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Mechanisms of neural precursor cell apoptosis by microglia-derived cytokines

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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MECHANISMS OF NEURAL PRECURSOR CELL APOPTOSIS INDUCED BY MICROGLIA-DERIVED CYTOKINES

by

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctorate of Philosophy

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Abstract

The persistence of neural precursor cells (NPCs) in distinct niches of the adult brain and spinal cord provides an important opportunity for regeneration in the affected nervous system. In the adult brain, neural precursor cells (NPCs) generate new neurons that can be integrated into the CNS circuitry to replace damaged or lost neurons, and contribute to learning and memory processes. Deregulated neurogenesis has been observed under both acute and chronic neurological conditions including stroke, Alzheimer's disease, and Parkinson's disease. The extent to which neurogenesis contributes to brain repair is severely limited by the neuroinflammatory processes associated with these neurodegenerative conditions. During injury, microglia, the CNS resident immune cells become activated and produce a number of anti- and pro-inflammatory factors that can modulate neurogenesis and survival of neural precursor cells. The goal of this study was to identify mechanisms of neural precursor cell apoptosis induced by microglia-derived cytokines. Using a conditioned media model, we have identified that activation of the TNFα, IL-1β and Fas signaling pathways induces death of neural precursor cells via the intrinsic pathway of apoptosis *in vitro*. Activation of TNFR1 by TNFα activates Puma and NPC apoptosis via an NF-κB-dependent mechanism. Activation of the IL-1β pathway, by microglia-derived or exogenous IL-1β induces cell cycle arrest and apoptosis via p53-dependent upregulation of p21 and Puma, respectively. IL-1β can also induce an increased expression of the death receptor Fas via an NF-κB-dependent pathway. Fas signaling in NPCs also culminates in activation of Puma and induction of mitochondrial-dependent apoptosis of NPCs. The BH3-only protein Puma appears to be a dominant regulator of cytokine-induced neural precursor cell apoptosis *in vitro*, as well as in an *in vivo* model of spinal cord injury. This study implicates microglia-derived TNFα and IL-1β as potent inducers of the BH3-only protein Puma through activation of the NF-κB and p53 pathways, respectively. Furthermore, these findings provide novel molecular targets to improve the survival of both endogenous and transplanted NPCs in regenerative therapies for acute and chronic neurological conditions.

Keywords

Neural precursor cells, microglia, neuroinflammation, apoptosis, pro-inflammatory cytokines, Bcl-2 family, Puma

Co-Authorship Statement (where applicable)

Chapter 2 is a published body of work. Mouse spinal cord injury, and immunohistochemistry of spinal cord sections was performed by Dr. X Xu. Immunoblotting for cleaved caspase-3 in chapter 3 was performed by P. Swan. Neurosphere diameter measurements and cell counts were performed by Dr. Rasha Shaikh. All other experimental work presented here was performed by J. Guadagno.

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γIFN	Gamma-interferon
AC-DEVD-AFC	N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl coumarin
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
aMCM	Activated microglia conditioned media
AP-1	Activator protein-1
APAF-1	Apoptosis protease activating factor -1
ATP	Adenosine tri-phosphate
Aβ	Amyloid-β
BAK	Bcl-1 homologous antagonist killer
BAX	Bcl-2-associated X-protein
BBB	Blood brain barrier
BCL-2	B-cell lymphoma 2
BCL-XL	Bcl-2 extra long
BDNF	Brain derived neurotrophic factor
BH3	Bcl-2 homology domain-3

bHLH	Basic helix loop helix
BID	BH3 interacting-death death agonist
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
cFLIPL	Caspase-8 like inhibitor protein
cIAP	Cellular inhibitor of apoptosis protein-1
CNS	Central nervous system
CRD	Cysteine rich domain
CXCL10	c-x-c motif chemokine 10
CXCR3	c-x-c motif chemokine receptor 3
DAMP	Damage associated molecular pattern
Dcx	Doublecortin
DD	Death domain
DED	Death effector domain
DG	Dentate gyrus
DISC	Death inducing signaling complex
DMEM-F12	Dulbecco’s modified eagles medium F-12
DNA	Deoxyribonucleic acid
DR4/5	Death receptor-4/5
EdU	5-ethynyl-2’-deoxyuridine

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinases
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
ICE	Interleukin converting enzyme
IKK	Inhibitor of kappa b kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1Ra	Interleukin-1 Receptor antagonist
IL-1RAcP	Interleukin-1 receptor associated protein
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IRAK	Interleukin-1 receptor-activated protein kinase
IκBα	Inhibitor of kappa b alpha
IκBβ	Inhibitor of kappa b beta

IκBε	Inhibitor of kappa b epsilon
JNK	c-Jun-N-terminal kinase
K1-15-EGFP	Keratin complex-1 enhanced green fluorescent protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCM	Microglia conditioned media
MCP-1	Monocyte chemoattractant protein-1
MDM2	Mouse double minute 2 homolog
mFasL	Membrane bound Fas ligand
MHC	Major histocompatibility complex
MMP	Matrix mettaloproteinase
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTNFα	Membrane bound tumour necrosis factor alpha
MYD88	Myeloid differentiation primary response gene 88
NEMO	Nuclear factor b essential modulator
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NIK	NF-κB inducing kinase

NPC	Neural precursor cell
NSAID	Non-steroidal anti-inflammatory drug
NSC	Neural stem cell
NT-3/4	Neurotrophin-3/4
P53	Tumour protein 53
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PSA-NCAM	Polysialylated neural cell adhesion molecule
PUMA	P53 upregulated modulator of apoptosis
RIP1	Receptor interacting protein-1
RIP3K	Receptor interacting protein-3 kinase
rTNFα	Recombinant tumour necrosis factor alpha
RT-PCR	Reverse transcriptase polymerase chain reaction
SCI	Spinal cord injury
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
sFasL	Soluble Fas ligand
SGZ	Subgranular zone
Shh	Sonic hedgehog
SOD-1	Superoxide dismutase

SVZ	Subventricular zone
TACE	TNFα converting enzyme
TBS-T	Tris buffered saline with tween
TGFβ	Transforming growth factor beta
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNFR	Tumour necrosis factor receptor
TNFα	Tumour necrosis factor alpha
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
TRAILR1/2	TNF related apoptosis inducing ligand receptor 1/2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
uMCM	Unconditioned microglia conditioned media
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein
Y-VAD-CMK	N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone
Z-VAD-FMK	Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone

Chapter 1

1 Introduction

1.1 Brief introduction to neurogenesis

The mature nervous system consists of three major differentiated cell types; neurons, astrocytes, and oligodendrocytes (glial cells) which arise from neural stem cells during development. Neural stem/progenitor cells arise from a population of neuroepithelial progenitor cells, very early in the development of the CNS. As development progresses, neural stem cell pools begin to undergo asymmetric division cycles, giving rise to progenitor cells with a restricted lineage. From this division, neural and glial progenitors are formed, which subsequently give rise to neurons, and glial cells (astrocytes and oligodendrocytes), respectively (Louis & Weiss, 2013; Kempermann et al., 2004).

In the field of neural stem cell research, cells are often referred to by three different names; neural progenitor cells, neural stem cells, and neural precursor cells. Neural progenitor cells are defined as an undifferentiated cell that has the capacity to proliferate and differentiate into more than one neural cell type, however unlike a stem cell, it does not exhibit self-renewal. A neural stem cell (NSC) possesses the defining characteristics of a stem cell; the ability to self-renew while maintaining an undifferentiated state, and potency; the capacity to differentiate into specialized cell types. Neural precursor cells (NPCs) contain a mixed population of cells that includes both neural stem cells, and neural progenitor cells (Louis & Weiss, 2013).

The transition of proliferative and multipotent NSCs to fully differentiated neurons and glia is called neurogenesis and gliogenesis, respectively. Neurons are generated from early embryonic development until early postnatal stages, with only a few neurogenic zones remaining active in the adult. In contrast, gliogenesis starts during late embryogenesis and continues in postnatal stages, with low but widespread production of both astrocytes and oligodendrocytes also occurring throughout the adult brain.

All post-natally derived neurons arise from GFAP-positive neural stem cells, which originate in the subependymal zone. Adult NSCs have longer cell cycle times, and have been shown to become more quiescent with age. In contrast to adult NSCs, embryonic NSCs exhibit greater proliferation, which allows for the generation of CNS cells throughout development. During development, a wave of neurogenesis preceeds gliogenesis, a cell-intrinsic property which allows for the development of each of the main CNS cell types (Kornblum et al., 2007).

1.1.1 Neurogenesis in the adult brain

It was originally thought that following the later stages of CNS development, neurogenesis was complete, and new brain cells could not be formed through mitotic division. However, in 1965, Altman and Das demonstrated the presence of newly generated neurons using [^{3}H]-thymidine labeling experiments (Altman & Das, 1965). It was not until 1992 when Reynolds and Weiss determined *in vitro* that a rare population of cells exhibited the characteristics of stem cells; the ability to self-renew, and form cells of multiple lineages (Reynolds & Weiss, 1992). Neural stem cells in the adult brain were found to be centralized into two main regions; the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles. In the hippocampal dentate gyrus, Type I neural stem cells represent quiescent NSCs expressing the markers glial-fibrillary, acidic protein (GFAP), Sox2, and Nestin. These cells generate type 2 NSCs that can self-amplify and express Sox2 and Nestin, which can then give rise to doublecortin (Dcx)-positive neuroblasts which differentiate into dentate granule cells (Yao et al., 2012; Faigle & Song, 2012). In the SVZ, there are three types of NSCs; termed A, B and C. Type B cells are quiescent NSCs, and correspond with type 1 cells of the SGZ, and type C cells express Sox2 and Nestin, similar to type 2 cells of the SGZ. In the SVZ there is also a large population of neuroblasts that express the markers Dcx and PSA-NCAM that only give rise to neurons, known as type A cells. These cells can proliferate through symmetric division, or undergo asymmetric division to produce neural progenitor cells which migrate through the rostral migratory stream to the olfactory bulb (Yao et al., 2012; Faigle & Song, 2012).

1.1.2 Factors regulating neurogenesis and NSC fate

Many extrinsic factors in the neurogenic niche act to regulate the lineage fate of NSCs. Astroglia play a role in the structural part of the niche, but also act to regulate self-renewal, migration, differentiation, fate specification and integration of new neurons into the CNS microenvironment (Mathieu et al., 2010). Astrocyte-derived Wnt-signaling mediates neuroblast proliferation and neuronal differentiation of adult hippocampal progenitors (Lie et al., 2005). NeuroD1 is a pro-neurogenic basic helix-loop-helix (bHLH) transcription factor that functions downstream of Wnt-signaling to mediate neurogenesis in the adult hippocampus by promoting the survival and maturation of newly born neurons (Gao et al., 2009). Other extracellular factors such as sonic hedgehog (Shh), and bone morphogenic proteins (BMPs) can also mediate NSC processes. Shh functions to favour neurogenesis by promoting self-renewal and proliferation of adult NSCs (Han et al., 2008). In contrast, BMPs act inhibit neuronal fate specification, favouring glial differentiation in the adult neurogenic niche (Lim et al., 2000). Endogenously produced Noggin, can act as an inhibitor to BMPs, to promote the formation of new neurons in the SVZ, and similar to Shh also promotes self-renewal and proliferation of NSCs in the hippocampus (Bonaguidi et al., 2008). Transcription factors such as members of the Sox family of transcription factors can have effects on neurogenesis; specifically Sox2 which is required for neuronal maturation, formation of dendrites, and differentiation of GABAergic neurons in the olfactory bulb (Cavallaro et al., 2008). Furthermore, growth factors and neurotrophic factors are also important in the maintenance and survival of the NSC pool, among these fibroblast growth factors (FGFs) and brain-derived neurotrophic factor (BDNF) have been found to contribute to proliferation and development of new neurons in the CNS (Yao et al., 2012; Mathieu et al., 2010).

1.1.3 Neurogenesis under pathological conditions

In the adult brain. Neural stem cells exist in two areas; the subventricular zone of the lateral ventricle (SVZ) and the subgranular zone of the dentate gyrus (DG) in the hippocampus.

NSCs from the SVZ migrate through the rostral migratory stream to the olfactory bulb, where neurogenesis occurs, whereas NSCs in the DG undergo neurogenesis in the DG of the hippocampus (Zhao et al., 2008; Gage, 2002). While under normal conditions, neurogenesis occurs exclusively in these two brain areas, following injury or disease, neurogenesis can be observed in non-neurogenic areas. Importantly, studies in animal models have demonstrated that progenitor cells in the SVZ can migrate to regions of neuronal damage or disease to potentially compensate for the loss of healthy cells and contribute to regenerative processes (Arvidsson et al., 2002; Yao et al., 2012). The ability of progenitor cells to migrate is evidenced by the presence of proliferating progenitor cells in the area between the site of damaged tissue and healthy brain regions following acute CNS injury such as spinal cord injury and stroke (Carmichael et al., 2003; Zhang et al., 2004; Martino & Pluchino, 2006). Similarly, in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, progenitor cells of the SVZ migrate along the rostral migratory stream to the spinal cord and into areas of damage, where they differentiate into glial cells, replacing lost myelin (Picard-Riera et al., 2002; Martino & Pluchino, 2006).

1.1.4 Limitations to neurogenesis

The ability of NPCs to proliferate, migrate and differentiate into cells of the CNS, has lead to a plethora of research devoted to the development of NPC-based therapies in animal models of nervous system disorders including stroke, Parkinson's disease, Huntington's disease, multiple sclerosis and spinal cord injury (Chen et al., 2014; Hermann et al., 2014; Mine et al., 2013; Svendsen et al., 1997; Mu & Gage, 2011). However the potential benefits of these therapies are confounded by factors that limit the repair capabilities and functional integration of NPCs. One such factor limiting the regenerative capacity of NPCs is the harsh inflammatory environment of the CNS during disease or following injury. The effects of inflammation on NPCs is not always negative. Inflammation can either promote or limit neurogenesis, and this likely depends on the stimulus for activation of microglia, and the duration of inflammation. Microglia activated with IL-4 or low levels of IFN-γ induced both neurogenesis and oligodendrogenesis of adult mouse neural progenitor cells

(Butovsky et al., 2005). In contrast, LPS-activated microglia can affect the proliferation and survival of NPCs through the release of soluble factors including pro-inflammatory cytokines, chemokines, and ROS (Monje et al., 2003; Cacci et al., 2008; Russo et al., 2011). In a mouse model of status epilepticus, the increased neurogenesis observed following injury is attenuated by microglia activation and inflammation (Ekdahl et al., 2003).

1.2 Neuroinflammation

1.2.1 General characteristics of neuroinflammation

Inflammation is a complex biological response that occurs in the body as a result of physiological or pathological conditions. Inflammation in the broad sense can occur systemically, or can be focused in a particular area such as the CNS, this being termed neuroinflammation. The CNS has often been considered an "immune-privileged" site as it contains very low levels of systemic immune/inflammatory cell types and products due to its protection by the blood-brain-barrier (BBB). Under normal physiological conditions, the selective permeability of the BBB allows only T cells, dendritic cells, and macrophages to enter the CNS (Graeber et al., 2011; Whitney et al., 2009). The CNS however does contain its own resident immune cell/macrophage termed microglia. Microglia, similar to peripheral macrophages are part of the innate immune system and thus are not antigen-specific, but respond to injury, or pathogens. Microglia are important not only in the pathological CNS but also to the homeostasis of the CNS during development, adulthood, and ageing (Luo & Chen, 2012; Walter & Neumann, 2009). Following damage or exposure to a pathogen, an inflammatory cascade is initiated by the activation of microglia and astrocytes, as well as lymphocytes, which release a variety of pro- and anti-inflammatory factors as well as chemokines, neutrotransmitters and reactive oxygen species. These factors can lead to distruption of the BBB which allows recruitment of peripheral immune cells into the CNS. Recruitment of peripheral immune cells contributes to an inflammatory feedback loop whereby newly recruited cells become activated, releasing more inflammatory factors which results in the further activation of microglia, as well as

neuronal damage and alterations in neurogenesis, in some cases (Hickey, 1999; Whitney et al., 2009).

1.2.2 Microglia: the central regulators of inflammation

1.2.3 Origin of microglia

Murine microglia originate from monocyte/Mφ precursor cells that migrate from the yolk sac into the CNS during embryogenesis. Once in the CNS, precursor cells proliferate and give rise to the microglial cell pool (reviewed in Town et al., 2005). During development and maturation of the CNS, microglia function to phagocytose the apoptotic bodies and cellular debris that results from the synaptic pruning and apoptosis of neurons and glial cells (Perry et al., 1985). During this period, microglia also play a role in promotion of axonal growth and guidance, neural differentiation, cortical precursor cell development and astrocyte proliferation (Rezaie et al., 2003; Aarum et al., 2003; Nakanishi et al., 2007; Czeh et al., 2011).

1.2.4 Microglia in the healthy CNS

In the healthy, mature CNS, microglia exist in a resting (quiescent) state, and are often termed 'surveying microglia' for their role in monitoring the environment of the CNS. Resting microglia are characterized by a small cell body with long, ramified (branched) processes used to monitor the microenvironment. Microglia also display a down-regulated phenotype, exhibiting low expression of the cell surface antigens CD45 and MHC class II (major histocompatibility complex class II), as well as Fc receptors; all of which are important for the innate immune response (Gautier et al., 2012; Perry et al., 2013). In a steady-state environment, neurons and astrocytes in the microenvironment contribute to this down-regulated phenotype through soluble factors such as nerve growth factor (NGF) released by neurons which inhibit MHC class II expression on microglia. Membrane bound factors such as the non-signaling molecule CD200 can interact with their receptors on the surface of microglia and inhibit downstream immune signaling (Perry et al., 2013;

Neumann et al., 1998; and Cardona et al., 2006). Mice lacking neuronal CD200 exhibited increased levels of activated microglia (Chitnis et al., 2007). This interplay between neurons and microglia plays an important role in microglial activation. In cases of neuronal damage or death, inhibitory signals from neurons are lost, thereby releasing microglia from their tonic down-regulated state.

1.2.5 Microglia activation

Changes in the quiescent microglia are activated rapidly by changes in the environment of the CNS. These changes can occur as a result of pathological conditions, traumatic injury, or invading pathogens. Surface molecules on microglia play an important role in maintaining their resting state. Cytokine and chemokine receptors, scavenger receptors, as well as pattern recognition receptors (PRRs) on the microglial cell membrane function to identify and bind ligands secreted by healthy neurons, astrocytes, and other cell types. In conditions of infection or inflammation, these receptors can also recognize pro-inflammatory stimuli, transforming resting microglia into activated microglia (Figure 1.1) (Kierdorf & Prinz, 2013). For example, damaged neurons release ATP (adenosine-5'-triphosphate) which attracts microglia to the site of damage by binding to purinergic receptors (Sanz et al., 2009; Walter & Neumann, 2009). Chemokines such as CXCL10 (chemokine motif ligand 10) also attract microglia via binding to chemokine receptors (CXCR3) on the microglial membrane; thus creating a chemokine gradient that directs migration of microglia (Rappert et al., 2004; reviewed in Czeh et al., 2011).

1.2.6 Activated microglia

Resting microglia have a distinct ramified morphology, however upon activation, microglia transform to an amoeboid phenotype, which allows them to migrate more efficiently to the site of damage (reviewed in Czeh et al., 2011). Proliferation of microglia is also observed following activation, as is the release of anti- and pro-inflammatory cytokines, chemokines, and growth factors (Haenish & Kettenmann, 2007; Graeber et al.,

2010; Czeh et al., 2011). Upon activation, the downregulated phenotype of microglia is also modified, such that immunologically relevant molecules such as CD45 as well as MHCII are upregulated, which is necessary for antigen presentation. As cells of the innate immune system, microglia recognize molecular structures of pathogens through pathogen-associated molecular patterns (PAMPs) through cell surface receptors termed Toll-like receptors (TLRs). The bacterial endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria can induce signaling pathways in microglia via a toll-like receptor (TLR4) expressed on the microglial membrane. Signaling events elicited by LPS activation of TLR4 can lead to the activation of NF-κB/MAPK and production and release of pro-inflammatory cytokines from microglia (Figure 1.1) (Lehnhardt et al., 2003; Lu et al., 2008).

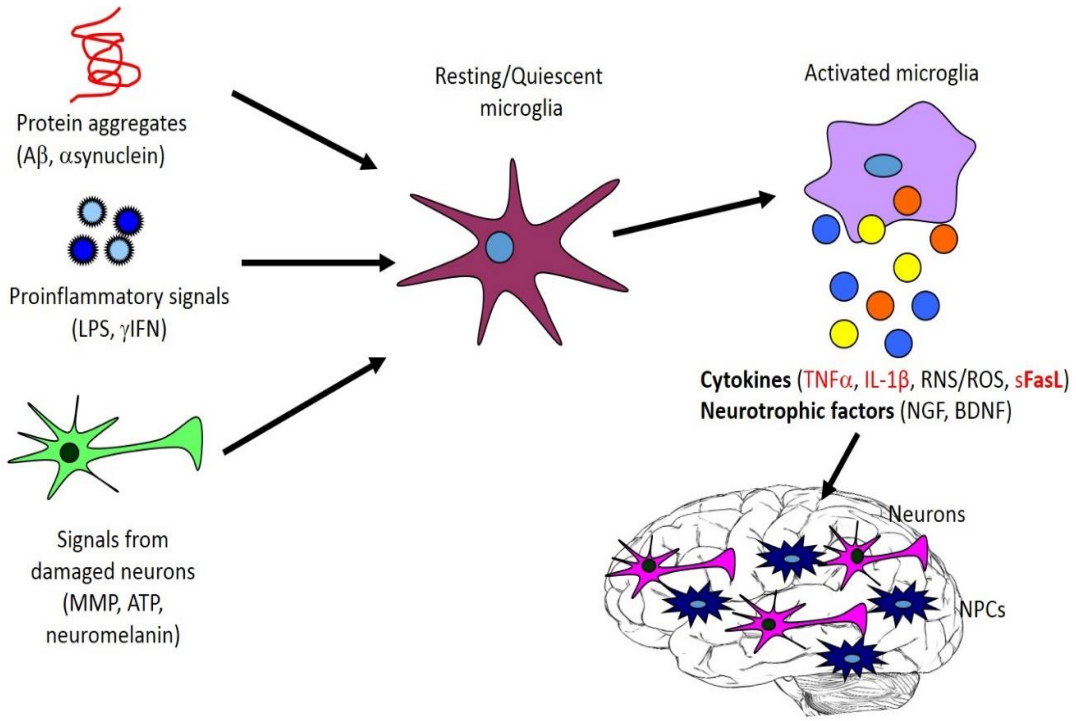

Figure 1.1

Figure 1.1. Microglia activation. Microglia can be activated by various factors including protein aggregates, pro-inflammatory signals, and signals from damaged neurons. Upon activation, microglia are converted from a resting quiescent state to an amoeboid activated

phenotype that can release various cytokines and factors that can have effects on cells in the microenvironment of the CNS.

1.2.7 Microglia polarization

Microglia, similar to macrophages of the peripheral immune system, can have two different activation states, which dictate their function in the inflammatory response (Figure 1.2). Classically activated microglia, or M1 polarized microglia, exacerbate inflammation, whereas alternatively activated, or M2 microglia function to alleviate inflammation. M1 polarization is activated by the T helper 1 (T_H1) cytokine, interferon-γ, or by exogenous sources such as the bacterial endotoxin lipopolysaccharide (LPS), which acts through toll-like receptor 4 (TLR4). Pro-inflammatory M1 microglia produce high levels of oxidative metabolites (NO and superoxide) and pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 (Czeh et al., 2011; Walter & Neumann, 2009). Pro-inflammatory microglia play an important role in host defence against pathogens or invading tumour cells, via the release of cytokines. However, these cytokines may also contribute to neuronal and glial cell damage or death. The 'alternatively activated', M2 polarized microglia are activated by anti-inflammatory factors such as IL-4, and IL-13. Anti-inflammatory microglia function to dampen the inflammatory response and promote tissue repair/regeneration and angiogenesis through the release of anti-inflammatory cytokines (IL-4, IL-10), and growth factors such as TGFβ (Kierdorf & Prinz, 2013) (Figure 1.2).

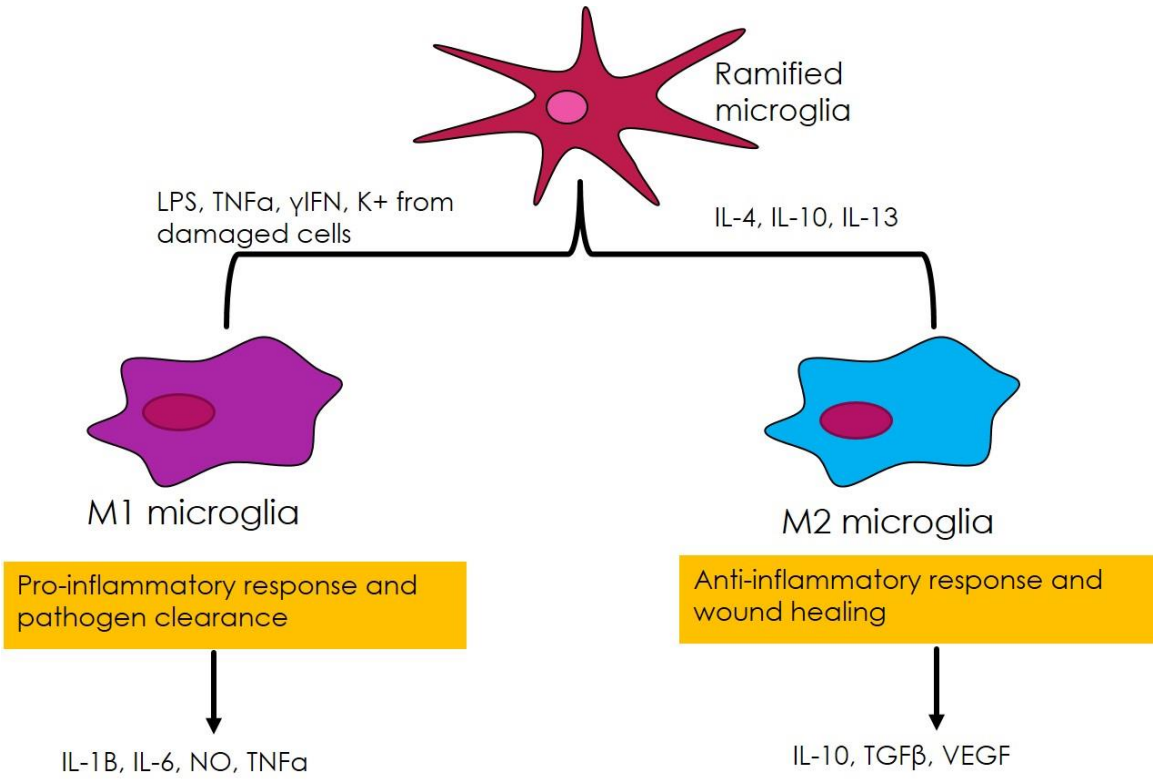

Figure 1.0.1

Figure 1.2. Microglia polarization. Depending on the stimulus of activation, microglia can be polarized to an M1 or M2 state. Pro-inflammatory factors such as LPS, can polarize microglia to an M1 phenotype. M1 polarized microglia promote a pro-inflammatory response through the release of pro-inflammatory cytokines. Signals such as IL-4, and IL-10 can polarize microglia to an M2 phenotype, which promotes an anti-inflammatory response and wound healing through the release of anti-inflammatory cytokines, and trophic factors.

1.2.8 Pro-inflammatory cytokines

1.2.8.1 Tumour necrosis factor-α (TNFα)

Tumour necrosis factor (TNF) was first discovered following the regression of tumours after treatment with the bacterial endotoxin LPS, which caused a hemorrhagic regression of tumours. Scientists later discovered that these effects were due to a factor in the serum that was named tumour necrosis factor (TNF) (Aggarwal, 2003). The TNF family is the largest characterized family of cytokines to date. Members of the TNF superfamily exert their biological effects through the interaction with transmembrane receptors of the TNFR superfamily. TNF family members are characterized by the presence of an extracellular cysteine rich domain, which is responsible for binding of specific ligands. The intracellular domain of TNF superfamily receptors separates them into two distinct groups; a group of death receptors, named for the presence of an intracellular death-inducing domain, and a second group of receptors which lack this death domain. Receptors of the TNF superfamily require the recruitment of adaptor proteins in order to activate intracellular pathways following ligand binding (Aggarwal, 2003; Baud & Karin, 2001). Adaptor proteins, similar to the receptors themselves are also divided into two groups, based on the presence or absence of a death domain. The first group of adaptor proteins include TRADD and FADD, which contain a death domain and are implicated in death receptor signaling. The second group of adaptor proteins lack a death domain, and interact with receptors directly through a TIM domain, or indirectly with other adaptor proteins. Members of this second group include the TNF-receptor associated factor (TRAF) proteins (Cabal-Hierro & Lazo, 2012). The binding of different adaptor proteins to TNFRs suggests that different signaling pathways may be activated by ligands leading to the activation of transcription factors such as NF-κB and AP-1, or cell death through apoptosis or necroptosis, or alternatively lead to cell survival.

Tumour necrosis factor α (TNFα) is a potent pro-inflammatory cytokine produced by many cell types including macrophages, microglia, monocytes, lymphocytes, and fibroblasts in response to infection, inflammation or injury. TNF is synthesized as a 26kDa transmembrane type II protein (mTNF) consisting of 233 amino acids, the first 76 acting as the signal peptide to direct the protein to the membrane for binding to cell surface receptors. Membrane-bound TNF is processed by the metalloproteinase TACE (TNFα converting enzyme; also known as ADAM17) which cleaves full-length TNF at the Ala66-Val67 amide bond of mTNF leading to the formation of a 17kDa soluble protein, sTNF (Cabal-Hierro & Lazo, 2012; Baud & Karin, 2001). TNF exerts its effects by interacting with two different receptors: TNFR1 and TNFR2. mTNF can bind and activate both TNFR1 and TNFR2, whereas sTNF only efficiently activates TNFR1. TNFR1 is expressed in almost all cell types and is part of the death receptor subfamily of the TNFR superfamily, as it contains an intracellular death domain. In contrast, TNFR2 is expressed only in oligodendrocytes, astrocytes, T cells, myocytes, and endothelial cells, and does not possess an intracellular death domain. Binding of TNF to TNFR1 and TNFR2 induces receptor trimerization and recruitment of adaptor proteins and signaling molecules to the cytosolic domain of the receptor. In the case of TNFR2, activation of the receptor results in direct recruitment of TRAF2 (TNF-receptor-associated factor-2) which then recruits TRAF1. TRAF2 is a common adaptor molecule between the two receptor signaling pathways and can lead to activation of IKK (NF-κB), and MAPK (JNK and p38) signaling (Baud & Karin, 2001; Liu et al., 1996).

Signaling through TNFR1 reflects a more complex regulatory network that can lead to induction of proliferation, apoptosis, or necroptosis depending on the cellular context and conditions of the microenvironment (Vanlangenakker et al., 2011). Binding of TNF to TNFR1 involves the formation of two distinct TNF receptor complexes separated both temporally and spatially (Figure 1.3). Complex I controls the expression of transcription of various genes, whereas Complex II (also known as the DISC) triggers cell death pathways. Following TNF association with TNFR1, the adaptor protein TRADD (TNF receptor-associated death domain) is recruited to the receptor and binds TNFR1 through its death domain. TRADD in turn functions as a platform for the recruitment of additional signaling molecules; receptor-interacting protein-1 (RIP1), TRAF2, cIAP1, and cIAP2, which

together comprise Complex I. Interaction with TNFR1, leads to ubiquitination of RIP1, which is an essential event allowing for downstream activation of the IKK complex. Formation of this complex is necessary for phosphorylation of IκBα, the NF-κB inhibitor protein which must be degraded via the ubiquitin-proteasome to allow NF-κB to translocate to the nucleus and initiate transcription (Figure 1.3) (Cabal-Hierro & Lazo, 2012).

Binding of TNF to TNFR1 can also induce internalization of the receptor and formation of Complex II. In this scenario, RIP1 can be deubiquitinated and recruited with RIP3 kinase (RIP3K) to form a new molecular complex with TRADD, FADD (Fas-associated death domain) and procaspase-8. This complex (Complex II) is also known as the death-inducing signaling complex (DISC). Procaspase-8 can inactivate both RIP1 and RIP3, leading to the induction of other caspases and triggering apoptosis. However, when caspase-8 is deleted or inhibited, the DISC is unable to trigger apoptosis, and this may lead to activation of necroptosis in certain cell types (Figure 1.3).

TNFα can trigger apoptosis through more than one pathway. The predominant pathway activated by TNFα appears to involve the adaptors TRADD and FADD, leading to activation of caspase-8, downstream activation of caspase-3, and apoptosis. However, FADD-deficient cells treated with TNFα still demonstrate an apoptotic response, suggesting that there is likely another pathway that can contribute to TNFα-induced apoptosis.

