animals at 1 d and 7 d after ICH, which were normalized to 1.0.

2.6. Functional assays

IL-1β and IL-10 release and production were measured using enzyme-linked immunosorbent assays (ELISA; R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Specifically, a 96-well microtiter plate was coated with the capture antibody at 4°C overnight. On the following day, a blocking agent, which contained IgG-free BSA or heat-inactivated fetal bovine serum, was applied to block non-specific binding. The samples and standards were then applied and cytokines were further bound to a biotin-linked detection antibody and then streptavidin-linked horseradish peroxidase. This caused a colorimetric reaction when substrate solution (tetramethylbenzidine and hydrogen peroxide, from R&D Systems) was applied. Absorbance was read at 450 nm with a λ correction at 570 nm using an EL311SX microplate reader (Bio-Tek Instruments, Winooski, VT). Concentration values were interpolated from a standard curve generated by Microsoft Excel. The minimum detection for both the IL-1β and the IL-10 assay was 20 pg/mL. To measure release, the supernatant was analyzed; to measure

production, the cells were lysed in non-denaturing cell lysis buffer containing 20 mM Tris-HCl pH 7.5, 1% Triton-X-100, 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA, and then those samples were analyzed. I subtracted proper controls of 2% MEM for cytokine release and cell lysis buffer for intracellular cytokine production. A cautionary note is that although the antibodies used in the IL-1β ELISA are more specific for the mature form of IL-1β, they are known to bind the pro-form as well.

Nitrite levels were measured using the Griess Assay (Griess, 1879). Slight modifications were used following concerns of Sohn and Fiala (1999). Therefore the Griess reagents used were: 0.1% N-(1-naphthalenediamine) (NAD) and sulfanillic acid (5% sulfanilamide in 3N HCl). 10 μL of sulfanillic acid was added to wells containing 100 μL of supernatant followed by 10 μL of the NAD solution. Standards were made using sodium nitrite and I found that this assay could detect concentrations of nitric oxide as low as 0.5 μM. I only measured nitrite levels and not nitrates since cadmium or nitrate reductase were not used.

2.7. Cell number, viability and proliferation assays

Alamar Blue was used as a simple, sensitive, and non-cytotoxic method to measure cell viability and cell proliferation (Nakayama et al., 1997). Alamar Blue, or resazurin, is a blue, non fluorescent compound that can undergo chemical reduction to become a red fluorescent compound named resorufin (O'Brien et al., 2000). Its use lies its reduction by many cellular enzymes, such as mitochondrial, cytosolic, and microsomal enzymes including NADH and NADPH reductases and cytochrome, thereby giving a measure of cell activity (Gonzalez and Tarloff, 2001). Specifically, a 1:10 concentration of Alamar Blue (Serotec) was distributed into the microglia-containing 96-well microtiter plate for 150 min at 24 h after stimulation and then

absorbance was measured at both 570 nm and 600 nm with the EL311SX microplate reader. The percent reduction of Alamar Blue was calculated according to the manufacturer-supplied equation 2 using a no-cell medium plus Alamar Blue negative control.

Propidium iodide (PI) is a membrane impermeant dye that increases its fluorescence when it intercalates with double-stranded DNA or RNA. Therefore, we used it to measure cell death. To do so, I applied 1 μg/mL of PI to microglia-containing wells and no-cell, medium control wells, and read the fluorescence after 15 min by exciting at 544 nm and measuring emission at 608 nm using a Victor3 plate-reader (Perkin Elmer, Waltham, MA). I also used PI to measure DNA/RNA content in the adherent cells by removing the cells and adding cell lysis buffer (see section 2.6) with 1 μg/mL of PI for 15 min and then measuring fluorescence.

2.8. Intracerebral Haemorrhage

All procedures were executed in accordance with guidelines established by the Canadian Council on Animal Care. An intracerebral hemorrhage was induced in the striatum of male Sprague-Dawley rats (3 months old; Charles River) using a method developed by Rosenberg *et al.* (1990) and modified by Wasserman and Schlichter (2007a). Rats were anesthetized using isofluorane (3% induction, 1.5% maintenance) and placed in a stereotaxic frame. A 30-gauge needle was inserted into the right caudate putamen (+0.2 A-P, -6.0 D-V, 3.0 L). 0.125 U of bacterial type IV collagenase (Sigma, Oakville, ON) in 0.5 μL saline, at 250 nL/min was injected using a micropump (Micro4, World Precision Instruments, Sarasota, FL). The needle was left in place for 5 min following injection to avoid reflux. Sham-operated animals underwent the same surgical procedure, but with 0.5 μL pure saline. The core temperature of animals was maintained

at 37 °C using an electric heating pad throughout surgery and recovery, which occurred within 10 min. No animals died as a result of the ICH or surgery.

2.9. Statistical Analyses

All data is represented as mean ± standard deviation. Significance is always expressed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Each treatment group was compared to its respective control using an unpaired 2-tailed *t* test using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Power analysis results were generated using an online tool by DSS Research (Fort Worth, TX): http://www.dssresearch.com/toolkit/sscalc/size_a2.asp.

3. Results

3.1. IFNγ is increased after ICH

Cytokines are known to play a large role in inflammation *in vivo* (Hopkins, 2003). Specifically, *Tnfα* and *Ifnγ* transcription has been shown to increase early after stroke (6 and 12 h, respectfully) and remain elevated for a few days (3 and 6 days, respectfully) (Li et al., 2001; Wasserman and Schlichter, 2007a). Albeit many studies have shown that TNFα is an early cytokine released following most types of cerebral injury, little is known about the existence and the importance of IFNγ following injuries where peripheral T lymphocytes do not infiltrate the brain (Baird, 2006; Yilmaz et al., 2006). Specifically, IFNγ levels have been consistently shown to be high in EAE and infections, though the extent to which T lymphocytes infiltrate the brain and to which IFNγ is produced in injuries such as trauma and stroke remains unclear (Baker et al., 1991; Frei et al., 1988; Gallo et al., 1989; Lambertsen et al., 2004; Li et al., 2001; Merrill et al., 1992; Traugott and Lebon, 1988; Yilmaz et al., 2006). Therefore, *Ifnγ* mRNA was examined in the collagenase model of ICH. Using quantitative real-time RT-PCR, *Ifn*γ mRNA was shown to be elevated in the ipsilateral striatum (expressed as fold change from sham-operated controls, 2.36 ± 1.25 on day 1 and 2.88 ± 0.98 on day 7) compared to the contralateral striatum (0.61 ± 0.12 on day 1 and 1.04 ± 0.23 on day 7, $p < 0.05$ for both days; $n = 5$; Fig. 5). Interestingly, an anti-inflammatory drug, minocycline, did not diminish the *Ifn*γ mRNA levels in the ipsilateral striatum. To the contrary, minocycline increased the difference in *Ifn*γ transcription between the contralateral and ipsilateral striatum (from 1.05 ± 0.42 to 3.75 ± 1.00 fold over sham when

minocycline was administered for 3 d or from 1.05 ± 0.56 to 3.93 ± 2.09 fold over sham when it was administered for the full 7 d, $p < 0.001$ and $p < 0.01$, respectively; n = 5; Fig 5).

Figure 5

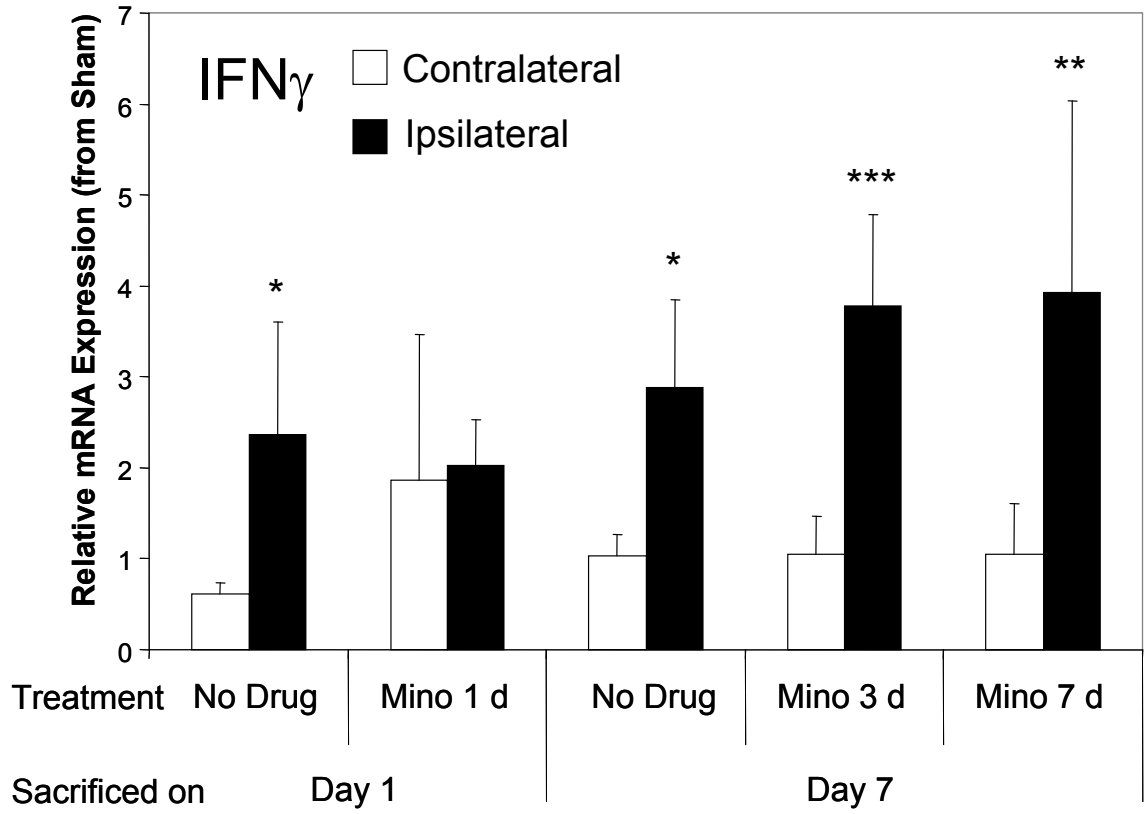

Figure 5: Effects of ICH and minocycline on *Ifnγ* expression. *Ifnγ* expression was measured by quantitative real-time RT-PCR in the ipsilateral (injured) striatum at 1 day and 7 days after ICH onset. Transcript expression for IFNγ is shown as the fold increase relative to sham-operated (saline-injected) time-matched control rats. ICH-induced changes in gene expression are indicated as: *p < 0.05, **p < 0.01, ***p < 0.001; n = 5 for each treatment group. IFNγ is increased in the ipsilateral striatum compared to the contralateral striatum. Minocycline-treated animals received injections at 6 h if sacrificed on day 1 [Mino 1 d], at 6 h, 1 and 2 days, if sacrificed at 3 days [Mino 3d], or at 6 h and then every day until day 7 [Mino 7d]. Minocycline further increased the expression of *Ifnγ* in the ipsilateral striatum whether it was administered for 3 or 7 days. ICH surgeries and tissue preparation were done by Jason Wasserman. qRT-PCR was executed by Xiao-Ping Zhu.

3.2. TNFα and IFNγ differentially affect microglia proliferation

Since *Ifnγ* transcription was elevated at 1 day *in vivo* after ICH, and since IFNγ is often used as a priming stimulus with LPS *in vitro* to increase the LPS-induced release of inflammatory molecules, I found its physiological relevance in infections and trauma worth exploring further in an *in vitro* model (Paludan, 2000). In addition, TNFα and IFNγ have been shown to have synergistic effects (Paludan, 2000; Spanaus et al., 1998; Suk et al., 2001). Therefore, I used TNFα and IFNγ alone and together as stimuli to study microglial release of pro- and anti-inflammatory molecules *in vitro*. Prior to measuring the release of inflammatory mediators from stimulated microglia, I wanted to examine changes in cell activity and cell numbers after stimulation with TNFα, IFNγ or both. To do so, I looked at metabolic activity, proliferation, and death. Specifically, I used Alamar Blue as a simple, sensitive, and non-cytotoxic method to measure metabolic activity (Nakayama et al., 1997). Cells were plated with or without 50 ng/mL TNFα and 20 ng/mL IFNγ, alone or together. I found that TNFα or TNFα+IFNγ significantly increased the percent reduction of Alamar Blue when compared to control cells (from 100 ± 22% to 129 ± 16% control, $p < 0.05$ and to 148 ± 23% control, $p < 0.01$, respectively; Fig.6A). This shows that TNFα, but not IFNγ, increases the metabolic activity of microglia. Because this increased Alamar Blue reduction could be caused by a higher number of cells, I estimated proliferation using propidium iodide to measure the amount of DNA and RNA in the wells for each treatment. Again, cells were plated with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both. At 24 h, the cells were lysed with buffer and propidium iodide was added. Only TNFα was found to significantly increase the DNA and RNA content in wells (from 100 ± 23 to 173 ± 50% of control, $p < 0.05$; Fig 6B). Finally, death was assessed by directly adding the membrane impermeant propidium iodide to the wells (Fig. 6C). No significant differences in microglia

death were observed following any of the treatments. In sum, I found that TNFα increased metabolic activity and proliferation, while TNFα+IFNγ only increased metabolic activity. IFNγ alone had no effect on metabolic activity or proliferation. Cell death was not elevated after applying any stimulus.