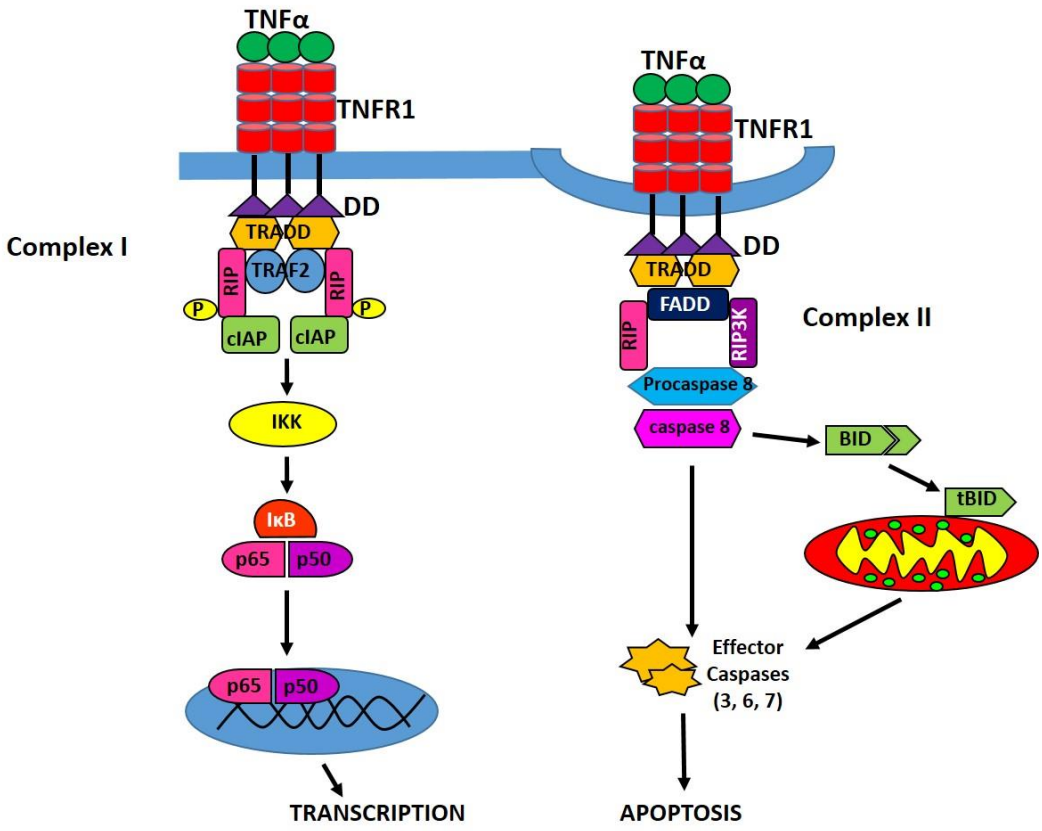

Figure 1.0.2

Figure 1.3. TNFα pathway. TNFα binding to TNFR1 induces the formation of two distinct TNFR complexes. Complex I involves recruitment of signaling molecules to the intracellular death domain. Formation of Complex I leads to downstream activation of the IKK complex, and subsequent degradation of IκBα, allowing NF-κB subunits to translocate to the nucleus and initiate transcription of various genes. Binding of TNFα to TNFR1 can also lead to internalization of the receptor and formation of Complex II. Complex II is also known as the death-inducing signaling complex (DISC) containing caspase-8, which can activate Bid, leading to downstream mitochondrial outer membrane permeabilization and apoptosis by activation of effector caspases.

1.2.8.1.1 Fas signaling

The death receptor Fas (CD95), is a ubiquitously expressed receptor, of particular abundance in the liver, heart, kidney, brain, thymus, and lymphoid tissues. Fas is a prototypical death receptor consisting of three extracellular cysteine rich domains (CRDs) and an intracellular region known as the "death domain" (Kaufmann et al., 2012). Fas is activated by binding of its cognate ligand FasL which is mainly expressed on the plasma membrane of activated immune cells, but can be cleaved by metalloproteinases to generate a soluble form (sFasL). Upon ligation of FasL, Fas receptors multimerize in the cell membrane which leads to a conformational change in the intracellular domain of the receptor. This leads to the recruitment of the adaptor molecule FADD (Fas-associated death domain) which in turn recruits procaspase-8 forming the death-inducing signaling complex (DISC). As previously described, recruitment of procaspase-8 to the DISC results in its auto-activation into an active protease. Fas signaling can lead to activation of caspases and apoptosis in two distinct manners, classifying cells as either Type I or Type II, depending on the mechanism of apoptosis induction. In type I cells, activated caspase-8 directly activates downstream effector caspases such as caspase-3, -6, and -7 resulting in apoptosis. Alternatively, in type II cells, activated caspase-8 cleaves the BH3-only protein Bid to its truncated form (tBid) which can translocate to the mitochondria and through its interactions with other Bcl-2 family proteins, induce release of cytochrome c (Figure 1.4). The other factor that dictates whether cells undergo type I or type II Fas-mediated apoptosis is the level of X chromosome-linked inhibitor of apoptosis protein (XIAP), an inhibitor of caspase-3, -7 and -9. Fas stimulation leads to a reduction of XIAP in type I cells, but an increase in XIAP in type II cells by a mechanism that is still unclear (Kaufmann et al., 2012; Lavrik & Krammer, 2012; Brint et al., 2013) (Figure 1.4). Signaling through Fas has been shown to activate the three main MAPK pathways, p38, JNK1/2, and ERK1/2 as well as the transcription factor NF-κB leading to cell proliferation, migration, and inflammation.

Activation of Fas signaling in a variety of non-lymphoid cells can lead to expression and release of inflammatory factors *in vitro* and *in vivo*. Examples of cytokines and chemokines induced by Fas signaling include IL-6, IL-8, IL-1β, TNFα and MCP-1. Release of

inflammatory factors can lead to the recruitment of additional inflammatory cells, exacerbating the inflammatory response. (Guicciardi & Gores, 2009; Brint et al., 2013). Fas/FasL-mediated inflammation has been implicated in various diseases including arthritis, cancer, multiple sclerosis, and spinal cord injury. Studies in animal models of SCI demonstrate that following SCI, expression of Fas and FasL are increased at the lesion site, and neutralization of FasL reduces apoptotic cell death and improves functional outcome (Demjen et al., 2004).

1.2.8.1.2 Interleukin-1β (IL-1β)

The IL-1 family plays an important role in inflammation, and host defence against pathogens, and more recent evidence also suggests that IL-1 can contribute to a wide range of neurodegenerative conditions such as AD, and stroke (Whitney et al., 2009, Gabay et al., 2010). Interleukin-1 consists of two separate ligands; IL-1β and IL-1α, which are products of two separate genes. Cells of the innate immune system such as monocytes and macrophages (microglia) are the major source of IL-1α and β, however other cell types such as epithelial cells, endothelial cells and fibroblasts can also produce IL-1α and β (Weber et al., 2010). IL-1α is primarily membrane anchored and signals through autocrine or juxtacrine mechanisms, whereas IL-1β is secreted from cells and can act in a paracrine manner or systemically. For the purposes of this thesis, I will focus on IL-1β going forward.

The production and release of IL-1β is induced a variety of stimuli such as Pathogen-associated molecular pattern molecules (PAMPs) and Damage-associated molecular pattern molecules (DAMPs). Invading pathogens such as bacteria, are recognized by PAMPs; whereas signals from damaged cells such as ATP are recognized by DAMPs both of which lead to the activation of toll-like receptors (TLRs) or nod-like receptors (NLRs). Pro-IL-1β is synthesized as a large biologically inactive protein that requires cleavage by caspase-1, also known as Interleukin converting enzyme (ICE), to produce a 17kDa protein which is secreted by a non-classical secretory pathway (Eder et al., 2008; Allan et al., 2005). Caspase-1 is a cysteine protease that exists in cells as a latent zymogen that is

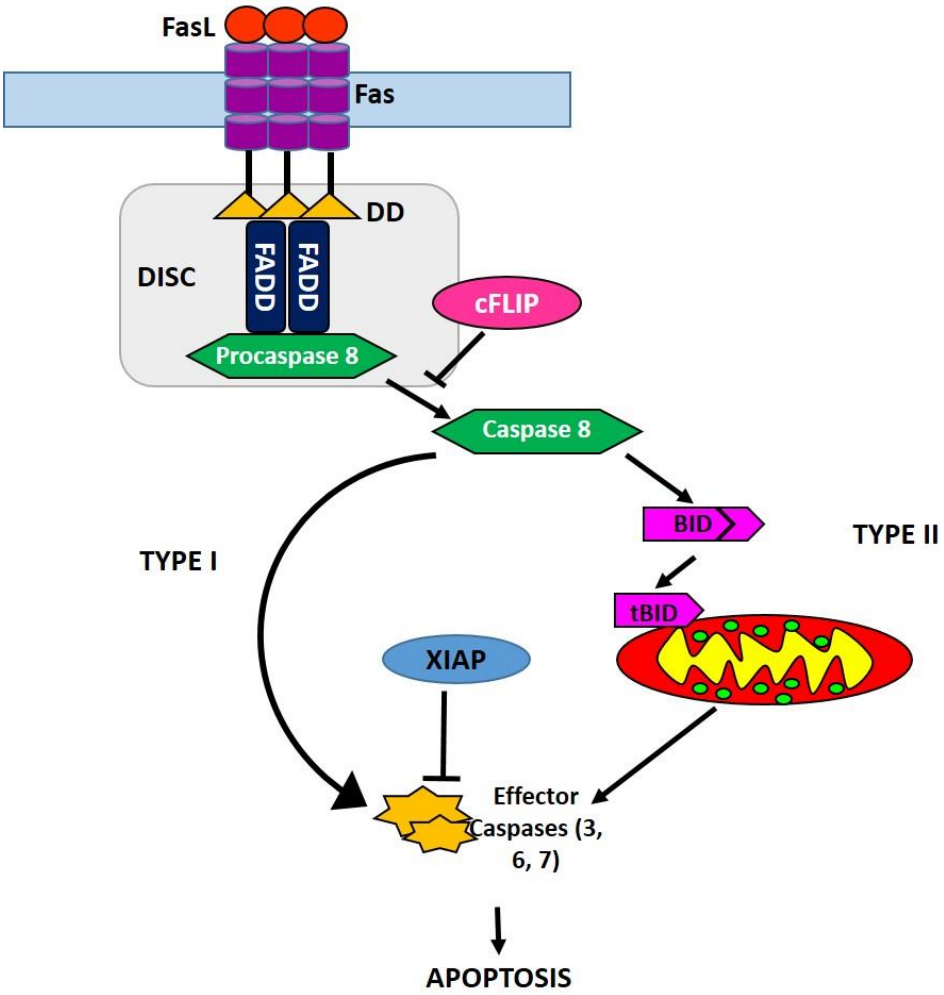

Figure 1.0.3

Figure 1.4. Fas signaling pathway. Activation of Fas by FasL induces homotrimerization of the receptor and recruitment of the adaptor molecule FADD and pro-caspase to form the DISC. Activation of caspase-8 can lead to direct activation of effector caspases (Type I cells); or will lead to cleavage and activation of Bid which will translocate to the mitochondria and lead to mitochondrial outer membrane permeabilization and apoptosis by activation of effector caspases (Type II cells).

activated through a regulated proteolytic cascade. Caspase-1 activation requires the assembly and activation of an inflammasome; a multi-protein complex consisting of a pattern-recognition receptor, an adaptor protein, and caspase-1 (Walsh et al., 2014).

Active IL-1β exerts its effects by binding to the membrane-bound type I IL-1 receptor (IL1-R1). The IL-1 receptor belongs to the family of IL-1 and Toll-like receptor (TLR) superfamily which are characterized by the presence of an intracellular Toll/IL-1 receptor (TIR) domain. Binding of IL-1β to IL-1R1 triggers the association IL-1R1 with the IL-1 receptor accessory protein (IL-1RAcP) which acts as a co-receptor, required for signal transduction. This heterodimeric complex recruits a number of intracellular adaptor molecules including myeloid differentiation factor 88 (MYD88), interleukin-1 receptor-activated protein kinases (IRAK) and TNF receptor-associated factor 6 (TRAF6), resulting in a complex series of signaling events. Activation of IL-1R1 can lead to downstream activation of NF-κB, p38, c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs) and mitogen-activated protein kinases (MAPKs). This leads to the transcription of several inflammation-associated genes that encode cytokines (IL-6 and TNFα), chemokines (CXCL8), and adhesion molecules (E-selectin and ICAM1) (Figure 1.5) (Gabay et al., 2010; Allan et al., 2005; Weber et al., 2010). In addition to IL-1α and IL-1β, the IL-1 family also consists of a third ligand, IL-1-receptor antagonist (IL-1RA). IL-1RA is produced as three intracellular isoforms and one secreted isoform that functions as a competitive antagonist that binds IL-1R1 but does not trigger signal transduction. Mice lacking IL-1RA exhibit excessive inflammatory responses and develop joint and skin inflammation as well as an increased occurrence of auto-inflammatory reactions (Gabay et al., 2010).

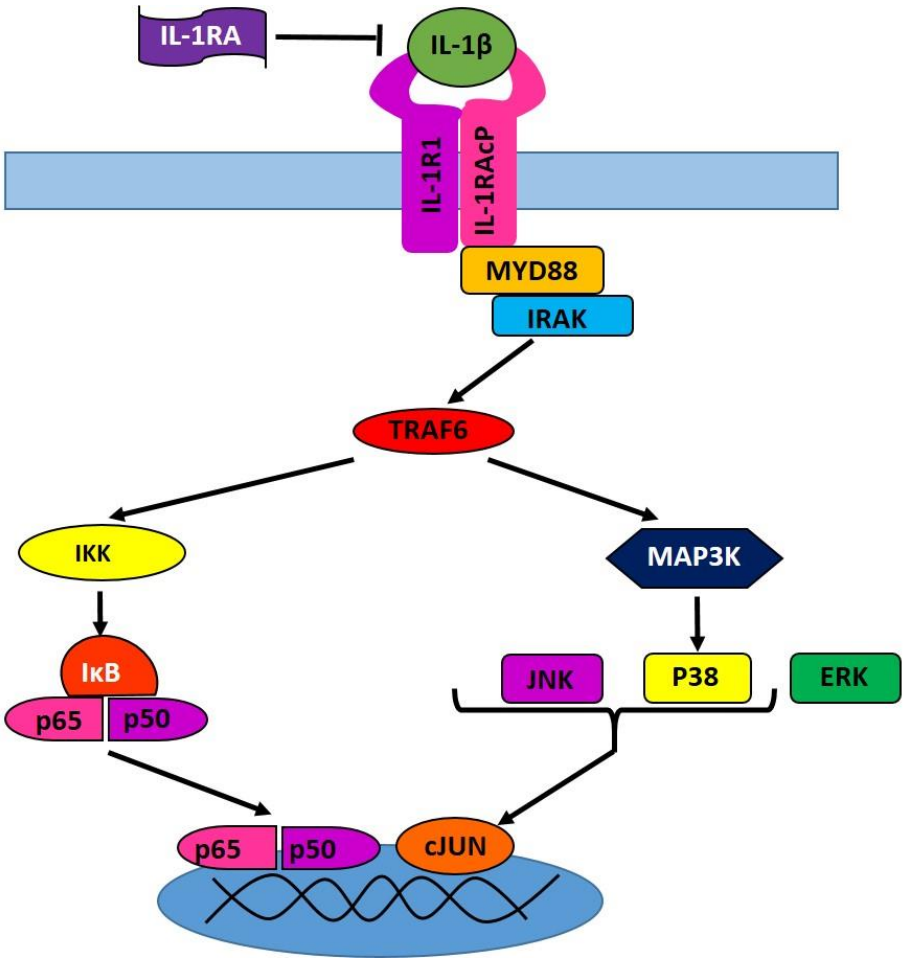

Figure 1.0.4

Figure 1.5. Interleukin-1β signaling pathway. Association of IL-1β with IL-1R1 and the co-receptor IL-1RAcP leads to recruitment of intracellular adaptor proteins, MYD88, IRAK, and TRAF6, resulting in activation of intracellular signaling cascades. Activation of IL-1R1 can lead to downstream activation of NF-κB, p38, and MAPK pathways to activate transcription of many target genes

1.2.9 Beneficial effects of neuroinflammation

The implications of microglial activation and inflammation in the CNS vary in a context and duration-dependent manner. Furthermore, many of the functions of microglia, such as phagocytosis or release of soluble factors can be both beneficial, and detrimental to cells in the CNS. Microglia play an important role in the homeostasis of the CNS based on their ability to survey the environment in search of potentially harmful substances. One of the most important roles of microglia is their ability to act as phagocytes of the brain, entering damaged brain regions to remove toxic by-products, pathogens or debris from damaged cells. Following injury to cells of the CNS, phosphatidylserine (PtdSer) residues on the plasma membrane of apoptotic cells serve as a recognition signal for phagocytic cells such as microglia. Phagocytic removal of debris and damaged cells is an essential function of microglia. (Walter & Neumann, 2005). This function is of particular importance in the removal of myelin following neuronal damage, as removal of myelin limits repair and regeneration by inhibiting oligodendrocyte recruitment and differentiation (Kotter er al., 2006).

Microglia can also have a beneficial role through their release of neurotrophic factors and anti-inflammatory molecules. Studies have shown that transplantation of microglia could help enhance neurite growth and functional recovery after CNS injury. Neurotrophic factors released from microglia include NGF, BDNF, neurotrophin-3 (NT-3), and NT-4 (Elkabes et al., 1996; Parkhurst et al., 2013). Neurotrophic factors have a variety of functions such as mediating neuronal survival, axonal and dendritic growth, synaptic plasticity, and learning and memory (Parkhurst et al., 2013; Kerschensteiner et al., 2009).

Inflammation and microglial-derived factors can also have beneficial effects in neurodegenerative conditions including Alzheimer's disease (AD), or prion disease. Simard et al., (2006) found that microglia in a transgenic model of Alzheimer's disease could reduce neurotoxicity of Amyloid-β plaques. Also in relation to AD, microglia promote protection via the secretion of proteolytic enzymes that degrade amyloid-β, as well as the clearance of Aβ plaques (Walter & Neumann 2009). TGFβ released by microglia was found to reduce plaque load in a model of AD. Similarly, TGFβ was also neurprotective in a rodent models of prion disease where inhibition of TGFβ resulted in

cerebral inflammation (Boche et al., 2006; Wyss-Coray et al., 2001; cited in Czeh et al., 2011). Similar to neurodegenerative diseases, Lalancette-Hebert et al., (2007) demonstrated that after ischemic insult, ablation of microglia resulted an increased infarct size and number of apoptotic neurons in the ischemic penumbral region. Production of the anti-inflammatory cytokine IL-4 in the CNS was found to be important for tissue repair in an animal model of MS. Mice deficient in CNS IL-4 showed exacerbated inflammation in an EAE model of MS (Ponomarev et al., 2007). IL-4 also attenuated pathology in a mouse model of Alzheimer's disease (Kiyoto et al., 2010).

Pro-inflammatory cytokines can themselves have dual roles in promotion of survival or death, contributing to the debate of whether inflammation is beneficial or detrimental. For example TNFα, a pro-inflammatory cytokine that is normally associated with neurotoxic effects, has been shown to exert neuroprotective effects on neurons following ischemia by binding to TNF Receptor 2 (TNFR2) (Lambertsen et al., 2009). Furthermore, Arnett et al., demonstrated that binding of TNF to TNFR2 was necessary for oligodendrocyte regeneration and proliferation in a model of demyelinating disease (Arnett et al., 2001). Release of the cytokine IL-6 can lead to impaired hippocampal neurogenesis, and can also act on astrocytes to promote tissue repair in the early stages following injury (Whitney et al., 2009).

1.2.10 Beneficial effects of inflammation on neurogenesis

Inflammation is often thought of as a negative consequence of neuronal injury or disease, however, in certain injury models it is well established that inflammation can also play a beneficial role through its effects on neurogenesis. Transient global ischemia increased proliferation and neural differentiation of NSCs in the dentate gyrus as well as the SVZ, shown by an increase in the number of Bromodeoxyuridine (BrdU)-positive cells co-labeled with neuronal markers. It was hypothesized that the increase in neurogenesis following stroke could be partially due to the expression of vascular endothelial growth factor (VEGF) which can stimulate proliferation, migration and survival of neurons (Hanson et al.,2008; Arvidsson et al., 2002; Whitney et al., 2009). While some studies

report an increase in proliferation and neurogenesis, it has been proposed that inflammatory changes accompanying ischemic injury cause death of neuroblasts and newly born striatal neurons in the first several weeks after injury (Arvidsson et al., 2002; Kokaia et al., 2006).

Another example of a beneficial role for microglia and inflammation comes from studies of electrically-induced status epilepticus. Status epilepticus results in neuronal loss, microglial activation and chronic inflammation, however an increase in neurogenesis is also observed. New neurons generated following status epilepticus induction were found in the hippocampus up to 6 months after injury, however it was noted that neurogenesis and survival of new neurons was heavily dependent on the severity of seizure, with severe seizures resulting in decreased differentiation (Bonde et al., 2006; Iosif et al., 2008; Whitney et al., 2009).

1.2.11 Detrimental effects of neuroinflammation

Neurogenesis occurs throughout the adult life in areas such as the hippocampus, the brain area important for learning and memory. Loss of hippocampal neurogenesis has been implicated as a contributor to cognitive decline observed in normal aging, as well as neurodegenerative diseases such as AD, and PD (Streit et al., 2004; Lazarov et al., 2010; Mu & Gage, 2011). Activated microglia have been discovered in patients with Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic Lateral Sclerosis (ALS). The activation of microglia in neurodegenerative diseases may occur as a result of misfolded proteins, or protein aggregates such as Aβ plaques in AD, α-synuclein in PD, or SOD-1 in the case of ALS (Boillee & Cleveland, 2008; Zhang et al., 2005; Floden & Combs, 2006). It is hypothesized that the degenerative process involving inflammation may be a result of a feed-forward loop whereby disease states activate microglia leading to the production of pro-inflammatory cytokines which in turn leads to neuronal death and further degeneration (Akiyama, 2000; Perry et al., 2010).

In models of acute damage, such as stroke, evidence suggests that the inflammatory response regulated by microglia exacerbates lesion size and tissue loss. Activation of

microglia following injury leads to the production of pro-inflammatory cytokines, which in turn leads to the release of chemokines and subsequent recruitment of other immune cells, such as neutrophils and monocytes from the blood. This recruitment of peripheral immune cells intensifies the inflammatory response (Perry et al., 2010).

1.2.12 Detrimental effects of inflammation on neurogenesis

A study by Monje and colleagues demonstrated that the impairment in neurogenesis caused by cranial radiation injury could be blocked by the addition of the anti-inflammatory drug indomethacin, a common nonsteroidal anti-inflammatory drug (NSAID). They reported that LPS-activated microglia induced a significant decrease in neurogenesis of hippocampal progenitors both in vitro and in vivo. This was thought be a result of an increase in pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 released by activated microglia. Simultaneous treatment with indomethacin abrogated the inflammatory inhibition of neurogenesis (Monje et al., 2003). In a similar study, Ekdahl et al., found that increased neurogenesis triggered by brain insult such as status epilepticus was attenuated when accompanied by activation of microglia caused by tissue damage or LPS injection. Importantly, it was also demonstrated that addition of the anti-inflammatory drug minocycline could restore neurogenesis in the hippocampus of adult rats under inflammatory conditions. (Ekdahl et al., 2003). In a rodent model of stroke, immunosuppression, either genetically or by administration of the immunosuppressive drug Cyclosporine A (CsA) resulted in increased migration of neural stem and progenitor cells to the site of injury, regeneration of cortical tissue, and improved behavioural functionality (Erlandsson et al., 2011).

Effects of inflammation on neurogenesis is largely influenced by the activation state of microglia. While microglia activated by IL-4 or low level IFN-γ resulted in increased neural stem cell proliferation and differentiation, whereas microglia activated with LPS or cytokines such as IL-1β are shown to limit neurogenesis, (Russo et al., 2011). Furthermore,

proliferation and survival was significantly decreased when neural precursor cells were cultured in conditioned media from LPS-activated microglia, but not quiescent microglia (Cacci et al., 2008). This detrimental effect on NPCs was likely due to the presence of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-18 as well as reactive nitrogen and oxygen species in the media from activated microglia.

1.2.13 TNFα and neurogenesis

Exposure to recombinant TNFα (20-100ng/ml) was found to decrease neurogenesis of hippocampal NSCs, and reduce proliferation and survival of NSCs from the SVZ (Monje et al., 2003; Ben-Hur et al., 2003). While TNFα appears to have a negative role on neurogenesis and survival of NSCs, other studies have examined the role of TNFα as positive regulator of neurogenesis. Widera et al., demonstrate that neurospheres derived from the adult rat SVZ exhibit an increase in proliferation when treated with low levels of TNFα (10ng/ml) (Widera et al., 2006). The effect of TNFα on NSCs and neurogenesis is multi-factorial, depending on both the levels of the cytokine, as well as relative expression of the two TNF receptors; TNFR1 and TNFR2. Signaling through the two TNF receptors has a differential effect on NSCs whereby signaling through TNFR1 can suppress neural progenitor proliferation and neurogenesis, and signaling of TNFα through TNFR2 increases the survival of new neurons (Iosif et al., 2006; Mathieu et al., 2010).

1.2.14 Fas in neurogenesis

The expression of the death receptor Fas is increased in the nervous system at critical developmental times of neuronal differentiation and apoptosis, suggesting an important role in programmed cell death in the development of the nervous system (Knight et al., 2010). The effects of Fas on neural precursor cells and neurogenesis is controversial, and

may be dependent on the stimulus for Fas activation. In response to ionizing radiation, Fas-deficient neural progenitor cells exhibit increased survival relative to wild-type neural progenitor cells (Semont et al., 2004). In contrast, lack of hippocampal Fas resulted in a reduction of neurogenesis and working memory mouse models (Corsini et al., 2009).

1.2.15 IL-1β and neurogenesis

Interleukin-1 is a pro-inflammatory cytokine that consists of two distinct proteins IL-1α and IL-1β, which bind to the receptor IL-1R1. Adult hippocampal NPCs express IL-1R1 and undergo cell cycle arrest when exposed to IL-1β *in vitro.* In vivo, prolonged expression of IL-1B in the adult hippocampus results in a significant decrease in hippocampal neurogenesis, and a corresponding decrease in BrdU-positive cells. The decrease in neurogenesis observed following IL-1β exposure may be a result of NPCs being directed toward an astrocyte or glial lineage (Koo & Duman, 2008). The anti-proliferative effects of IL-1β may occur via activation of signaling pathways such as NF-κB, c-Jun, JNK, or p38 MAPK (Mathieu et al., 2010). The administration of an antagonistic protein (IL-1Ra) can block the decrease in proliferation in the SGZ. Furthermore, it was found that transplantation of IL-1Ra-overexpressing NPCs into the hippocampus could rescue the impairment in neurogenesis observed in a mouse model of Alzheimer's disease (Ben Menachem-Zidon et al., 2014). IL-1β has been suggested to play a role in the deficiency in hippocampal-dependent learning that occurs during aging. An upregulation of IL-1β correlates with impairment in long-term potentiation, a process involved in learning and memory (Nolan et al., 2005; cited in Russo et al., 2011). IL-1β is also hypothesized to supress hippocampal neurogenesis via epigenetic regulation of the neural progenitor marker NeuroD (Kuzumaki et al., 2010).

1.3 Apoptosis

1.3.1 Intrinsic pathway of apoptosis

Apoptosis is a genetically programmed cell death pathway that has been observed in chronic and acute neurodegenerative conditions. Both the extrinsic (death-receptor mediated), and the intrinsic (mitochondrial) pathway involve the activation of caspases and induce morphological changes within the cell. The intrinsic pathway is activated by various stimuli including developmental cues or cytotoxic insults such as viral infection, DNA damage, and growth factor withdrawal. The intrinsic (mitochondrial) pathway is tightly regulated by the B-cell lymphoma-2 (Bcl-2) family of proteins (Figure 1.6).

1.3.1.1 The B-cell lymphoma-2 (Bcl-2) family

The Bcl-2 family is divided into three main subfamilies based on the presence of Bcl-2 homology (BH) domains. The anti-apoptotic proteins, Bcl-2, Bcl-xL and Mcl-1 possess BH domains 1-4. A second subfamily, consisting of Bax, and Bak possess three BH domains (BH1-3) promote apoptosis. Of these pro-apoptotic members of the Bcl-2 family, Bax is considered to be the most significant mediator of mitochondrial apoptosis, however it has been suggested that in neural precursor cells, Bax and Bak play redundant roles in the regulation of apoptosis (Lindsten et al., 2003). Finally the third group, named the BH3-only group possess only the BH3 domain, as the name suggests, and is represented by the proteins Puma, Noxa, Bim and Bid. In the healthy cell, the pro-apoptotic Bcl-2 protein Bax and Bak are held in a cytosolic complex by the anti-apoptotic Bcl-2 proteins, preventing their translocation to the mitochondria. However, upon encountering a cellular stressor, Bax/Bak become activated and are released from their anti-apoptotic complex, allowing them to translocate to the mitochondrial membrane.

1.3.1.2 BH3-only Bcl-2 family members

The activation of BH3-only proteins is a critical step in the intrinsic apoptotic pathway. The activity of these proteins is regulated at multiple levels. Under normal, healthy conditions, levels of some BH3-only proteins is low or undetectable (eg. Puma and Noxa), whereas others are constitutively expressed and can be activated by phosphorylation or cleavage events (eg. Bad and Bid). Following stressors or apoptotic stimuli, a number of transcription factors have been identified as transcriptional activators of BH3-only family members, including CHOP, Foxo3a, c-Jun, and p53 family members in a cell-type and stimulus-specific manner (Reviewed in Puthalakath & Strasser, 2002).

1.3.1.3 Bax activation

It remains a topic of controversy whether the activation of Bax by BH3-only proteins is via a direct or indirect mechanism. The indirect model of Bax activation proposes that in healthy cells, Bax is sequestered by anti-apoptotic Bcl-2 proteins, and that BH3-only proteins must engage and neutralize anti-apoptotic proteins, freeing Bax to translocate to the mitochondrial membrane. The direct theory of Bax activation suggests that in healthy cells, 'activator' BH3-only proteins are sequestered by anti-apoptotic proteins (Bcl-2, BclX, Mcl1), however, when activated by cytotoxic stimuli, 'sensitizer' BH3-only proteins (Bad, Hrk, Noxa), bind to anti-apoptotic family members, allowing the 'activator' BH3's (Puma, Bid, and Bim) to engage and activate Bax (Villunger et al., 2011; Cheng, 2001. In the absence of cytotoxic stimuli, the pro-apoptotic protein Bax exists in the cytosol as a monomer, however, direct activation of Bax by BH3-only proteins (ie. Puma) induces a conformational change in Bax to promote homo-oligomerization and targeting of Bax to the mitochondrial outer membrane. The BH3 domain of Puma binds transiently to the $\alpha 1$ helix of Bax to induce this structural reorganization, thereby promoting mitochondrial targeting (Cartron et al., 2004; Youle & Strasser, 2008). Once at the mitochondria, Bax induces MOMP, allowing soluble proteins such as cytochrome c, DIABLO, and AIF to diffuse into the cytosol. Cytochrome c can then bind to APAF1, leading to the assembly of a heptameric protein ring called the 'apoptosome', which can bind pro-caspase-9 and

induce its activation through a conformational change (Youle & Strasser, 2008). Activation of the 'initatior' caspase-9, leads to subsequent activation of 'effector' caspases such as caspase-3, culminating in apoptosis of the cell (Figure 1.6).

1.3.2 Extrinsic pathway of apoptosis

The extrinsic pathway of apoptosis, also known as the death receptor mediated pathway of apoptosis is triggered by the binding of specific pro-apoptotic ligands to death receptors. The death receptors are type I transmembrane proteins and are members of the TNF superfamily of receptors. These receptors share homology in their extracellular region, which is characterized by up to 6 cysteine-rich domains (CRDs) that define their ligand specificity. Perhaps the most characteristic feature of these receptors is the presence of an intracellular region known as the "death domain" (DD) that enables the receptors to initiate cytotoxic signals when activated by cognate ligands (Guicciardi et al., 2009; Schmitz, et al., 2000).

1.3.2.1 Death receptors and the death inducing signaling complex

The most extensively studied death receptors are Fas (CD95/APO-1), TNF-receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptor 1 [TRAIL-R1/ Death Receptor 4 (DR4)], and receptor 2 (TRAIL-R2/DR5/APO-2/KILLER). Death receptors are activated by ligands that are part of a group of cytokines belonging to the TNF protein family. These ligands can either be membrane bound (eg. mFasL, mTNFα) or released as soluble cytokines (eg. sFasL, sTNFα) through proteolytic cleavage. Prior to binding of the ligands to the receptors, death receptors form receptor complexes through the interaction of the first cysteine-rich domain of the extracellular tail of the receptor, known as the preligand assembly domain (PLAD). PLAD is not directly involved in ligand binding, however the oligomerization and formation of complexes is a crucial step for receptor-ligand interaction (Guicciardi & Gores, 2009). Ligand binding causes a conformational change in the receptor complex, leading to the recruitment of adaptor proteins to the intracellular region of the receptor. Adaptor proteins such as TRADD and

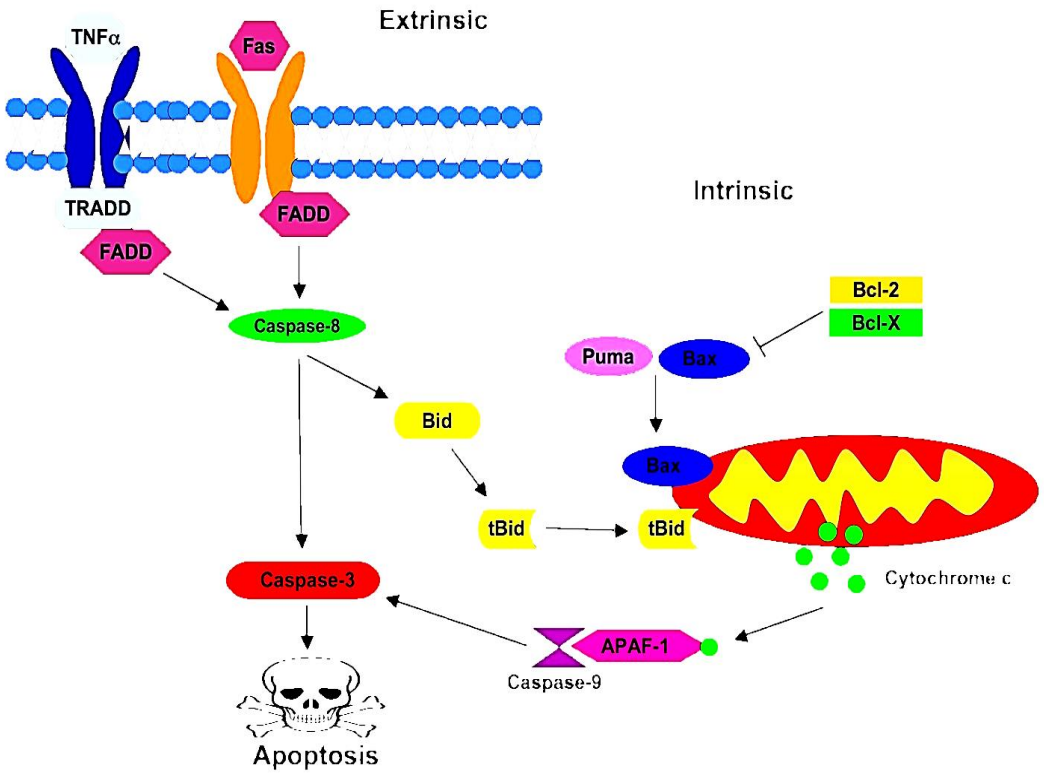

Figure 1.0.5

Figure 1.6. Pathways of Apoptosis. There are two main pathways of apoptosis; the extrinsic, and intrinsic pathway. The extrinsic pathway is characterized by activation of death receptors, such as TNFR1 and Fas, followed by activation of caspase 8. Caspase 8 can directly activate effector caspases, or can cleave and activate Bid, which translocates to the mitochondria and drives cells through a mitochondrial-dependent apoptotic pathway. The intrinsic pathway involves activation of Bcl-2 family members leading to mitochondrial outer membrane permeabilization, release of pro-apoptotic factors and activation of effector caspases.