Figure 6

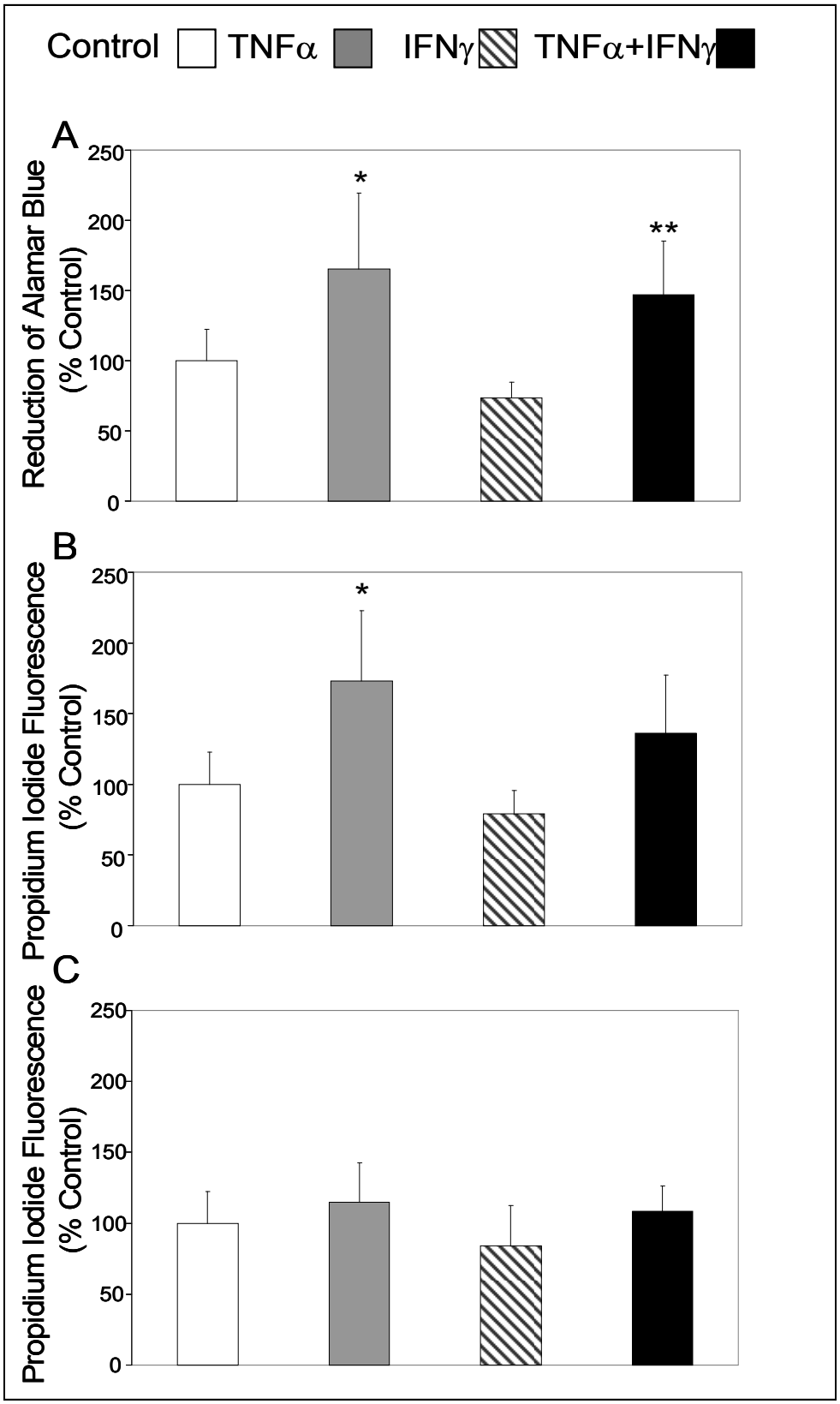
Control
TNFα
IFNγ
TNFα+IFNγ
A
Reduction of Alamar Blue (% Control)
250
200
150
100
50
0
*
**
B
Propidium Iodide Fluorescence (% Control)
250
200
150
100
50
0
*
C
Propidium Iodide Fluorescence (% Control)
250
200
150
100
50
0

Figure 6: Metabolic activity, proliferation, and death of 5.0 x 10^4 plated cells was measured Cells were cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, for 24 h. A) Metabolic activity was measured using Alamar Blue. Both TNFα alone and TNFα/IFNγ were found to increase the reduction of Alamar Blue compared to control (*$p <$ 0.05 and $p = 0.01$, respectfully; $n = 5$). B) The DNA and RNA content of each well was measured using propidium iodide. After cell lysis, 1 µg/mL PI was applied for 15 min. The wells were excited at 544 nm and emission was measured at 608 nm. Only TNFα alone significantly increased the DNA and RNA content of each well (*$p < 0.05$; $n = 5$). C) Cell death was measured using propidium iodide. 1 µg/mL PI was applied for 15 min without lysing the cells. The wells were excited at 544 nm and emission was measured at 608 nm. No significant differences in death were found; $n = 6$. For all experiments, each n comes from a different litter and is an average of 3 wells.

3.3. TNFα and IFNγ differentially activate microglia

The different activation states of microglia have been studied extensively (Nakamura, 2002). This is largely because defining the specific type of activation can be an important clue to their function. Therefore, to further characterize the activation state of microglia following stimulation with cytokines, changes in mRNA expression for genes related to different types of microglia activation were measured. These included major histocompatibility complex class II molecules (*Mhc II*), complement receptor 3 (*Cr3*), and mannose receptor 1 (*Mr1*). MHC II receptor expression is essential for antigen presentation to antigen-specific T lymphocytes (Neumann, 2001). MHC II receptors are important for the development of an adaptive immune response. *Mhc II* mRNA expression was significantly increased from unstimulated microglia after treatment with IFNγ alone (in fold difference from control: from 1.00 ± 2.20 to 45.7 ± 17.7, $p <$ 0.05) and together with TNFα (to 99.8 ± 44.0 fold, $p < 0.05$; Fig. 7). This is supported by previous studies showing that murine microglia acquire antigen-presenting function after stimulation with IFNγ both *in vitro* and *in vivo* (Frei et al., 1988; Lassmann et al., 1993; Panek and Benveniste, 1995; Steiniger and van der Meide, 1988; Suzumura et al., 1987; Vass and Lassmann, 1990; Wong et al., 1983; Xu and Ling, 1994). TNFα has been previously shown to

leave *Mhc II* levels unaffected, whether used alone or in conjunction with IFNγ (Panek and Benveniste, 1995). Similarly, TNFα did not further enhance the IFNγ-induced *Mhc II* up-regulation.

Next, *Cr3* mRNA expression was assessed. CR3 has been shown to be increased in microglia following injury and has been proposed to be a marker of their maturation as phagocytes (Abraham and Lazar, 2000; Graeber et al., 1988; Rupalla et al., 1998). *Cr3* mRNA expression was significantly increased only by TNFα+IFNγ (from 1.00 ± 0.41 to 2.04 ± 0.51, $p < 0.05$; Fig. 7).

Finally, I was interested in examining a marker of alternative activation. MR1 is a transmembrane protein expressed at the surface of phagocytes, which allows them to clear mannosylated residues from the external environment (Stahl and Ezekowitz, 1998). Mannosylated residues are present on the surface of prokaryotic cells but solely on intracellular components of eukaryotic cells. Hence, these could be released into the extracellular milieu after cell death and activate MR1. *Mr1* expression was found to be significantly decreased by the combination of TNFα and IFNγ (from 1.00 ± 0.42 to 0.25 ± 0.33, $p < 0.05$). Since *Mr1* was reduced and since *Cr3* and *Mhc II* were increased, the microglia are likely in a phagocytic activation state following activation with a combination of TNFα and IFNγ.

Figure 7

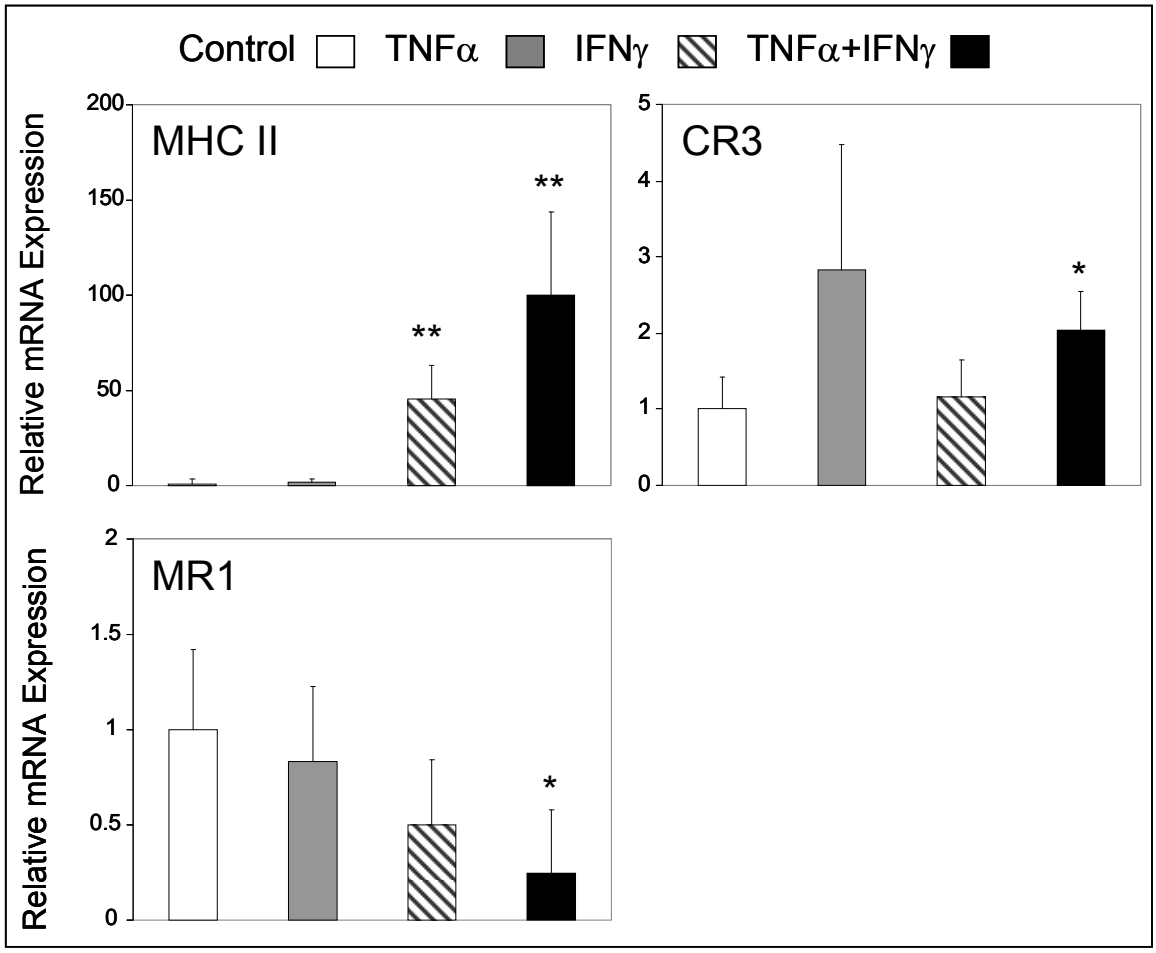

Figure 7: The effect of cytokine stimulation on microglia activation markers. Gene expression was measured by quantitative real-time RT-PCR in cells cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, for 24 h. *Mhc II*, *Cr3*, and *Mr1* mRNA transcripts were measured and transcript expression for each gene is shown as the fold increase relative to the 2% FBS control. TNFα with IFNγ was found to significantly change all markers (increasing *Cr3* and *Mhc II* and decreasing *Mr1*), while TNFα alone increased *Mhc II* only: $^{*}p < 0.05$, $^{**}p < 0.01$; n = 3-4. qRT-PCR was executed by Xiao-Ping Zhu.

3.4. TNFα with or without IFNγ elevates IL-1β

IL-1β is an orchestrator of the inflammatory response and is released by microglia early following injury (Basu et al., 2004). It has also been shown to be released specifically by

phagocytic microglia and to induce cytotoxicity in surrounding cells (Giulian and Ingeman, 1988). I have shown that TNFα and TNFα+IFNγ-stimulated microglia had up-regulated *Cr3* transcription, which is important for phagocytosis. Therefore, I examined whether TNFα and IFNγ alone or together could cause the release of IL-1β. I found that IL-1β release was elevated significantly only with TNFα alone (from 146 ± 93 to 386 ±162 pg/mL, $p < 0.05$; $n = 5$; Fig. 8). Furthermore, even though the average IL-1β released by the TNFα+IFNγ-treated group was higher than control, its difference from control did not reach significance ($p = 0.07$). I also measured the intracellular IL-1β levels by lysing the cells with a buffer containing a non-selective caspase-1 inhibitor (20 μM z-VAD fmk) in order to prevent cleavage of pro-IL-1β by caspase-1 activation by the lysis buffer. Intracellular IL-1β was found to be significantly higher in TNFα-treated cells (738 ± 495 pg/mL, $p < 0.05$) and in TNFα+IFNγ-treated cells (631 ± 150 pg/mL, $p < 0.01$) as compared to control (204 ± 106 pg/mL, $n = 5$ for all groups). Therefore, I found that total IL-1β production (including intracellular and released IL-1β) was significantly increased from control in TNFα-stimulated microglia (from 370 ± 70 pg/mL to 1227 ± 435 pg/mL, $p < 0.01$, $n = 5$ for all groups) and TNFα- and IFNγ-stimulated microglia (to 929 ± 121 pg/mL, $p < 0.001$). In sum, both TNFα alone and TNFα+IFNγ cause more IL-1β to be produced; IFNγ alone does not have an effect on this production.