FADD are able to associate with receptors through the homotypic interaction of their death domain (DD) to the DD of the receptor. Adaptor molecules may also contain an additional domain, known as a DED (death-effector domain) which mediate the recruitment of caspases and cFLIPL (caspase-8-like inhibitor protein) through their DEDs. This complex is referred to as the death-inducing signaling complex (DISC), which can generate an apoptotic signaling cascade, however the mechanism for this remains a topic of debate.

1.3.2.2 Caspase activation in extrinsic apoptosis

The first proposed model of caspase-activation is known as the 'induced-proximity model'. In this model, the recruitment of FADD to the receptor complex results in clustering of initiator procaspases (procaspase-8) at the DISC, which leads to their self-processing into the active form which is able to cleave caspase-3 and Bid. The second model, known as the 'proximity-induced dimerization model' suggests that accumulation of caspases at the DISC promotes their dimerization which results in their activation. The final model known as the 'induced-conformation model' suggests that activation of the initiator caspases occurs through a conformational change in their active site following interaction with the adaptor protein complex. Activation of caspase-8 at the DISC can be regulated by cFLIP, possibly by competing for binding and recruitment to FADD (Kantari & Walczak, 2011; Guicciardi et al., 2009). Following activation, caspase-8 is released into the cytosol as an active heterotetrameric form containing two large and two small subunits. The downstream signaling events initiated by caspase-8 are likely dependent on the amount of active caspase 8 released into the cytosol, whereby large amounts of caspase-8 can directly process and activate effector caspases such as caspase-3 to induce apoptosis. In cases where small amounts of caspase-8 are released, cleavage of the BH3-only protein Bid is required to induce apoptosis (Kantari & Walczak, 2011).

1.3.2.3 The BH3-only protein Bid

The BH3-only protein Bid acts as a molecular bridge between the extrinsic and intrinsic pathway of apoptosis. In its uncleaved, full-length form, Bid is thought to be inactive. Following death receptor stimulation and activation of caspase-8, Bid is cleaved by

caspase-8 to its truncated form tBid. tBid is a 15 kDa protein that is capable of translocating to the mitochondria to induce mitochondrial outer membrane permeabilization (MOMP) through activation of Bax (Yin, 2006) (Figure 1.6). As previously described, permeabilization of the mitochondrial outer membrane results in cytochrome c release and downstream activation of effector caspases, culminating in apoptosis. The requirement for MOMP to elicit apoptosis following engagement of death receptors separates cells into two categories. In type I cells, activation of the extrinsic pathway is sufficient to induce death, and they do not require cleavage of Bid and MOMP. Alternatively, type II cells have weak DISC formation capability and therefore cleavage of Bid and activation of the mitochondrial pathway are required to induce apoptosis (Kantari & Walczak, 2011; Schmitz et al., 2000).

1.3.3 Detection of apoptosis

Whether cell death is induced by intrinsic or extrinsic factors, the physical and biochemical hallmarks for apoptosis remain the same. Both the extrinsic and intrinsic pathway end with the activation of caspases which results in degradation of nuclear material by cytoplasmic endonuclease, and degradation of nuclear and cytoskeletal proteins by proteases (Elmore et al., 2007). Activation of caspases can be detected by western blot, immunohistochemistry or caspase-activity assays. Other characteristics of apoptotic cells include DNA fragmentation, plasma membrane blebbing, and cell shrinkage and disintegration into packaged apoptotic bodies. The nuclear features of apoptosis can be detected using DNA frangementation assays such as Terminal dUTP Nick End-Labeling (TUNEL) assay, or nuclear staining by Bisbenzimide (Hoechst) stain (Kroemer et al 2007; Elmore, 2007). As a specific measure of intrinsic apoptosis, mitochondrial depolarization can be detected using MitoTracker Red, which accumulates in cells with an intact mitochondrial membrane. Immunostaining for cytochrome c can also be used to detect engagement of the intrinsic pathway, as permeabilization of the outer mitochondrial membrane results in release of cytochrome c, and therefore a decrease in the number of cytochrome c positive cells.

1.4 Tumour protein p53 (p53)

The tumour suppressor p53 belongs to a small family of related proteins that also includes p63 and p73. p63 and p73 have clear roles in normal development, whereas p53 has evolved in higher organisms for tumour suppression (Vousden & Lu, 2002; Jacobs et al., 2006). In general tumour suppressors function to maintain growth and homeostasis by regulating one of more processes that mediate anti-proliferative effects. P53 can be activated by a variety of stimuli including DNA damage, hypoxia, or aberrant oncogene expression. Several responses can be elicited by p53 including cell cycle arrest, senescence, differentiation, and apoptosis (Fridman & Lowe, 2003; Vousden & Lu, 2002). Which of these options occurs in the cell is greatly dependent many factors including cell type, stimulus, and environmental factors. Disruption of p53 results in cell-cycle checkpoint defects, genomic instability, cellular immortalization, and inappropriate survival of damaged cells. Due to the many transcriptional targets of p53, it can have effects on many signal transduction pathways within the cell, and thus is an important determinant of cell/tissue fate. Such effects include activation of cell cycle checkpoints leading to growth arrest; promotion of DNA repair; as well as stimulation of effectors of growth arrest (p21), and cell death (Vousden & Prives, 2009; Jacobs et al., 2006; Lanni et al., 2012). p53 both protects the genome by promoting repair of lesions in the DNA, and also eliminates or arrests the proliferation of damaged or mutant cells by apoptosis and cellular senescence (Figure 1.7).

The stability and activity of p53 is primarily regulated through the modulation of the interaction between p53 and its negative regulators such as Mdm2 which function to suppress the transcriptional activities of p53 by destabilizing p53. In response to cellular stressors, such as DNA damage, the stability and activity of p53 is increased, allowing p53 to influence transcription of genes. P53 can also be regulated by post-translational modifications, most notably by phosphorylation on Thr21 and Ser 23 which disrupts the interaction between p53 and Mdm2, leading to the activation of p53 (Liu et al., 2010; Xu, 2003).

1.4.1 P53 in cell cycle regulation

p53 may be an important checkpoint in the balance between cell survival versus cell death due to its role in cell cycle arrest at G0/G1 and G2/M phases, either allowing cell repair or activating cell death pathways. While dysfunctional activity of p53 is involved in cancer progression, it is also observed in aging and Alzheimer's disease, as neurodegeneration was correlated with neurons re-entering a lethal cell cycle (Yang et al., 2001; Lanni et al., 2012). P53 can effectively inhibit cell cycle progression by activating the transcription of the cyclin-dependent kinase inhibitor p21. P21 inhibits both the G1-to-S and the G2-to-M transitions of the cell cycle. The induction of p21 can occur in response to low levels of p53, suggesting that a temporary block in cell cycle by acute or mild stress may act as a protective mechanism for the cell until the stressor has been removed. However, in cases of prolonged stress, this may lead to the activation of cellular senescence; an irreversible cell cycle arrest (Vousden & Prives; 2009).

1.4.2 P53 in apoptosis

One of the most studied functions of p53 is its ability to induce apoptosis. Numerous studies have shown that endogenous p53 could control apoptosis, and that loss of apoptosis was correlated with tumour progression in p53-null transgenic mice (Fridman & Lowe, 2003). The link between p53 and its ability to control apoptosis may come from its ability to regulate transcription of pro-apoptotic Bcl-2 family members, including Bax, and BH3-only members Puma, Noxa, and Bid, as well as other factors including apoptotic protease activating factor-1 (APAF-1), Tp53INP-1, Death receptor 5 (DR5), and Fas (CD95) (Fortin et al., 2001; Peuget et al., 2014; Fridman & Lowe, 2003). The promoters of these genes contain p53 response elements that can bind p53 *in vitro*. The influence of p53 on apoptosis may not be solely through transcriptional regulation. p53 may also control apoptosis through transcription-independent mechanisms, for example by activation of Bax at the mitochondria (Fridman & Lowe, 2003).

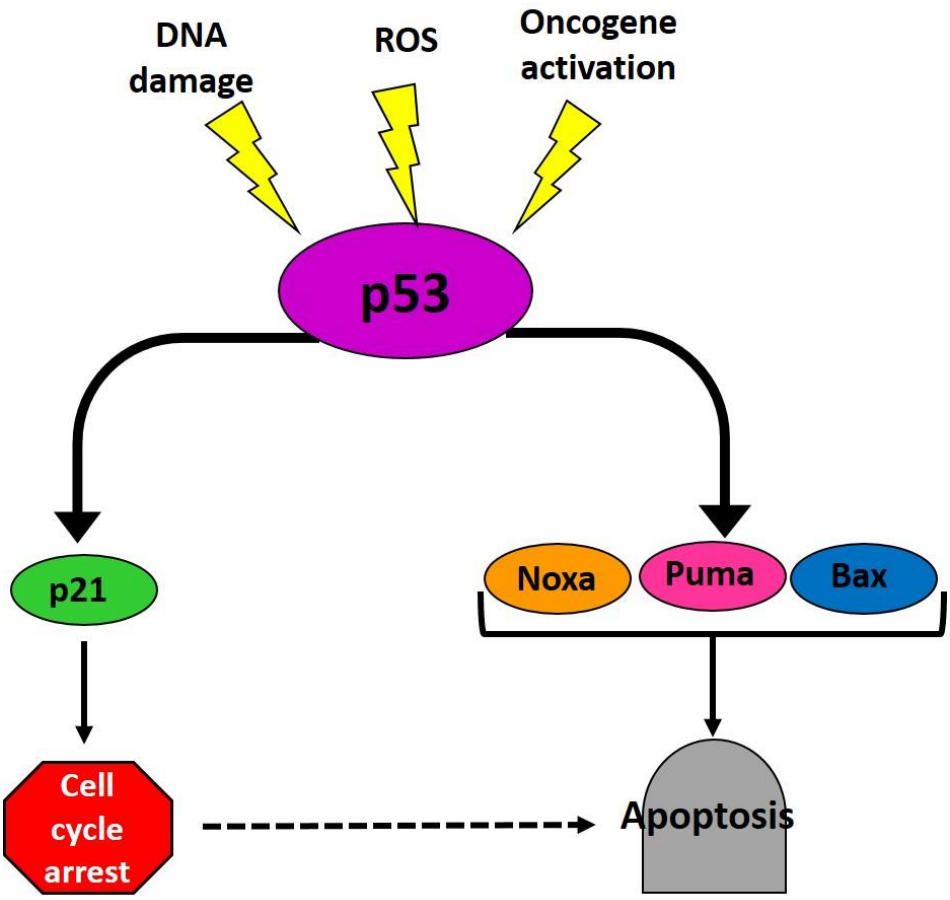

Figure 1.0.6

Figure 1.7. p53 activation and outcomes. The tumours suppressor p53 can be activated by various stimuli including DNA damage, reactive oxygen species (ROS), or oncogene activation. P53 can play dual roles in regulating the cell cycle and apoptosis through the regulation of target genes.

P53-mediated apoptosis occurs predominantly through the mitochondrial apoptotic pathway as p53 can control factors that act upstream of the mitochondria and induce perturbations in mitochondrial dynamics. The ability of p53 to transactivate Bid may facilitate cross-talk between the extrinsic and intrinsic pathways (Sax et al., 2002; Fridman

& Lowe, 2003). Overexpression of p53 alone is sufficient to induce neuronal cell death. Studies have demonstrated that Puma is required for p53-induced neuronal death, and furthermore that p53-induced cell death in neurons involves a Bax-caspase-3 dependent pathway (Cregan et al., 1999).

1.4.3 P53 in neural precursor cells

p53 is expressed within the neurogenic niche in the lateral ventricles, one of the sites of neurogenesis in the adult CNS. Moreover, nuclear p53 is observed within cells of the subventricular zone including astrocytes and progenitor cells, suggesting that p53 may play a role in regulating adult neurogenesis. Neurospheres cultured from the adult subventricular zone of $p53^{-/-}$ mice proliferate more rapidly, have an increased capacity for self-renewal and are less apoptotic than those cultured from $p53^{+/+}$ adult SVZ. Furthermore, $p53^{-/-}$ neurospheres generate an increased number of neuronal progenitors relative to glial progenitors, suggesting that p53 may play a role in negative regulation of neurogenesis (Mendrysa et al., 2011; Gil-Perotin et al., 2006; Meletis et al., 2006). Analysis of the transcriptional effects of p53 on neural stem cells identified that dysregulation or absence of p53 in neurospheres lead to a reduction in p21; a protein involved in cell cycle arrest through its inhibition of CDKs. Absence of p21 leads to increased proliferation of neural stem cells in the lateral ventricle wall of adult mice. This finding was also observed in a model of ischemia, whereby proliferation was increased in the brain of $p21^{-/-}$ mice compared to wildtype mice (Qiu et al., 2004; Meletis et al., 2005). p53-deficient NPCs show reduced caspase-3 activation and cell death following exposure to genotoxic stressors (Akhtar et al., 2006). Stabilization of p53 can interact with both pro- and anti-apoptotic molecules suggesting that p53 can mediate transcription-independent as well as dependent death in NPCs (Akhtar et al., 2006). Akhtar et al., demonstrated that p53 could activate intrinsic apoptosis directly through Bax at the mitochondria by acting as a ‘BH3 analog’ in response to treatment with the broad kinase inhibitor staurosporine (Akhtar et al., 2006). Activation of p53 can lead to the transcription of many genes, but can also lead to the suppression of genes such as Nanog, a factor required for the self-renewal of embryonic stem cells. p53 was found to bind to the promoter of Nanog and suppress Nanog expression

following DNA damage (Lin et al., 2005). DNA damage-activated p53 has also been implicated in depletion of adult stem cells and segmental progeria; a model of premature aging (Liu et al., 2010).

1.5 Nuclear factor- kappa B (NF-κB)

The transcription factor NF-κB plays an important role in homeostasis as it is a critical regulator of various cellular functions including; inflammation, differentiation, stress responses, and survival/apoptosis. There are five members of the NF-κB family; RelA (p65), RelB, c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52 respectively. Each of these NF-κB proteins shares a Rel homology domain which is responsible for DNA binding and dimerization (Oeckinghaus et al., 2011). With the exception of RelB, NF-κB proteins can homodimerize, or form heterodimers with each other, the most prevalent active form being a heterodimer consisting of p50 or p52 subunit and a p65 subunit (Tak & Firestein, 2001). Studies have identified distinct roles for each member of the NF-κB family such as immunity, development and differentiation, and production of cytokines. Specifically, p50 and p65 heterodimers are involved in the activation of inflammatory genes by IL-1 or TNFα in monocytes, as well as constitutive production of IL-6 in rheumatoid arthritis (Tak & Firestein, 2001). Furthermore, the predominant transcriptionally active form of NF-κB in the central nervous system is the p50/p65 heterodimer (Gutierrez & Davies, 2011).

At rest, NF-κB exists inactive in the cytoplasm, associated with regulatory proteins called inhibitors of κB (IκBα, IκBβ and IκBε), which sequester NF-κB dimers preventing nuclear translocation. Degradation of IκB proteins occurs via phosphorylation by the IκB kinase (IKK) complex, which consists of two active kinases, IKKα and IKKβ and the regulatory subunit IKKγ (NEMO). Activation of IKK initiates the phosphorylation of IκBα at specific NH2-terminal serine residues which targets the IκB protein for degradation by the proteasome. This phosphorylation and degradation is a critical step in the activation of NF-κB as it frees NF-κB dimers from the inhibitory IκB complex, allowing them to

translocate to the nucleus (Tak & Firestein, 2001; Oeckinghaus et al., 2011; Viatour et al., 2005).

There are two main pathways that lead to the activation of NF-κB in cells. The canonical pathway is induced by most NF-κB stimuli including pro-inflammatory cytokines such as TNFα and signals that originate from cytokine receptors such as TNFR, IL-1R, and PAMP receptors such as TLR4. This pathway involves recruitment of various adaptor proteins including TRADD, RIP, and TRAF2 to the cytoplasmic membrane of the cell. This leads to recruitment and activation of the IKK complex which ultimately functions to degrade IκB. As a result of this the canonical pathway is defined as being dependent on IKKβ and NEMO for the activation and nuclear translocation of mostly p65-containing heterodimers. The second pathway, referred to as the non-canonical or 'alternative' pathway is NEMO-independent and is activated by cytokines such as lymphotoxin β, CD40 ligand, and BAFF (B-cell activating factor). The non-canonical pathway relies on recruitment of TRAF proteins and on the NF-κB-inducing kinase (NIK) resulting in downstream cleavage of p100 to produce NF-κB protein p52, which heterodimerizes with RelB to influence gene transcription.

1.5.1 Effects of NF-κB activation

Activation of NF-κB can have many different outcomes in the cell and lead to the transcription of a variety of genes with diverse functions. Specifically in the nervous system, NF-κB regulates an array of genes that are critical in the cellular response to injury as well as in neuronal plasticity (Mattson & Camandola, 2001). One important role of NF-κB is in inflammation where it acts as central regulator through its ability to induce the transcription of pro-inflammatory genes TNFα, IL-1β, IL-6, and IL-8. In human diseases, NF-κB is activated at sites of inflammation. Such diseases include Rheumatoid arthritis, Atherosclerosis, Multiple sclerosis, Asthma, and Inflammatory bowel disease (Viatour et al., 2004;Tak & Firestein, 2001). .

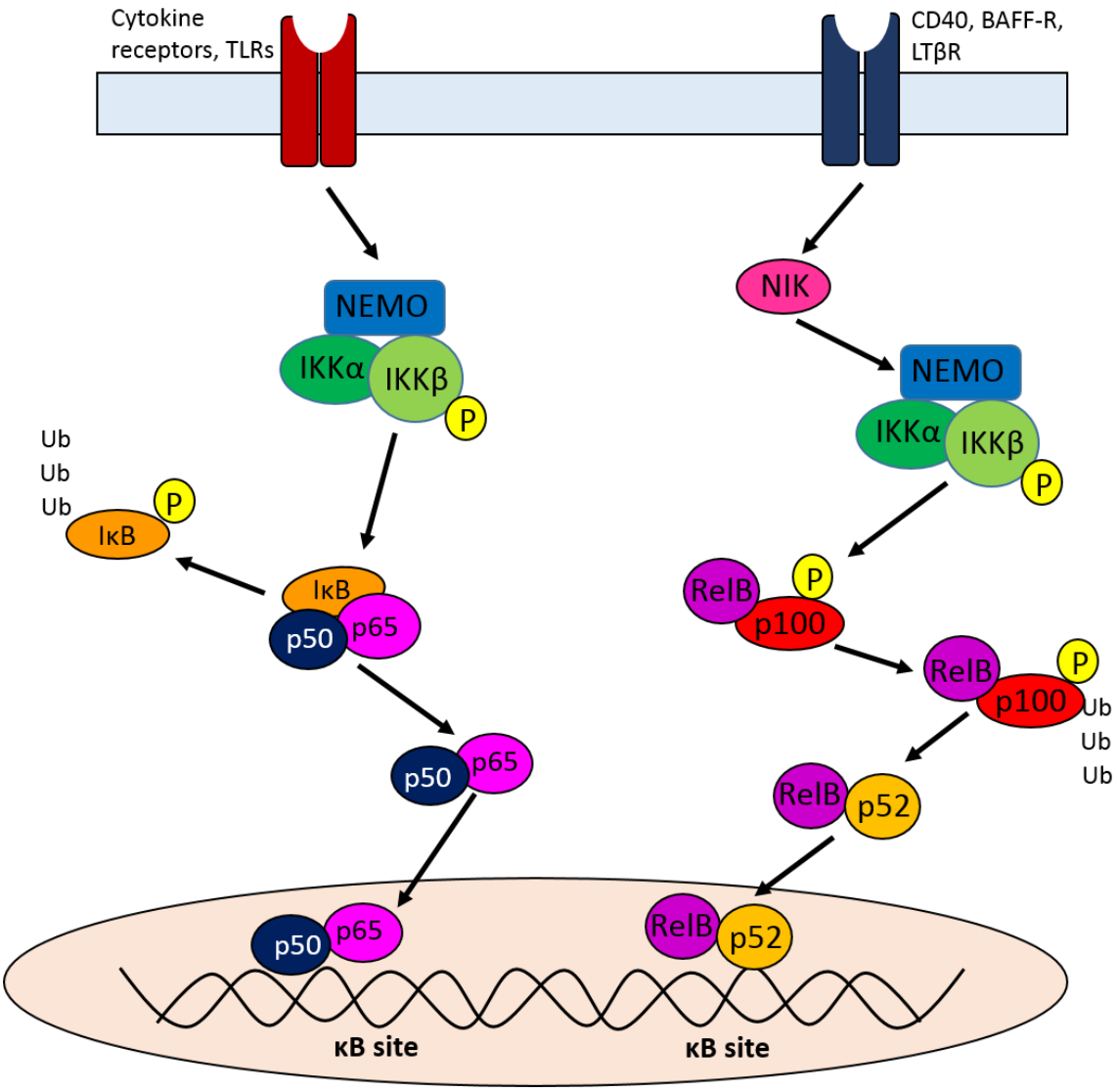

Figure 1.0.7

Figure 1.8. NF-κB signaling pathway. The NF-κB pathway can be engaged by activation of various inflammation-associated receptors including TLRs, cytokine receptors, BAFF-R, and CD40. Signaling can proceed through a canonical pathway, culminating the activation of the subunits p50 and p65; or through a non-canonical pathway leading to activation of RelB and p52. NF-κB signaling can result in transcription of numerous genes; including pro-inflammatory cytokines, pro-survival factors, as well as pro-death genes.

NF-κB activation can also be involved in apoptosis, though whether it promotes cell survival or cell death is highly dependent on the cell type and stimulus for activation. NF-κB activation can lead to the transcription of pro-survival genes including inhibitor of apoptosis proteins (IAPs), Bcl-2, Bcl-x, as well as genes involved in cell cycle and proliferation (Mattson & Camandola, 2001; Glasgow et al., 2000). Conversely, NF-κB may also promote apoptosis during normal development, and following injury or exposure to inflammatory stimuli. Recent studies have identified the pro-apoptotic gene Puma as a target of NF-κB activation following TNFα exposure in multiple cancer cell lines (Wang et al., 2009).

1.5.2 NF-κB and neurogenesis

The effect of NF-κB on neural stem/precursor cells and neurogenesis is somewhat controversial. While some studies report that NF-κB promotes differentiation and asymmetric division, others identify NF-κB as a negative regulator of neurogenesis (Koo et al., 2010; Zhang et al., 2011; Widera et al., 2006). A study by Zhang et al., suggested that NF-κB signaling has a critical role in the regulation of the very early stages of neurogenesis. It was shown that inhibition of NF-κB signaling blocks asymmetric division and neural differentiation at an early stage, leading to accumulation of NSCs (Zhang et al., 2011). Consistent with the promotion of neurogenesis by NF-κB, Widera and colleagues found that activation of the canonical NF-κB pathway by TNFα resulted in increased proliferation of NSCs (Widera et al., 2006). Contrary to these findings, in models of stress-impaired neurogenesis, NF-κB was activated by chronic exposure to stress, and this impaired proliferation of NSCs in the adult hippocampus (Koo et al., 2010). Thus it appears that the effects of NF-κB on neural stem cells and neurogenesis may be stimulus and context dependent.

1.6 Rationale

While previous studies have demonstrated that inflammation can limit neurogenesis and the survival of neural precursor cells, the mechanisms of how inflammatory factors can regulate neural precursor cell processes remain unclear. The goal of this study is to identify the inflammatory mediators that contribute to the detrimental effects on NPCs and elucidate the mechanism(s) of NPC death during inflammatory conditions. The overall hypothesis of this study is that pro-inflammatory cytokines, released from activated microglia act on NPCs to activate apoptotic pathways, thereby limiting their survival. I used the following experimental approach to investigate the effects of specific pro-inflammatory (M1) microglia derived factors on NPC proliferation and survival (Figure 1.9). Microglia were left unactivated or activated with LPS and IFNϒ to induce a pro-inflammatory phenotype and cultured in neural stem cell media. Primary neural precursor cells (NPCs) were isolated from wild-type mice and various transgenic mice and cultured in the unactivated or activated microglia conditioned stem cell media to determine the effects of soluble factors released by microglia on NPCs. Based on our preliminary findings using this conditioned media model of neuroinflammation we have focused on examining the role of three prominent pro-inflammatory factors: TNFα, IL-1β, and Fas.

1.6.1 Objectives

1. Examine the role and mechanisms of TNFα –mediated NPC death.

2. Examine the role and mechanisms of IL-1β-induced effects on NPC survival and proliferation.

3. Evaluate the significance of Fas upregulation and signaling in NPCs during inflammatory conditions.

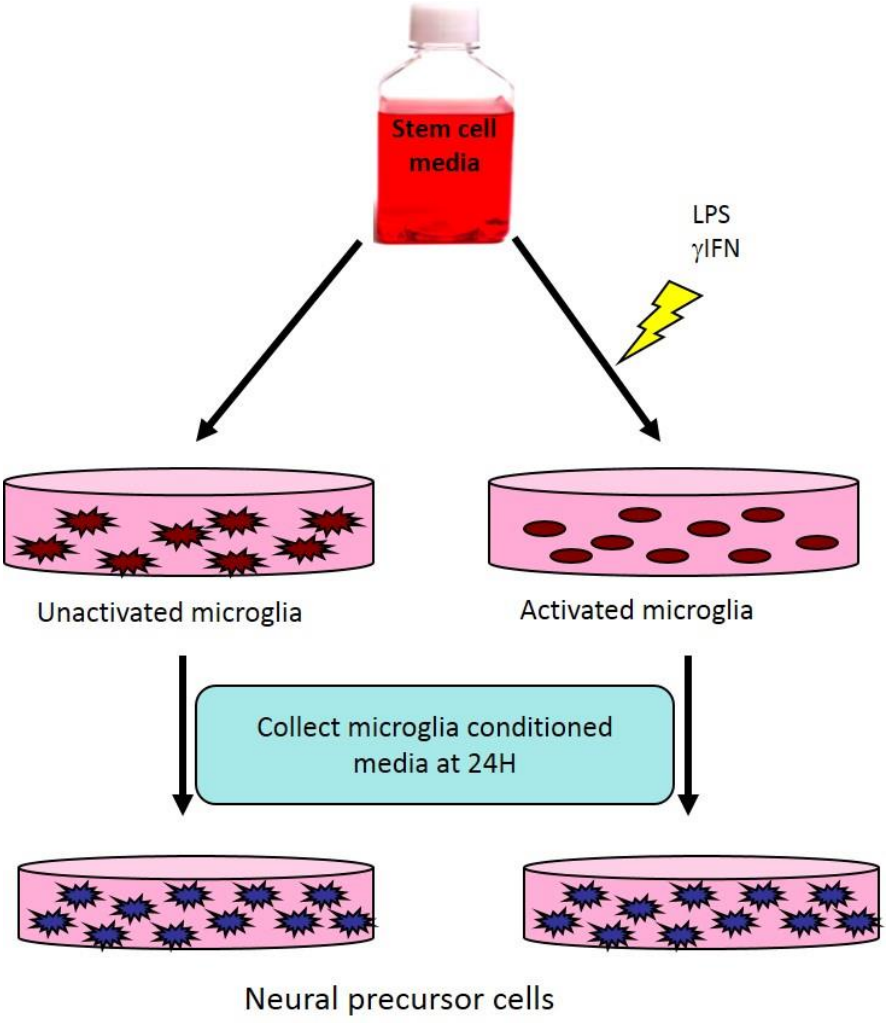

Figure 1.0.8

Figure 1.9. Schematic of *in vitro* inflammation model. Cultured microglia are incubated in stem cell media and either activated with LPS/γIFN or left unactivated. After 24 hours, conditioned media is collected and added to neural precursor cells.

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Chapter 2

2 Microglia derived TNFα induces apoptosis in neural precursor cells via transcriptional activation of the Bcl-2 family member Puma

This chapter is published in Cell Death and Disease (Guadagno et al., 2013). The focus of this study was to examine the mechanism of TNFα-induced apoptosis of neural precursor cells.

2.1 Introduction

The persistence of neural precursor cells (NPCs) in distinct niches of the adult brain and spinal cord suggests the potential for regeneration in the affected nervous system (Taupin and Gage, 2002). Indeed, numerous studies have reported evidence of increased neurogenesis in animal models of cerebral ischemia, epilepsy, and spinal cord injury, as well as in models of neurodegenerative disease including Alzheimer's, Parkinson's, and Huntington's disease (Whitney et al., 2009). However, the hostile environment of the injured or degenerating nervous system is known to be detrimental to the survival of NPCs and newborn neurons thereby limiting the capacity for regeneration and repair (Ekdahl et al., 2002;Arvidsson et al., 2002).

Neuroinflammation is a common feature of neurodegenerative diseases including Alzheimer's and Parkinson's disease, as well as acute neurological conditions such as stroke and spinal cord injury (Perry et al., 2010). Neuroinflammatory processes can have both beneficial and detrimental effects on neurogenesis in the affected nervous system depending on the nature and duration of the inflammatory response (Ekdahl et al., 2009; Whitney et al., 2009). Microglia cells are the innate immune cells of the central nervous system and are primary regulators of neuroinflammatory responses (Tambuyzer et al., 2009). During brain injury microglia cells become activated and produce a number of anti- and pro-inflammatory factors that can modulate neurogenesis (Whitney et al., 2009). On the one hand, it has been reported that microglia can produce growth factors and chemokines that can promote the proliferation and recruitment of NPCs to sites of injury (Aarum et al., 2003;Walton et al., 2006). On the other hand, microglia can also produce

pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 as well as reactive oxygen species that can inhibit neurogenesis and induce NPC apoptosis (Cacci et al., 2008;Ekdahl et al., 2003;Hoehn et al., 2005;Monje et al., 2003). TNFα can signal through its cognate receptors TNFR1 and TNFR2 to promote cell survival or cell death (Cabal-Hierro and Lazo, 2012;Micheau and Tschopp, 2003). Moreover, depending on the cellular context TNFα can induce cell death via caspase-8-mediated apoptosis or RIPK1-mediated necroptosis (Degterev et al., 2005;Vandenabeele et al., 2010). However, the mechanism by which TNFα signaling affects NPC survival has not been defined.

The Bcl-2 gene family consists of pro-apoptotic and pro-survival members that interact physically and functionally to regulate apoptosis (Youle and Strasser, 2008). In response to apoptotic stimuli the Bcl-2 family members Bax and/or Bak oligomerize in the mitochondria and induce membrane permeabilization leading to the release of factors such as cytochrome-c and Smac/Diablo that promote caspase activation and apoptotic cell death (Zou et al., 1999;Wei et al., 2001). Bax/Bak activation requires the actions of a third group of Bcl-2 family proteins known as the BH3-domain-only subfamily that promote apoptosis by binding to and neutralizing pro-survival Bcl-2 proteins such as Bcl-2, Bcl-XL and Mcl-1 (Cheng et al., 2001). Several BH3-domain only proteins have been identified and specific members can be activated through transcriptional mechanisms and/or post-translational mechanisms (Puthalakath and Strasser, 2002). For example, the BH3-only family member Puma is known to be regulated through p53-mediated transcriptional activation whereas the BH3-only protein Bid is regulated primarily through proteolytic cleavage (Li et al., 1998;Han et al., 2001;Nakano and Vousden, 2001). The existence of multiple BH3-domain only proteins and activation pathways is thought to underlie the cell type and stimulus-specific nature of apoptosis regulation. Importantly, the Bcl-2 family proteins involved in the regulation of NPC apoptosis induced by neuroinflammatory conditions has not been investigated and is the focus of this study.

The nuclear factor kappaB (NF-kB) family of transcription factors are ubiquitously expressed and can be activated by a diverse array of stimuli (Hayden and Ghosh, 2012). NF-kB complexes can regulate the expression of genes that either promote survival or cell death depending on the cellular milieu (Dutta et al., 2006;Perkins and Gilmore, 2006). In

the present study we demonstrate that TNFα produced by lipopolyssacharide-activated microglia induces NPC apoptosis via a mechanism involving the NF-kB dependent transcriptional induction of the BH3-only family member Puma. Furthermore, we demonstrate that Puma plays a critical role in regulating NPC apoptosis induced by activated microglia in vitro and in the neuroinflammatory environment of the injured spinal cord in vivo.

2.2 Materials and Methods

2.2.1 Animals

Mice carrying a targeting null mutation for Bax were obtained from Jackson Laboratories (Bar Harbor, ME) and were genotyped as previously described (Cregan et al., 1999). Mice carrying a targeted null mutation for Puma were generated and maintained on a C57/BL6 background in the laboratory of Dr. Andreas Strasser (WEHI, Victoria, Australia) and genotyping of these mice was performed as previously described (Villunger et al., 2003). K1-15-EGFP and ACTB-EGFP transgenic mice that express enhanced green fluorescent protein (EGFP) under the control of the mouse keratin complex-I gene 15 promoter and the chicken beta-actin promoter respectively were obtained from Jackson Laboratories. For the transplantation experiments transgenic ACTB-EGFP mice were crossed with $Puma^{-/-}$ mice to generate $Puma^{+/-}$/EGFP mice. These progeny were then crossed to generate $Puma^{+/+}$/EGFP and $Puma^{-/-}$/EGFP littermates from which NPCs were harvested and expanded for spinal cord transplantation experiments. Timed pregnant wild-type CD1 mice were purchased from Charles River Laboratories.

2.2.2 Neural precursor cell cultures

Neural precursor cells were dissociated from the striatum of E13.5 mice and grown as neurospheres for 7 days in neural stem cell media consisting of DMEM-F12 containing d-

glucose (6 mg/ml), l-glutamine (2 mM), penicillin/streptomycin, insulin (20 µg/ml), apotransferrin (100 µg/ml), progesterone (0.02 nM), putrescine (20 nM), sodium selenite (30 nM), heparin (0.3 nM) and bFGF (10 ng/ml) as previously described (Tropepe et al., 1999). Neurospheres were then dissociated into a single cell suspension using the NeuroCult chemical dissociation kit (Stem Cell Technologies, Vancouver, BC) and plated on dishes coated with poly-l-ornithine and laminin (Sigma, Oakville, ON).