Since there was much more intracellular IL-1β than released IL-1β, I tested whether release could be induced. Indeed, IL-1β release is known to be stimulated by the activation of P_2X_7 receptors (Bianco et al., 2005; Brough et al., 2002; Cheneval et al., 1998; Ferrari et al., 1997; Gudipaty et al., 2003; Kahlenberg and Dubyak, 2004; MacKenzie et al., 2001; Pelegrin and Surprenant, 2006; Perregaux and Gabel, 1994; Sanz and Di Virgilio, 2000). Consequently, I examined whether 30 min stimulation of P_2X_7 receptors with BzATP could induce further release

of IL-1β from stimulated microglia. P_2X_7 receptor activation causes an outward flux of K^+ ions, which presumably activates a tyrosine kinase that phosphorylates phospholipase A to cleave pro-caspase-1 from its pro-form (Cheneval et al., 1998; Kahlenberg and Dubyak, 2004; Perregaux and Gabel, 1994; Sanz and Di Virgilio, 2000). In addition, the inward flux of Ca^{2+} through the P_2X_7 pore increases the processing of pro-IL-1β into mature IL-1β and increases the release of IL-1β-containing microvesicles (Gudipaty et al., 2003; MacKenzie et al., 2001). To activate P_2X_7 receptors specifically, I used BzATP, a non-hydrolyzable P_2X_7 agonist that has been shown to have a similar effect as ATP on these receptors at 10-fold lower concentrations (Raouf et al., 2007). After application of 300 μM BzATP for 30 min, there was no increase in IL-1β release, in addition the effect of TNFα alone increasing IL-1β was significantly reduced (from 386 ±162 pg/mL to 169 ± 48.4 pg/mL, $p < 0.05$; $n = 5$; Fig. 8).

Figure 8

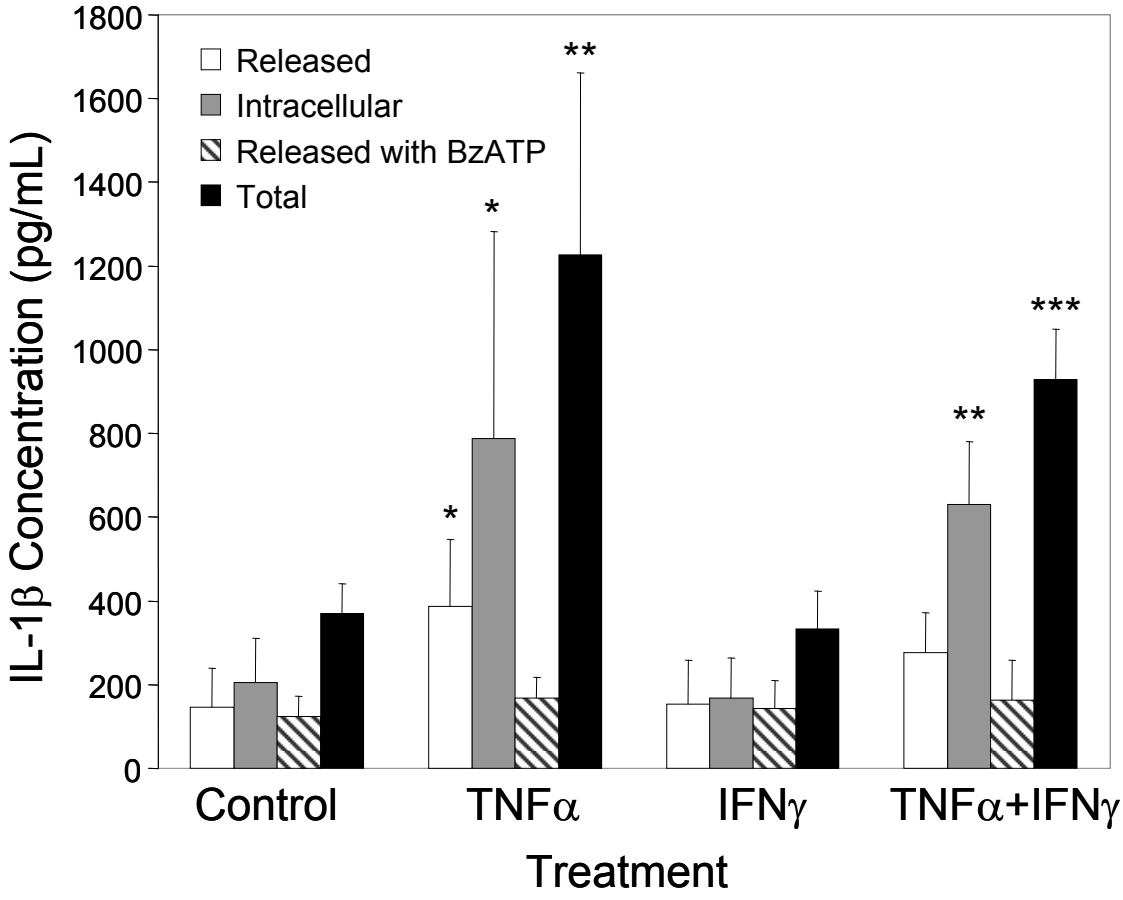

Figure 8: Total Il-1β, including intracellular contents (after cell lysis) and extracellular release, were measured using an ELISA. BzATP (300 μM) was applied 30 min before supernatant collection to measure whether it could stimulate release. Cells were cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together for 24 h. TNFα alone and with IFNγ were able to increase IL-1β, significant changes are indicated as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n = 5$, each n comes from a different litter and is an average of 3 wells.

3.5. TNFα with IFNγ increases nitrite release

After showing that microglia activation markers were increased following stimulation with TNFα+IFNγ, and that TNFα alone and TNFα+IFNγ caused increased production of IL-1β, I wanted to examine the effect of these cytokines on another pro-inflammatory molecule, nitric

oxide. Nitric oxide is a free radical that readily reacts with other molecules, which can lead to oxidative stress and cytotoxicity (Ortega Mateo and Amaya Aleixandre de, 2000). Activated microglia have been highly implicated in the production and release of NO after injury (Block et al., 2007). Therefore, I measured nitrite in the supernatant of cells stimulated with either TNFα, IFNγ alone or TNFα+IFNγ. I found that TNFα or IFNγ alone did not increase nitrite levels from control ($p = 0.28$ and $p = 0.60$, respectively) whereas together, they increased nitrite release significantly (from 1.26 ± 0.83 to 13.6 ± 3.18 μM, $p < 0.0001$; $n = 6$; Fig. 9). This suggests that nitrite release from microglia requires multiple stimuli acting together, possibly activating different intracellular pathways.

Figure 9

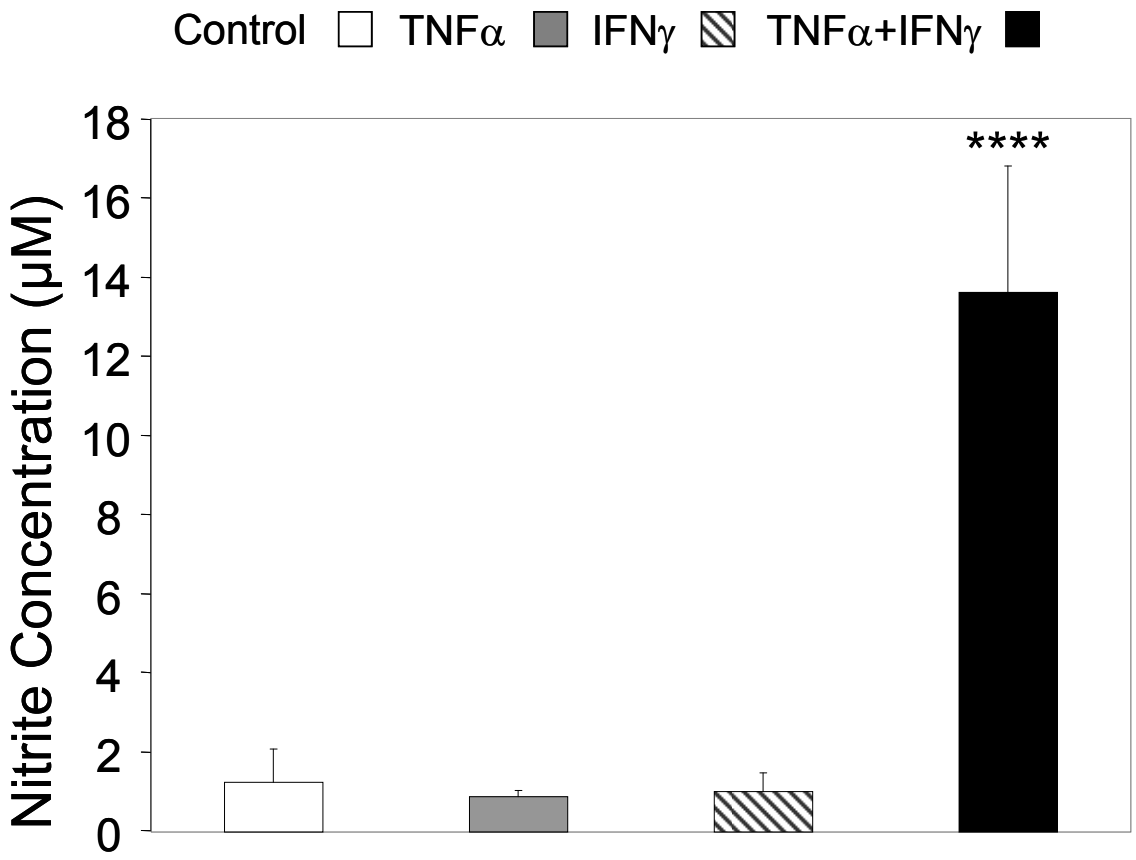

Figure 9: Nitrite released from 5.0 x 10^4 cells plated in 100 μL of supernatant was measured using the Griess Assay. Cells were cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, for 24 h. Only TNFα with IFNγ was found to increase the release of nitrite significantly ($p < 0.0001$; $n = 5$, each n comes from a different litter and is an average of 3 wells).

3.6. TNFα and IFNγ effect on IL-10

An anti-inflammatory cytokine, IL-10, known to be associated with microglia deactivation and with immunosuppression was examined to see whether its release or its intracellular concentration would be changed after stimulation with TNFα or IFNγ, alone or in combination. No significant changes in either intracellular IL-10 or released IL-10 were measured from

control following stimulation with these cytokines. However, a large amount of intracellular IL-10 was measured (for control: 156 ± 24 pg/mL, *n* = 3; Fig. 10) even though release of this cytokine was low (for control: 28 ± 9 pg/mL, *n* = 4; Fig. 10).

Figure 10

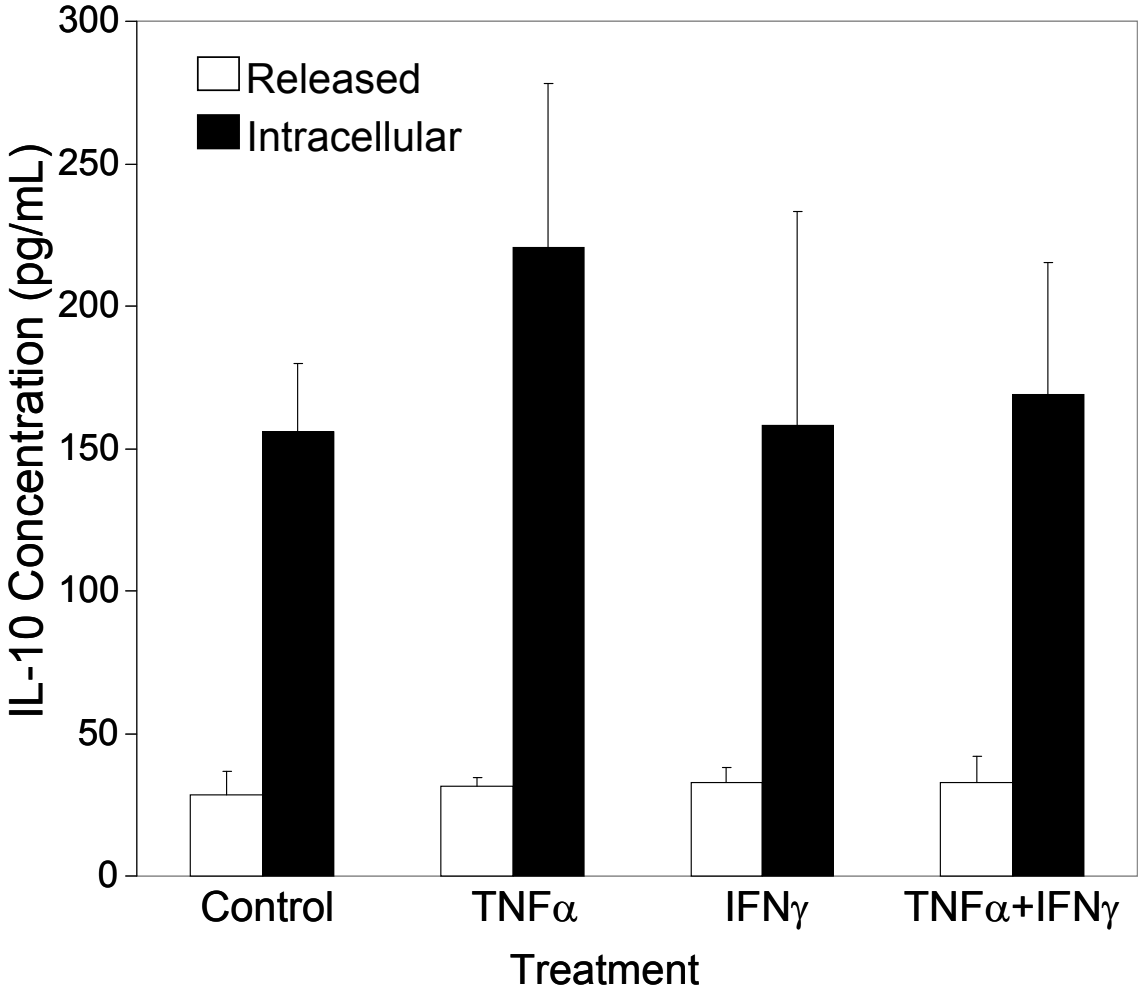

Figure 10: Released and intracellular IL-10 were measured using an ELISA. Cells were cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, for 24 h. No significant changes were measured following any treatment in either released or intracellular IL-10 (*n* = 3-4, each *n* comes from a different litter and is an average of 3 wells).

Other studies have shown low basal levels of released IL-10 in primary microglia *in vitro* (Seo et al., 2004; Seo et al., 2008). However, this is the first evidence that unstimulated microglia produce large amounts of IL-10, which they are not releasing. To further investigate these elevated basal levels of intracellular IL-10 in microglia, I stained control cells and cells stimulated with TNFα+IFNγ for 24 h with antibodies directed at IL-10 and CD 63. CD 63 is a lysosomal marker for macrophages and microglia that is up-regulated with activation (Graeber et al., 1988). I also performed 'no primary' negative controls with no primary antibody to ensure low background staining (Fig 11A). I observed that, regardless of the treatment, ramified cells with less intense CD 63 staining possessed less IL-10 (Fig. 11B) than more rounded, brightly CD 63 stained, and presumably more activated cells (Fig. 11C). There were no obvious differences in the number of cells containing high IL-10 between the control group, although no quantification was executed to confirm this (Fig. 11D) and the TNFα with IFNγ-stimulated group (Fig. 11E).

Figure 11

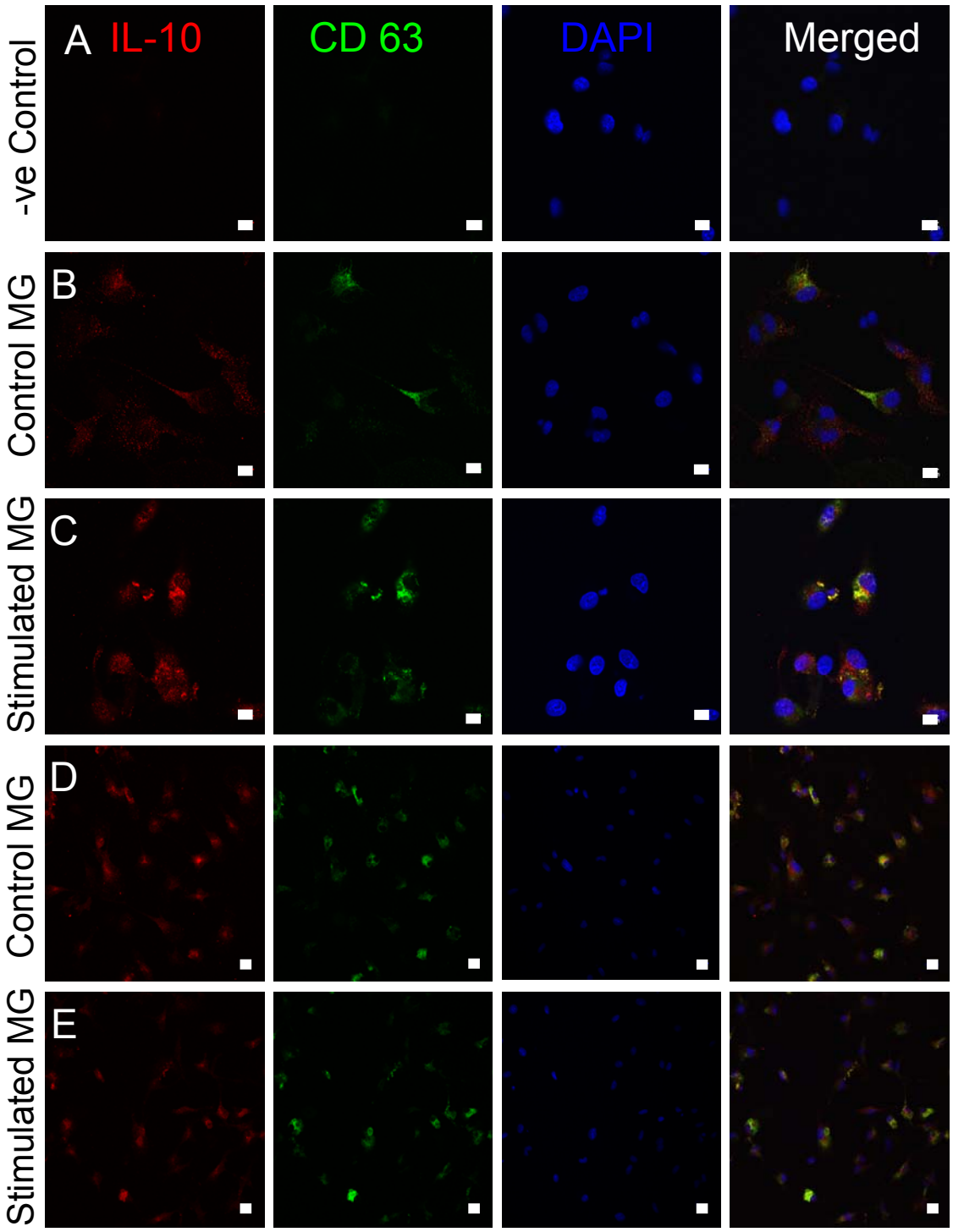

Figure 11: IL-10 is increased in more activated microglia. Cells were labeled with a primary antibody against IL-10 and CD 63, and with DAPI. Shown are no-primary antibody controls with DAPI (A), less activated cells (B) and more activated cells (C) at high magnification, and unstimulated cells (D) and cells stimulated with 50 ng/mL TNFα and 20 ng/mL IFNγ for 24 h (E) at low magnification. Scale bars for A, B, C = 10 µm; for D and E =20 µm.

3.7. Changes in the expression levels of immunomodulators after TNFα and IFNγ stimulation

To support the functional assays examining IL-1β, NO, and IL-10 release and production in this thesis, gene transcription levels were examined using qRT-PCR. Because TNFα and IFNγ have been implicated in iNOS elevation, which directly increases COX-2 levels, *Cox-2* was also monitored (Nathan, 1992; Nathan and Xie, 1994; Nogawa et al., 1998). Similarly to the ELISA results on release and intracellular IL-1β contents, *Il-1β* mRNA transcription was increased from control by TNFα alone (in fold change, from 1.00 ± 0.56 to 11.4 ± 1.22, $p < 0.001$; $n = 3$-4; Fig. 12) and by TNFα+IFNγ (to 9.13 ± 1.34, $p < 0.001$; $n = 3$-4; Fig. 12). The expression levels of *iNos* also followed the same trends as nitrite release. Indeed, *iNos* was increased from control only by TNFα+IFNγ (as fold change, from 1 ± 1.19 to 66.9 ± 26.2, $p < 0.01$; $n = 3$-4; Fig. 12). Although both *Il-10* and *Cox-2* mRNA levels were measured after stimulation, no differences were found between control and treated cells (Fig. 12). Since all real-time qRT-PCR results matched functional assay results, it is likely that the changes measured in the functional assays are at the transcriptional level and not occurring due to post-transcriptional modifications.

Figure 12

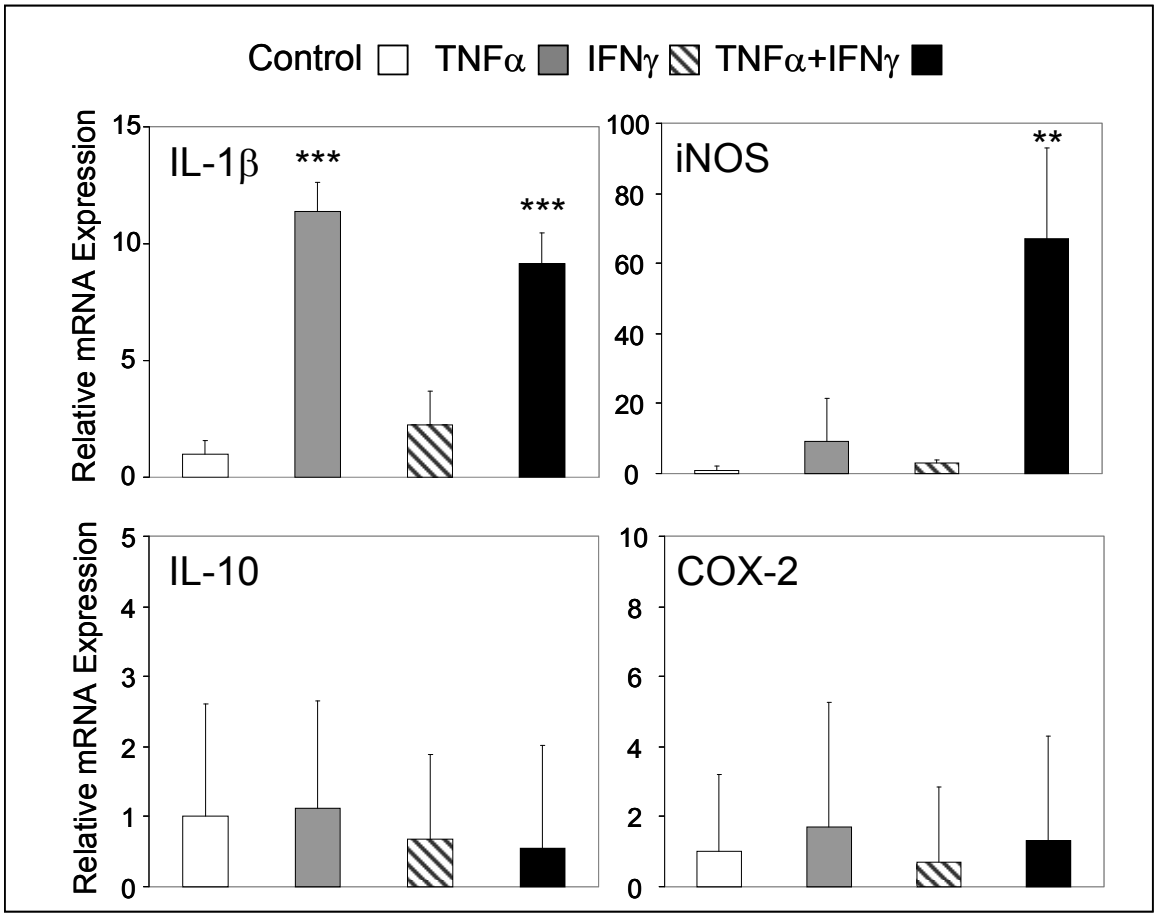

Figure 12: The effect of cytokine stimulation on the transcription of inflammatory genes. Gene expression was measured by quantitative real-time RT-PCR in cells cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, for 24 h. *Il-1β*, *iNos*, *Il-10*, and *Cox-2* mRNA transcripts were measured and transcript expression for each gene is shown as the fold increase relative to the 2% FBS control. TNFα alone and with IFNγ were found to significantly increase *Il-1β* but solely the combination of the two increased *iNos* transcription, though no changes in *Il-10* and *Cox-2* were measured: $^{**}p < 0.01$; n = 3-4. qRT-PCR was executed by Xiao-Ping Zhu.

3.8. Phosphorylation of CREB induced by stimulation by TNFα and IFNγ

Studies have shown that pCREB increases in activated microglia both *in vitro* and *in vivo* (Ajmone-Cat et al., 2003; Herdegen et al., 1992; Sanagi et al., 2005). However, CREB phosphorylation in microglia in a stroke model has never been examined. Hence, I looked at the phosphorylation of CREB in microglia following ICH induced using the collagenase model. In the contralateral side, resting microglia with very low CD 11b staining had very weak pCREB staining (Fig. 13A, C). On the other hand, pCREB staining varied much more in microglia in the ipsilateral side (Fig. 13B, D). Some cells were found to have high pCREB while some had a pCREB intensity similar to the microglia on the contralateral side. It should be noted that the antibody against pCREB also recognizes the activating transcription factor-1 when it is phosphorylated at Ser63 because the phosphorylation sequences of these two members of the bZIP family are homologous (Iordanov et al., 1997; Mayo et al., 2001).