2.2.3 Microglia cell culture and preparation of microglia conditioned media

The mouse micrgolial cell line EOC20 was obtained from the American Type Culture Collection (ATCC CRL-2469). Cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 4mM l-glutamine and 20% conditioned medium from bone-marrow derived Ladmac cells (ATCC CRL-2420) as a source of colony stimulating factor-1. To prepare microglia conditioned media (CM), EOC20 cells were grown to 60% confluence at which point their media was removed and replaced with neural stem cell media (lacking bFGF and heparin). To activate microglia, NPC media was supplemented with 10 ng/ml lipopolysacchardide (LPS, Sigma, Oakville, ON) for 24 hours. Microglia conditioned media (MCM) was collected, centrifuged and filtered through a 0.2 µm filter to remove cells and debris. MCM was then supplemented with10 ng/ml bFGF and 0.3 nM heparin and immediately used for NPC cultures. In indicated experiments LPS was added to non-activated MCM or unconditioned stem cell media immediately before adding to NPC culture. LPS-activated MCM was added to NPCs either undiluted (100%) or in indicated experiments diluted with conditioned media from unactivated microglia to yield 25, 50 or 75% activated MCM.

2.2.4 NPC treatments and TNFα neutralization experiments

NPCs were treated with microglia conditioned media or recombinant mouse TNFα (rTNFα, R&D Systems) 2 days after plating as a monolayer. In the indicated experiments, the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-

fluoromethylketone (z-VAD-FMK, Santa Cruz Biotech) or the NF-kB inhibitor Bay 11-7082 (Santa Cruz Biotech) was added to NPC cultures simultaneously with the switch to MCM or rTNFα treatment. For TNFα neutralization experiments, MCM was supplemented with 10 µg/ml anti-mouse TNFα/TNFSF1A (R&D Systems) or 10 µg/ml normal goat IgG as a control and incubated for 1 hour before adding to NPC cultures.

2.2.5 Cell death assays

NPC apoptosis was assessed by examining nuclear morphology in Hoechst 33342 stained cells as previously described (Steckley et al., 2007). NPCs were stained with 1 µg/ml Hoechst 33342 (Sigma, Oakville, ON) and the fraction of cells exhibiting an apoptotic nuclear morphology characterized by chromatin condensation and/or apoptotic bodies, was determined. In certain experiments NPC death was determined by Live/Dead assay according to manufacturer's instructions (Invitrogen) to account for apoptotic and non-apoptotic cell death. Briefly NPCs were stained with Calcein-AM (2 µM) and ethidium homodimer (4 µM) for 20 minutes and the fraction of live (Calcein-AM positive) and dead (ethidium positive) cells was scored. NPCs were visualized by fluorescence microscopy (IX70, Olympus) and images were captured with a CCD camera (Q-imaging, Burnaby, BC) and Northern Eclipse software (Empix Imaging, Mississauga, ON). Images were captured and scored by an observer blinded to the treatment. A minimum of 500 cells from 5 randomly selected fields were analyzed for each treatment and data represents the mean and SEM from a minimum of 4 independent experiments.

2.2.6 Determination of mitochondrial depolarization by Mitotracker Red staining

Mitochondrial membrane potential was assessed using the potentiometric dye Mitotracker Red as per manufacturer's instructions (Molecular Probes Inc, Eugene, OR). Mitotracker Red is selectively incorporated into mitochondria with an intact transmembrane potential

and loss of mitochondrial staining can be used as indicator of mitochondrial depolarization. NPCs were incubated with Mitotracker Red (50nM) for 30 minutes at 37°C and counterstained with Hoechst 33342. Images were captured as described above and the fraction of Mitotracker Red positive and negative cells relative to the total cell number (Hoechst labeled) was scored. Data is presented as the fraction of cells exhibiting mitochondrial depolarization (Mitotracker Red negative). A minimum of 500 cells from 5 randomly selected fields were analyzed for each treatment and data represents the mean and SEM from a minimum of 4 independent experiments.

2.2.7 Caspase-3-like activity assay

NPCs were harvested in lysis buffer (1 mM KCl, 10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 1 mM PMSF, 5 µg/ml leupeptin, 2 µg/ml aprotinin, and 10% glycerol) and 10 µg of protein was used in caspase-3-like activity assay as previously described (33). Briefly protein samples were added to caspase reaction buffer [25 mM HEPES (pH 7.4), 10 mM DTT, 10% sucrose, 0.1% CHAPS, and 10 µM N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC)] and fluorescence produced DEVD-AFC cleavage was measured on a SpectraMax M5 fluorimeter (excitation 400 nm, emission 505 nm) over a 1h interval. Caspase-3-like activity is reported as the ratio of the fluorescence output in NPCs cultured in microglia conditioned media to NPCs cultured in unconditioned stem cell media.

2.2.8 TNFα ELISA

Conditioned stem cell media from microglia either left unstimulated or stimulated with 10 ng/ml LPS (Sigma) was collected at 0, 4, 12, or 24 hours. TNFα levels were detected using the Quantikine ELISA kit (R&D Systems) as per manufacturer's instructions. Briefly, conditioned media samples were added to microplates pre-coated with mouse polyclonal TNFα antibody. Following incubation and washes to remove unbound TNFα, an enzyme-

linked mouse polyclonal antibody was added. The addition of the substrate yields a colorimetric product and the absorbance (450 nm) was measured using a microplate reader. Samples were assayed in duplicate and TNFα concentrations were determined from a standard curve using SoftmaxPro software.

2.2.9 Quantitative real-time RT-PCR

RNA was isolated using Trizol reagent as per manufacturer's instructions (Invitrogen) and 10 ng of RNA was used in one-step Sybr green reverse transcription (RT)-PCR (QuantiFast, Qiagen). RT-PCR was carried out on a Chromo4 system (MJ Research BioRad) and changes in gene expression were determined by the Δ(ΔCt) method using S12 transcript for normalization as previously described (Steckley et al., 2007). Data is reported as fold increase in mRNA levels in treated samples relative to corresponding untreated control cells for each transcript. All PCR's exhibited high amplification efficiency (>90%) and the specificity of PCR products was confirmed by sequencing. Primer sequences used for gene specific amplification are available on request.

2.2.10 Western blot analysis

To prepare whole cell lysates NPCs were incubated in lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH8, 1 mM EDTA, 1µM DTT and protease and phosphatase inhibitor cocktail (Invitrogen) for 20 minutes on ice and soluble extract was recovered by centrifugation. Protein concentration was determined by BCA assay (Pierce, Rockford, IL) and 40 µg of protein was separated on 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked for 1h in TBS-T (10mM Tris, 150mM NaCl, 0.05% Tween-20), followed by overnight incubation in primary antibodies to Bid/ tBid (R&D Systems), Puma, phospho(ser536)-p65 (Cell Signalling Technology), Tnfr1 or Actin (Santa Cruz Biotech) in TBS-T containing 5% skim milk. Membranes were washed with TBS-T and incubated for 1h with the appropriate

HRP-conjugated secondary antibodies and developed by enhanced chemiluminescence system according to manufacturer's instructions (ThermoScientific).

2.2.11 Mouse model of spinal cord injury (SCI) and NPC transplantation

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the Guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. A total of 12 adult female C57B6-Kr15-EGFP mice weighing 18-22g were anesthetized with Ketamine (100mg/kg) and xylazine (10mg/kg) and subjected to spinal cord injury as previously described (Xu et al., 2011b). Kr15-EGFP mice which express EGFP exclusively in hair follicle bulge cells were used in these experiments to avoid a potential immunogenic response induced by transplantation of EGFP expressing NPCs. Briefly, the vertebral column was stabilized at T6 and T10 and a laminectomy was performed by exposing the dura matter at spinal cord level T7-8. Spinal cord contusion was performed using the Infinite Horizon Impactor (Precision Systems and Instrumentation, Fairfax, VA) with 50kdyn and 1 second dwelling time. After surgery, mice were kept at 37°C and closely monitored. Urine was expressed manually twice per day, and mice were checked daily to monitor their overall health. Neural precursor cell transplantation was performed 7 days after SCI. The dura was incised with the tip of an injection needle exposing the surface of the injured spinal cord. $Puma^{+/+}$/EGFP NPCs ($3x10^4$ cells/3µl, 6 mice) or $Puma^{-/-}$/EGFP NPCs ($3x10^4$ cells/3 µl, 6 mice) were injected into the cord at the lesion site using a spinal stereotaxic frame by means of a glass pipette (tip diameter 100 µm) configured to a 10 µl Hamilton syringe. Each SCI mouse was transplanted with NPCs derived from an independent $Puma^{-/-}$/EGFP or $Puma^{+/+}$/EGFP donor mouse (n=6 for each genotype).

2.2.12 Immunohistochemistry

At 21 d after injury, mice were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). The T2-L1 vertebral segments, which included the site of the contusion injury, were removed and processed for cryo-sectioning and immunohistochemistry as previously described (Xu et al., 2011a). Transverse sections (16 µm) of spinal cord were serially collected and mounted on glass slides. Every 10^{th} section (25 sections out of 250 sections from each cord), spanning approximately 4 mm in length across the lesion site (~2 mm caudal to ~2 mm rostral to the lesion epicenter), was stained for EGFP expression. The slides were incubated with rabbit anti-EGFP antibody (1:300; Invitrogen, Carlsbad, CA) in a humidified chamber at 4°C overnight and the signal was visualized by a peroxidase-DAB reaction (Zymed, Carlsbad, CA) and hematoxylin counter-stain. The immuno-stained sections were examined using an Olympus epifluorescence microscope (BX51) and the total number of EGFP+ NPCs in the 25 sections/ animal was counted.

2.2.13 Data analysis

Data is reported as mean and standard error of the mean. The n value represents the number of independent experiments and/or number of mice from which independent NPC cultures were prepared. Data was analyzed by one-way ANOVA followed by Tukeys post-hoc test and differences were considered significant at $p<0.05$. All statistical analyses were conducted using GraphPad Prism software.

2.3 Results

2.3.1 Soluble factors released by activated microglia induce NPC apoptosis

To investigate the mechanism by which microglia cells trigger neural precursor cell (NPC) apoptosis we utilized the EOC-20 mouse microglia cell line as a homogeneous and renewable source of microglia cells (Walker et al., 1995). We first examined whether EOC-20 microglia cells when activated by the bacterial endotoxin lipopolysaccharide (LPS) to induce a pro-inflammatory phenotype secrete factors that promote NPC apoptosis. To examine the effects of microglia derived factors on NPC survival, culture media was removed from adherent NPCs and replaced with unconditioned stem cell media or conditioned stem cell media from either unactivated microglia or LPS-activated microglia and apoptosis was assessed by Hoechst 33342 staining. As shown in figure 2.1A and 2.1B, the fraction of apoptotic cells was increased in NPCs cultured in LPS-activated microglia conditioned stem cell media (MCM) in a concentration-dependent manner (Fig. 2.1A and 2.1B). In contrast, NPC apoptosis was not increased by conditioned media from unactivated microglia or unconditioned media directly supplemented with LPS (Fig. 2.1A and 2.1B). Consistent with this, NPCs treated with conditioned media from activated microglia but not unactivated microglia exhibited a significant increase in caspase-3-like activity (Fig. 2,1C). Furthermore, cell death induced by activated microglia conditioned media as assessed by Live/Dead assay was markedly reduced in the presence of the pan-caspase inhibitor z-VAD-FMK consistent with a predominate role of apoptotic cell death in these conditions (Fig. 2.1D). Taken together these findings indicate that soluble factors released from activated microglia can trigger apoptosis in NPCs.

Figure 2.1. LPS-activated microglia release soluble factors that induce NPC apoptosis.

NPCs were cultured for 2 days in stem cell media and then media was replaced with either unconditioned stem cell media (C) or microglia conditioned stem cell media from unactivated microglia (0% MCM) or LPS-activated microglia (25-100% MCM). Activated microglia conditioned media was left undiluted (100%) or diluted to 75%, 50%, or 25% with unactivated microglia conditioned media. NPCs maintained in unconditioned stem cell media and directly treated with LPS (10ng/ml) are labeled as LPS. **(A)** NPCs were stained at 72 hours with Hoechst 33342 and the fraction of apoptotic cells was determined by examining nuclear morphology (n=4, *$p<0.05$). **(B)** Representative images of Hoechst stained NPCs maintained in unconditioned stem cell media (Ctrl), or treated with conditioned media from unactivated microglia (MCM) or LPS-activated microglia (a-MCM) for 72 hours. Note the marked increase in NPCs exhibiting chromatin condensation and pyknotic/fragmented nuclei following incubation in conditioned media from LPS-activated microglia. Nestin immunostaining (green) demonstrates that the vast majority of NPCs remain in an undifferentiated state. Scale bar, 20μm. **(C)** Protein extracts were obtained from NPCs at 72 hours and assayed for caspase-3-like activity. Caspase-3 activity in NPCs treated with microglia conditioned media is reported as fold increase over that in NPCs cultured in unconditioned stem cell media (n=3, *$p<0.05$). **(D)** NPCs were cultured in unconditioned stem cell media (C), conditioned media from unactivated microglia (MCM) or conditioned media from LPS-activated microglia (a-MCM) in the presence of the pan-caspase inhibitor z-VAD-FMK (100μM) or DMSO as a vehicle control. The fraction of dead (ethidium positive) NPCs was determined by Live/Dead assay at 48 hours (n=4, *$p<0.05$).

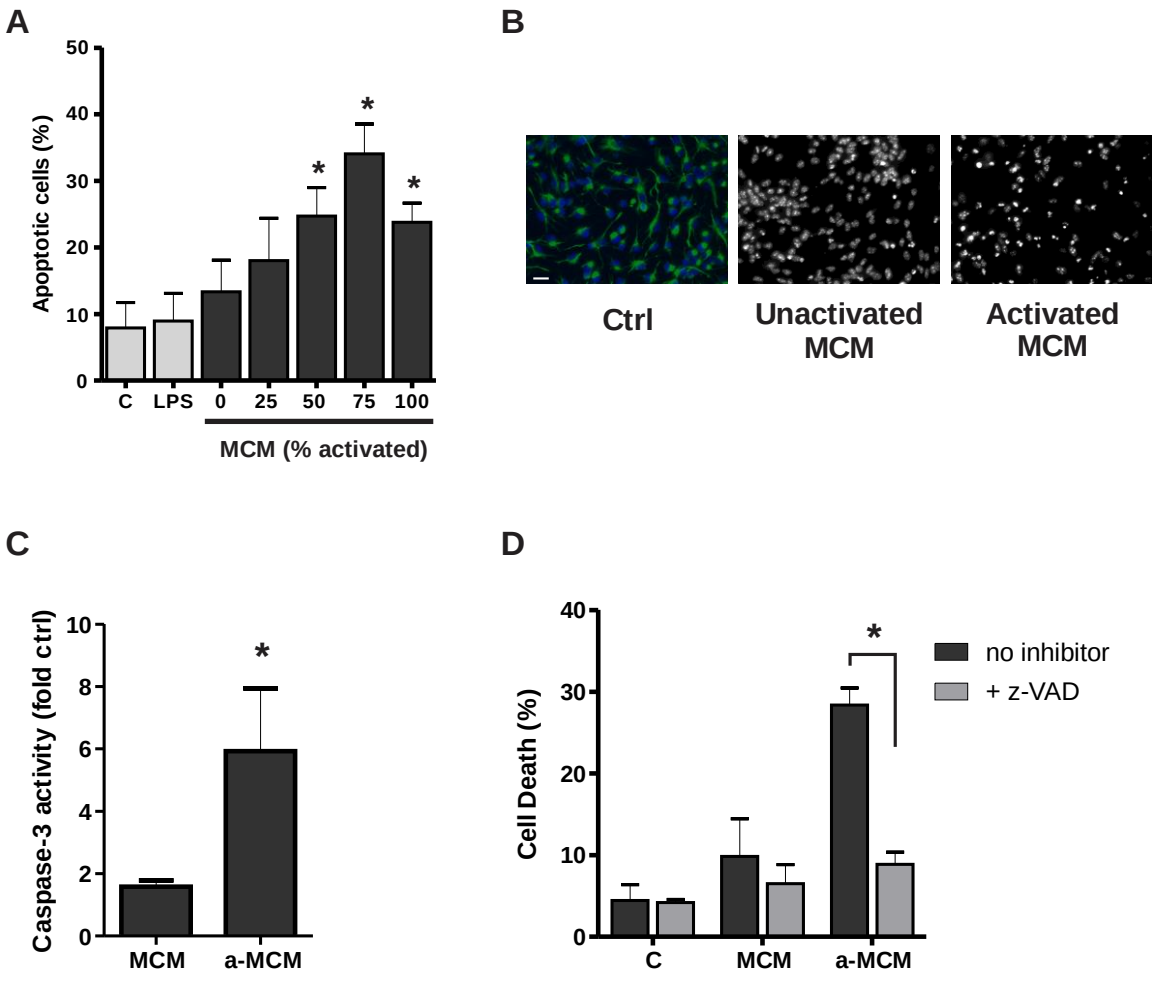
A
Apoptotic cells (%)
50
40
30
20
10
0
C
LPS
0
25
50
75
100
MCM (% activated)
B
Ctrl
Unactivated MCM
Activated MCM
C
Caspase-3 activity (fold ctrl)
10
8
6
4
2
0
MCM
a-MCM
D
Cell Death (%)
40
30
20
10
0
C
MCM
a-MCM
no inhibitor
+ z-VAD

Figure 2.1

2.3.2 TNFα released from activated microglia induces NPC apoptosis

Activated microglia release a variety of soluble factors including reactive oxygen/nitrogen species, chemokines, and both pro- and anti-inflammatory cytokines (Tambuyzer et al., 2009). ELISA analysis of conditioned media from LPS-stimulated microglia revealed a significant increase in TNFα levels as well as several other pro-inflammatory factors in conditioned media from LPS-stimulated microglia as with unactivated microglia (Fig. 2.2A, data not shown). TNFα is a potent pro-inflammatory cytokine that has been reported to affect neurogenesis (Ben-Hur et al., 2003;Cacci et al., 2005;Liu et al., 2005;Monje et al., 2003;Wong et al., 2004) and exerts its effects on cells by activating two receptor subtypes on target cell membranes: TNF receptor-1 (TNFR1), and TNF receptor-2 (TNFR2) (Cabal-Hierro & Lazo, 2012). We examined the expression of TNFR1 and TNFR2 in NPCs treated with microglia conditioned media by qRT-PCR and western blot. NPCs exposed to microglia conditioned media exhibited a marked increase in Tnfr1 mRNA levels (Fig. 2.2B) as well as a corresponding increase in TNFR1 protein levels (Fig. 2.2C). In contrast, Tnfr2 mRNA levels were not altered by treatment with microglia conditioned media (Fig. 2.2B) and TNFR2 protein was not detectable in NPCs. Given this increase in TNFR1 expression we examined whether TNFα produced by activated microglia contributes to NPC apoptosis. To address this we pre-treated microglia conditioned media with a TNFα neutralizing antibody or IgG antibody as a control and then examined NPC apoptosis. The efficacy of the antibody to neutralize TNFα in microglia conditioned media was verified by ELISA (Fig. 2.2A). As shown in figure 2.2D, NPC apoptosis induced by activated microglia conditioned media was significantly reduced in the presence of the TNFα neutralizing antibody but not IgG control antibody. Furthermore, we found that addition of recombinant TNFα (rTNFα) was sufficient to induce NPC apoptosis and that this could be attenuated by the addition of the TNFα neutralizing antibody or the pan-caspase inhibitor zVAD-FMK (Fig. 2.2E and 2.2F). Taken together these results indicate that TNFα is a key mediator of NPC apoptosis induced by activated microglia.

Figure 2.2 Activated microglia derived TNFα induces NPC apoptosis.

(A) Conditioned stem cell media from unactivated microglia (MCM) or LPS-activated microglia (a-MCM) was collected at the indicated times and incubated in the presence or absence of TNFα neutralizing antibody (10μg/ml) and then assayed for TNFα levels by ELISA (n=4). **(B)** RNA was harvested from NPCs incubated with increasing concentrations of activated microglia conditioned media (MCM) for 24 hours and TNFR1 and TNFR2 mRNA levels were determined by qRT-PCR. TNFR1/2 mRNA levels in NPCs incubated in microglia conditioned media are reported as fold increase over that in NPCs incubated in unconditioned media (n=3). **(C)** NPCs were incubated in unconditioned media (C) or activated microglia conditioned media (a-MCM) for 24 hours and TNFR1 protein levels were determined by western blot. Representative blot from 3 independent experiments is shown. **(D)** NPCs were cultured in unconditioned stem cell media (C) or LPS-activated microglia conditioned media (a-MCM) in the presence of TNFα neutralizing antibody or IgG control antibody (10μg/ml) and the fraction of apoptotic cells was determined by Hoechst 33342 staining at 72 hours (n=5, *$p<0.05$). **(E)** NPCs were treated with the indicated concentrations of recombinant TNFα (rTNF) in the presence of TNFα neutralizing antibody or IgG control antibody (10μg/ml) and the fraction of apoptotic cells was determined by Hoechst staining at 72 hours (n=4, *$p<0.05$). **(F)** NPCs were treated with rTNFα (10ng/ml) in the presence or absence of the pan-caspase inhibitor zVAD-FMK (100μM) and the fraction of dead (ethidium positive) cells was determined by Live/Dead assay at 72 hours (n=4, *$p<0.05$).

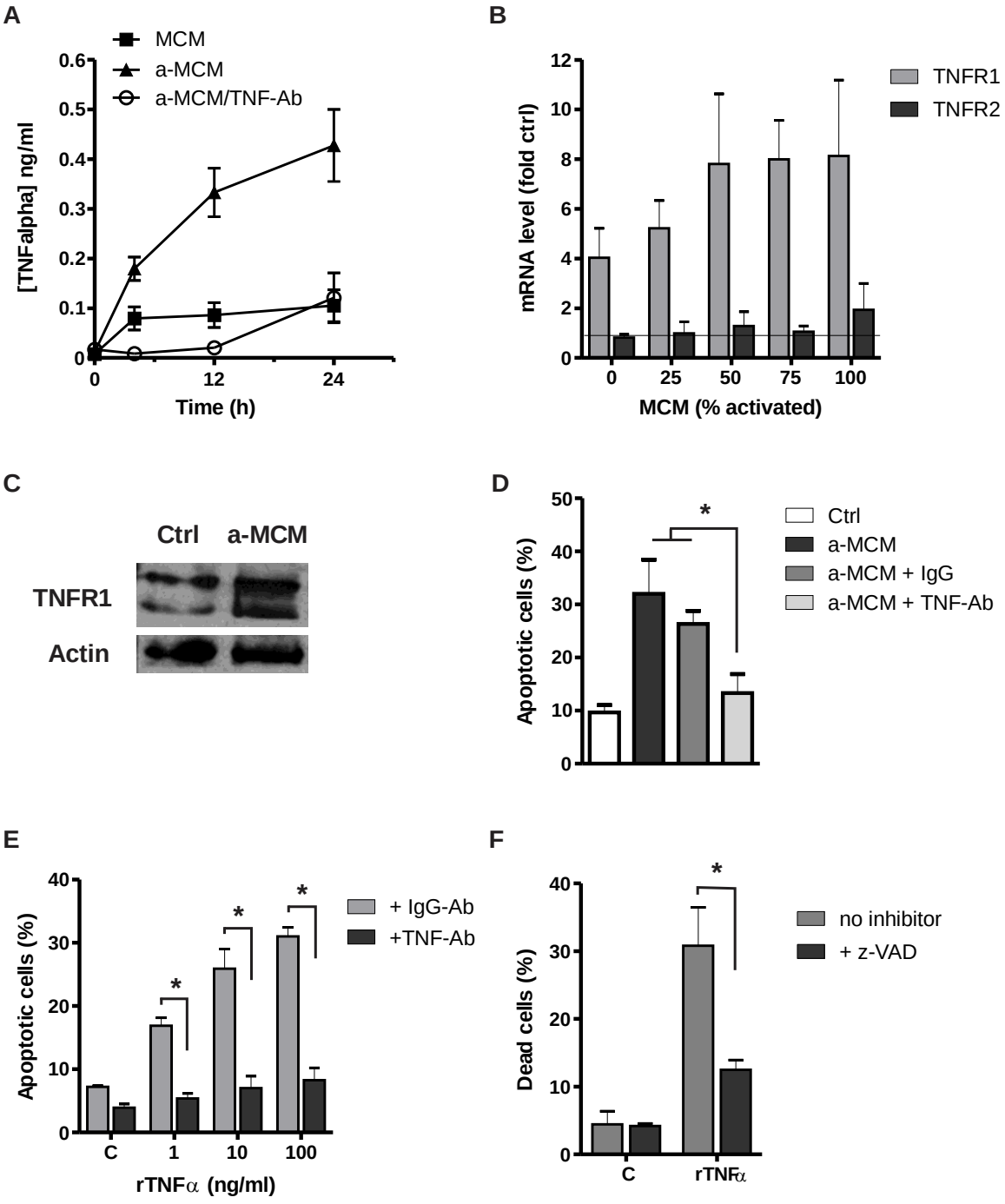
A
MCM
a-MCM
a-MCM/TNF-Ab
[TNFalpha] ng/ml
Time (h)
B
TNFR1
TNFR2
mRNA level (fold ctrl)
MCM (% activated)
C
Ctrl
a-MCM
TNFR1
Actin
D
Ctrl
a-MCM
a-MCM + IgG
a-MCM + TNF-Ab
Apoptotic cells (%)
E
+ IgG-Ab
+TNF-Ab
Apoptotic cells (%)
rTNFα (ng/ml)
F
no inhibitor
+ z-VAD
Dead cells (%)
rTNFα

Figure 2.2

2.3.3 Activated microglia/TNFα induces NPC apoptosis via a Bax mediated mitochondrial pathway

Depending on the cell type, TNFα has been reported to trigger apoptosis through either a mitochondrial-dependent pathway or a mitochondrial-independent pathway involving direct activation of caspase-3 by caspase-8 (van Raam & Salveson, 2012). The pro-apoptotic Bcl-2 family proteins Bax and Bak are known to be essential regulators of mitochondrial mediated apoptotic pathways (Wei et al., 2001). Therefore, to determine whether microglia derived TNFα induces NPC apoptosis via a mitochondrial pathway we examined survival in NPCs derived from Bax-deficient mice and wild type littermates by Live/Dead assay. As shown in figure 2.3, NPC death induced by either LPS-activated microglia conditioned media or rTNFα was markedly reduced in Bax-deficient NPC cultures. Furthermore, Mitotracker Red staining revealed that both a-MCM and rTNFα treatments caused mitochondrial depolarization in a significant portion of wild type NPCs but not Bax-null NPCs (Fig. 2.3B). These results suggest that activated microglia derived TNFα induces NPC apoptosis primarily through a mitochondrial dependent pathway regulated by the Bcl-2 protein family.

2.3.4 Activated microglia derived TNFα induces Puma expression via an NF-κB-dependent pathway

BH3-only proteins are known to play a key role in regulating Bax activation with distinct family members being activated in a cell type and stimulus specific manner (Youle and Strasser, 2008). The BH3-only protein Bid has previously been implicated in death receptor mediated apoptotic pathways (Gross et al., 1999; Li et al., 1998). It has been proposed that when engaged, death receptors such as Fas and TNFR recruit and activate caspase-8 which can then cleave Bid into its active, truncated form tBid. Therefore, we examined tBid production in NPCs treated with LPS-activated MCM. While we could detect tBid in NPCs treated with activated MCM the exposure time required to detect tBid was much longer than that for full length Bid suggesting that the amount of tBid produced was very modest

Figure 2.3. Activated microglia/TNFα-induced NPC apoptosis via a Bax mediated mitochondrial pathway.

NPCs derived from $Bax^{+/+}$ and $Bax^{-/-}$ embryos were maintained in unconditioned stem cell media (C) or treated with LPS-activated microglia conditioned media (a-MCM) or recombinant TNFα (10ng/ml). **(A)** Cell death was assessed at 72 hours by Live/Dead assay for 72 hours and is reported as the percentage of dead (ethidium positive) cells (n=4, *$p<0.05$). Representative images of Live/Dead staining of $Bax^{+/+}$ and $Bax^{-/-}$ NPCs treated with activated microglia conditioned media (a-MCM) for 72 hours are shown. Scale bar, 20μm. **(B)** Mitochondrial membrane potential was assessed at 72 hours by MitoTracker Red staining and the percentage of MitoTracker negative NPCs is reported (n=4, *$p<0.05$). Representative images of MitoTracker Red and Hoechst 33342 staining in $Bax^{+/+}$ and $Bax^{-/-}$ NPCs treated with activated microglia conditioned media (a-MCM) for 72 hours are shown. Scale bar, 20μm.

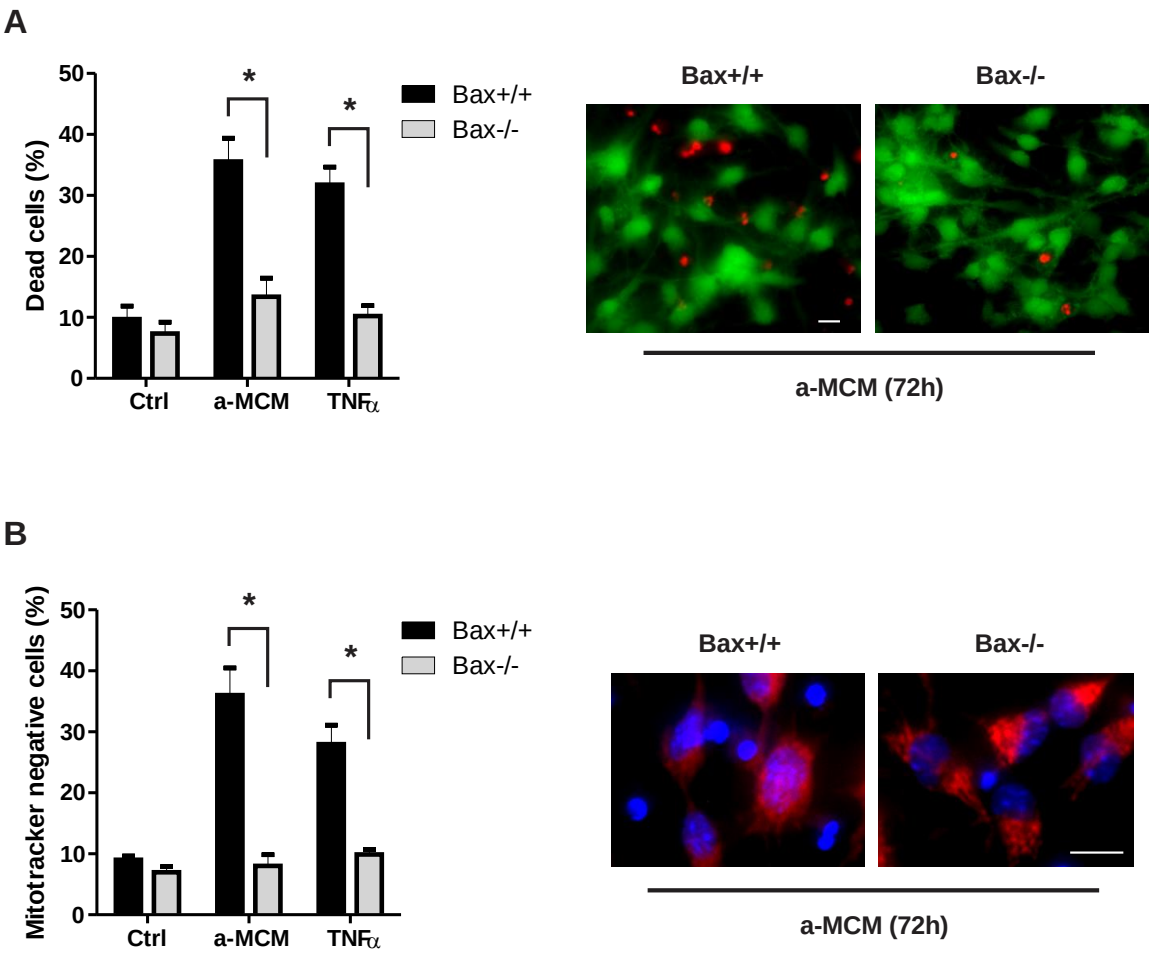
A
Dead cells (%)
50
40
30
20
10
0
Ctrl
a-MCM
TNFα
*
*
Bax+/+
Bax-/-
Bax+/+
Bax-/-
a-MCM (72h)
B
Mitotracker negative cells (%)
50
40
30
20
10
0
Ctrl
a-MCM
TNFα
*
*
Bax+/+
Bax-/-
Bax+/+
Bax-/-
a-MCM (72h)

Figure 2.3

(Fig. 2.4A). Consistent with this activated MCM did not cause an appreciable decrease in the level of full length Bid. Furthermore, tBid production did not appear to be related to TNFα (or cell death) as it was not blocked by the TNFα neutralizing antibody (Fig. 2.4A) and was not detected in NPCs treated with rTNFα. Therefore, we examined the expression levels of other BH3-only family members and interestingly found that Puma protein levels were consistently increased in response to both activated microglia conditioned media and rTNFα treatment (Fig. 2.4A). Furthermore, we found that neutralization of TNFα blocked the induction of Puma in response to microglia conditioned media (Fig. 2.4A). Since Puma expression is generally regulated at the transcriptional level we examined Puma mRNA levels by quantitative RT-PCR. As shown in figure 2.4B Puma mRNA levels were not affected in conditioned media from unactivated microglia but were markedly induced by activated MCM. Furthermore, the increase in Puma mRNA observed in response to activated microglia conditioned media was significantly reduced in the presence of the TNFα neutralizing antibody (Fig. 2.4C). These results indicate that activated microglia derived TNFα promotes Puma induction in NPCs.

NF-κB is a well-known mediator of the cellular response elicited by TNFα signaling and has been shown to regulate the transcription of both pro-survival and pro-apoptotic genes depending on the cellular context (Dutta et al., 2006;Perkins and Gilmore, 2006). Therefore, we investigated whether activated MCM induces Puma expression in NPCs via an NF-κB dependent mechanism. To test this we used the pharmacological inhibitor BAY 11-7082 that inhibits NF-κB activation by blocking cytokine induced phosphorylation of IkB-α, which binds to NF-κB subunits keeping them sequestered in an inactive state in the cytoplasm (Karin, 1999). As an indicator of NF-κB activity, we examined protein levels of serine-536 phosphorylated (active) p65 subunit. As shown in figure 2.5A phosphorylated-p65 protein levels were increased in NPCs following treatment with either MCM or rTNFα and this was abrogated in the presence of BAY 11-7082. Importantly, we found that NF-κB inhibition attenuated Puma induction in response to activated MCM and rTNFα (Fig. 2,5A and 2.5B). Consistent with this, BAY 11-7082 also significantly reduced MCM and rTNFα induced apoptosis in NPCs (Fig. 2.5C). Taken together these results suggest that NF-κB regulates Puma induction and NPC apoptosis induced by activated microglia derived TNFα.

Figure 2.4. Activated microglia derived TNFα induces the expression of the pro-apoptotic Bcl-2 family member Puma.