Figure 13

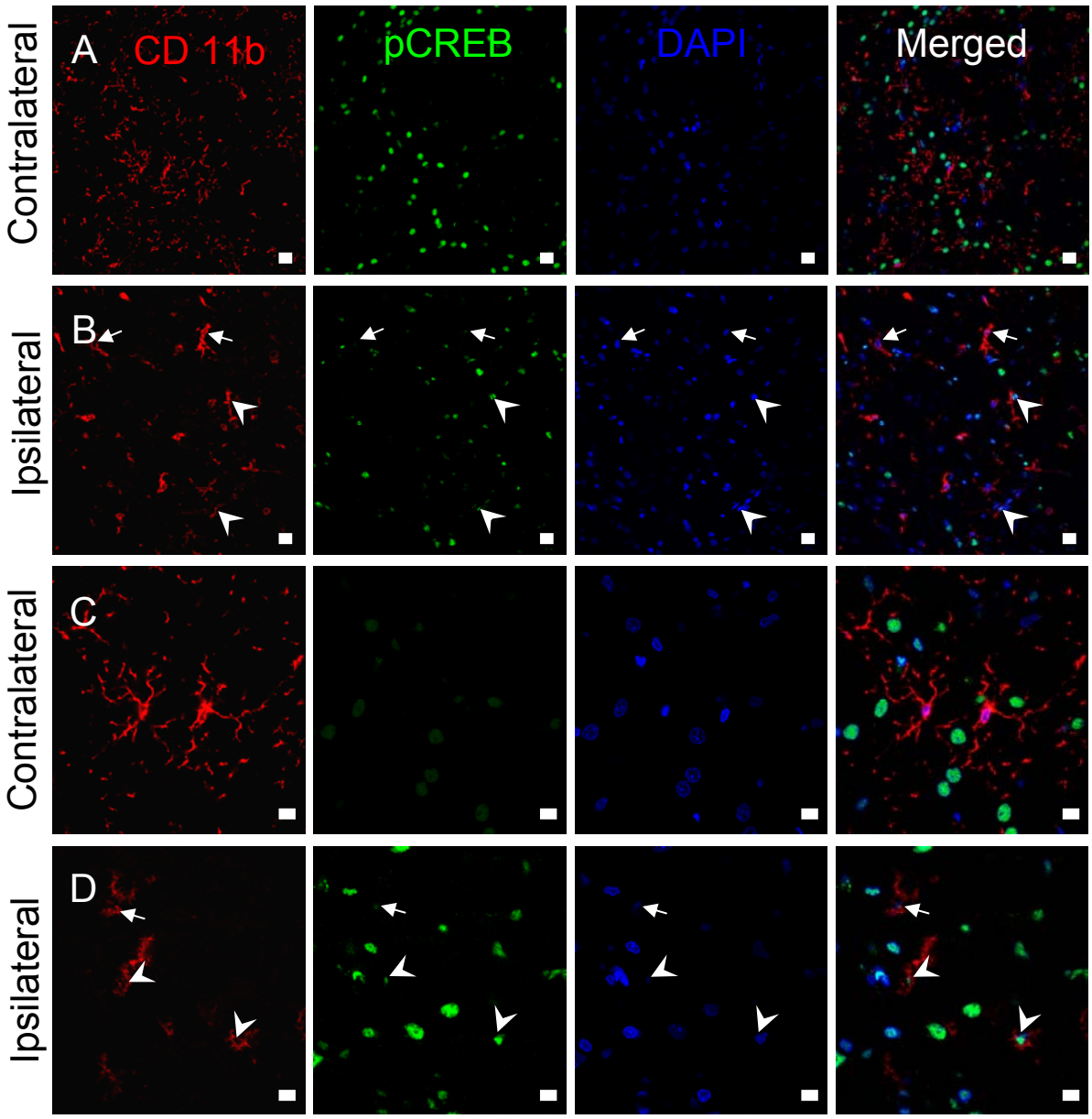

Figure 13: pCREB changes in activated microglia *in vivo*. Brain sections were stained using primary antibodies directed against pCREB and CD 11b, and with DAPI 3 d after intracerebral haemorrhage. Shown are low magnification pictures of the contralateral (A) and ipsilateral (B) side (scale bars = 20 µM), and high magnification pictures of the contralateral (C) and ipsilateral (D) side (scale bars = 10 µM). ICH surgeries and immunohistochemistry were done with the help of Jason Wasserman.

Based on these results *in vivo*, I then examined the phosphorylation of CREB following the activation of microglia by cytokines. Therefore, cells were stimulated with TNFα or IFNγ alone or combined for 10, 30, 60, or 120 min. Then, they were fixed in PFA for pCREB staining. The average intensity of pCREB was measured in each nucleus using Image J 1.38 (see Methods). The mean intensity of pCREB was not found to be significantly different between control and any of the stimulated groups (Fig. 14).

Figure 14

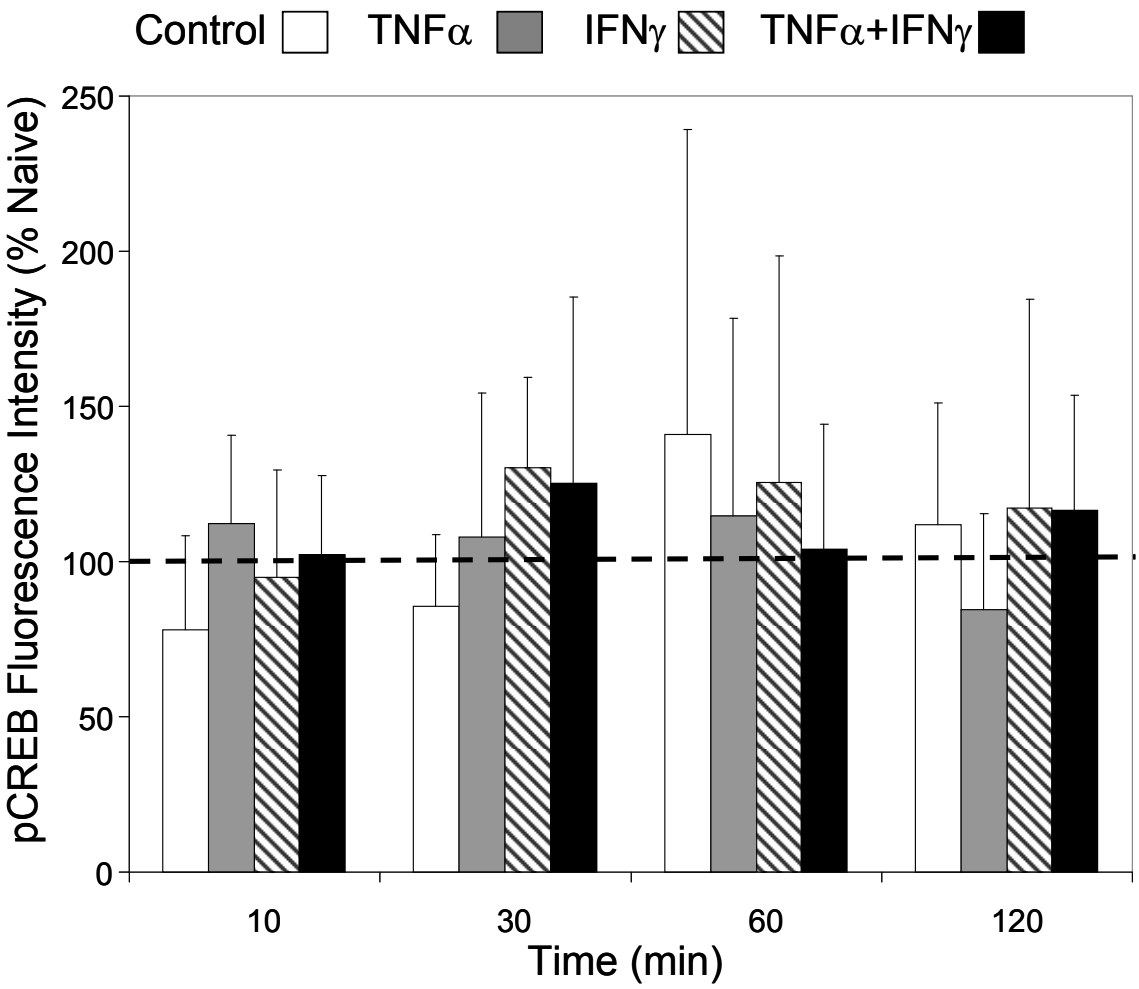

Figure 14: Mean pCREB intensity per cell following microglia stimulation. The average pCREB fluorescence intensity was measured in cells cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both TNFα and IFNγ together at 10, 30, 60, and 120 min. Results are shown as % control where the control cells are cells that were fixed at 0 min, with no change in media; n = 3-4, each *n* comes from a different litter and is the average intensity value from 3 coverslips, which are themselves an average of 3 fields per coverslip.

However, the data *in vivo* and *in vitro* suggested two populations of cells: cells with very high pCREB and cells with very low pCREB. I wondered whether the number of these cells was changed by stimulation with TNFα and IFNγ. Therefore, I did frequency counts of cells in 3 different intensity ranges (low: 0-50%, average: 50-150%, or high: > 150% intensity of naïve

cells). I found that, at 60 there was a significantly smaller number of cells with a pCREB intensity similar to that of naïve cells in cells stimulated with TNFα+IFNγ (57.2 ± 8.7% of all cells in control *vs.* 34.2 ± 7.0% of all cells in TNFα+IFNγ, $p < 0.05$; $n = 3\text{-}4$; Fig 15). This was also true at 120 min (50.6 ± 3.5% of all cells in control vs. 38.5 ± 3.2% in TNFα+IFNγ, $p < 0.01$; $n = 3\text{-}4$; Fig 15). Also, at 60 min there were significantly more cells with a high pCREB intensity in the TNFα-stimulated group compared to controls at 60 min (13.4 ± 8.7% of all cells in control *vs.* 30.8 ± 7.9% of all cells, $p < 0.05$; $n = 3\text{-}4$; Fig 15).

Figure 15

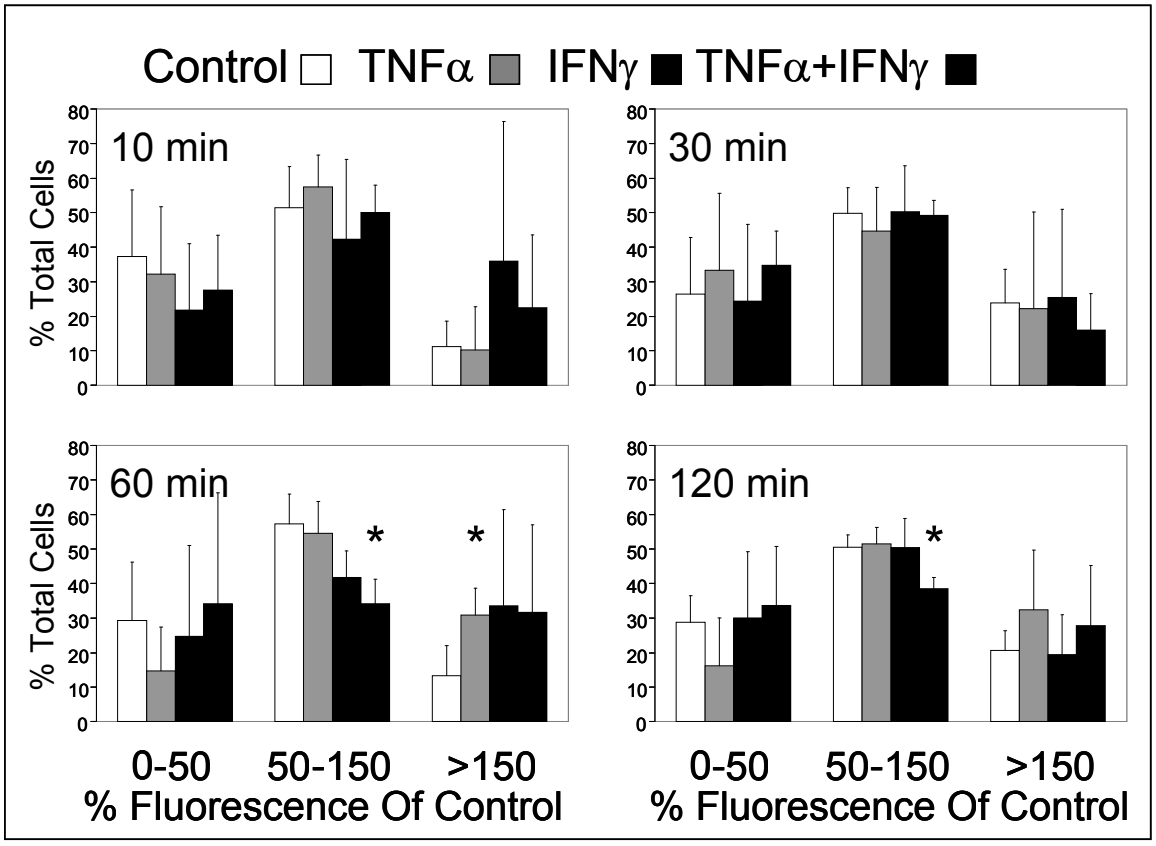

Figure 15: Percentage of cells with a pCREB intensity in three different ranges (low, average, and high) compared to control. Cells cultured in MEM with 2% FBS with or without 50 ng/mL TNFα, 20 ng/mL IFNγ, or both together, were fixed for immunocytochemistry at 10, 30, 60, and 120 min. Results are shown as % of cells having a pCREB fluorescence intensity between 0-50 % that of control, 50-150 % of control or over 150 % of control, where the control cells are cells that were fixed at 0 min, with no change in media. Significance is expressed as * $p < 0.05$ ** $p < 0.01$; n = 3-4, each n is an average of the intensity values from 3 coverslips, which are themselves an average of 3 fields per coverslip.

Shown in Fig. 16 are higher magnification descriptive pictures of pCREB, CD 63, and DAPI staining in control cells and TNFα-stimulated cells fixed at 60 min. It is clear that most of the control cells have similar pCREB intensities (Fig. 16B); whereas more of the TNFα-stimulated cells have either higher or lower pCREB (Fig. 16C). 'No primary' antibody negative controls

are also shown (Fig. 16A). Taken together these results suggest that TNFα stimulation causes differential activation of microglia.

Figure 16

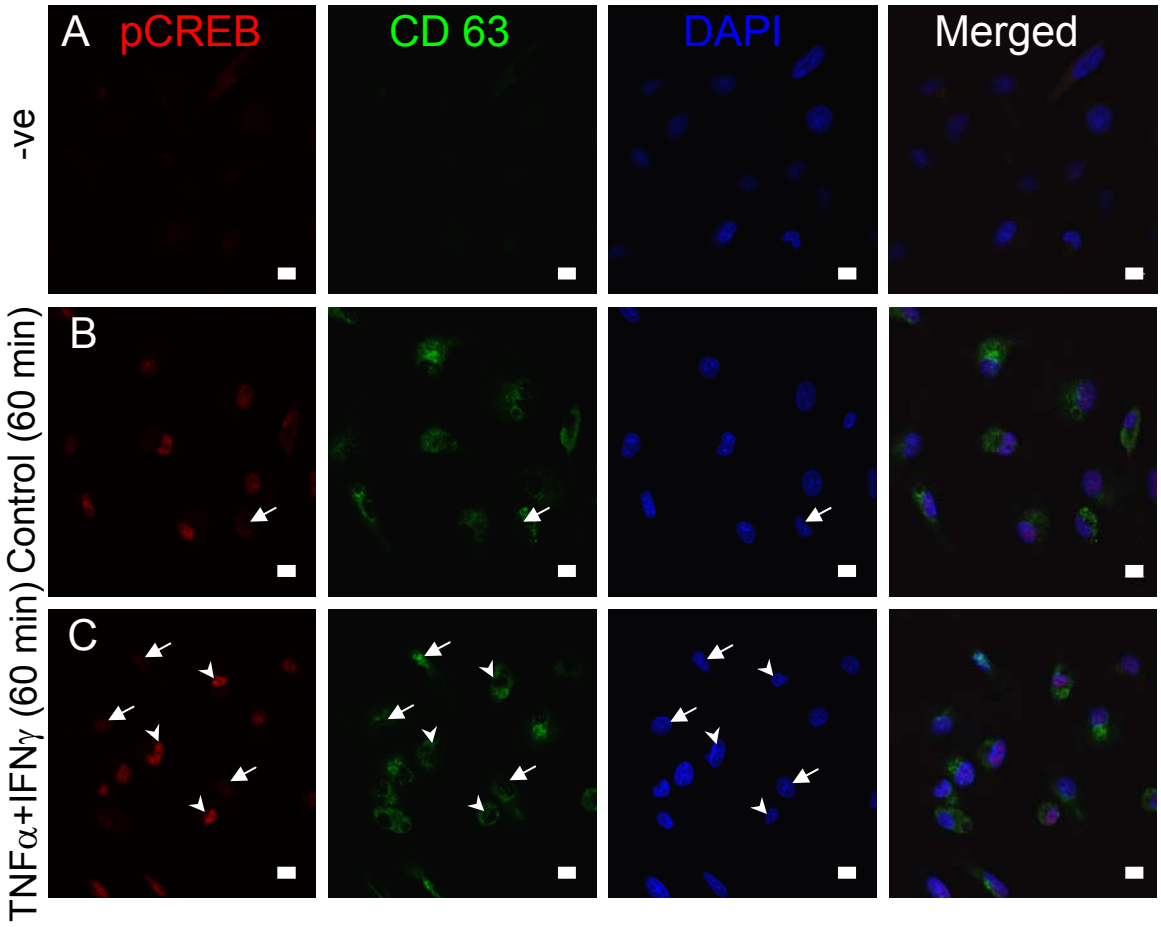

Figure 16: pCREB intensity changes in stimulated microglia. Cells were labeled with a primary antibody against pCREB and CD 63, and with DAPI. Shown are no-primary antibody controls with DAPI (A), non-activated cells (B), and cells activated with 50 ng/mL TNFa for 60 min (C) in vitro. In vivo, microglia were stained with CD 11b and pCREB on the contralateral side of an ICH (D) and on the ipsilateral side (E). Block arrows designate cells with a lower than average intensity while arrowheads designate cells with a higher than average intensity. Scale bars = 10 μm.

4. Discussion

This thesis details the inflammatory phenotype acquired by microglia after stimulation with cytokines. Although many studies have used other physiological stimuli to induce microglia activation (i.e. Aβ fragments, gp41/Tat protein, prion protein), these are pathology-specific and generally involved with neurodegenerative diseases (Nakamura, 2002). In this thesis, I chose to design an *in vitro* model that would represent an acute model of brain injury. I described microglia activation in response to two cytokines released early after many different types of insult: TNFα and IFNγ.

4.1. Inflammatory cytokine expression following ICH

In order to determine which cytokines would be most relevant to studying an acute brain injury model *in vitro*, I examined the inflammatory profile of the ICH injury. ICH is a good model to study acute brain injury because it is a reproducible model with a well characterized inflammatory response (Wasserman and Schlichter, 2007a; Wasserman and Schlichter, 2007b; Wasserman et al., 2007). Indeed, TNFα, IL-1β, and IL-6 transcription were found to be significantly enhanced within 6 h and remained significantly elevated until 3 d after injury (Wasserman et al., 2007). TNFα, IL-1β, and IL-6 are early pro-inflammatory cytokines known to be involved in many different acute injuries and neurodegenerative diseases (Werner and Grose, 2003). Specifically, studies have established the importance of TNFα early following insult. This includes both its release from inflammatory cells and its release by injured neurons (Barone and Feuerstein, 1999; Lavine et al., 1998; Liu et al., 1994). However, one cytokine that

was never examined after ICH is IFNγ. IFNγ has been used widely *in vitro* as a primer to up-regulate the release of inflammatory mediators produced by LPS. It is an interesting molecule because it regulates inflammation in neurodegenerative diseases. However, it is rarely examined in acute brain injuries (Beck et al., 1988; Issazadeh et al., 1995; Merrill et al., 1992). Therefore, *Ifnγ* levels were measured after ICH. *Ifnγ* transcription was found to be higher in the injured striatum than the non-injured striatum. The increased *Ifnγ* transcription after ICH in the ipsilateral striatum was particularly intriguing because no T lymphocytes were detected in the ICH model (unpublished results). Since only *Ifnγ* transcription was measured and since no T lymphocytes were seen in the brain after ICH, the cells that up-regulate *Ifnγ* must be resident CNS cells. This is consistent with previous studies that have shown IFNγ production by microglia, astrocytes, and possibly even by neurons in the brain (Kawanokuchi et al., 2006; Lau and Yu, 2001; Neumann et al., 1997; Neumann, 2001; Olsson et al., 1989). In addition, the elevation of *Ifnγ* by minocycline was surprising because Panitch *et al.* (1987) have shown that an IFNγ injection worsens MS lesions, and Wasserman and Schlichter (2007a) showed that administering minocycline after ICH reduced inflammation and oedema. That higher levels of *Ifnγ* after minocycline treatment after ICH was beneficial contradicts the detrimental effect of IFNγ in MS. However, it is possible that the cellular sources of IFNγ determine the nature of the ensuing inflammatory response.

4.2. Proliferation of TNFα+IFNγ-stimulated microglia

Early after ICH, microglia become activated, proliferate, and surround the injury. I proposed that the early up-regulation of TNFα and IFNγ in ICH might play a role in these actions. Therefore,

metabolic activity, proliferation, and death were assessed in response to stimulation of microglia with these cytokines *in vitro*. TNFα was found to induce proliferation, which likely also explained the increase in total metabolic activity because the difference from control for both of these variables was similar. TNFα+IFNγ did not further increase metabolic activity. Although the increase in proliferation measured with TNFα+IFNγ did not reach significance, there was a trend for the two cytokines to cause a similar effect to TNFα alone. Hence, it is likely that TNFα accounts for all the proliferation occurring following stimulation with the combination of TNFα+IFNγ.

That TNFα alone had an effect on proliferation was contrary to previous results by Kloss *et al.* (1997) who found no effect of recombinant murine TNFα on the proliferation of primary rat microglia grown on an astrocyte monolayer. However, other studies have shown that TNFα is able to induce proliferation in microglia. Giulian and Ingeman (1988) found a very small increase in proliferation, and Mander *et al.* (2006) found a large enhancement of proliferation following TNFα application. The main difference between these studies appears to be the degree to which the cells appear resting and ramified. Indeed, in co-cultures with astrocytes, the ability of TNFα to induce proliferation appears to be diminished (Giulian and Ingeman, 1988; Kloss et al., 1997).

Astrocytes have previously been shown to stimulate microglia proliferation by secreting colony stimulating factors, and to induce ramification (Gebicke-Haerter et al., 1989; Shafit-Zagardo et al., 1993; Tanaka and Maeda, 1996). On the other hand, in isolated microglia, which are often more activated, TNFα stimulated proliferation through a pathway requiring NADPH oxidase-mediated production of hydrogen peroxide (Ganter et al., 1992; Mander et al., 2006). Hence, I

suggest that when microglia are already proliferating, such as when in co-culture with astrocytes, TNFα cannot induce further proliferation. However, when they are isolated from astrocytes and basal proliferation is lower, TNFα alone is able to induce proliferation.

4.3. Characterizing the activation state of TNFα+IFNγ-stimulated microglia

4.3.1. TNFα+IFNγ induce increased transcription of *Cr3* and *Mhc II*

It is important, when studying primary isolated microglia *in vitro*, to not only define whether they are resting or activated, but also to define the specific type of activation. Based on the current literature, different microglia activation types can be divided in five categories: innate, humoral, classical, and alternative activation, and innate/acquired deactivation (Gordon, 2003). CR3 is known to be linked to the general activation of microglia and thus, would be expected to decrease if the cells were becoming deactivated. MHC II is associated with the acquisition of antigen-presenting functions that are linked to acquired immunity and not to innate immunity. MR1 is a marker of alternative activation, which is associated with beneficial inflammation and/or immunosuppression.

For this thesis, I wanted to further characterize the physiological model of TNFα+IFNγ-induced stimulation by determining which activation state the microglia were likely to be in. Therefore, qRT-PCR was carried out to measure changes in different types of activation markers: *Cr3*, *Mhc II*, and *Mr1*. The *Mhc II* expression increase seemed to be largely mediated by IFNγ while the *Cr3* expression increase seemed mostly mediated by TNFα. With TNFα+IFNγ, *Mhc II* and *Cr3*

expression were significantly increased while *Mr1* expression was decreased. This suggests that microglia activated by TNFα+IFNγ are neither in either an innate activation state or an innate/acquired deactivation state, since *Mhc II* increased nor are they alternatively activated, since *Mr1* was decreased.

It was surprising that IFNγ alone did not significantly decrease the expression of *Mr1* because of literature showing lower expression of the mannose receptor in murine microglia and in macrophages stimulated with IFNγ alone (Harris et al., 1992; Schreiber et al., 1993; Zimmer et al., 2003). However, Zimmer *et al.* (2003) showed that, in microglia, MR1 was most down-regulated 3 d after stimulation and only with the highest IFNγ concentrations and Shreiber *et al.* (1993) showed that macrophages down-regulated MR1 binding maximally at high IFNγ doses and at 48 h. In fact, the only study to show a pronounced effect of IFNγ on *Mr1* mRNA transcription at a shorter time point (6 h) used slightly higher IFNγ concentrations (~ 50 ng/mL) and studied the effect on a macrophage cell line (Harris et al., 1992).

It is also possible that the basal activation state of the cells is important in examining changes in *Mr1* expression. This is supported by a study in human macrophages where the extent of inhibition of uptake mannosyl, fucosyl-terminated glycoconjugates (through mannose receptors) depended on the IFNγ dose and the individual source of the cells (Mokoena and Gordon, 1985). Taken together, I suggest that the basal activation state of the microglia, combined with the differences in IFNγ concentration and time of exposure, explain why some studies show a significant decrease in *Mr1* expression whereas significance was not reached in my experiments.

4.3.2. Pro-inflammatory mediators are elevated by TNFα+IFNγ

Not only do activated microglia up-regulate transcription of activation-specific genes, they also produce and release immunomodulators, which can be pro- or anti-inflammatory. Furthermore, the nature of the activation: innate, humoral or classical, is defined by these immunomodulators, which can include IL-1β, IL-10, and NO. Indeed, innate and humoral activation are characterized by the production of pro- and anti-inflammatory cytokines even though only innate activation causes the release of reactive oxygen and nitrogen species. Whereas classical activation does not the release anti-inflammatory cytokines, pro-inflammatory cytokines and reactive nitrogen species are produced (Gordon, 2003). The release of IL-1β, IL-10, and nitrite from TNFα+IFNγ-stimulated microglia was measured to specify their activation state. The respective mRNA levels of these modulators were also measured to determine whether changes were transcriptional or post-transcriptional. For all immunomodulators examined, mRNA changes matched results from functional assays, therefore, only the functional assays will be discussed.

4.3.2.a IL-1β

IL-1β production was significantly elevated by TNFα+IFNγ. In addition, some important findings from these experiments were that IL-1β release and intracellular accumulation seemed largely dependent on TNFα; P_2X_7 activation could not induce the release of accumulated intracellular IL-1β, and P_2X_7 activation blocked the TNFα-induced IL-1β release.

The observation that TNFα induced IL-1β production is consistent with the finding that addition of recombinant TNFα to human mononuclear cells leads to an elevation in IL-1 release

(Dinarello et al., 1986). I, however, demonstrate, for the first time, that this effect also occurs in primary microglia. On the other hand, Dinarello *et al.* (1986) also measured an IFNγ-induced additive effect on the TNFα-induced IL-1 release, which was not the case in this thesis. It is likely that the reason for this is that the increased IL-1 release was IL-1α and not IL-1β. Therefore, since the ELISA used to measure IL-1β in my experiments does not detect IL-1α, I did not detect an increase. Supporting this is a transcriptional study of human microglia activated by IFNγ, which showed a 3.4 fold increase in IL-1α transcription 24 h after stimulation (Rock et al., 2005). TNFα-mediated effects are largely dependent on NF-κB signalling (Aggarwal, 2004; Dos Santos et al., 2007). Conversely, IFNγ mediates most of its effects via Jak-STATs (Schroder et al., 2004). Since IL-1β is largely up-regulated by NF-κB, it was not surprising to measure a TNFα-mediated IL-1β increase and no effect of IFNγ on IL-1β production.