(A) NPCs were incubated in unconditioned stem cell media (C), unactivated microglia conditioned media (MCM) or LPS-activated microglia conditioned media (a-MCM) in the presence or absence of TNFα neutralizing antibody (10μg/ml). NPCs were harvested after 48 hours and protein extracts were subjected to SDS-PAGE and immunoblotted for Bid/tBid, Puma or Actin as a loading control.Representative blots from 3 independent experiments are shown. It should be noted that much longer exposure times were required to detect tBid relative to full length Bid. **(B)** RNA was harvested from NPCs treated with increasing concentrations of LPS-activated microglia conditioned media (MCM) for 24 hours and Puma mRNA levels were determined by qRT-PCR. Puma mRNA levels in NPCs treated with MCM is reported as fold increase over NPCs incubated in unconditioned media (n=4). **(C)** NPCs were cultured in unconditioned stem cell media or treated with LPS-activated microglia conditioned media (a-MCM) in the presence of TNFα neutralizing antibody or IgG control antibody (10μg/ml)for 24 hours and Puma mRNA levels were determined by qRT-PCR. Puma mRNA levels in NPCs treated with microglia conditioned media (+/- antibody) is reported as fold increase over NPCs incubated in unconditioned media (n=4, *$p<0.05$).

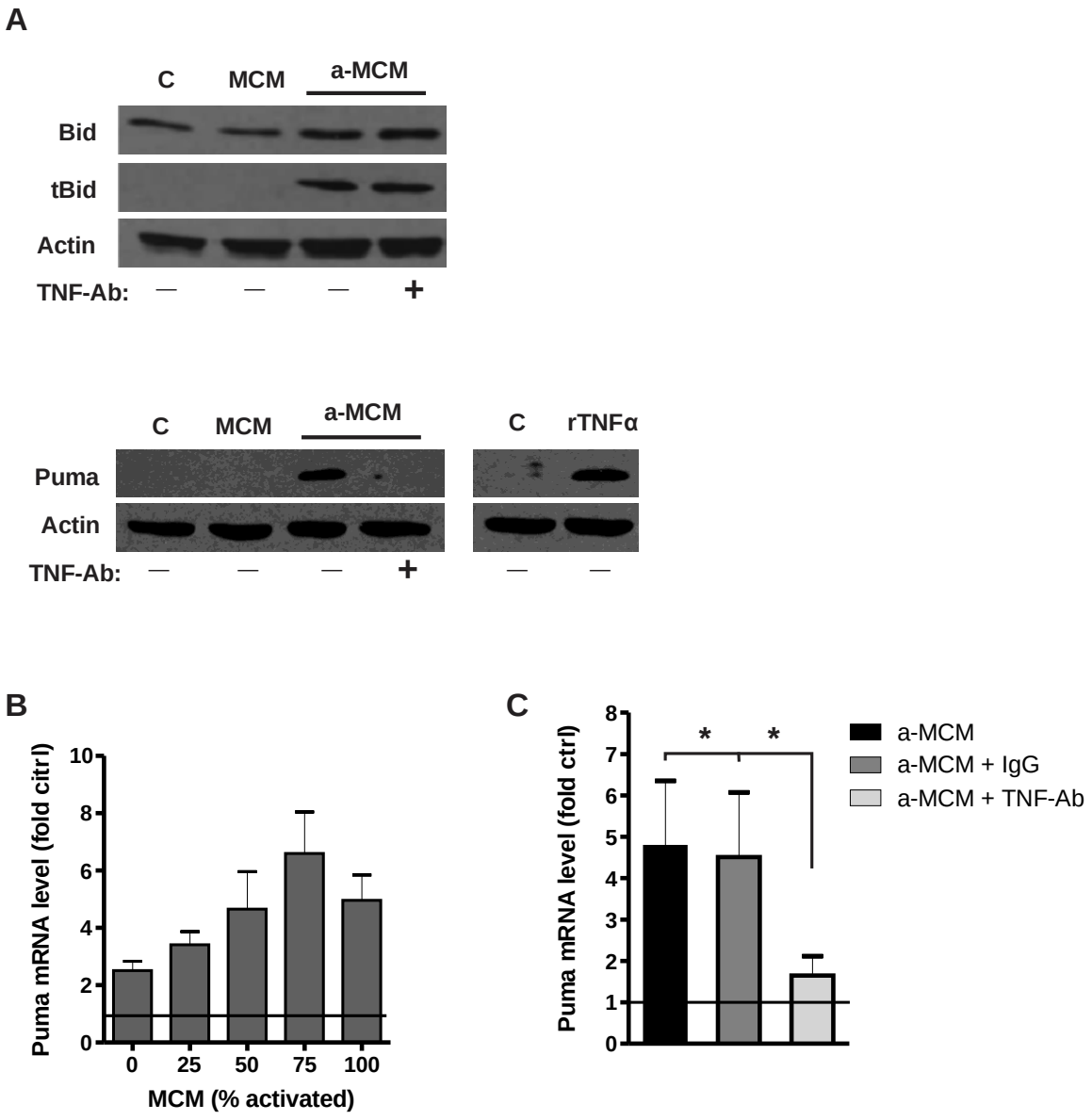
A
C
MCM
a-MCM
Bid
tBid
Actin
TNF-Ab:
Puma
rTNFα
B
Puma mRNA level (fold ctrl)
MCM (% activated)
C
a-MCM
a-MCM + IgG
a-MCM + TNF-Ab

Figure 2.4

Figure 2.5. Activated microglia derived TNFα induces Puma expression in NPCs via an NF-kB dependent pathway.

(A) NPCs were cultured in unconditioned stem cell media (C) or treated with LPS-activated microglia conditioned media (a-MCM) or rTNFα (10ng/ml) in the presence of the NF-kB inhibitor BAY-11-7082 (10μM) or DMSO (0.1%) as a vehicle control. NPCs were harvested at 48 hours and protein extracts were subjected to SDS-PAGE and immunoblotted for phoshphorylated-p65 (Ser536), Puma, and Actin as a loading control. Representative images from 3 independent experiments are shown. **(B)** NPCs were treated with LPS-activated microglia conditioned media (a-MCM) or rTNFα (10ng/ml) in the presence or absence of the NF-kB inhibitor BAY 11-7082 (10μM) for 24 hours and Puma mRNA levels were determined by qRT-PCR. Puma mRNA levels are reported as fold increase over untreated controls (n=4, *$p<0.05$). **(C)** NPCs were treated with activated microglia conditioned media (a-MCM) or rTNFα (10ng/ml) in the presence or absence of BAY 11-7082 (10μM) and the fraction of apoptotic cells was determined at 48 hours by Hoechst 33342 staining (n=4, *$p<0.05$).

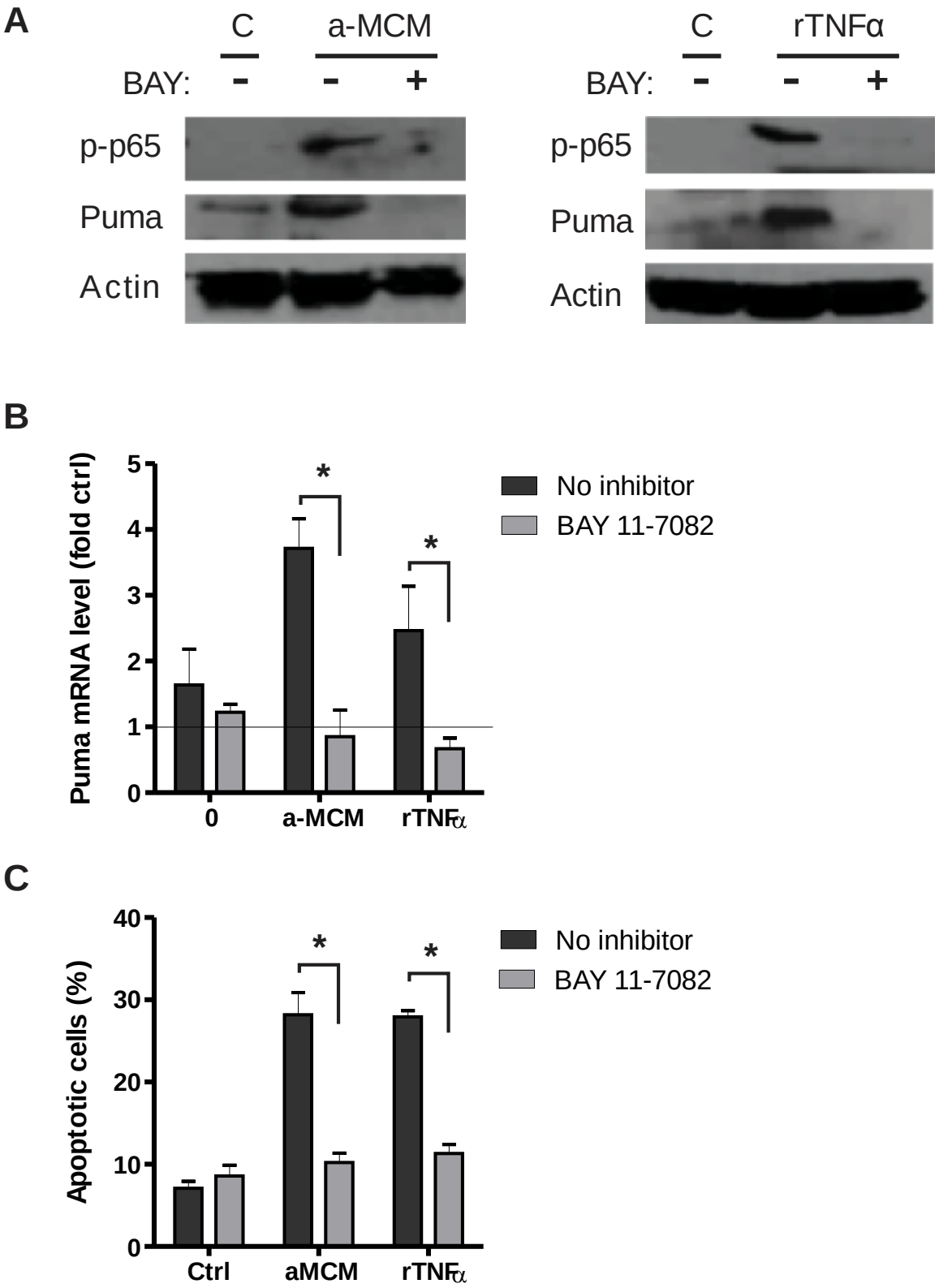
A
C
a-MCM
BAY:
-
-
+
p-p65
Puma
Actin
C
rTNFα
BAY:
-
-
+
p-p65
Puma
Actin
B
Puma mRNA level (fold ctrl)
5
4
3
2
1
0
*
*
0
a-MCM
rTNFα
No inhibitor
BAY 11-7082
C
Apoptotic cells (%)
40
30
20
10
0
*
*
Ctrl
aMCM
rTNFα
No inhibitor
BAY 11-7082

Figure 2.5

2.3.5 Puma is required for NPC apoptosis induced by neuroinflammation *in vitro* and *in vivo*

We next examined whether Puma is necessary for microglia/TNFα induced NPC apoptosis. To address this we compared apoptotic frequencies in NPCs derived from Puma$^{-/-}$ mice and their wild type littermates following treatment with conditioned media from unactivated microglia or increasing concentrations of conditioned media from LPS-activated microglia. As shown in Figure 2.6A and 2.6B, apoptosis induced by activated MCM was markedly reduced in Puma$^{-/-}$ NPCs as compared to wild type NPCs. Similarly, we found that rTNFα induced apoptosis was attenuated in Puma-deficient NPCs (Fig. 2.6C). Taken together these results indicate that Puma is required for the induction of NPC apoptosis by activated microglia derived TNFα. Spinal cord injury induces an inflammatory response associated with the infiltration of microglia and peripheral immune cells and is associated with elevated levels of a variety of inflammatory cytokines including TNFα (Conti et al., 2003;Genovese et al., 2008;Pineau and Lacroix, 2007a). Furthermore, several studies have reported that inflammatory processes in the injured spinal cord markedly reduce the survival of transplanted NPCs (Lee et al., 2009;Oh et al., 2011). As we have identified Puma as a key regulator of microglia induced NPC death *in vitro*, we next examined whether Puma plays an important role in regulating NPC death in the inflammatory environment of the injured spinal cord *in vivo*. To address this we first bred Puma$^{-/-}$ mice with transgenic mice ubiquitously expressing enhanced green fluorescent protein under control of the chicken beta-actin promoter (ACTB-EGFP). Puma$^{+/-}$/EGFP+ progeny were then bred to generate Puma$^{+/+}$/EGFP+ and Puma$^{-/-}$/EGFP+ embryos from which EGFP-labeled NPCs were harvested and expanded for transplantation. K1-15-EGFP transgenic mice that express EGFP exclusively in keratinocytes were used as recipient mice for NPC transplants to avoid potential immunogenic responses to EGFP expression by transplanted NPCs. K15-EGFP mice received a contusion injury at the T7-8 segment of the spinal cord, and one week after injury the mice received an intraspinal injection of equal numbers of NPCs isolated from either Puma$^{-/-}$/EGFP mice or Puma$^{+/+}$/EGFP mice. Three weeks after spinal cord injury mice were euthanized and the number of surviving EGFP+ NPCs remaining in the lesioned spinal cords of mice transplanted with Puma$^{+/+}$/EGFP+ or Puma$^{-/-}$/EGFP+ NPCs was evaluated. As shown in Figure 2.7A and 2.7B, a substantial number

Figure 2.6. Puma is required for activated microglia/TNFα-induced NPC apoptosis.

(A) NPCs derived from $Puma^{+/+}$ and $Puma^{-/-}$ embryos were maintained in unconditioned stem cell media (C) or treated with increasing concentrations of LPS-activated microglia conditioned media (MCM) and the fraction of apoptotic cells was determined by Hoechst staining at 72 hours (n=5, *p<0.05, **p<0.01). **(B)** Representative images of Hoechst staining in $Puma^{+/+}$ and $Puma^{-/-}$ NPCs treated for 72 hours with LPS-activated microglia conditioned media (a-MCM). Scale bar, 20μm. **(C)** $Puma^{+/+}$ and $Puma^{-/-}$ NPCs were treated with the indicated concentrations of rTNFα and the fraction of apoptotic cells was determined by Hoechst staining at 72 hours (n=5, *p<0.05).

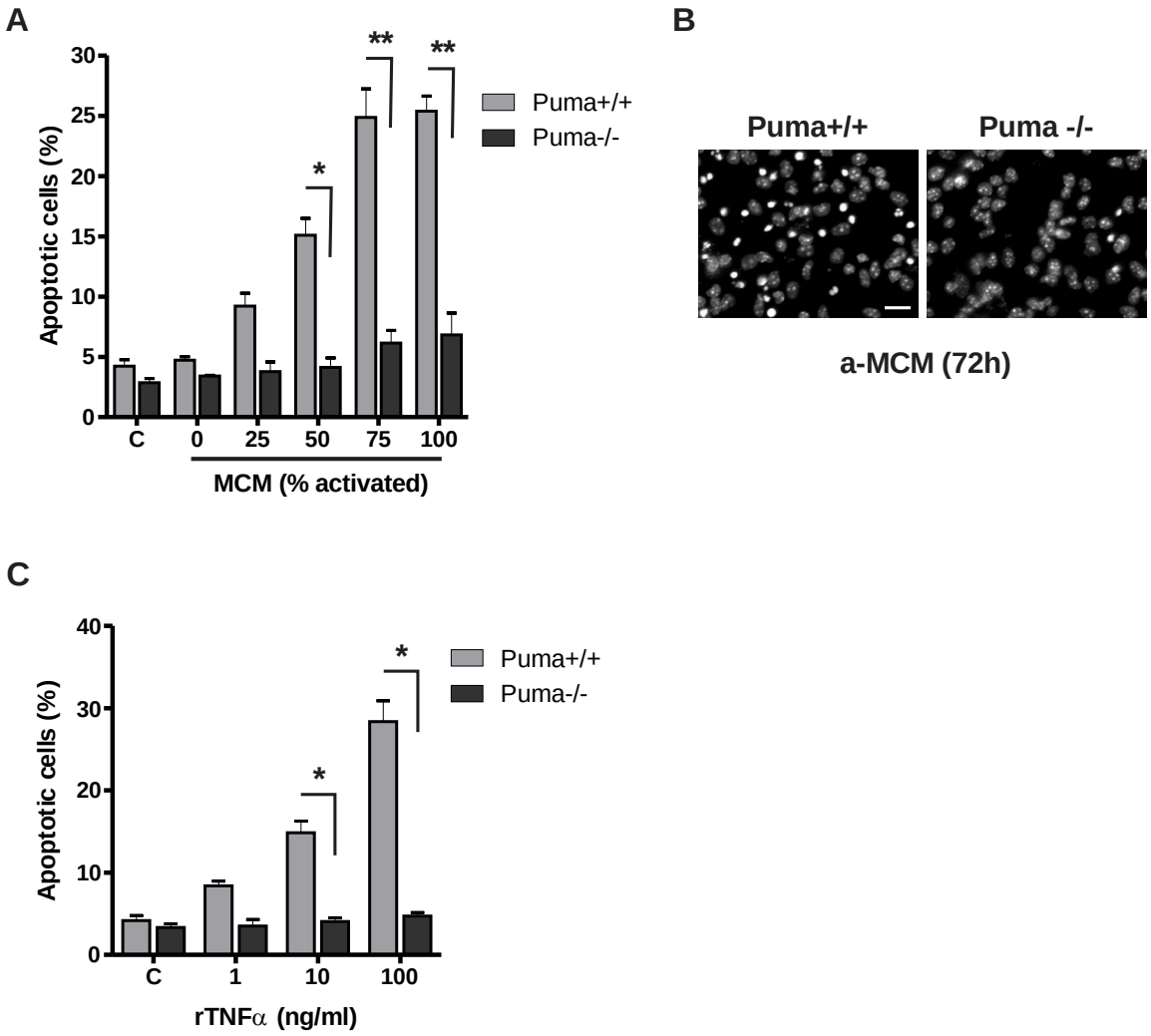
A
Apoptotic cells (%)
30
25
20
15
10
5
0
**
**
*
Puma+/+
Puma-/-
C
0
25
50
75
100
MCM (% activated)
B
Puma+/+
Puma -/-
a-MCM (72h)
C
Apoptotic cells (%)
40
30
20
10
0
*
*
Puma+/+
Puma-/-
C
1
10
100
rTNFα (ng/ml)

Figure 2.6

of transplanted Puma$^{-/-}$/EGFP+ NPCs remained in the lesioned region of the cord and the majority of the engrafted cells were located at the lesion epicenter. In contrast, transplanted Puma$^{+/+}$/EGFP+ NPCs were rarely detected at the lesion epicenter and only a few were found at the interface between the lesion epicenter and the spared neural tissue (Fig. 2.7C and 2.7D). Indeed, the number of engrafted Puma$^{-/-}$ NPCs remaining in the lesioned spinal cord was approximately 13-fold greater than that of Puma$^{+/+}$ NPCs (3828 ± 1219 vs 297 ± 149, Fig. 2.7E). These results suggest that similar to the situation *in vitro*, Puma plays a prominent role in regulating NPC survival during neuroinflammation *in vivo*.

In summary, we have determined that TNFα produced by activated microglia induces NPC apoptosis via the NF-κB mediated induction of the pro-apoptotic Bcl-2 family member Puma. Furthermore, we demonstrate that Puma plays a key role in the regulation of NPC survival during neuroinflammatory responses both *in vitro* and in the injured nervous system *in vivo*.

2.1 Discussion

Neuroinflammation is a common feature of acute neurological injuries as well as many chronic neurodegenerative conditions and several studies have demonstrated that during the acute phase neuroinflammatory processes can induce apoptosis in NPCs and immature neurons and inhibit neurogenesis (Ekdahl et al., 2003;Hoehn et al., 2005;Liu et al., 2007;Monje et al., 2003). Microglia are key regulators of neuroinflammation and depending on the nature of their activation can produce anti-inflammatory and/or pro-inflammatory factors and exert either beneficial or detrimental effects on neurogenesis (Ekdahl et al., 2009;Whitney et al., 2009). Previous studies have demonstrated that microglial cells stimulated with LPS to induce a pro-inflammatory response secrete factors that induce NPC apoptosis although the underlying mechanisms were not examined (Cacci et al., 2008;Monje et al., 2003). Importantly, while previous studies have suggested a correlation between TNFα and NPC death our study is the first to directly implicate TNFα

Figure 2.7. Transplanted Puma-deficient NPCs exhibit increased survival in the injured mouse spinal cord.

K15-EGFP mice received a contusion injury at the T7-8 segment of the spinal cord. One week after injury, animals received an intraspinal injection of NPCs isolated from either $Puma^{+/+}$/EGFP mice ($3x10^4$ cells) or $Puma^{-/-}$/EGFP mice ($3x10^4$ cells) at the lesion site. Three weeks following injury mice were sacrificed and spinal cord sections spanning the lesion site were prepared and immunostained with EGFP antibody and visualized by DAB reaction and hematoxylin counter-stain. Representative images of EGFP stained sections from the lesion site of spinal cord injured mice transplanted with $Puma^{-/-}$/EGFP NPCs **(A, B)** or $Puma^{+/+}$/EGFP NPCs **(C, D)**. Scale bars, 100µm and 10µm (insets). **(E)** The number of EGFP+ cells remaining in the injured spinal cord of mice transplanted with $Puma^{+/+}$/EGFP NPCs (n=6 mice) and $Puma^{-/-}$/EGFP NPCs (n=6 mice) was counted in 25 sections/ animal and the data is reported as the mean ± sem (n=6, *$p<0.05$).

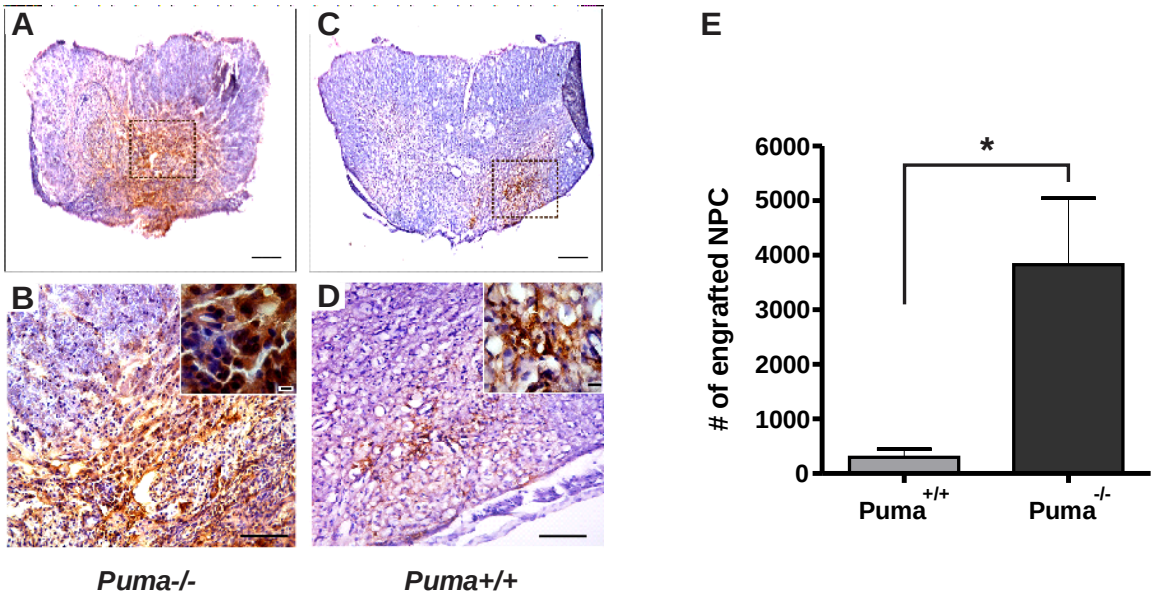
A
C
B
D
Puma-/-
Puma+/+
E
# of engrafted NPC
6000
5000
4000
3000
2000
1000
0
*
Puma+/+
Puma-/-

Figure 2.7

as a key determinant in microglia induced NPC apoptosis. Specifically, we demonstrate that neutralization of microglia derived TNFα blocks NPC apoptosis induced by activated microglia conditioned media and that recombinant TNFα is sufficient to induce NPC death. Consistent with our findings it has been reported that acutely activated microglia that produce high levels of pro-inflammatory cytokines such as TNFα induce NPC apoptosis whereas chronically activated microglia that produce low levels of TNFα and increased levels of anti-inflammatory factors do not induce significant apoptosis (Cacci et al., 2008). In another study, it was shown that microglia activated in the presence of the anti-inflammatory factor IL-4 resulted in decreased TNFα production and instead promoted neurogenesis (Butovsky et al., 2006). TNFα is known to exert its effects on cells by activating two receptor subtypes on target cell membranes: TNF receptor-1 (TNFR1), which can mediate pro-death signaling through an intracellular death domain, and TNF receptor-2 (TNFR2), which lacks an intracellular death domain and typically activates pro-survival signals (Cabal-Hierro and Lazo, 2012). Interestingly, we found that TNFR1 expression is specifically upregulated in NPCs exposed to activated microglia conditioned media and that this is not mediated by TNFα as it is not blocked by TNFα neutralization. This suggests that TNFR1 induction is triggered by additional microglia derived factors that may contribute to NPC death. One interesting possibility is interleukin-6 as Monje and colleagues previously reported that IL-6 contributes to the anti-neurogenic effects of LPS activated microglia (Monje et al., 2003). Interestingly, Iosif and colleagues have reported that TNFR1 knockout mice exhibit increased neurogenesis in models of epileptic seizure and cerebral ischemia, although NPC death was not assessed in these in vivo contexts (Iosif et al., 2006;Iosif et al., 2008). While it is likely that TNFR1 induction potentiates the apoptotic response of NPCs this is yet to be formally tested.

Depending on the cell type death receptors such as TNFR, Fas and Trail can trigger apoptosis via a caspase-8 dependent mechanism resulting in direct caspase-3 activation or through an indirect pathway involving Bax/Bak-dependent mitochondrial permeabilization (Micheau and Tschopp, 2003). We have determined that microglia derived TNFα as well as rTNFα induce NPC apoptosis predominately via a Bax-dependent mitochondrial pathway. Specifically, we demonstrate that conditioned media from activated microglia or rTNFα treatment induces mitochondrial depolarization and caspase-3 activation in NPCs

and that this is attenuated in Bax-deficient NPCs. BH3-domain-only Bcl-2 family proteins are known to play an important role in regulating Bax activation (Cheng et al., 2001). However, the specific BH3-only family members involved is dependent on the cell type and nature of the apoptotic stimulus (Puthalakath and Strasser, 2002). Extracellular death ligands including TNFα have been shown to induce apoptosis by engaging their respective receptors on the cell surface of target cells leading to the assembly of a death inducing signaling complex (DISC) on the intracellular domain of the receptor that mediates the activation of caspase-8 (Schneider-Brachert et al., 2004). Caspase-8 in turn has been proposed to cleave the BH3-only family member Bid into its active truncated form (tBid) which can then trigger Bax/Bak activation (Gross et al., 1999;Li et al., 1998). However, we have found that NPCs exposed to activated MCM exhibited only a very modest increase in tBid levels and that this was not blocked by the anti-TNF neutralizing antibody. Furthermore, we did not detect an increase in tBid in NPCs treated with rTNFα. NPCs appear to express full length Bid thus the lack of Bid cleavage suggests that the DISC is not efficiently activated by TNFα in NPCs although the reason for this is not clear. These results suggest that tBid is not likely required for activated microglia/TNFα induced apoptosis in NPCs. On the other hand we found that NPCs exposed to conditioned media from activated microglia exhibited a marked increase in the expression of the BH3-only family member Puma that appears to be mediated by TNFα as it is suppressed by a TNFα-neutralizing antibody. Consistent with this we found that Puma expression was also induced in NPCs treated with recombinant TNFα. Importantly, we have demonstrated that Puma-deficient NPCs are remarkably resistant to apoptosis induced by activated MCM and rTNFα indicating that Puma induction is required for cell death.

We further examined the importance of this cell death pathway in an in vivo model of spinal cord injury known to involve a marked inflammatory response including extensive microglia activation and elevated levels of pro-inflammatory cytokines such as TNFα (Pineau and Lacroix, 2007). Previous studies have demonstrated that the majority of stem cells transplanted into the injured spinal cord undergo cell death (Lee et al., 2009;Oh et al., 2011;Xu et al., 2011). Importantly, we have determined that three weeks after transplantation the number of Puma-deficient NPCs remaining in the injured spinal cord was >13-fold higher than that of wild type NPCs. These results are consistent with the high

propensity of NPCs to undergo apoptotic cell death in a neuroinflammatory environment and emphasizes the importance of Puma activation in mediating NPC cell death in vivo.

Puma expression can be induced by diverse apoptotic stimuli and is typically regulated through transcriptional mechanisms (Han et al., 2001). Pro-inflammatory cytokines including TNFα are known to activate NF-kB family transcription factors (Hayden and Ghosh, 2012). Indeed, we found that NPCs exposed to activated MCM exhibited a marked increase in p65 phosphorylation consistent with NF-kB activation. Importantly, we demonstrated that the NF-kB inhibitor BAY-11,7082 significantly reduced Puma induction and NPC apoptosis indicating that NF-kB is a key mediator of Puma induction in NPCs exposed to activated MCM. Similar to our findings it has recently been demonstrated that pro-inflammatory cytokines including TNFα can induce Puma expression via an NF-kB mediated pathway in colorectal cancer cells and islet beta-cells (Gurzov et al., 2010;Omori et al., 2011;Wang et al., 2009). NF-kB has been reported to promote survival or cell death depending on the context (Dutta et al., 2006;Perkins & Gilmore, 2006). The protective effects of NF-kB have been attributed to its ability to induce the expression of anti-apoptotic factors such as Bcl-2, Bcl-XL, and cIAP2 (Hayden & Ghosh, 2012). Conversely, NF-kB has been shown to promote apoptosis in certain conditions by inducing the expression of pro-apoptotic proteins such as death receptor-5, Fas/Fas ligand and p53 (Dutta et al., 2006). Several studies have suggested that p65 and p53 can co-operate to activate the expression of pro-apoptotic genes and promote cell death (Fujioka et al., 2004;Ryan et al., 2000). Puma was originally identified as a p53 responsive gene (Han et al., 2001;Nakano and Vousden, 2001) therefore it is possible that p53 and NF-kB could act together to promote Puma induction in NPCs exposed to microglia derived pro-inflammatory factors.

In summary, we have identified a key signaling pathway that regulates neuroinflammation induced apoptosis in NPCs that could potentially be targeted to promote regeneration and repair in diverse injury and neurodegenerative conditions.

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Chapter 3

3 Microglia-derived IL-1β triggers p53-mediated cell cycle arrest and apoptosis in neural precursor cells

3.1 Introduction

In the adult brain, neural precursor cells (NPCs) generate new neurons that can be integrated into the CNS circuitry to replace damaged or lost neurons, and contribute to learning and memory processes (Shors et al. 2001; Aimone et al. 2014). Dysregulation of adult neurogenesis has been observed in animal models of stroke and epilepsy, and neurodegenerative diseases including Alzheimer's, Huntington's and Parkinson's disease (Nakatomi et al. 2002; Phillips et al. 2005; Parent 2007; Crews et al. 2008; Lazarov and Marr 2010; Winner et al. 2011). However, the extent to which neurogenesis contributes to brain repair is severely limited by the neuroinflammatory processes associated with these neurological conditions (Arviddson et al. 2002; Monje et al. 2003; Ekdahl et al. 2003; Hoehn et al. 2005; Biscaro et al. 2012). Microglia are the resident immune cells of the central nervous system and are the primary regulators of neuroinflammatory responses. During injury and pathological conditions, microglia cells become activated and depending on the nature and duration of the stimulus can produce either anti-inflammatory or pro-inflammatory factors that can differentially affect neurogenesis (Aarum et al. 2003; Monje et al. 2003; Ekdahl et al. 2009; Whitney et al. 2009). Microglia cells induced to exhibit a pro-inflammatory phenotype release cytokines such as TNFα, IL-6 and IL-1β and decrease neurogenesis and NPC survival *in vitro* and *in vivo* (Cacci et al. 2008; Guadagno et al. 2013).

Interleukin-1β (IL-1β) is synthesized in microglia as an inactive precursor protein that requires cleavage by caspase-1 (also known as interleukin-1 converting enzyme or ICE) to be transformed into its mature, biologically active form. IL-1β exerts its effects on target cells by binding the cell surface IL-1 type-1 receptor (IL-1R1) leading to the activation of a signaling cascade that results in the activation of mitogen activated protein kinases and transcriptional regulators such as NF-κB (Sims and Smith 2010). Embryonic and adult NPCs express IL-1R1 and undergo cell cycle arrest when exposed to IL-1β in vitro (Green

et al. 2012; Wang et al 2007). Furthermore, it has been shown that hippocampal neurogenesis is impaired in mice chronically exposed to IL-1β *in vivo* and that IL-1β induced inhibition of hippocampal progenitor cell proliferation was blocked by the IL-1R1 antagonist IL-1RA (Goshen et al. 2008; Koo & Duman 2008). Similarly, it has been demonstrated that hippocampal neurogenesis is markedly reduced in transgenic mice engineered to inducibly express human IL-1β in the hippocampus (Wu et al. 2012). While these data demonstrate that IL-1β has anti-neurogenic properties the mechanisms by which it exerts these effects remains unknown.

Control of cell division and cell death during neurogenesis is critical for the generation of new neurons. Amongst other functions the tumour suppressor protein p53 has dual roles in the regulation of cell cycle and apoptosis. p53 is a sequence-specific transcription factor that can regulate the expression of genes involved in a number of cellular processes including cell cycle checkpoint control, metabolism, autophagy and apoptotic cell death (Vousden and Prives 2009). Specifically, the cyclin-dependent kinase inhibitor p21 is a target gene known to play a key role in p53-mediated cell cycle arrest (Dulic et al. 1994; El-Deiry et al. 1993). P53 has also been shown to induce the expression a number of genes involved in promoting apoptosis including Trp53INP1, Fas, Noxa and Puma (Riley et al. 2008). Recent evidence suggests that P53 plays a role in regulating neurogenesis in the developing and adult brain (reviewed in Bartesaghi and Salomoni 2013). Indeed, p53 expression is enriched in NPCs during development and in adult neurogenic regions such as the subventricular zone and subgranular zone (van Lookeren Campagne et al. 1998; Meletis et al. 2006). Postnatal p53-deficient mice exhibit increased proliferation within the SVZ and increased neurogenesis (Meletis et al. 2006). Furthermore, NPCs derived from p53-null mice exhibit reduced apoptosis and enhanced proliferation (Gil-Perotin et al. 2006). However, the potential role of p53 in regulating NPC proliferation and survival during neuroinflammation has not been investigated.