Previous studies have shown that P_2X_7 receptor stimulation induces the processing and release of IL-1β from macrophages and microglia. In this study, P_2X_7 receptor activation did not further increase the release of IL-1β following TNFα or TNFα+IFNγ stimulation. This discrepancy is likely due to the time at which I examined release. Indeed, I chose a 24 h time point because this is a time at which IL-1β release is high with LPS activation (Nakamura et al., 1999). However, Sanz and Di Virgilio (2000) showed that the maximal difference in microglia IL-1β release from microglia activated with LPS alone or with an additional 5 mM ATP for 30 min was at 6 h. When they looked at 24 h, adding 5 mM ATP for 30 min did not have an effect on LPS-induced IL-1β release. This is different from the study by Brough *et al.* (2002) who observed a large increase in LPS-induced IL-1β at 24 h when they pre-incubated cells for 3 h with 5 mM ATP, but they found this concentration of ATP to kill almost all the cells. Hence, it is possible that part of the increase they observed with ATP was due to the release of intracellular IL-1β from dying

cells. This is supported by their finding that 1 mM ATP, which was not cytotoxic, had no effect on IL-1β release.

P_2X_7 receptor activation induces the release of IL-1β following LPS stimulation (Bianco et al., 2005; Brough et al., 2002; Cheneval et al., 1998; Ferrari et al., 1997; Gudipaty et al., 2003; Kahlenberg and Dubyak, 2004; MacKenzie et al., 2001; Pelegrin and Surprenant, 2006; Perregaux and Gabel, 1994; Sanz and Di Virgilio, 2000). Similarly, TNFα stimulation induces IL-1β release (Dinarello et al., 1986). That P_2X_7 receptor activation combined with TNFα stimulation caused a decrease in the amount of IL-1β release was entirely unexpected. Although simultaneous P_2X_7 and TNFα receptor activation could lead to competition between transcription factors, the BzATP stimulation in this study was very short (30 min). Therefore, it is unlikely that the decreased IL-1β release is due to competing transcription factors. However, IL-1β levels can decrease if it is degraded after release. Indeed, IL-1β can be degraded by proteases such as matrix metalloproteinases (MMPs), which are up-regulated by TNFα (McGeehan et al., 1994). Moreover, ATP has been shown to elevate TNFα release (Gu and Wiley, 2006). Therefore, it is possible that the combination of stimuli lead to increased TNFα release, increased MMP activation, and increased IL-1β degradation. A likely candidate is matrix metalloproteinase-9 (MMP-9), which is activated by TNFα and which also cleaves and activates TNFα (Gearing et al., 1994; Gottschall and Deb, 1996; McGeehan et al., 1994). Because activation of P_2X_7 receptors can more than double the release of TNFα by microglia within 2 h, and their activation also causes the rapid release of MMP-9, it is possible that TNFα and BzATP together cause a self-enhancing propagation of MMP-9 and TNFα release (Gu and Wiley, 2006; Suzuki et al., 2004; Trembovler et al., 1999). Large MMP-9 concentrations in the supernatant could then cause the degradation of IL-1β (Ito et al., 1996).

4.3.2.b Nitrite

Nitrite is a source of NO, a diffusible factor that, combined with superoxide, leads to the production of peroxynitrite, which induces cytotoxicity (Minghetti and Levi, 1998). In this study, the combination of TNFα+IFNγ was found to induce nitrite release from microglia. This is contrary to a study by Colasanti *et al.* (1995) who showed that TNFα alone could induce nitrite release in primary ramified human microglia. However, these human cells had undergone over 50 passages; therefore, they were likely to have a very different inflammatory profile than the primary rat microglia I used. Similar to the results described in this thesis, other studies have shown a synergistic effect of TNFα and IFNγ on macrophages or microglia, which causes the release of nitric oxide (Anderson et al., 1995; Banati et al., 1993; Corradin et al., 1993; Liew et al., 1990). Indeed, TNFα and IFNγ activate different transcription factors through their respective signalling pathways and these can interact to affect transcription of *iNos*. Briefly, IRF-1 produced by IFNγ and NF-κB translocated to the nucleus by TNFα can interact physically in the nucleus to enhance transcription of certain genes (Paludan, 2000; Suk et al., 2001).

My results suggest that the interplay between different modulators of inflammation is crucial to maintain an appropriate, balanced response. The fact that TNFα+IFNγ elevated the release of nitrite but that neither alone increased *iNos* transcription and release of nitrite is probably related to the importance of NO in cytotoxicity. Undoubtedly, following acute brain injury *in vivo*, injured cells release activators of microglia to communicate the extent of the injury. Presumably, the specific combination and concentration of each of these activators will determine whether microglia release protective molecules to promote repair or cytotoxic molecules like NO.

4.3.2.c IL-10

IL-10 is the major anti-inflammatory cytokine released following acute brain injury. It causes immunosuppression and promotes the release of repair and regeneration-related molecules (Planas et al., 2006). In this thesis, no change in IL-10 release was measured with any of the treatments. However, a few studies had previously found that TNFα alone can increase IL-10 release from monocytes (Daftarian et al., 1996; Platzer et al., 1995; Wanidworanun and Strober, 1993). This effect was specifically seen when low concentrations of TNFα (< 10 ng/mL) were used. At higher concentrations, transcription of *Il-10* and protein levels remained unchanged (Bondeson et al., 1999; Platzer et al., 1995; Wanidworanun and Strober, 1993).

Sheng *et al.* (1995) is the only study contradicting this result. They showed a dose-dependent elevation in IL-10 release following stimulation of foetal human microglia with 4-100 ng/mL TNFα. Specifically, Sheng *et al.* (1995) measured IL-10 released by 5 x 10^4 cells in 500 µL (same number of cells in 2.5x the volume of that in the present study) and that IL-10 release rose from basal levels of 1 ng/mL to 22 ng/mL in cells stimulated with 100 ng/mL TNFα. These concentrations of IL-10 release were higher than in any other report using any type of stimulus. In fact, one of the better stimulators of IL-10 release is LPS. The highest LPS-induced IL-10 release in cells of monocyte lineage were measured by Wanidworanum and Strober (1993) who showed an increase from < 20 pg/mL to 8700 pg/mL. In neonatal primary rat microglia, the LPS-induced IL-10 concentrations were even lower, rising from 40 to 600 pg/mL at 24 h (Seo et al., 2004). Therefore, I can conclude that the difference in TNFα-induced IL-10 transcription or

release between the previous studies and this thesis is likely due to higher than 10 ng/mL concentrations used in the present study.

Microglia contain elevated levels of intracellular IL-10. These levels were unchanged by TNFα and IFNγ stimulation. Similarly to most other studies, I measured very low levels of released IL-10 but when the cells were lysed, the concentration of IL-10 measured was much higher. This indicates that there must be a regulation of IL-10 release by microglia. Different stimuli have been found to modulate IL-10 transcription and release: up-regulating for LPS, TNFα, and IL-1β and down-regulating for IFNα, IFNγ, and IL-4 (Bondeson et al., 1999; Foey et al., 1998; Wanidworanun and Strober, 1993). Certainly, transcription must be modulated at the promoter level but IL-10 release may additionally be modulated independently of transcription levels. Accordingly, Seo *et al.* (2004) have shown that small doses of ADP or ATP can cause IL-10 release from LPS-stimulated microglia but that at these doses, TNFα was not induced.

Again, it is possible that mediators released from injured cells communicate the state of inflammation. Accordingly, at the beginning of an acute brain injury, cells die from necrosis and release ATP, TNFα, and glutamate into the extracellular space (Lipton, 1999a; Volonte et al., 2003). Subsequently, when the majority of cells dying from necrosis have been phagocytosed, lower levels of ATP, TNFα, and glutamate are released. This occurs directly (from decreased release from dying cells) and indirectly (since glutamate stimulates ATP release and ATP stimulates TNFα release) (Liu et al., 2006). These lowered levels of ATP and TNFα may then stimulate increased release and production of IL-10. This is supported by studies showing that lower concentrations of ATP and lower concentrations of TNFα induce IL-10 (Oswald et al., 1992). After that, IL-10 could act in a negative feed-back loop to further down-regulate TNFα

and suppress inflammation (Kim et al., 2002; Sawada et al., 1999; Tan et al., 1999). In this way, the switch from inflammation to immunosuppression is tightly regulated by the state of surrounding cells. Microglia clearly play a central role in this switch because, as seen in this model of acute brain injury, they can hold large amounts of IL-10.

In sum, in this thesis, I found no change in IL-10 release or transcription, which can be explained by the concentration of TNFα used as a stimulus. The elevated levels of IL-10 in unstimulated microglia have never before been described. I propose that other stimuli, which signal a switch to immunosuppression, are required to induce IL-10 release.

4.3.3. Microglia stimulation by TNFα+IFNγ is a model of classical activation

The up-regulation of *Mhc II*, *Il-1β*, and *iNos* transcription, in addition to the increased release of IL-1β and nitrite suggests that the model of microglia stimulation by TNFα+IFNγ is one of classical activation. I have shown evidence to support that this stimulation does not match any other models described in Gordon (2003). First, stimulation with TNFα+IFNγ diminished transcription of *Mr1* and increased *Mhc II*, thereby eliminating alternative activation and innate/acquired deactivation, both of which are associated with T helper lymphocyte Type 2 responses. Second, the elevation in *Mhc II* also suggested that microglia were not innately activated since antigen presentation is not involved in innate immune responses. Third, after measuring various immunomodulators including IL-1β, IL-10, and nitrite, only pro-inflammatory molecules were shown to be changed by TNFα+IFNγ. IL-10 remained the same, while both the transcription and the production of IL-1β and nitrite (linked to *iNos* transcription) were significantly elevated. Since both innate and humoral activation of microglia are

characterized by the release of pro- and anti-inflammatory cytokines, these types of activation probably do not define microglia stimulated by TNFα+IFNγ.

Classical activation is characterized by IFNγ stimulation in addition to a microbial trigger such as LPS. It causes the up-regulation of membrane MHC II receptors on top of provoking the release of pro-inflammatory mediators such as IL-1β and NO. In this thesis, I used a physiological stimulus, TNFα, instead of LPS, and described a similar inflammatory profile: high MHC II, IL-1β, and NO, and low IL-10 and MR1 (Fig. 17). Classical activation has been shown to cause tissue damage in different models (Bianca et al., 1999; Melton et al., 2003). Hence, this model may be appropriate to study signalling pathways activated in microglia that cause detrimental effects during different types of inflammation.

Figure 17:

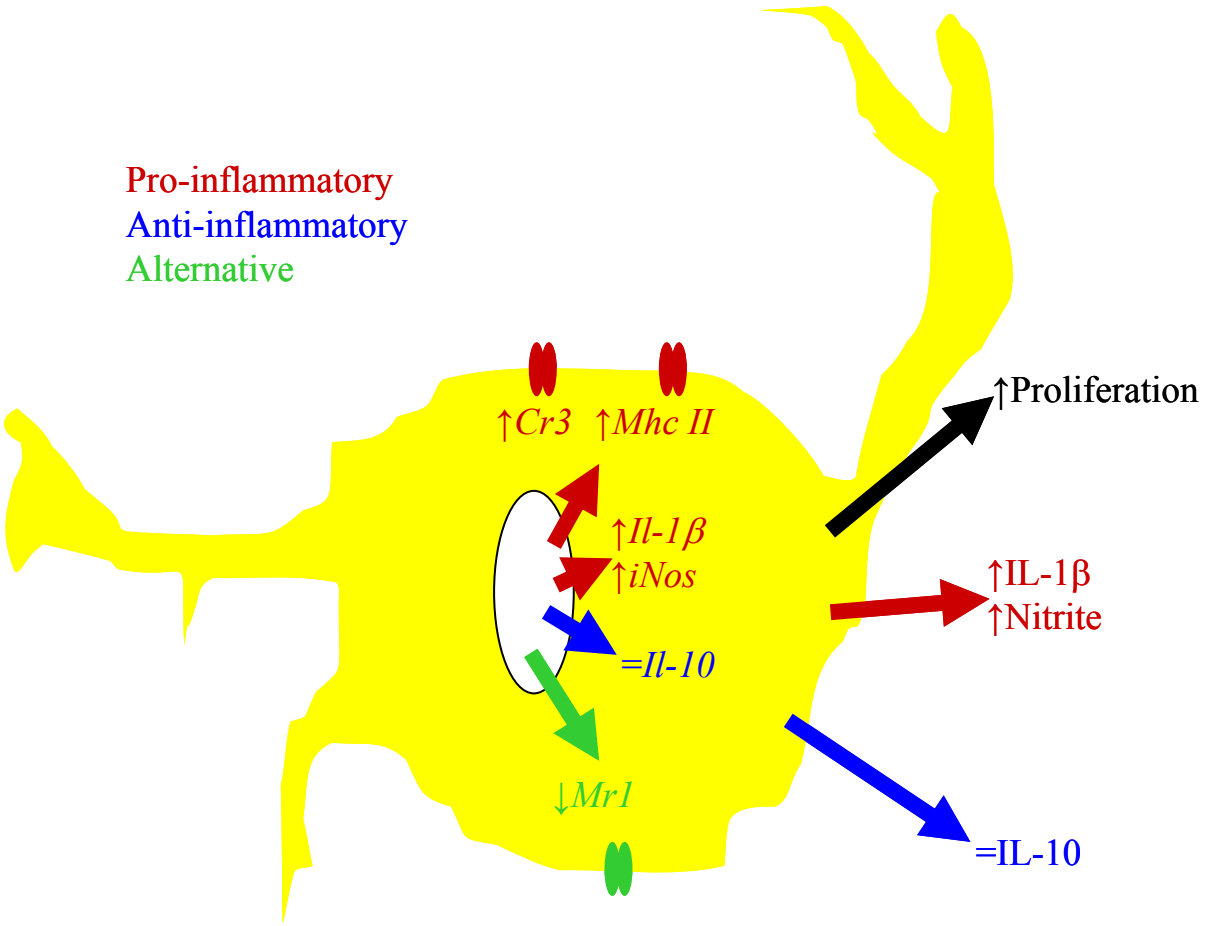

Figure 17: Responses of microglia stimulated by TNFα+IFNγ. Microglia stimulated with 50 ng/mL TNFα and 20 ng/mL IFNγ up-regulated their transcription of *Cr3*, *Mhc II*, *Il-1β*, and *iNos* and down-regulated their transcription of *Mr1* as measured using real-time qRT-PCR. They also increased their production of IL-1β (measured by ELISA) and their release of nitrite (measured using the Griess reagent assay). Finally, an increase in proliferation measured using propidium iodide and alamar blue was measured.

4.4. *pCREB is changed in microglia in vivo and in vitro*

Multiple different transcription factors have been studied in microglia in acute models of brain injury (Lopez-Neblina and Toledo-Pereyra, 2006; Planas et al., 2006; Zheng and Yenari, 2004). CREB is a transcription factor, which, albeit studied in neurons following injury, has never been

looked at in microglia *in vivo*. Therefore, I chose to examine the phosphorylation of CREB both *in vivo* and *in vitro*, in the classical activation model I defined. *In vivo*, after ICH, many cells with bright pCREB staining were observed. However, most of these cells were neurons. In microglia on the contralateral side, pCREB staining was very low. In contrast, on the ipsilateral side, microglia were observed with a wide range of pCREB intensity. This is the first report of changes in pCREB in microglia *in vivo*, after ICH. This is also the first report of different populations of microglia *in vivo*.

I then examined whether a similar a change in CREB phosphorylation could be identified in the model of TNFα+IFNγ stimulation. Since *in vivo*, pCREB staining in microglia was highly varied, I divided the cells into three different groups for my analysis *in vitro*: low pCREB (pCREB↓), average pCREB (pCREB~), high pCREB (pCREB↑). These intensities were based on the percent change from naïve cells, in which there was no change in solution. It should be noted that these cells did have visualized pCREB, which further supports the notion that *in vitro* cells are slightly activated even without stimulation. Following stimulation, I found that at 60 and 120 min, TNFα+IFNγ significantly decreased the number of cells with pCREB~. This suggests populations of microglia that can be differentiated based on pCREB intensity. Interestingly, this was also the result *in vivo*, which further supports the utility of the TNFα+IFNγ stimulation model as a model of microglia in acute brain injury.

Different populations of microglia were also observed when we stained for IL-10: a population of cells had high CD 63 and high IL-10 immunoreactivity and a population had low CD 63 and low IL-10 immunoreactivity. Since increases in pCREB have been linked to increases in IL-10 transcription, I wondered whether higher pCREB was also localized to cells with strong CD 63 staining (Platzer et al., 1999). However, no such correlation was observed from these images.

Nonetheless, it is possible that initial differences in the activation state of the cultured microglia lead to different responses following stimulation. This is supported by the fact that throughout this thesis, I measured large differences between different litters. Therefore, it would be valuable to better establish the basal activation of cells prior to beginning experimentation.

The decrease in pCREB~ cells provides evidence for the differentiation of microglia into separate populations after stimulation. It is possible that this is due to the activation of divergent signalling pathways associated to TNFα *vs.* IFNγ. Additionally, the timing involved in activation of these pathways might be especially relevant. Indeed, TNFα has been shown to induce phosphorylation of CREB in endothelial cells as early as 10 min after application and this induction is maintained for at least 30 min (Gustin et al., 2004). IFNγ has been shown to induce pCREB at 60 min (Mead et al., 2003). Therefore, the decrease in the number of pCREB~ cells at 60 min following TNFα+IFNγ stimulation was likely due to a combination of both stimuli acting together to increase pCREB. The decrease in the number of pCREB~ cells at 120 min may be due to cells entering a cycle of CREB activation and inhibition (described below).

Following IFNγ-induced pCREB, there is also an increase in the detection of the inducible cAMP early repressor (ICER), which begins at 1 h and peaks at 4 h after stimulation. It is well known that ICER is an endogenous competitive antagonist of CREB; it can bind to CREB and form heterodimers or bind directly to CRE sequences (Mayr and Montminy, 2001; Mioduszewska et al., 2003). In addition, the gene for ICER has a CRE sequence in its promoter and therefore is elevated by CREB-mediated transcription (Mioduszewska et al., 2003). ICER can also bind to its own promoter to repress its own activation (Molina et al., 1993). This allows CREB and ICER to carefully regulate transcription of genes containing CRE sequences via

negative feed-back loops. Hence, I can speculate that the decrease observed in the number of pCREB~ cells is due to cells entering a cycle of CREB phosphorylation and then repression by ICER at different times. Even though ICER forming heterodimers with CREB has not been shown to dephosphorylate it, this has been proposed (Bodor et al., 2000). It is equally possible that this binding causes the phosphorylation site to be masked from antibody binding during immunocytochemistry. In sum, I think the decrease in number of pCREB~ cells after stimulation with TNFα+IFNγ is due to some cells entering stages of pCREB↑ and pCREB↓ at different times after stimulation. This may also be the case *in vivo*, after ICH.

5. Conclusions and Future Directions

In this thesis, I stimulated primary microglia with two cytokines: TNFα and IFNγ and characterized the response, which I found to be consistent with a model of classical activation. Furthermore, I showed that both TNFα and IFNγ are relevant in a model of acute brain injury. Similarly to all *in vitro* models, our results are limited in their representation of the full spectrum of inflammatory events that occur *in vivo*. However, this *in vitro* model was representative of changes in CREB phosphorylation *in vivo*.

Another advantage of this model was that physiological stimulators were used to activate microglia. In contrast, most other studies stimulate microglia use LPS, which induces a different inflammatory profile than physiological stimuli (Kaushal et al., 2007; Kaushal and Schlichter, 2008). On the other hand, the present model of classical activation is simple, specific, and may be more relevant to study microglia-mediated events following activation of well-defined cytokine-activated intracellular pathways. The reproducibility of *in vivo* events in this model was demonstrated by the similar different microglia pCREB intensity populations following TNFα+IFNγ stimulation in culture and three days after ICH. In Figure 18, I represent some of the possible interactions between the molecules I examined.

Figure 18

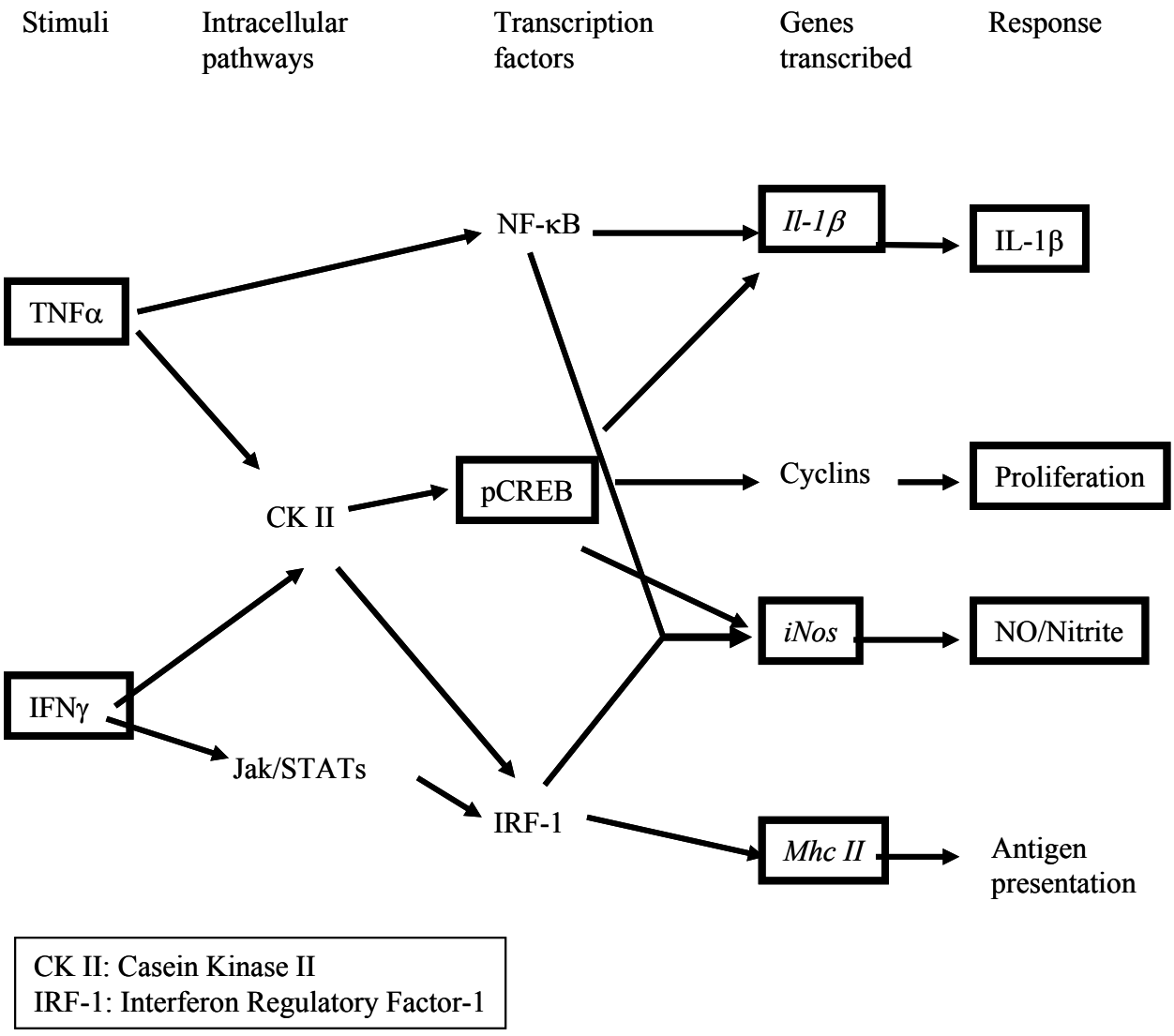

Figure 18: Model of possible interactions between molecules examined. TNFα may cause IL-1β production alone by activating NF-κB although IL-1β could also be elevated by TNFα and IFNγ acting on CKII, which could increase CREB phosphorylation and *Il-1β* transcription. Through this same mechanisms, *iNos* and cyclin transcription could be increased, which would lead to increased NO production and proliferation, respectfully. Finally, IRF-1 activated by Jak/STATs could increase *MhcII*, which may lead to more efficient antigen presentation abilities.

Another interesting conclusion from these results was that, although microglia may produce high amounts of anti-inflammatory cytokines, their release might be tightly regulated by the concentrations of different pro-inflammatory mediators. For example, I reported for the first time

is that, even though *in vitro* microglia release very little IL-10 basally, they contain large amounts of it intracellularly. Unstimulated microglia *in vivo* do not contain intracellular IL-10 (unpublished results). Furthermore, following collagenase-induced ICH in rats, *Il-10* transcription is unchanged on the ipsilateral side during the early stages of the injury. However, *Il-10* transcription increases significantly at seven days (Wasserman and Schlichter, 2007a). In microglia *in vitro*, I observed high amounts of intracellular IL-10, which further supports the notion that isolated primary microglia are basally activated in culture. That TNFα+IFNγ-stimulation did not induce any change in these levels is similar to the lack of transcription in the early stages of ICH. It is possible that in both the *in vitro* cytokine-stimulation model and the *in vivo* collagenase-induced ICH model, a stimulus signals the switch from a pro- to anti-inflammatory microglia phenotype. Based on my results and past studies, low levels of ATP and TNFα, and the change from necrotic death to apoptotic death may be sufficient to induce an anti-inflammatory phenotype (De Simone et al., 2003). The exact stimulus or combination of stimuli that cause this switch in phenotype needs future study.

Additionally, this is the first report that the combination of TNFα and BzATP completely abolished the TNFα-mediated enhancement of IL-1β release. I proposed that this could be due to a self-propagating increase in TNFα, which causes MMP-9 to be released and, possibly, to degrade extracellular IL-1β. This may be of particular relevance *in vivo* because following neuron death, TNFα and ATP are both released, which suggests that the reductive nature of many *in vitro* studies ignores the synergistic effect of many modulators *in vivo*. Further studies should measure MMP-9 levels or block MMP-9 activity to check whether the measured IL-1β release increases.

Finally, I observed a change in the distribution of microglia populations dependent on pCREB intensities both *in vitro* and *in vivo*. To my knowledge, this is the first study to show different microglia populations defined by different levels of a transcription factor. Whether there are differences in the roles of each population following inflammation remains to be elucidated. The TNFα+IFNγ *in vitro* model described in this thesis will be useful for describing the changes occurring in microglia following brain injury *in vivo*.

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