In the present study we demonstrate that IL-1β produced by LPS/ϒ-IFN-activated microglia decreases NPC proliferation and survival. We show that IL-1β upregulates p53 and p53-mediated gene expression leading to cell cycle inhibition and Puma/Bax-mediated apoptosis in NPCs. Moreover, we demonstrate that p53-deficient NPCs are resistant to

apoptosis and proliferation defects induced by microglia-derived IL-1β, suggesting that p53 is a key regulator of NPC proliferation and survival during neuroinflammatory conditions.

3.2 Materials and methods

3.2.1 Animals

Mice carrying a targeted null mutation for Bax were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were genotyped as previously described (Cregan et al. 1999). Mice carrying a targeted null mutation for Puma were generated and maintained on a C57/BL6 background in the laboratory of Dr. Andreas Strasser (WEHI, Victoria, Australia). Genotyping of these mice was performed as previously described (Villunger et al. 2003). Timed pregnant wild-type CD1 mice were purchased from Charles River Laboratories (Sherbrooke, QC, Canada).

3.2.2 Neural precursor cell culture

NPCs were dissociated from the striatum of E13.5 mice and grown as neurospheres for 7 days in neural stem cell media consisting of DMEM-F12 containing D-glucose (6 mg/ml), L-glutamine (2 mM), penicillin/streptomycin, insulin (20 mg/ml), apotransferrin (100 mg/ml), progesterone (0.02 nM), putrescine (20 nM), sodium selenite (30 nM), heparin (0.3 nM), and bFGF (10 ng/ml) as previously described (Tropepe et al. 1999). Neurospheres were then dissociated by incubation in 0.05% trypsin-EDTA and trituration with glass pipette. Trypsin inhibitor was added and the single cell suspension was centrifuged at 300xg for 5 minutes. Single cell NPCs were plated on dishes coated with poly-L-ornithine and laminin (Sigma, Oakville, ON, Canada).

3.2.3 Microglia cell culture and preparation of MCM

The mouse microglial cell line EOC-20 was obtained from the American Type Culture Collection (ATCC CRL-2469, Manassas, VA, USA). Cells were maintained at 37^0C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 0.5%

penicillin/streptomycin, 4mM L-glutamine, and 20% conditioned medium from bone-marrow-derived Ladmac cells (ATCC CRL-2420) as a source of colony stimulating factor-1. For preparation of microglia conditioned media (MCM), EOC-20 cells were grown to 60% confluence at which point their media was removed and replaced with neural stem cell media (lacking bFGF and heparin). To activate microglia, stem cell media was supplemented with 10ng/ml LPS (Sigma, Mississauga, ON) and 10ng/ml recombinant mouse ϒ-IFN (R&D Systems, Minneapolis, MN, USA) for 24 hours. Microglia conditioned media (MCM) was collected, centrifuged and filtered through a 0.2-mm filter to remove cells and debris. MCM was then supplemented with 10 ng/ml bFGF and 0.3 nM heparin and immediately used for NPC cultures. In the indicated experiments, LPS/ϒ-IFN was added to non-activated MCM or unconditioned stem cell media immediately before adding to NPC culture.

3.2.4 Neurosphere size quantification

NPCs were cultured for 7 days in suspension to form neurospheres in unconditioned stem cell media or conditioned stem cell media from either unactivated or LPS/ϒ-IFN activated microglia. Phase contrast micrographs were taken at 100X total magnification using a microscope equipped with a digital camera. Five representative images were taken for each treatment and analysis was done using Northern Eclipse 7.0 software by manually measuring the diameters of neurospheres using the straight line measurement tool which provides an arbitrary pixel length. Pixel length was then converted to μm using the Calibrate for Distance tool. Two hundred spheres were measured per treatment and results are representative of three separate experiments.

3.2.5 NPC treatments and IL-β neutralization experiments

NPCs were treated with MCM or recombinant mouse IL-1β (rIL-1β; R&D Systems, Minneapolis, MN, USA) 2 days after plating as a monolayer. In the indicated experiments, 50ng/ml recombinant mouse IL-1 Receptor antagonist (IL-1RA; R&D Systems,

Minneapolis, MN, USA) was added to NPC cultures simultaneously with the switch to MCM or rIL-1β treatment. For inhibition of IL-1β cleavage in microglia, 20μM of the caspase-1 inhibitor N-Ac-Tyr-Val-Ala-Asp-chloromethyl ketone (y-VAD-CMK; Sigma) was added to microglia at the time of activation, and conditioned media was collected after 24 hours.

3.2.6 IL-1β ELISA

Conditioned stem cell media from microglia either left unstimulated or stimulated with 10 ng/ml LPS (Sigma)/ 10ng/ml ϒ-IFN was collected at 24 h. IL-1β levels were detected using the Mouse IL-1β ELISA-Max; BioLegend, San Diego, CA, USA) as per the manufacturer's instructions. Briefly, microglia conditioned media samples were added to microplates pre-coated with mouse polyclonal IL-1β antibody. Following incubation and washes to remove unbound IL-1β an enzyme-linked mouse polyclonal antibody was added. The addition of the substrate yields a colorimetric product and the absorbance (450 nm) was measured using a microplate reader. Samples were assayed in duplicate and IL-1β concentrations were determined from a standard curve using SoftmaxPro software (Molecular Devices, Sunnyvale, CA, USA).

3.2.7 EdU labeling experiments

Proliferation studies were performed by EdU (5-ethynyl-2'-deoxyuridine) labeling using the Click-iT EdU AlexaFluor 594 Imaging kit (Invitrogen) as per manufacturer protocol. Briefly, monolayer NPCs were labeled with EdU for 1 hr prior to fixation with 4% paraformaldehyde. Cells were then washed twice with PBS containing 3% BSA, and permeabilized with 0.5% TritonX-100 in PBS. Cells were then incubated with ClickIT reaction cocktail containing AlexaFluor 594 azide for detection of EdU labeling. Cells were counterstained with Hoechst 33258 (1μg/ml), and images were captured using fluorescence microscopy. A minimum of 400 cells/well were counted and the number of EdU labeled cells was calculated as a fraction of total cells.

3.2.8 Cell death assays

Apoptosis of NPCs was assessed by examining nuclear morphology in Hoechst 33342-stained cells as previously described (Steckley et al. 2007). Briefly, NPCs were stained with 1 mg/ml Hoechst 33342 (Sigma) and the fraction of cells exhibiting an apoptotic nuclear morphology characterized by chromatin condensation and/or apoptotic bodies, was quantified. In certain experiments, NPC death was determined by Live/Dead assay according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, NPCs were stained with Calcein-AM (2 µM) and ethidium homodimer (4 µM) for 20 min and the fraction of live (Calcein-AM positive) and dead (ethidium positive) cells was scored. NPCs were visualized by fluorescence microscopy (Zeiss, Toronto, ON, Canada) and images were captured with a Zeiss Axio-Cam camera (Zeiss). Images were captured and scored by an observer blinded to the treatment. A minimum of 500 cells from five randomly selected fields were analyzed for each treatment and data represent the mean and S.E.M. from a minimum of four independent experiments

3.2.9 Quantitative real-time PCR

RNA was isolated using Trizol reagent as per the manufacturer's instructions (Invitrogen) and 10 ng of RNA was used in one-step Sybr green reverse transcription (RT)-PCR (QuantiFast, Qiagen, Mississauga, ON, Canada). RT-PCR was carried out on a Chromo4 system (MJ Research Bio-Rad, Mississauga, ON, Canada) and changes in gene expression were determined by the $\Delta(\Delta Ct)$ method using S12 transcript for normalization. Data are reported as fold increase in mRNA levels in treated samples relative to untreated control cells. All PCR's exhibited high amplification efficiency (>90%) and the specificity of PCR products was confirmed by sequencing.

3.2.10 Western blot analysis

Whole cell lysates were prepared by incubating NPCs in lysis buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8, 1mM EDTA, 1 mM DTT,

and protease and phosphatase inhibitor cocktail (Invitrogen) for 20 min on ice. The soluble extract was recovered by centrifugation at 14,000xg. Protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA) and 50 μg of protein was separated on 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked for 1 h in TBS-T (10mM Tris, 150mM NaCl, 0.05% Tween-20), followed by overnight incubation in primary antibodies to p53, cleaved (active) caspase-3, GAPDH (Cell Signaling Technology, Danvers, MA, USA), Puma (Sigma), p21 or Actin (Santa Cruz Biotech) in TBS-T containing 5% skim milk. Membranes were washed with TBS-T and incubated for 1 h with the appropriate HRP-conjugated secondary antibodies and developed by enhanced chemiluminescence system according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada).

3.2.11 Cytochrome-c immunostaining

Neural precursor cells were fixed in 4% paraformaldehyde, washed in three changes of PBS and then incubated overnight with a monoclonal antibody directed against cytochrome-c (BD PharMingen, San Diego, CA) as described previously (Steckley et al. 2007). Cells were then washed and incubated for 1 hour with Alexa-488 conjugated goat anti-mouse IgG secondary antibody (Invitrogen) and counterstained with Hoechst 33258 (1 μg/ml). To evaluate mitochondrial membrane permeabilization cells were visualized by fluorescence microscopy and cells exhibiting punctate, cytoplasmic cytochrome-c staining were considered to have maintained membrane integrity. Images were captured and scored by a blinded observer and a minimum of 400 cells were analyzed per well.

3.2.12 Data analysis

Data are reported as mean and standard error of the mean. The n value represents the number of independent experiments and/or number of mice from which independent NPC cultures were prepared. Data were analyzed by one-way ANOVA followed by Tukeys or Bonferroni post hoc test and differences were considered significant at $P<0.05$. All

statistical analysis were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

3.3 Results

3.3.1 Activated microglia derived IL-1β induces cell cycle arrest and apoptosis in neural precursor cells

NPCs isolated from the mouse striatum can be expanded ex vivo in stem cell media containing EGF/FGF to form neurospheres (Tropepe et al. 1999). To examine the effects of microglia-derived inflammatory factors on NPC proliferation we cultured NPCs for 7 days in unconditioned neural stem cell media or conditioned stem cell media from either unactivated microglia or LPS/ϒIFN-activated microglia. As shown in figure 1, NPCs cultured in conditioned media from LPS/ϒIFN-activated microglia produce significantly smaller neurospheres after 7 days in culture, as compared to those cultured in naive stem cell media or conditioned media from unactivated microglia (Figure 3.1A and B). Furthermore, the number of NPCs obtained following dissociating of the week-old neurospheres was also found to be significantly reduced following culture in activated microglia conditioned media (aMCM) (Figure 3.1C). We next examined whether the decrease in proliferation was due to effects on cell cycle progression and/or cell death. To determine whether microglia-derived factors affected cell division, NPCs were grown as an adherent monolayer and pulse labeled with the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU). As shown in figure 3.1D, the fraction of dividing (EdU positive) cells was markedly reduced when NPCs were cultured in activated MCM as compared to unactivated MCM. Furthermore, NPCs cultured in activated MCM exhibited a significant increase in cell death as determined by Live/Dead assay (Figure 3.1E). These results suggest that pro-inflammatory microglia-derived factors inhibit NPC proliferation through the induction of both cell cycle arrest and apoptotic cell death.

Upon activation by pro-inflammatory stimuli microglia produce a number of soluble factors that can influence cells in the microenvironment including TNFα, IL-6 and IL-1β (Monje et al. 2003; Cacci et al. 2008; Guadadgno et al. 2013). A number of studies have demonstrated that Interleukin-1β (IL-1β) in particular can inhibit neurogenesis in vitro and in vivo (Wang et al. 2007; Koo & Duman 2008; Goshen et al. 2008; Green et al. 2012). Therefore, we examined whether IL-1β contributed to the anti-proliferative actions of microglia on NPCs using two different approaches. In the first approach, we added the caspase-1/ICE inhibitor, y-VAD-CMK, to microglia during LPS/ϒIFN stimulation to prevent the processing and production of mature IL-1β (Figure 3.2A). In a second approach, we specifically blocked IL-1R signaling in NPCs using the natural IL-1 receptor antagonist (IL-1RA) that competitively inhibits binding of IL-1β to the IL-1R1 receptor (Hannum et al. 1990; Sims and Smith 2010). As shown in figure 3.2B and 3.2C, the inhibitory effect of aMCM on NPC proliferation as assessed by EdU-labeling, was significantly reduced by treatment of NPCs with IL-1RA. Furthermore, we found that inhibition of IL-1β by either y-VAD or IL-1RA significantly reduced activated MCM induced NPC apoptosis (Figure 3.2D). These results indicate that IL-1β released by activated microglia induces both cell cycle arrest and apoptosis in NPCs.

3.3.2 Microglia derived IL-1β induces p53 activation in NPCs

The tumour suppressor p53 is a transcription factor that has been implicated in the regulation of genes involved in the control of cell cycle and apoptosis. P53 is expressed in NPCs and has been suggested to play a role in the regulation of neurogenesis in the adult brain (Meletis et al 2006; Bartesaghi & Salomoni 2013). Since our initial findings indicated that aMCM induces both cell cycle inhibition and cell death in NPCs we examined whether P53 was activated in NPCs under neuroinflammatory conditions. Protein levels of p53 were consistently increased in NPCs in response to treatment with activated MCM (Figure 3.3A). We also observed marked increases in the expression of several p53 target genes known to be involved in the regulation of cell cycle arrest and apoptosis. P21 is a cyclin-dependent kinase inhibitor and known target gene of p53 that functions as a negative regulator of cell cycle progression at the G1-S phase (Dulic et al.

Figure 3.1. Conditioned media from LPS/ϒIFN-activated microglia inhibits proliferation and induces cell death of NPCs.

NPCs were cultured as neurospheres for 7 days in either unconditioned stem cell media (c, ctrl), or microglia conditioned stem cell media from either unactivated microglia (0% MCM) or LPS/ϒIFN-activated microglia (25-100% MCM). Activated microglia conditioned media was left undiluted (100% MCM) or diluted to 75%, 50%, or 25% with unactivated microglia conditioned media. **(A)** Representative images of NPCs grown as neurospheres for 7 days in naïve stem cell media or increasing concentrations of activated MCM. **(B)** The mean diameter of neurospheres was measured after 7 days in culture in unconditioned or microglia conditioned stem cell media (n=4, *$p<0.05$). **(C)** Neurospheres were dissociated after 7 days in culture and the number of NPCs were counted and reported as a percentage of NPCs obtained from neurospheres grown in control stem cell media (n=4, *$p<0.05$). **(D, E)** After 7 days in culture, neurospheres grown in complete stem cell media were dissociated and plated as a monolayer. NPCs were then incubated for indicated time points in complete stem cell media (Ctrl) or conditioned stem cell media from activated microglia (aMCM). **(D)** To assess proliferation, NPCs were pulse labeled with EdU at the indicated times and counterstained with Hoechst 33342. NPCs were visualized by fluorescence microscopy and the fraction of Edu-positive cells was determined (n=4, *$p<0.001$). **(E)** The fraction of dead (ethidium positive) NPCs was determined by Live/Dead assay following incubation with aMCM or unconditioned stem cell media (ctrl) for 72 hours (n=3; *$p<0.05$).

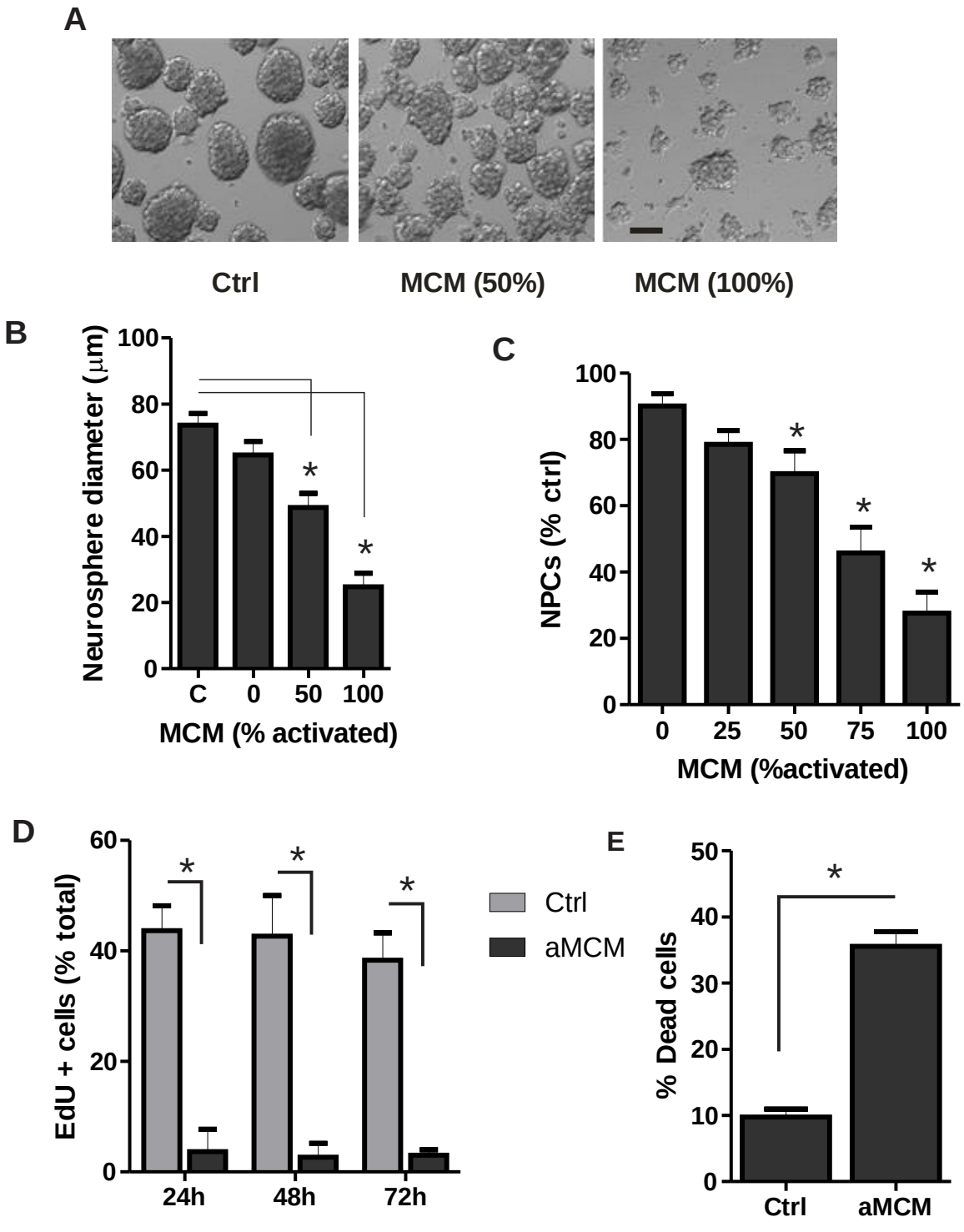

A
Ctrl
MCM (50%)
MCM (100%)
B
Neurosphere diameter (μm)
100
80
60
40
20
0
C
50
100
MCM (% activated)
C
NPCs (% ctrl)
100
80
60
40
20
0
0
25
50
75
100
MCM (%activated)
D
EdU + cells (% total)
60
40
20
0
24h
48h
72h
Ctrl
aMCM
E
% Dead cells
50
40
30
20
10
0
Ctrl
aMCM

Figure 3.1

1994). Puma and Noxa are pro-apoptotic members of the Bcl-2 gene family and are known to be transcriptionally regulated by p53 (Oda et al. 2000; Nokano and Vousden 2001; Yu et al. 2001). Interestingly, a robust increase in the expression of p21, Puma and Noxa mRNA was observed in NPCs treated with activated MCM (Figure 3.3B). Consistent with their induction being mediated by p53 we found that the expression of p21, Puma and Noxa was not induced in p53-deficient NPCs (Figure 3.3B). Similarly we found that p21 and Puma protein levels were upregulated in a p53-dependent manner in NPCs treated with activated MCM (Figure 3.3C).

We next sought to determine whether microglia induced p53 activation in NPCs was mediated by IL-1β. As shown in figure 3.4A, inhibition of IL-1β signaling by either the caspase-1 inhibitor y-VAD-CMK or the IL-1 receptor antagonist IL-1Ra blocked activated MCM induced p53 expression in NPCs. Inhibition of IL-1β production/ signaling also attenuated the induction of the p53 target genes p21 and Puma at both the mRNA and protein levels (Figure 3.4A and 3.4B). To further investigate the relationship between IL-1β and p53, we treated NPCs with recombinant interleukin-1β (rIL-1β) to determine whether this was sufficient to induce p53 activation. As shown in figure 3.4C, rIL-1β treatment markedly increased p53 protein levels in NPCs. Furthermore, we found that rIL-1β induced p21 and Puma expression in p53+/+ but not p53-/- NPCs (Fig. 4D).

It has previously been reported that IL-1β can stimulate the production of reactive oxygen species (ROS) in retinal epithelial cells and pancreatic β-cells (Yang et al 2007; Gurzov et al. 2009). P53 is known to be activated in response to oxidative damage and therefore we examined whether IL-1β triggers p53 activation in NPCs via an oxidative stress dependent mechanism. Consistent with this we found that the induction of p53 and its target genes P21 and Puma by activated MCM and rIL-1β was markedly reduced in the presence of the ROS scavenger N-acetyl-cysteine (NAC) (Figures 3.5A and 3.5B). Taken together these results suggest that microglia derived IL-1β induces p53 activation in NPCs via an oxidative stress dependent mechanism**.**

Figure 3.2. Microglia-derived IL-1β induces cell cycle arrest and apoptotic death of NPCs.

(A) Conditioned stem cell media from unactivated microglia (uMCM), LPS/ϒIFN-activated microglia (MCM), or microglia activated in the presence of the caspase-1 inhibitor y-VAD-CMK (20 μM) was collected after 24h and assayed for IL-1β levels by ELISA (n=3, *p<0.05). **(B)** NPCs were treated with unconditioned stem cell media (Ctrl) or conditioned media from LPS/ϒIFN-activated microglia in the presence or absence of IL-1RA (50 ng/ml) for 72 hours and then pulse labeled with EdU. NPCs were fixed and EdU was detected using AlexaFluor 594 azide and cells were counterstained with Hoechst 33342. The number of EdU-positive NPCs was counted and values are expressed as a percentage of the total number of cells (n=3; *p<0.01). **(C)** Representative images of EdU labeling of NPCs cultured in activated microglia conditioned media (MCM) in the presence or absence of IL-1RA (50 ng/ml). **(D)** NPCs were incubated for 72h in unconditioned stem cell media (Ctrl) or LPS/ϒIFN-activated microglia conditioned media (MCM) in the presence or absence of y-VAD (20 μM) or IL-1RA (50 ng/ml). NPCs were stained with Hoechst 33342 and the fraction of apoptotic nuclei was determined by examining nuclei morphology (n=4; *p<0.01).

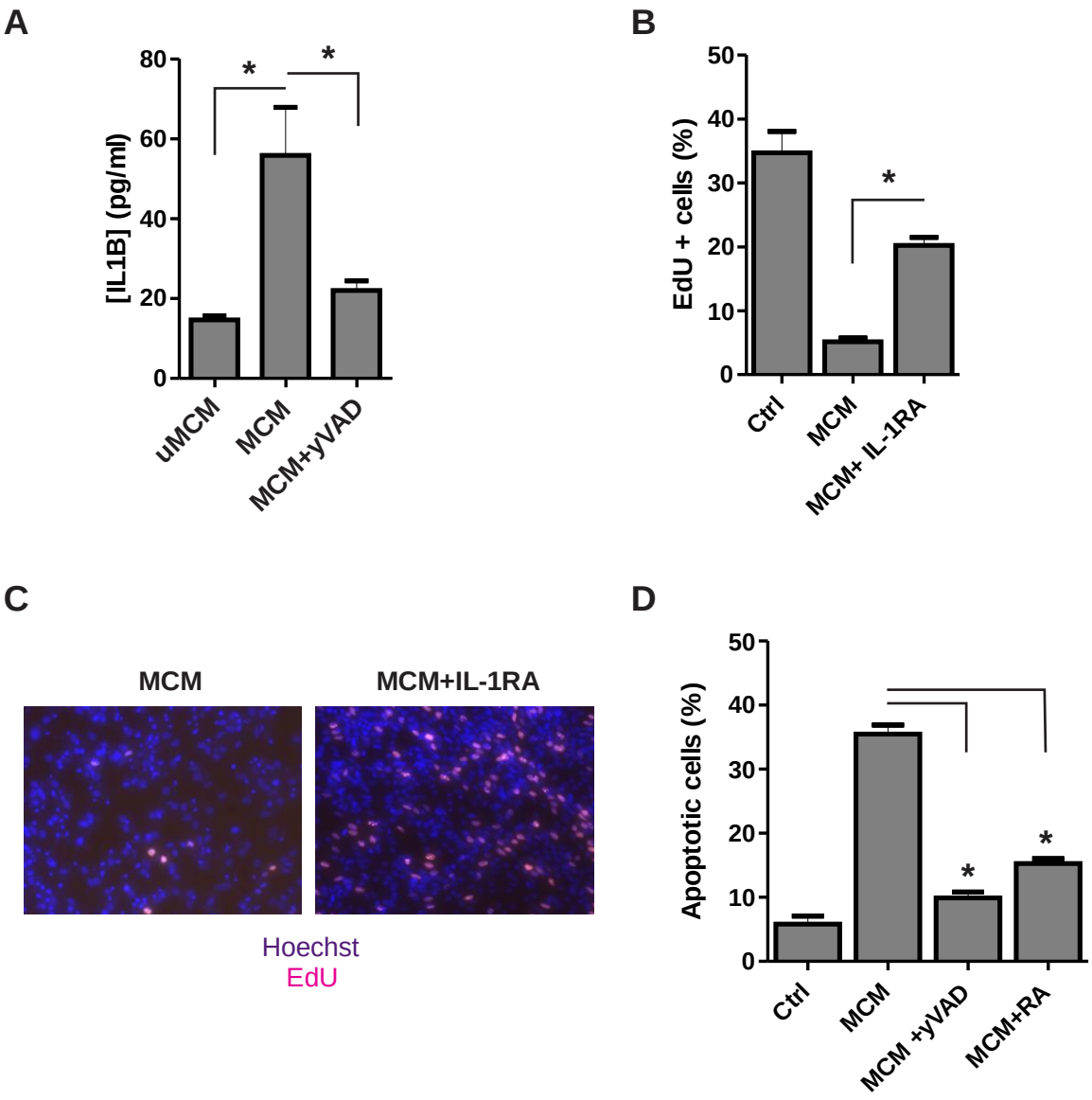

A
[IL1B] (pg/ml)
80
60
40
20
0
*
*
uMCM
MCM
MCM+yVAD
B
EdU + cells (%)
50
40
30
20
10
0
*
Ctrl
MCM
MCM+ IL-1RA
C
MCM
MCM+IL-1RA
Hoechst
EdU
D
Apoptotic cells (%)
50
40
30
20
10
0
*
*
Ctrl
MCM
MCM +yVAD
MCM+RA

Figure 3.2

Figure 3.3 Activated microglia conditioned media induces expression of p53 and p53 target genes in NPCs.

(A) NPCs were cultured in unconditioned stem cell media (Ctrl) or activated microglia conditioned media (MCM) for 24h and P53 protein levels were determined by western blot. A representative blot from three independent experiments is shown. **(B)** RNA was harvested from $p53^{+/+}$ and $p53^{-/-}$ NPCs incubated with MCM for 24h and p21, Puma, and Noxa mRNA levels were determined by qRT-PCR. mRNA levels are reported as fold increase over NPCs cultured in unconditioned stem cell media (n=4; *$p<0.05$). **(C)** Protein was extracted from $p53^{+/+}$ and $p53^{-/-}$ NPCs cultured for 24h in either unconditioned stem cell media (Ctrl) or conditioned media from LPS/ϒIFN-activated microglia (MCM) and Puma and p21 protein levels were determined by western blot. A representative blot from three independent experiments is shown.

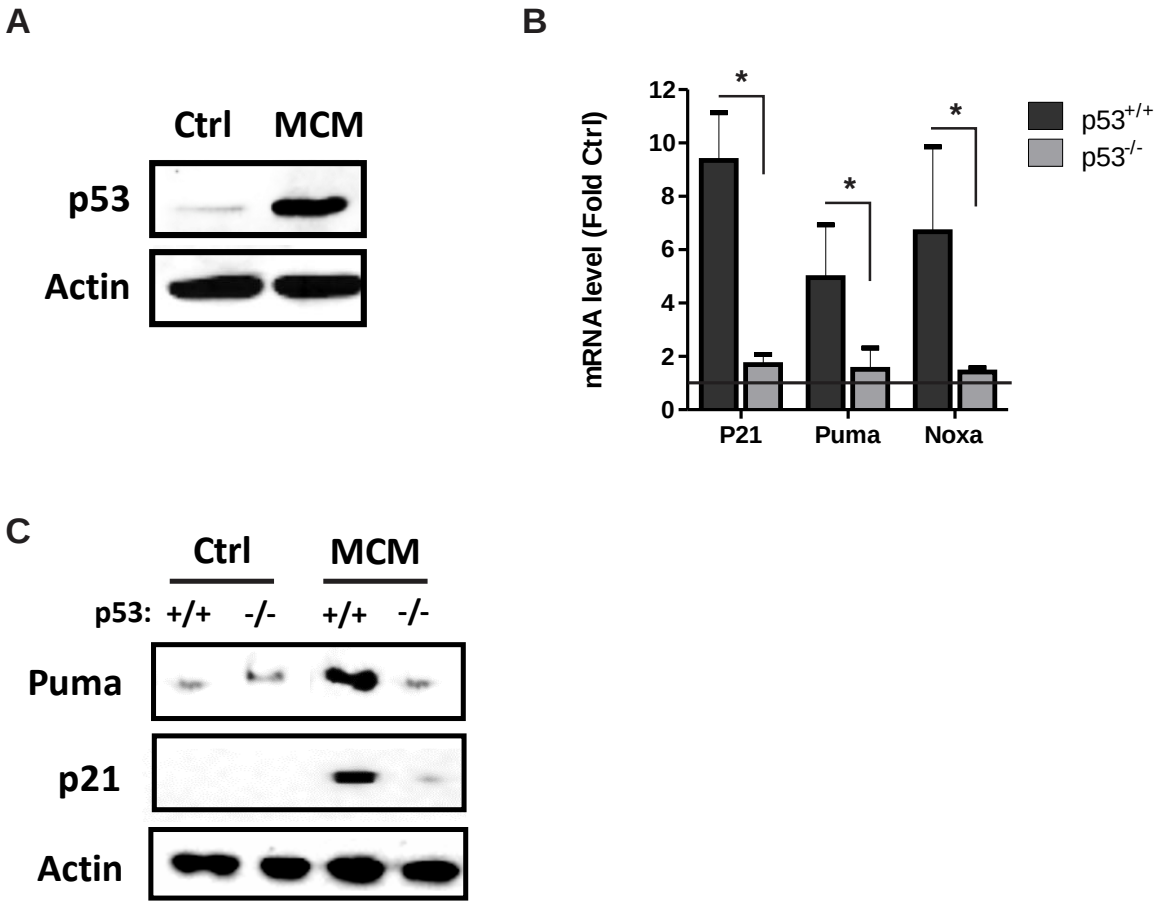

A
Ctrl
MCM
p53
Actin
B
mRNA level (Fold Ctrl)
12
10
8
6
4
2
0
*
*
*
P21
Puma
Noxa
p53+/+
p53-/-
C
Ctrl
MCM
p53: +/+ -/- +/+ -/-
Puma
p21
Actin

Figure 3.3

3.3.3 Microglia/IL-1β-induced cell cycle arrest and apoptosis in NPCs is mediated by p53.

We next examined whether p53 is required for the microglia induced effects on NPC proliferation and cell death. Consistent with this we found that activated MCM induced a significant reduction in the fraction of EdU+ cells in p53+/+ NPC cultures but not in p53-/- NPC cultures (Figure 3.6A and 3.6B). Moreover, we found that caspase-3 activation and apoptotic cell death induced by activated MCM was markedly reduced in p53-deficient NPCs (Figures 3.6C and 3.6D).

Since we had found that rIL-1β was sufficient to activate p53 we examined whether p53 was required for rIL-1β induced cell cycle arrest and apoptosis. Indeed, we found that rIL-1β significantly reduced the fraction of EdU-labeled NPCs in wildtype but not in p53-deficient NPCs (Figure 3.7A). Furthermore, we found that rIL-1β induced caspase-3 activation and apoptosis in NPCs and that these effects were essentially abolished in p53-null NPCs (Figure 3.7B and 3.7C). Puma is a pro-apoptotic member of the Bcl-2 protein family and has been shown to function by promoting Bax mediated mitochondrial permeabilization (Yu et al. 2003). We have found that Puma expression is induced by rIL-1β in a p53-dependent manner (Figure 3.7C), and consistent with the role of p53 in IL-1β mediated cell death, we found that both Puma-/- and Bax-/- NPCs are resistant to rIL-1β induced mitochondrial permeabilization as demonstrated by their maintenance of mitochondrial cytochrome-c staining (Figure 3.7D). Furthermore, we found that rIL-1β induced apoptosis was attenuated in both Puma-/- and Bax-/- NPCs (Figures 3.7E and 3.7F). Taken together, these results suggest that p53 plays an essential role in regulating microglia derived IL-1β induced cell cycle inhibition and apoptosis in NPCs, and that IL-1β induces NPC apoptosis via the p53-mediated activation of a Puma/Bax mediated mitochondrial pathway.

Figure 3.4. Microglia derived IL-1β and rIL-1β induce p53 and p53 target gene expression in NPCs.

NPCs were cultured in unconditioned stem cell media (Ctrl), conditioned media from microglia activated with LPS/ϒIFN in the presence or absence of yVAD-CMK (20μM), or LPS/ϒIFN-activated microglia conditioned media supplemented with IL-1RA (50 ng/ml). **(A)** NPCs were harvested after 48h and protein extracts were subjected to SDS-PAGE and immunoblotted for p53, Puma, p21, and Actin as a loading control. Representative blots from 3 independent experiments are shown. **(B)** RNA was harvested after 24h and mRNA levels of Puma, Noxa, and p21 were determined by qRT-PCR. mRNA levels are reported as fold increase over NPCs incubated in unconditioned media (n=4, *$p<0.05$). **(C)** NPCs treated with recombinant IL-1β (50ng/ml) were harvested after 48h and subjected to SDS-PAGE and immunoblotted for p53 and actin as a loading control. A representative blot from three independent experiments is shown. **(D)** RNA was harvested from $p53^{+/+}$ and $p53^{-/-}$ NPCs treated with rIL-1β for 24h and mRNA levels of Puma and p21 were examined by qRT-PCR. mRNA levels are reported as fold increase over control NPCs treated with vehicle (n=3; *$p<0.01$).

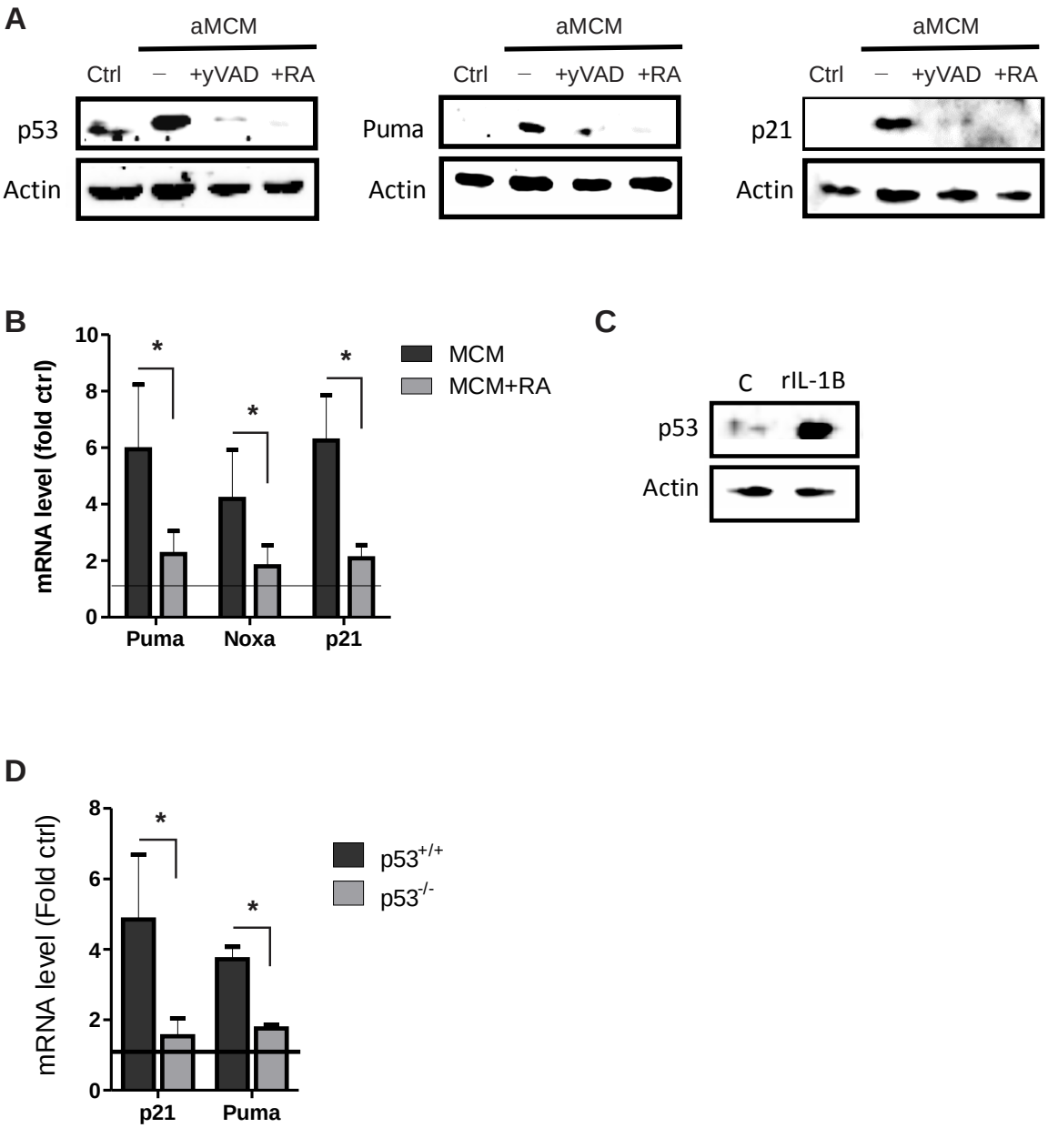

A
aMCM
Ctrl
–
+yVAD
+RA
p53
Actin
Puma
p21
B
mRNA level (fold ctrl)
MCM
MCM+RA
Puma
Noxa
p21
C
C
rIL-1B
p53
Actin
D
mRNA level (Fold ctrl)
p53+/+
p53-/-
p21
Puma

Figure 3.4

Figure 3.5. IL-1β activates P53 through an oxidative stress-dependent mechanism.

(A) NPCs were cultured in unconditioned stem cell media (Ctrl) or LPS/ϒIFN-activated microglia conditioned media (aMCM) in the presence or absence of N-acetylcysteine (5mM). Protein was extracted at 48 hours and p53, Puma, p21 and Actin expression was assayed by western blot. Representative blots from 3 independent experiments are shown. **(B)** NPCs were treated with rIL-1β (50 ng/ml) or left untreated (Ctrl) and protein was extracted after 48 hours and assayed for p53, p21 and GAPDH expression by western blot. Representative blots from 3 independent experiments are shown.

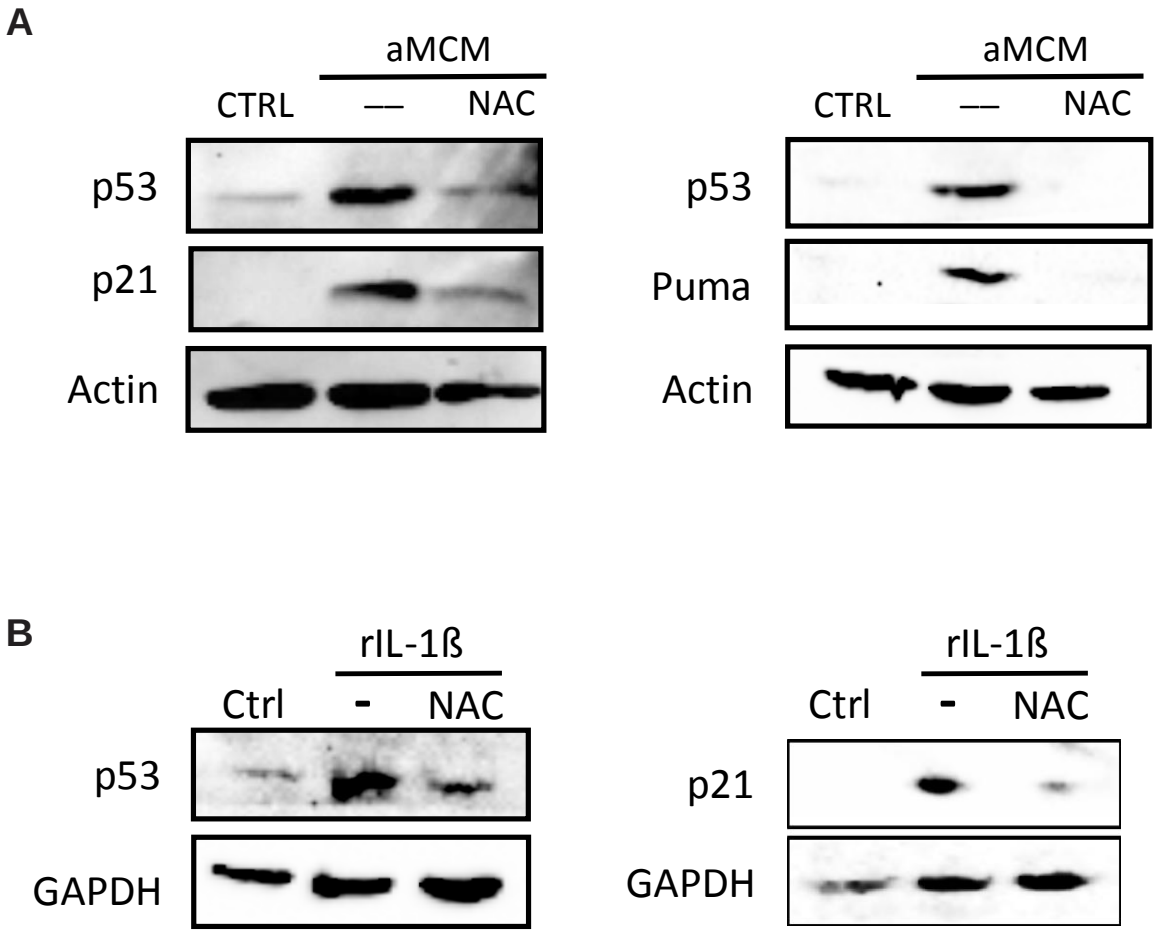

A
aMCM
CTRL
—
NAC
p53
p21
Actin
aMCM
CTRL
—
NAC
p53
Puma
Actin
B
rIL-1ß
Ctrl
-
NAC
p53
GAPDH
rIL-1ß
Ctrl
-
NAC
p21
GAPDH

Figure 3.5

Figure 3.6. Activated microglia induced cell cycle arrest and apoptosis in NPCs is mediated by P53.

(A) p53+/+ and p53-/- NPCs were cultured in unconditioned media (Ctrl) or activated microglia conditioned media (aMCM) for 72h and then pulse labeled with EdU for 1 hour. EdU was detected using AlexaFluor 594 azide and cells were counterstained with Hoechst 3342 nuclear dye. The number of EdU-positive cells were counted and expressed as a percentage of total cells (n=5, *$p<0.01$). **(B)** Representative images of EdU labeling of p53+/+ and p53-/- NPCs cultured in activated microglia conditioned media (aMCM) for 72h. **(C)** p53+/+ and p53-/- NPCs were incubated for 72h in unconditioned stem cell media (Ctrl) or LPS/ϒIFN-activated microglia conditioned media (aMCM). NPCs were stained with Hoechst 33342 and the fraction of apoptotic nuclei was determined by examining nuclei morphology (n=4; *$p<0.001$). **(D)** Protein was extracted from p53+/+ and p53-/- NPCs 48h of treatment with aMCM and active (cleaved) caspase-3 protein levels were determined by western blot. Representative blot of 3 independent experiments is shown.

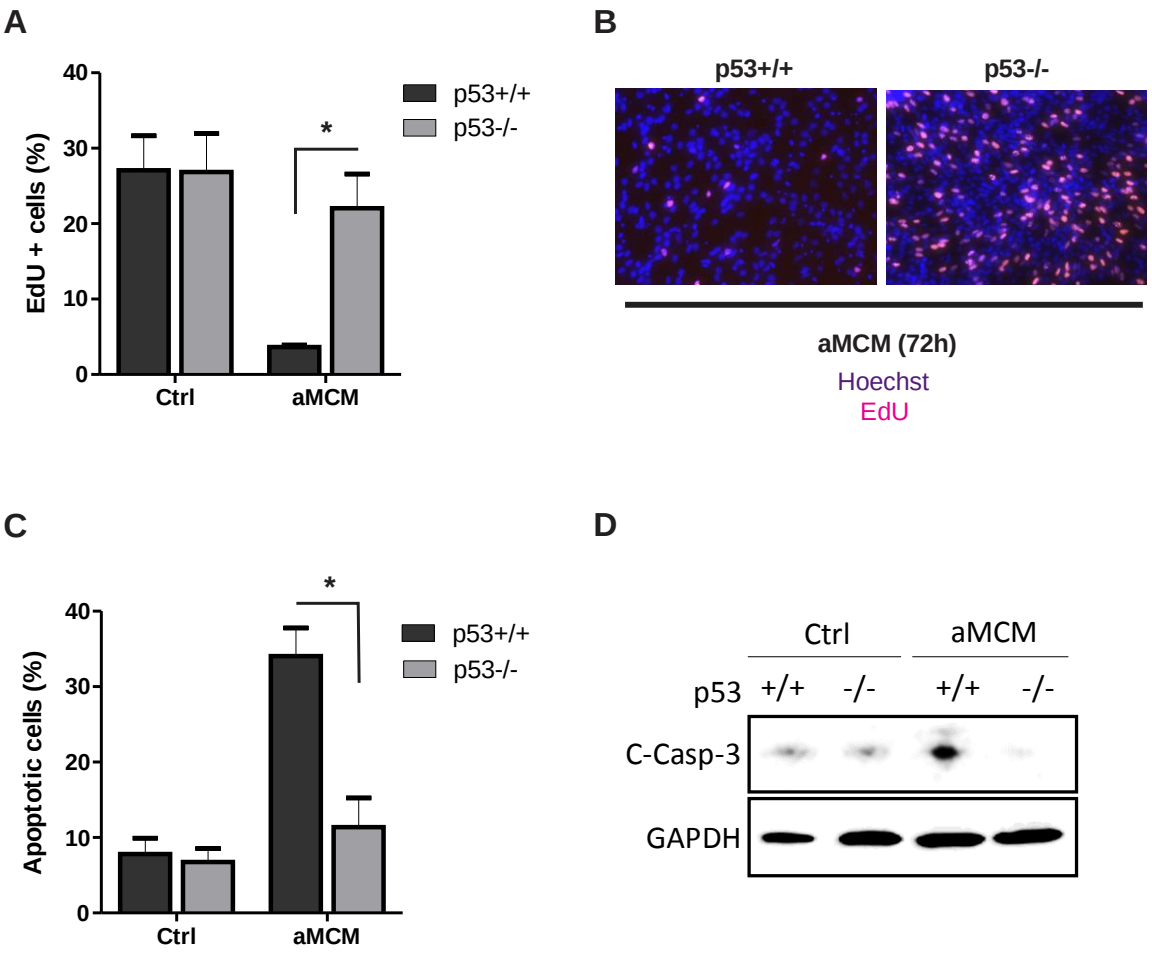
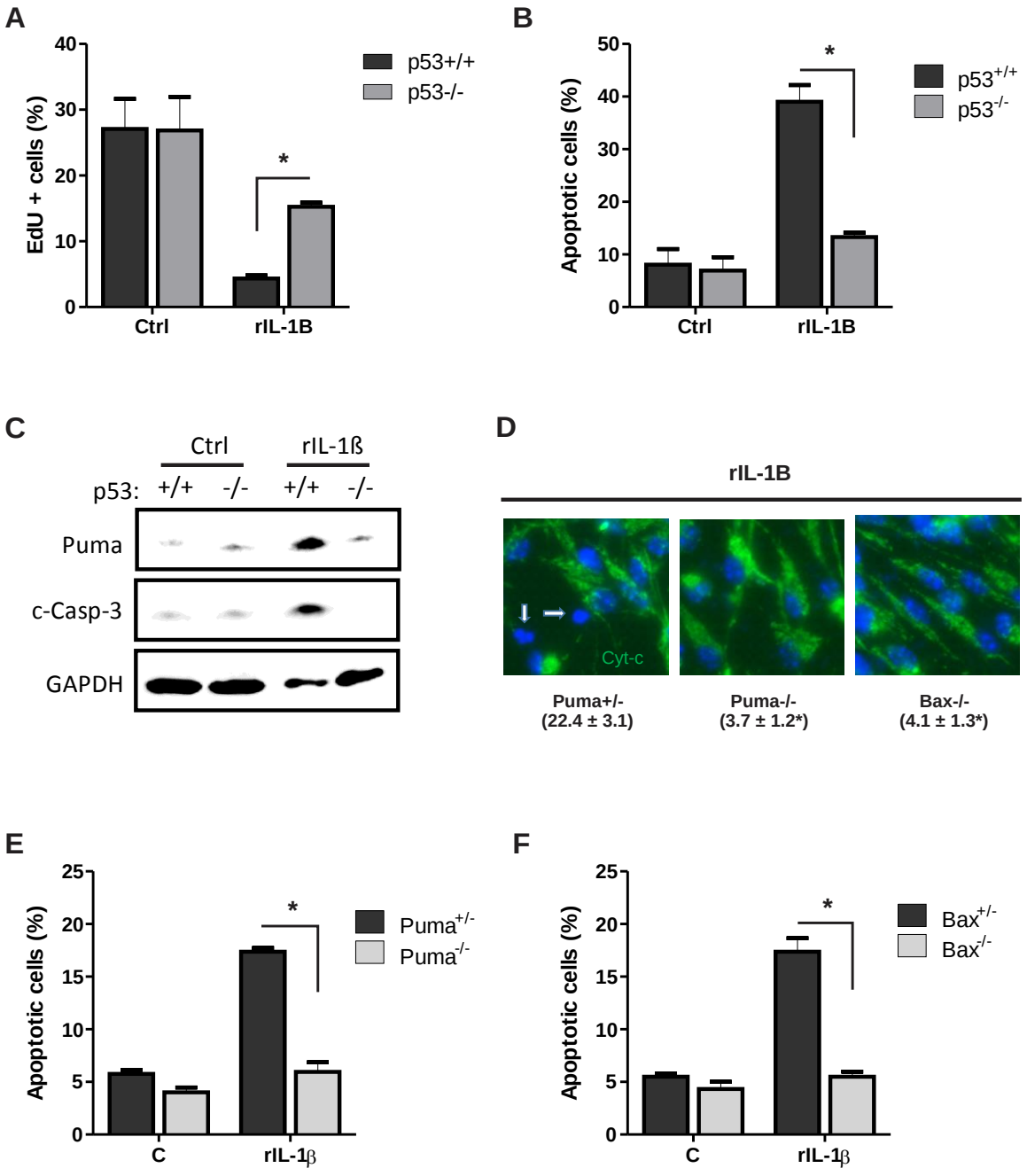

A
40
30
20
10
0
EdU + cells (%)
*
p53+/+
p53-/-
Ctrl
aMCM
B
p53+/+
p53-/-
aMCM (72h)
Hoechst
EdU
C
40
30
20
10
0
Apoptotic cells (%)
*
p53+/+
p53-/-
Ctrl
aMCM
D
Ctrl
aMCM
p53
+/+
-/-
+/+
-/-
C-Casp-3
GAPDH

Figure 3.6

Figure 3.7. IL-1β is sufficient to inhibit proliferation and induce NPC apoptosis via p53-mediated induction of Puma.

(A) $p53^{+/+}$ and $p53^{-/-}$ NPCs were treated with rIL-1β (50ng/ml) and after 72 hours NPCs were pulse labeled with EdU to detect dividing cells. The number of EdU-positive NPCs was counted and is reported as the percentage of the total number of cells (n=5; *p<0.05). **(B)** $p53^{+/+}$ and $p53^{-/-}$ NPCs were treated with rIL-1β (50ng/ml) for 72h and stained with Hoechst 33342. The fraction of apoptotic cells was determined by examining nuclear morphology (n=4, *p<0.01). **(C)** Protein was harvested from $p53^{+/+}$ and $p53^{-/-}$ NPCs treated with rIL-1β (50ng/ml) for 48h. Protein levels of Puma, active (cleaved) caspase-3, and GAPDH as a loading control were examined by western blot. A representative blot from three independent experiments is shown. **(D)** Representative images of cytochrome-c immunostaining in Puma+/-, $Puma^{-/-}$ and $Bax^{-/-}$ NPCs treated with vehicle or rIL-1β for 72 hours. Arrows highlight apoptotic cells lacking mitochondrial cytochrome-c indicative of mitochondrial permeabilization. The percentage of NPCs exhibiting loss of mitochondrial cytochrome-c for each genotype is indicated and represents the mean and SD (n=3, *p<0.01). **(E)** Puma+/- and Puma-/- NPCs were treated with rIL-1β (50ng/ml) for 72 hours and the fraction of apoptotic cells was determined by Hoechst 33342 staining (n=4, *p<0.01). **(F)** Bax+/- and Bax-/- NPCs were treated with rIL-1β and the fraction of apoptotic cells was determined after 72 hours by Hoechst 33342 staining (n=4, *p<0.01).


A
EdU + cells (%)
40
30
20
10
0
Ctrl
rIL-1B
p53+/+
p53-/-
*
B
Apoptotic cells (%)
50
40
30
20
10
0
Ctrl
rIL-1B
p53+/+
p53-/-
*
C
Ctrl
rIL-1ß
p53: +/+ -/- +/+ -/-
Puma
c-Casp-3
GAPDH
D
rIL-1B
Cyt-c
Puma+/-
(22.4 ± 3.1)
Puma-/-
(3.7 ± 1.2*)
Bax-/-
(4.1 ± 1.3*)
E
Apoptotic cells (%)
25
20
15
10
5
0
C
rIL-1β
Puma+/-
Puma-/-
*
F
Apoptotic cells (%)
25
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C
rIL-1β
Bax+/-
Bax-/-
*

Figure 3.7

3.4 Discussion

Neurogenesis occurs throughout life in two areas of the adult brain; the subventricular zone of the lateral ventricles, and the dentate gyrus of the hippocampus (Taupin and Gage 2002). Neurogenesis plays an important role in learning and memory (Shors et al. 2001; Aimone et al. 2014) and deficits in adult neurogenesis have been implicated in the cognitive impairments observed in rodent models of Alzheimer's disease (Verret et al. 2007; Demars et al. 2010). The persistence of neurogenesis in the adult brain also suggests the potential for regeneration and repair of the affected nervous system. Indeed, increased neurogenesis is observed following ischemic injury and status epilepticus as well as in models of neurodegenerative disease (Jin et al. 2001; Parent 2007; Verret et al. 2007; Winner et al. 2011). However, neuroinflammatory processes associated with these neurological conditions have been shown to inhibit neurogenesis thereby limiting the capacity for regeneration (Hoehn et al. 2005; Ekdahl et al 2003; Monje et al 2003; Liu et al 2007; Biscaro et al. 2012). Microglia are the primary regulators of neuroinflammatory responses and previous studies have demonstrated that activated microglia release pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 and decrease the proliferation and survival of NPCs (Monje et al., 2003; Cacci et al., 2008; Guadagno et al., 2013). However, the role and mechanism of action of specific microglia derived pro-inflammatory cytokines on NPCs has not been clearly defined. Importantly, in the present study we demonstrate that LPS/ϒIFN-activated microglia release the pro-inflammatory cytokine IL-1β which plays a pivotal role in inhibiting the proliferation and survival of NPCs. Specifically, we found that blocking IL-1β production in microglia using a caspase-1 inhibitor or blocking IL-1β signaling in NPCs with the receptor antagonist IL-1RA abrogated the effects of microglial on NPCs, resulting in restoration of proliferation and protection from apoptosis. Consistent with this, a number of studies have demonstrated that exogenous IL-1β can inhibit neurogenesis *in vitro* and *in vivo*. Both embryonic and adult NPCs express IL-1R1 and it has been demonstrated that recombinant IL-1β can decrease the proliferation of NPCs in culture (Wang et al. 2007; Green et al. 2012), and that hippocampal neurogenesis is impaired in mice chronically exposed to IL-1β in vivo (Goshen et al. 2008; Koo & Duman 2008). Elevated levels of IL-1β and impaired hippocampal neurogenesis has also been observed in rodent models of chronic stress and it has been shown that the anti-neurogenic

effects and behavioral symptoms induced by stress can be alleviated by IL-1RA or IL-1 receptor knockout (Goshen et al. 2008; Koo & Duman 2008). In yet another study it was shown that transplantation of IL-1RA-overexpressing NPCs into a mouse model of Alzheimer's disease rescued hippocampal neurogenesis and spatial memory disturbances (Ben-Menachem-Zidon et al., 2014). Taken together these studies implicate IL-1β as an anti-neurogenic factor and suggest that targeting IL-1β may promote neurogenesis and enhance regenerative capacity in diverse neurological conditions.

NPCs are tightly regulated in terms of proliferation, self-renewal, and survival processes. Recent studies demonstrate that p53 family members co-operate to regulate adult NPC pools (reviewed in Bartesaghi and Salomoni 2013). The p53 family of transcription factors consists of p53, p63, and p73. There are two major isoforms of p63 and p73; full length transactivation-competent (TA), and N-terminally truncated (ΔN) isoforms that lack transcriptional activity and suppress the function of p53 (Yang et al. 1998; Grob et al. 2001; DeYoung and Ellisen 2007). The most predominantly expressed p53 family members in NPCs are p53, ΔNp63, and TAp73 (Talos et al 2010; Fujitani et al. 2010). Studies have suggested that ΔNp63 promotes survival of NPCs by opposing the activation of pro-apoptotic p53 target genes, while TAp73 functions to promote self-renewal of NPCs (Talos et al. 2010; Fujitani et al 2010; Fatt et al., 2014; Cancino et al., 2013). Importantly, we have identified p53 as a negative regulator of NPC proliferation and survival in response to neuroinflammatory factors. Specifically, we demonstrate that microglial-derived IL-1β as well as recombinant IL-1β induce p53 expression in NPCs and trigger a p53-dependent increase in the expression of the cell cycle regulator p21. Studies have shown that p21-deficiency results in increased NPC proliferation in the lateral ventricle wall of adult mice, and in the hippocampus and subventricular zone following ischemic injury (Kippin et al. 2005; Qiu et al., 2004). Interestingly, a recent study found that haploinsufficiency of the p53 family member p73, or combined haploinsufficiency of p63 and p73 lead to increased levels of p21 and cellular senescence under basal conditions and more dramatically following genotoxic stress (Fatt et al., 2014). P73-deficient mice also possess fewer NPCs and exhibit dysregulated Sox2 and Notch signaling, as well as increased cellular senescence, suggesting p73 has a major role in NPC self-renewal and proliferation (Talos

et al. 2010; Fujitani et al 2010). These results suggest that p53 family members may work cooperatively or independently to regulate NPC pools. Thus, it would be interesting to determine whether the p53 family members p63 and p73 also play a role in the regulation of NPC proliferation and survival in neuroinflammatory conditions.

P53 activation can also result in activation of apoptosis in a cell-type, and stimulus-specific manner via transcriptional regulation of pro-apoptotic Bcl-2 family members, APAF-1, DR5, and Fas, as well as by transcription-independent mechanisms (Riley et al. 2008; Moll et al. 2005). Here we show that exogenous or microglial-derived IL-1β leads to increased expression of the pro-apoptotic BH3-only Bcl-2 family member Puma in a p53-dependent manner. Similar to our findings, IL-1β was also shown to induce the expression of Puma in pancreatic β-cells, although this appeared to occur through an NF-κB-dependent but p53-independent mechanism (Gurzov et al., 2010). Importantly, we demonstrate that deletion of Puma in NPCs confers significant protection from IL-1β-induced apoptosis. As further evidence of intrinsic (mitochondrial) apoptosis activation, Bax-deficient NPCs were also found to be resistant to IL-1β-induced death. Taken together these results indicate that IL-1β induces apoptosis through the mitochondrial pathway of apoptosis via p53-mediated upregulation of Puma.

In the present study we identify a novel link between IL-1β and p53 activation in the regulation of NPC proliferation and survival. P53 is known to be activated by oxidative stress (Liu et al. 2008) and IL-1β has been reported to induce ROS production in retinal epithelial cells through NADPH-oxidase activation (Yang et al. 2007) and in pancreatic β-cells via induction of iNOS (Gurzov et al. 2009). Consistent with this we found that p53 activation by exogenous and microglia-derived IL-1β was abrogated in the presence of the ROS scavenger N-acetylcysteine. Activation of IL-1R1 by IL-1β is also known to activate several signaling pathways that have been implicated in the anti-neurogenic effects of IL-1β including JNK, GSK3β and NF-kB (Green et al. 2012; Wang et al. 2007; Koo et al. 2010). It is unclear whether these pathways affect p53 activation or co-operate with p53 to affect NPC proliferation and cell death. However, previous studies have demonstrated that both JNK and GSK3β can regulate p53 activity (Charvet et al. 2011; Shi et al. 2014).

Furthermore, NF-kB has been shown to co-operate with p53 to regulate the induction of pro-apoptotic factors (Ryan et al. 2000; Fujioka et al. 2004).

In summary, we have identified a novel signaling pathway that regulates neuroinflammation induced decreases in NPC proliferation and survival. Importantly, we identify the transcription factor p53 as a critical mediator of NPC regulation by IL-1β leading to cell cycle arrest and apoptosis. This could potentially provide targets to promote neurogenesis and regeneration/ repair in the CNS following injury or neurodegenerative pathology.

3.5 References

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Chapter 4

4 Mechanisms of Fas upregulation and apoptosis of NPCs

4.1 Introduction

Neuroinflammation is a common hallmark in both acute and chronic models of neuronal injury and disease, such as spinal cord injury, ischemia, Alzheimer's disease, Parkinson's disease, and Huntington's disease (Luo & Chen, 2012; Whitney et al., 2009). The CNS is an immunologically privileged environment that is regulated by resident innate immune macrophages, known as microglia. Upon injury, or exposure to pathogens, microglia become 'activated', resulting in the production and release of anti- and pro-inflammatory cytokines, as well as growth factors and chemokines. Release of growth factors and chemokines can promote proliferation and migration of neural precursor cells to sites of injury which could potentially act as a regenerative mechanism to replace or repair damaged cells (Luo & Chen, 2012; Wee Yong,, 2010; Graeber et al., 2011). However, we and others have shown that microglial derived factors, can limit the survival of neural precursor cells, thus limiting their regenerative capacity. The neurotoxic effects of microglia may be due to the actions of pro-inflammatory cytokines such as interleukins IL-1α, IL-1B, IL-6, tumour necrosis factor-α (TNFα), and Fas ligand (sFasL), each of which has been shown to influence neurogenesis in the CNS (Monje et al., 2003; Ben-Hur et al., 2003; Cacci et al., 2008; Guadagno et al., 2013; Zhang et al., 2012).

Fas (CD95) along with TNFR1, DR3, DR4, DR5, and the two TRAIL receptors belong to the subgroup of the TNF family of receptors that possess an intracellular death domain, which is essential for transduction of the apoptotic signal. Cytotoxic T-cells, which express mFasL on their surface can kill target cells that express Fas. Neutralization of FasL with antibodies, has demonstrated that the ability of FasL to induce apoptosis is through its interaction with Fas (Kramer, 2000; Strasser et al., 2009). Furthermore, studies on mice lacking either Fas receptor ($Fas^{lpr/lpr}$) or Fas ligand ($Fas^{gld/gld}$) demonstrated that stimulation of already activated T-cells results in activation-induced cell death (AICD) via a Fas-FasL dependent mechanism (Krammer, 2000; Strasser et al., 2009). Increases in the expression

of pro-inflammatory cytokines and factors can increase the expression of Fas through activation of transcription factors including STAT1, p53 and NF-κB, in a cell-type and stimulus-specific manner (Curtin & Cotter, 2003; Ouaaz et al., 1999; Darville & Eizirik, 2001; Crescenzi et al., 2011). In addition to apoptotic signaling, signaling through Fas has been shown to activate the three main MAPK pathways, p38, JNK1/2, and ERK1/2 as well as NF-kB, leading to cell proliferation, migration, and inflammation (Beier & Schulz, 2009; Kreuz et al., 2004; Juo et al., 1997). Upon binding of FasL or a Fas agonist, Fas receptors multimerize in the cell membrane, leading to the recruitment of adaptor molecular FADD, and pro-caspase-8, forming a signaling complex known as the DISC. Pro-caspase-8 then oligomerizes and auto-activates through self-cleavage to release the active protease, caspase-8. Activation of caspase-8 can induce apoptosis through direct activation of effector caspases, or through a mitochondrial-mediated activation of effector caspases, classifying cells as either type I or type II. However, the outcome of Fas signaling may be determined by the form of ligand that activates the Fas receptor (Lavrik & Krammer, 2012; Guicciardi & Gores, 2009). Membrane-bound Fas ligand (mFasL) has been shown to have a more potent apoptotic effect than soluble Fas ligand (sFasL). mFasL can be cleaved from the cell surface by matrix metalloproteinases (MMPs), which may serve as a mechanism to dampen the activity of mFasL (Brint et al., 2013). The role of Fas in the immune system has been extensively studied and characterized, however the role of Fas in NPCs is still unclear. Fas expression is increased in the developing nervous system during critical periods of neuronal differentiation and apoptosis, and is therefore is thought to play a role in nervous system development by regulating programmed cell death pathways (Knight et al., 2010).

In this study we investigated the role of Fas signaling in neural precursor cells. We also examined the regulation of Fas in NPCs by inflammatory mediators. We demonstrate that NPCs are susceptible to Fas-induced apoptosis through the intrinsic apoptotic pathway in a Puma-dependent manner. We also identify IL-1β as an inducer of Fas transcription through the activation of NF-κB.

4.2 Materials and methods

4.2.1 Animals

P53 and Bax were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were genotyped as previously described. Mice carrying a targeted null mutation for Puma were generated and maintained on a C57/BL6 background in the laboratory of Dr. Andreas Strasser (WEHI, Victoria, Australia). $Fas^{lpr/lpr}$ mice were obtained from the laboratory of Dr. Renian Wang (Western University, London, ON, Canada) and maintained on a C57/BL6 background. Genotyping of these mice was performed as previously described (Cregan et al., 1999). Timed pregnant wild-type CD1 mice were purchased from Charles River Laboratories (Sherbrooke, QC, Canada).

4.2.2 Neural precursor cell culture

Neural precursor cells were dissociated from the striatum of E13.5 mice and grown as neurospheres for 7 days in complete neural stem cell media consisting of DMEM-F12 containing D-glucose (6 mg/ml), L-glutamine (2 mM), penicillin/streptomycin, insulin (20 mg/ml), apotransferrin (100 mg/ml), progesterone (0.02 nM), putrescine (20 nM), sodium selenite (30 nM), heparin (0.3 nM), and bFGF (10 ng/ml) as previously described. After 7 days, neurospheres were dissociated by incubation in 0.05% trypsin-EDTA. Trypsin inhibitor (Roche) was added and suspension was triturated with flame-polished glass pipette. The cell suspension was centrifuged at 300xg for 5 minutes. Single cell NPCs were plated on dishes coated with poly-L-ornithine and laminin (Sigma, Oakville, ON, Canada).

4.2.3 Microglia cell culture and preparation of microglia conditioned media

The mouse microglial cell line EOC-20 was obtained from the American Type Culture Collection (ATCC CRL-2469, Manassas, VA, USA). Cells were maintained at 37^0C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 4mM L-glutamine, and 20% conditioned medium from bone-marrow-derived Ladmac cells (ATCC CRL-2420) as a source of colony stimulating factor-1. For preparation of microglia conditioned media (MCM), EOC-20 cells were grown to 60% confluence at which point their media was removed and replaced with neural stem

cell media (lacking bFGF and heparin). To activate microglia, LPS and γIFN were added to NPC media at 10 ng/ml (Sigma) for 24 h. MCM was collected, centrifuged and filtered through a 0.2-mm filter to remove cells and debris. MCM was then supplemented with 10 ng/ml bFGF and 0.3 nM heparin prior to use on NPC cultures. In the indicated experiments, LPS was added to non-activated MCM or unconditioned stem cell media immediately before adding to NPC culture.

4.2.4 NPC treatments and TNFα/IL-1β neutralization experiments

NPCs were treated with MCM, the monoclonal antibody Jo2 (BD Biosciences), or recombinant mouse IL-1β (rIL-1β; R&D Systems, Minneapolis, MN, USA) 2 days after plating as a monolayer. In the indicated experiments**,** N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-FMK), TNFα neutralizing antibody (10μ/ml), or recombinant mouse IL-1 Receptor antagonist (IL-1RA; R&D Systems, Minneapolis, MN, USA) was added to NPC cultures simultaneously with the switch to MCM or rIL-1β. For inhibition of IL-1β cleavage from microglia, the caspase-1 inhibitor N-Ac-Tyr-Val-Ala-Asp-chloromethyl ketone (y-VAD-CMK; Sigma) was added to microglia at the time of activation, and conditioned media was collected after 24 hours.

4.2.5 Cell death assays

Apoptosis of NPCs was assessed by examining nuclear morphology in Hoechst 33342-stained cells as previously described (Steckley et al., 2007). Briefly, NPCs were stained with 1 μg/ml Hoechst 33342 (Sigma) and the fraction of cells exhibiting an apoptotic nuclear morphology characterized by chromatin condensation and/or apoptotic bodies, was quantified. NPCs were visualized by fluorescence microscopy (Zeiss, Toronto, ON, Canada) and images were captured with a Zeiss Axio-Cam camera (Zeiss). Images were captured and scored by an observer blinded to the treatment. A minimum of 500 cells from five randomly selected fields were analyzed for each treatment and data represent the mean and S.E.M. from a minimum of four independent experiments.

4.2.6 Caspase-3-like activity assay

NPCs were harvested in lysis buffer (1mM KCl, 10mM HEPES, pH 7.4, 1.5mM MgCl2, 1mM PMSF, 5 mg/ml leupeptin, 2 mg/ml aprotinin, and 10% glycerol), and 10 µg of protein was used in caspase-3-like activity assay as previously described. Briefly, protein samples were added to caspase reaction buffer (25mM HEPES (pH 7.4), 10mM DTT, 10% sucrose, 0.1% CHAPS, and 10 mM N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC)) and fluorescence produced DEVD-AFC cleavage was measured on a SpectraMax M5 fluorimeter (excitation 400 nm, emission 505 nm) over a 1-h interval. Caspase-3-like activity is reported as the ratio of the fluorescence output in NPCs treated with Jo2 to NPCs treated with vehicle control.

4.2.7 Quantitative real-time RT-PCR

RNA was isolated using Trizol reagent as per the manufacturer's instructions (Invitrogen) and 10 ng of RNA was used in one-step Sybr green reverse transcription (RT)-PCR (QuantiFast, Qiagen, Mississauga, ON, Canada). RT-PCR was carried out on a Chromo4 system (MJ Research Bio-Rad, Mississauga, ON, Canada) and changes in gene expression were determined by the $\Delta(\Delta Ct)$ method using S12 transcript for normalization. Data are reported as fold increase in mRNA levels in treated samples relative to untreated control cells. All PCR's exhibited high amplification efficiency (>90%) and the specificity of PCR products was confirmed by sequencing.

4.2.8 Western blot analysis

Whole cell lysates were prepared by incubating NPCs in lysis buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8, 1mM EDTA, 1 mM DTT, and protease and phosphatase inhibitor cocktail (Invitrogen) for 20 min on ice. The soluble extract was recovered by centrifugation at 14,000xg. Protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA) and 50 µg of protein was separated on 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked for 1 h in TBS-T (10mM Tris, 150mM NaCl, 0.05% Tween-20), followed by overnight incubation in primary antibodies to p53, p-p65, GAPDH (Cell Signaling Technology, Danvers, MA, USA), Bid (R&D Systems), Puma (Sigma), Fas or Actin (Santa

Cruz Biotech) in TBS-T containing 5% skim milk. Membranes were washed with TBS-T and incubated for 1 h with the appropriate HRP-conjugated secondary antibodies and developed by enhanced chemiluminescence system according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada).

4.2.9 Lentiviral constructs

For knockdown of p65, *p65* shRNA and control shRNA (scramble as well as empty shRNA) were designed using the TRC database (The RNAi Consortium, Broad Institute, MIT). Custom oligonucleotides were obtained from Sigma Aldrich. Forward and reverse oligonucleotides were annealed and phosphorylated and cloned into a pLB plasmid under the U6 promoter, co-expressing green fluorescent protein (GFP) (Addgene; Appendix Figure A2).

P65 target sequence:

Forward: 5'AACCCGGTCTGTGGACAACTCAGAGTTTTCAAGAGAAACTCTGAG TTGTCCACAGATTTTTTC 3'.

Reverse:5'AACCCGGTCTGTGGACAACTCAGAGTTTTCAAGAGAAACTCTGAGT TGTCCACAGATTTTTTC 3'.

4.2.10 Lentivirus production and transduction of NPCs

Lentiviruses were generated by transient co-transfection of HEK293T cells with a four plasmid combination as follows: sh-p65 in pLB (or sh-Scramble or empty pLB), pSL3/pMD2.VSVG, pSL4/pMDLg/pRRE#S4, and pSL5/pRSV-REV. Plasmids were transfected using Lipofectamine 2000 according to manufacturer's protocol (Invitrogen). After 6 hours of incubation, transfection media was removed and replaced with DMEM containing 10% FBS, 10mM sodium butyrate. At approximately 28 hours post-transfection media in flasks was collected and filtered into Amicon Ultra 15mL 100k MWCO centrifugal filter devices. Media was centrifuged for 30 minutes at 3,200xg at 4^0C. Neural precursor cells were transduced with lentivirus expressing a short hairpin for p65 (sh-p65) or scrambled sequence (sh-scr) at 1 day post plating as a monolayer at a concentration of 2 µl/ml and a transduction efficiency of ~70%.

4.3 Results

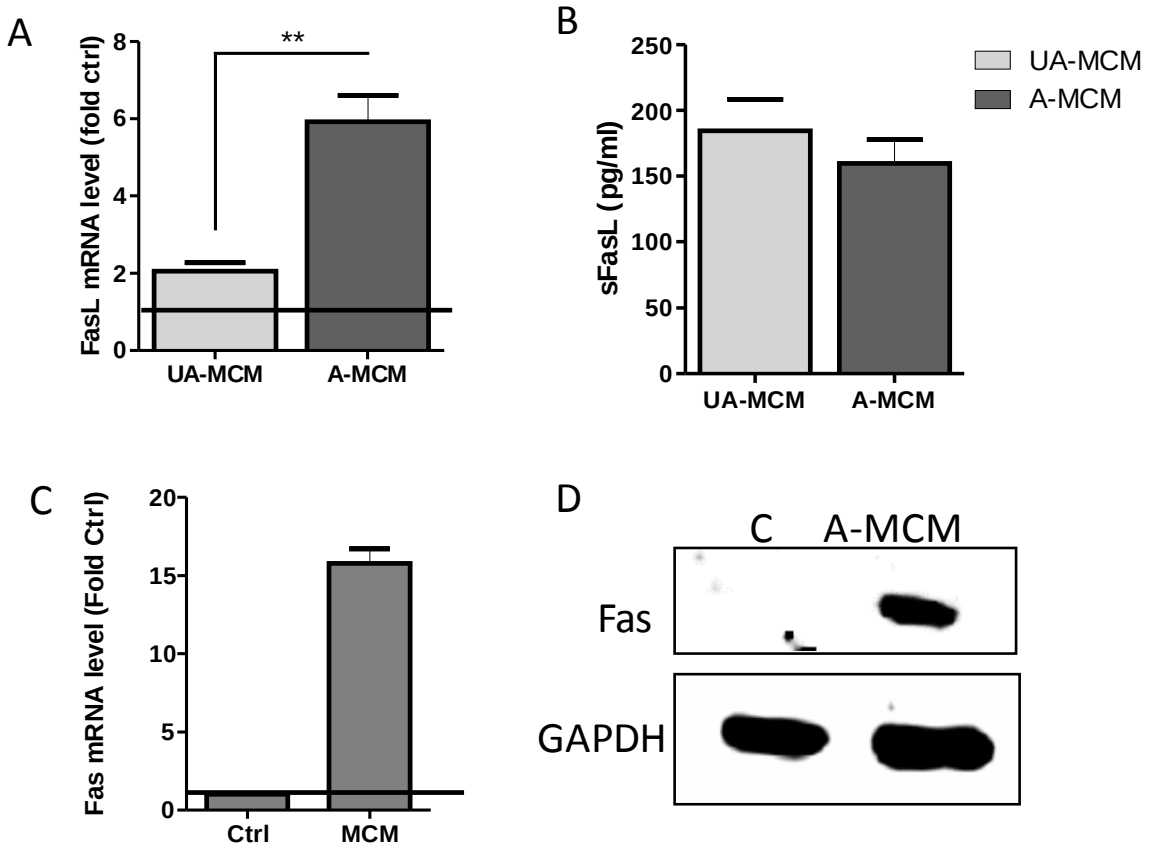
4.3.1 Conditioned media from LPS/γIFN-activated microglia induces Fas expression in NPCs

When activated by pro-inflammatory stimuli, microglia increase the expression and secretion of pro-inflammatory cytokines and soluble factors. Fas ligand (FasL) is a member of the TNF family of proteins that can trigger death of cells that express Fas receptors. The amount of FasL on the surface of microglia is correlated to the ability of microglial cells to induce apoptosis in Fas-positive cells (Frigerio et al., 2000). Evidence suggests that FasL is consitutively expressed at low levels on microglia, but can be upregulated in response to microglia activation (Frigerio, et al., 2000). Figure 4.1A demonstrates that mRNA levels of FasL were increased in microglia following activation with LPS/γIFN. The majority of FasL exists as a membrane-bound protein (mFasL), which can be cleaved by matrix metalloproteinases (MMPs) to yield a soluble form of the ligand (sFasL). We examined whether the upregulation of FasL expression in microglia resulted in increased levels of sFasL in microglia conditioned media (MCM). We did not however, observe detectable levels of sFasL in conditioned media from LPS-activated microglia as indicated by ELISA analysis (Figure 4.1B).

We next examined whether microglia-derived factors could modulate the expression of Fas in NPCs. Figure 4.1C illustrates that following treatment with MCM, there is a robust increase in the mRNA levels of Fas in NPCs (>15 fold), as detected by qRT-PCR. Corresponding to this, we also observe an increase in Fas protein levels in NPCs incubated for 24H in MCM (Figure 4.1D).

Figure 4.1: Fas is upregulated on NPCs following exposure to conditioned media from LPS-activated microglia.

(A) EOC-20 microglia were activated with LPS/γIFN or left unactivated and cultured in complete stem cell media. After 24 hours, RNA was harvested from unactivated (UA) or activated microglia (A) and levels of FasL were assessed by qRT-PCR (n=3, **P<0.01). (B) Conditioned media from LPS activated (A), or unactivated (UA) microglia was collected after 24 hours and levels of sFasL in conditioned media were analyzed by ELISA. Notice that levels of sFasL were not different in UA vs. A-MCM (n=5; N.S.). (C) NPCs were cultured in either unconditioned stem cell media (Ctrl), or conditioned stem cell media from LPS/γIFN -activated microglia (MCM). RNA was harvested from NPCs incubated in a-MCM for 24 hours and mRNA levels of Fas were measured by qRT-PCR. mRNA levels are reported as fold increase over NPCs incubated in unconditioned stem cell media (n=4). (D) NPCs were incubated in conditioned media from activated microglia for 48 hours and Fas protein levels were determined by western blot. Representative blot from three independent experiments is shown.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A-MCM
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sFasL (pg/ml)
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150
100
50
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UA-MCM
A-MCM
UA-MCM
A-MCM
C
Fas mRNA level (Fold Ctrl)
20
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Ctrl
MCM
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C
A-MCM
Fas
GAPDH

Figure 4.1

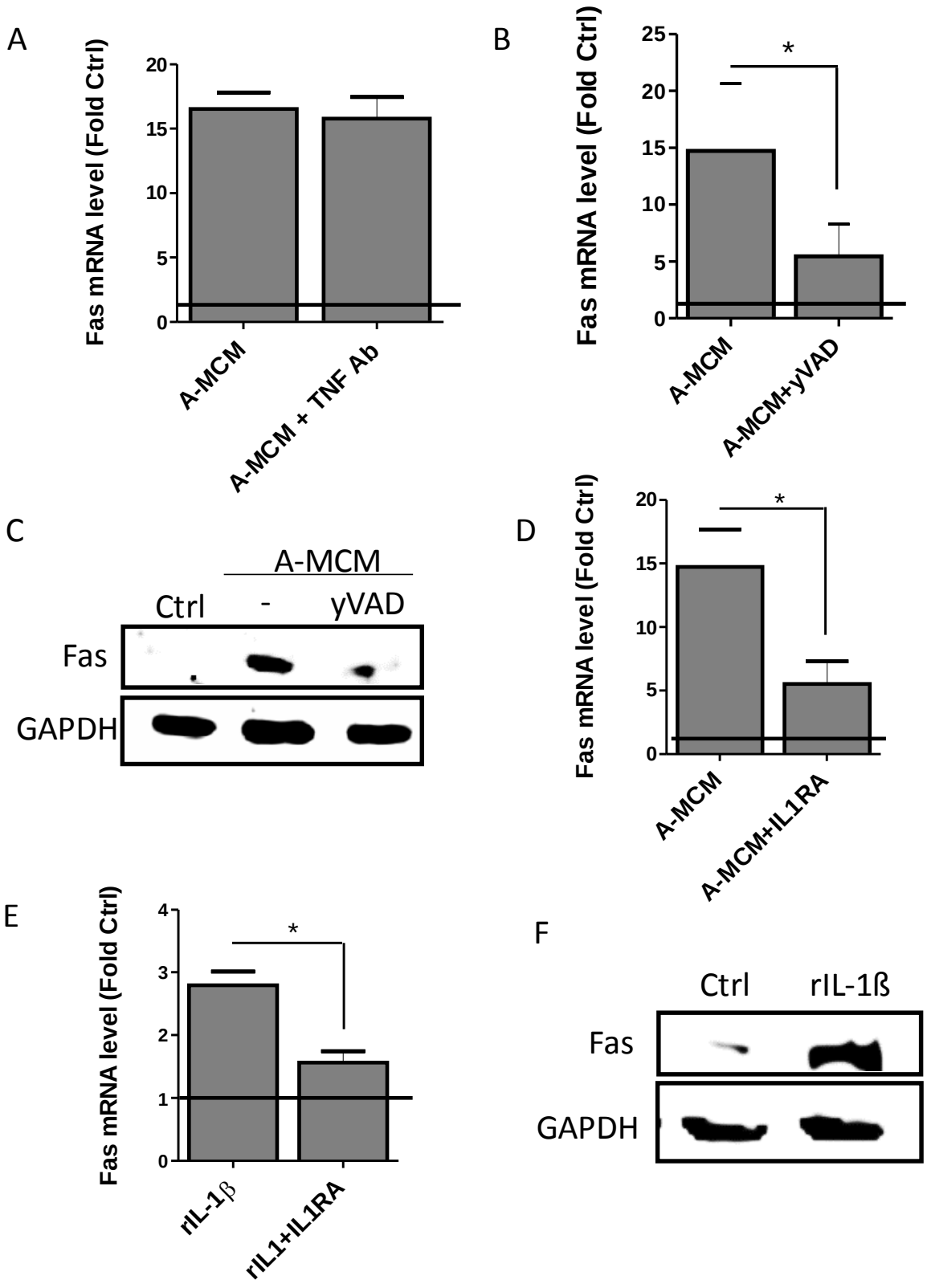
4.3.2 Blockade of IL-1β signaling attenuates Fas expression in NPCs

To determine the mechanism of Fas upregulation, we examined cytokines released from microglia that could be responsible for upregulation of Fas on NPCs. Previous studies investigating Fas signaling have indicated that TNFα and IL-1β were capable of inducing expression of Fas in sertoli cells and pancreatic β-cells, respectively (Starace et al., 2005; Darville & Eizirik, 2001). We have previously demonstrated that both TNFα and IL-1β are released by LPS-activated microglia, and neutralization of these factors in MCM attenuates apoptosis of NPCs exposed to MCM (Guadagno et al., 2013). We therefore investigated whether neutralization of these factors in MCM could mitigate the increase in Fas expression in NPCs. Figure 4.2A demonstrates that neutralization of TNFα using a TNF-neutralizing antibody does not have a significant effect on Fas mRNA levels in MCM-treated NPCs, suggesting that TNFα does not contribute to MCM-induced Fas expression in NPCs. Production and release of mature, active IL-1β relies on the cleavage of pro-IL-1β in microglia by caspase-1, also known as interleukin-1 converting enzyme (ICE). Consistent with this, we have previously shown that addition of the caspase-1 inhibitor y-VAD-CMK (y-VAD) reduces levels of IL-1β in MCM (Guadagno et al., 2014; unpublished data). As shown in Figures 4.2 B and C, activated MCM generated in the presence of y-VAD-CMK was significantly less effective at inducing Fas mRNA and protein expression in NPCs. Furthermore, we demonstrate that blockade of the IL-1 receptor in NPCs with exogenous IL-1 receptor antagonist (IL-1RA) diminished MCM induced Fas expression in NPCs (Figure 4.2D).

To further investigate the ability of IL-1β to induce Fas expression we examined whether exogenous application of IL-1β was sufficient to induce Fas expression. NPCs were treated with recombinant IL-1β (rIL-1β) and Fas mRNA and protein levels were examined by qRT-PCR and western blot, respectively. As shown in Figure 4.2 E and F the addition of rIL-1β is sufficient to induce expression of Fas in NPCs, and this is prevented by the co-application of IL-1RA (Figure 4.2 E,F). Takent together, these results suggest that IL-1β released from activated microglia contributes to Fas upregulation in NPCs.

Figure 4.2. Blockade of Interleukin-1B signaling attenuates activated microglia-induced Fas expression in NPCs

(A) NPCs were incubated in unconditioned stem cell media (control) or conditioned media from LPS/γIFN-activated microglia (MCM) in the presence or absence of TNFα neutralizing antibody (10µg/ml). RNA was harvested after 24 hours and Fas mRNA levels were determined by qRT-PCR. mRNA levels are reported as fold-increase over control (n=4; N.S.). (B) NPCs were incubated in conditioned media from LPS-activated microglia (MCM) in the presence or absence of caspase-1 inhibitor y-VAD-CMK (y-VAD; 20µM). RNA was harvested after 24 hours and Fas mRNA levels were determined by qRT-PCR. mRNA levels are reported as fold-increase over control (n=4; *P<0.05). (C) Protein was extracted from NPCs treated with MCM +/- y-VAD-CMK (20 µM) for 48 hours and Fas protein levels were analyzed by western blot. A representative image from three independent experiments is shown. (D) NPCs were incubated in MCM in the presence or absence of Interleukin-1 receptor antagonist (IL-1RA; 50ng/ml). RNA was harvested after 24 hours and Fas mRNA levels were determined by qRT-PCR. mRNA levels are reported as fold-increase over control (n=4; *P<0.05). (E) RNA was harvested from NPCs treated for 24 hours with rIL-1β (50ng.ml) in the presence or absence of IL-1RA, and Fas mRNA levels were determined by qRT-PCR (n=4, *P<0.05). (F) Protein was harvested from NPCs at 48 hours after treatment with rIL-1B +/- IL-1RA and Puma and Fas protein levels were determined by western blot analysis. Representative blot from three independent experiments is shown.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rIL-1ß
Fas
GAPDH

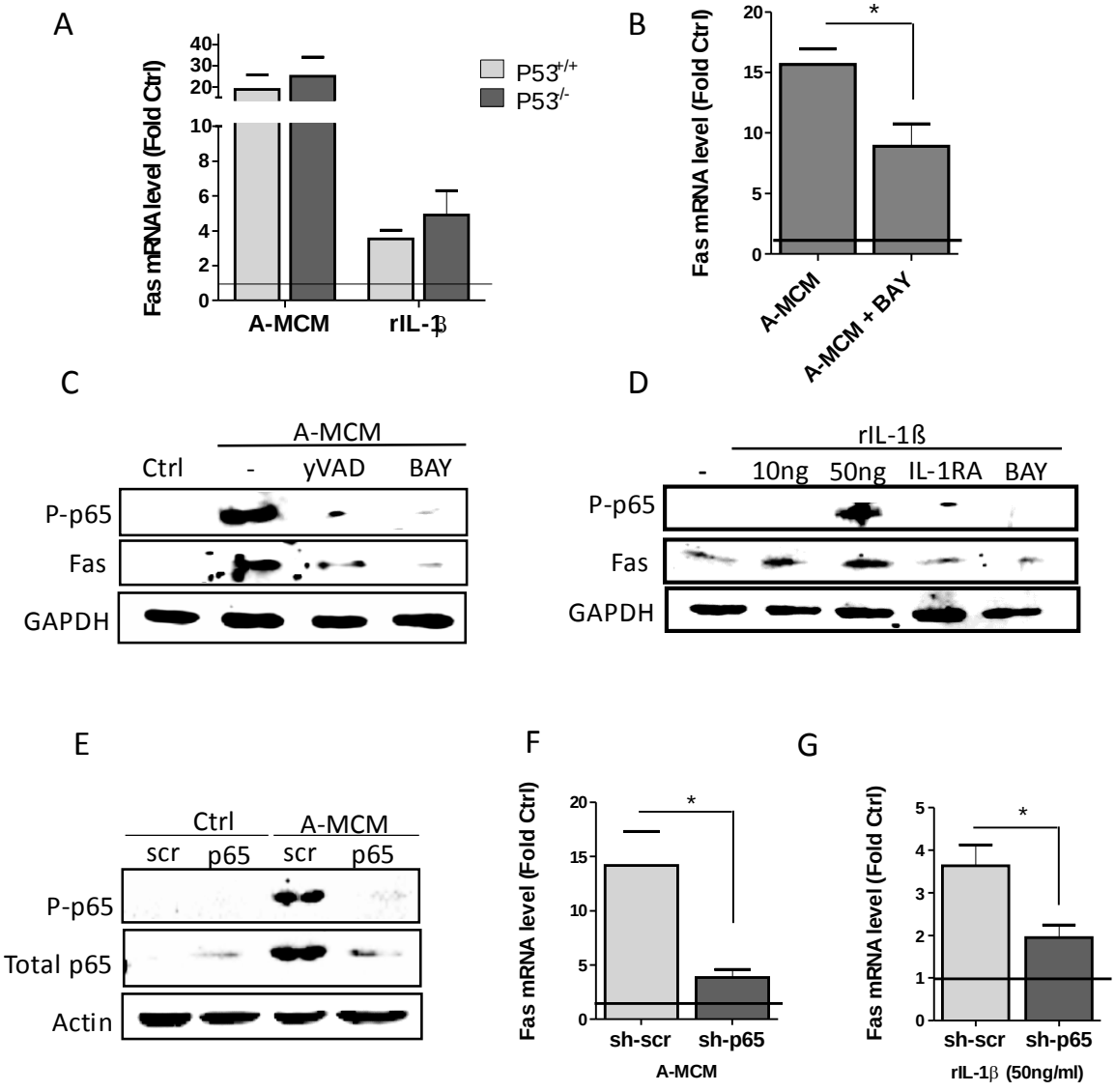
Figure 4.2

4.3.3 IL-1β induces Fas expression via an NF-κB-dependent pathway

The IL-1 receptor belongs to the IL-1 receptor and Toll-like receptor (TLR) superfamily, which is characterized by the presence of an intracellular Toll/IL-1Receptor (TIR) domain. Activation of the TIR domain can trigger signaling cascades that result in activation of downstream targets such as MAPKs, p53, and NF-κB (Allan et al., 2005). Previous studies have reported that p53 could activate Fas in NPCs in response to ionizing radiation. Therefore, we investigated a potential role for p53 in Fas upregulation using p53-deficient NPCs. However, as illustrated in Figure 4.3A we found that Fas induction by MCM and rIL-β is not dependent on p53. We next examined whether IL-1β-mediated NF-κB activation was involved in regulating Fas upregulation in NPCs. The pharmacological inhibitor BAY-117082 inhibits NF-κB activation by blocking cytokine induced phosphorylation of IκB-α, thereby blocking phosphorylation (activation) of p65 and preventing translocation to the nucleus (Chiarugi, 2002). Importantly, when we examined the levels of serine-536 phosphorylated (active) p65, we observed an increase in p-p65 protein levels following treatment with activated MCM or rIL-1β, indicating that IL-1R1 signaling triggers NF-κB activation in NPCs (Figure 4.3C and D). This increase in the active p65-subunit was inhibited by the addition of either IL-1RA or BAY-117082 (Figure 4.3C and D). Interestingly, the Fas promoter has been shown to contain NF-kB binding sites, therefore we sought to examine whether NF-kB could be responsible for Fas upregulation following MCM and r IL-1β treatment. Indeed, we found that addition of BAY-117082 to NPCs, resulted in a reduction of Fas mRNA and protein induction by a-MCM and rIL-1β (Figure 4.3B-D). To confirm our findings with the pharmacological inhibitor of NF-κB, we generated a lentivirus expressing a small-hairpin directed against the NF-κB subunit p65. Figure 4.3E shows that transduction of NPCs with the lenti-sh-p65 vector markedly decreased levels of total and serine-536 phosphorylated p65 following a-MCM treatment. Importantly, we also demonstrate that knockdown of p65 attenuates MCM and rIL-1β-induced Fas expression in NPCs (Figure 4.3F and G). Together, our findings suggest that microglia-derived IL-1β regulates Fas induction in NPCs throughactivation of NF-κB.

Figure 4.3: Interleukin-1β induces Fas expression via an NF-κB dependent pathway.

(A) $p53^{+/+}$ and $p53^{-/-}$ NPCs were treated with MCM or rIL-1β (50ng/ml). RNA was harvested after 24 hours, and Fas mRNA levels were determined by qRT-PCR. Values are expressed as fold-increase over control (N=4, N.S.) (B) NPCs incubated for 24 hours in LPS-activated microglia conditioned media in the presence or absence of BAY-117082 (10μM) or vehicle control. Fas mRNA levels were determined by qRT-PCR and are expressed as fold increase over NPCs cultured in unconditioned stem cell media (n=4, *$p<0.05$). (C,D) Protein levels of p-p65 and Fas were assessed by western blot following treatment with aMCM or rIL-1β in the presence or absence of BAY-117082 (10μM) or IL-1RA (50ng/ml) for 48 hours. A representative blot from three independent experiments is show. (E) NPCs were infected with lentivirus expressing either a non-coding shRNA sequence (sh-scr), or a shRNA targeting p65 (sh-p65) sequence after 1 day in culture. 1 day later NPCs were incubated with unconditioned stem cell media or conditioned media from LPS-activated microglia (a-MCM). Protein levels of P-p65, total p65 were assessed by western blot to confirm knockdown of p65. A representative blot from three independent experiments is shown. (F, G) NPCs transduced with lenti-sh-p65 or lenti-sh-scr were treated with activated MCM or rIL-1β (50ng/ml) and RNA was harvested after 24 hours. Fas mRNA levels were determined by qRT-PCR and are reported as fold increase over control NPCs cultured in unconditioned stem cell media (N=3, *$p<0.05$).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sh-scr
sh-p65
rIL-1β (50ng/ml)

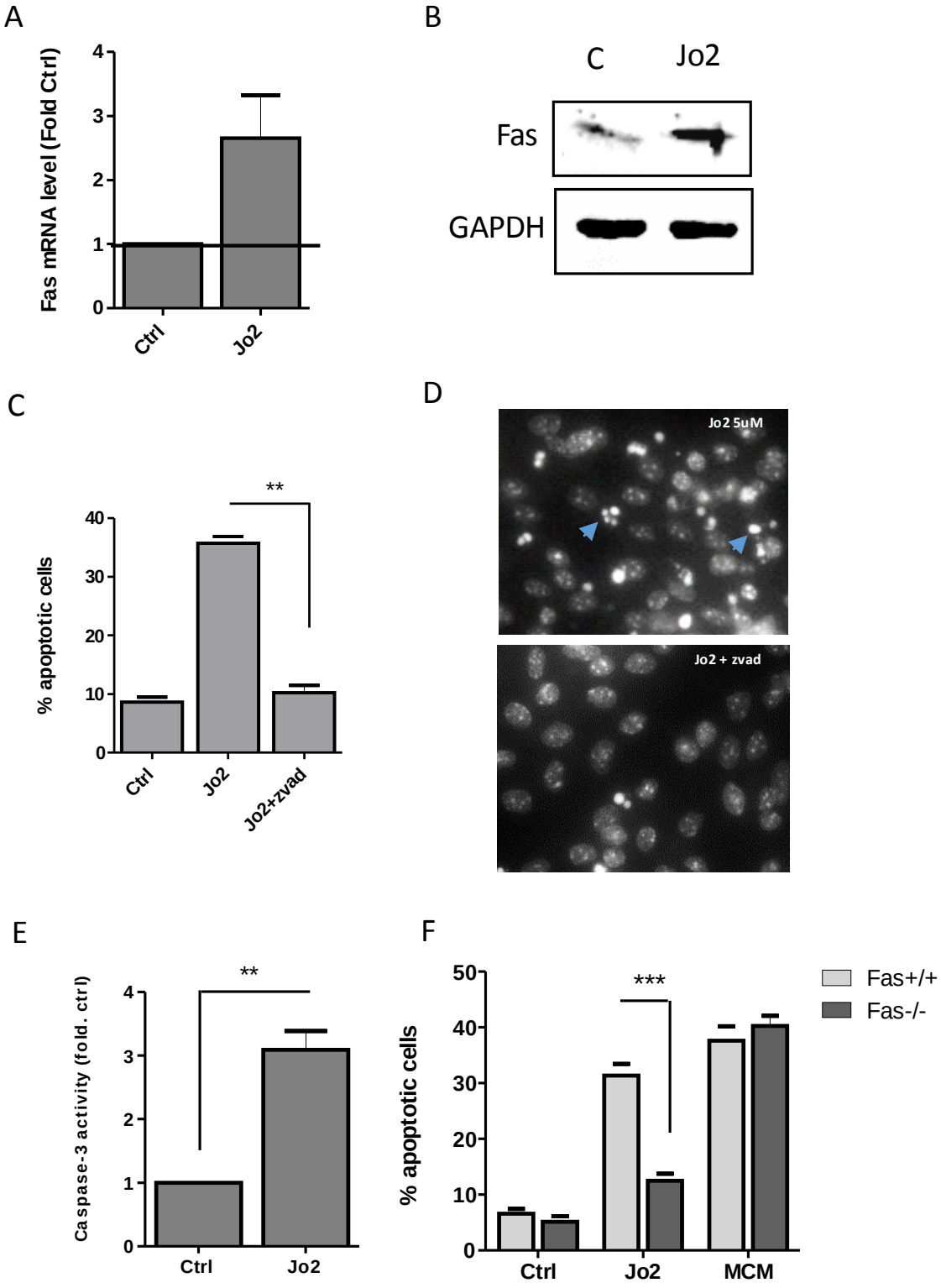
Figure 4.3

4.3.4 The monoclonal anti-Fas antibody Jo2 induces apoptosis in NPCs

The monoclonal antibody clone Jo2 has been shown to have cytolytic activity on cells expressing Fas. Moreover Edmond et al., (2012) suggested that upon binding Jo2 induces aggregation and homotrimerization of Fas receptor, which is necessary for the transmission of apoptotic signals (Edmond et al., 2012). We exploited the ability of Jo2 to act as an activating ligand for Fas to further examine the significance of Fas upregulation and the role of Fas signaling in NPCs. We first examined the mRNA and protein level of Fas on NPCs following treatment with Jo2 using qRT-PCR and western blot, respectively. NPCs exhibit an increase in the mRNA and protein levels of Fas when treated with Jo2 (Figure 4.4 A and B). Given this increase in Fas expression, we examined whether Jo2 could induce apoptosis in NPCs. Figure 4.4 C and D illustrates that treatment with Jo2 can induce apoptosis in NPCs, and that this could be abrogated with the pan-caspase inhibitor z-VAD-fmk. As further evidence of apoptosis, caspase-3 activity was increased in NPCs following Jo2 treatment, as indicated by caspase-3 activity assay (Figure 4.4E). While it appears that Jo2 is activtating a caspsase-dependent pathway, we cannot completely rule out a role for other cell death pathways, such as necroptosis, which is a mechanism of programmed necrotic death induced by engagement of death receptors. As a confirmation that the effects of Jo2 were a result of Fas activation and signaling we examined the effect of Jo2 on Fas-deficient ($Fas^{lpr/lpr}$) NPCs. We demonstrate that Fas-deficient NPCs are resistant to Jo2-induced cell death, indicating that the effects of Jo2 on NPCs are primarily mediated by Fas signaling (Figure 4.4F).

Figure 4.4: The Fas activating monoclonal antibody Jo2 induces apoptosis in neural precursor cells.

(A) NPCs were treated with the monoclonal anti-Fas antibody Jo2 (5μM) and harvested after 24 hours to examine RNA levels of Fas using qRT-PCR (n=4, *P<0.05). (B) Protein levels of Fas were analyzed by western blot after 48 hours of Jo2 (5 μM) treatment. A representative blot from three-independent experiments is shown. (C) NPCs were treated with Jo2 (5μM) in the presence or absence of z-VAD-FMK (50 μM) and stained after 72 hours with Hoechst 33342 to assess apoptosis. The fraction of apoptotic cells was determined by examining nuclear morphology (n=4, **P<0.01). (D) Representative images of Hoechst stained NPCs following treatment with Jo2 in the presence or absence of the pan-caspase inhibitor z-vad-fmk (50uM). (E) Protein extracts were obtained from NPCs at 72 hours and assayed for caspase-3-like activity. Caspase-3 activity is reported as fold increase over vehicle control treated NPCs (n=3, **p<0.01). (F) NPCs derived from $Fas^{lpr/lpr}$ and wild-type embryos were treated with, Jo2 (5uM) or vehicle control. Apoptosis was assessed at 72 hours with Hoechst staining (n=3, ***p<0.001).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% apoptotic cells
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10
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Ctrl
Jo2
Jo2+zvad
D
Jo2 5uM
Jo2 + zvad
E
Caspase-3 activity (fold. ctrl)
4
3
2
1
0
**
Ctrl
Jo2
F
% apoptotic cells
50
40
30
20
10
0
***
Ctrl
Jo2
MCM
Fas+/+
Fas-/-

Figure 4.4

4.3.5 Fas induces NPC apoptosis via a Puma, and Bax-dependent, mitochondrial pathway

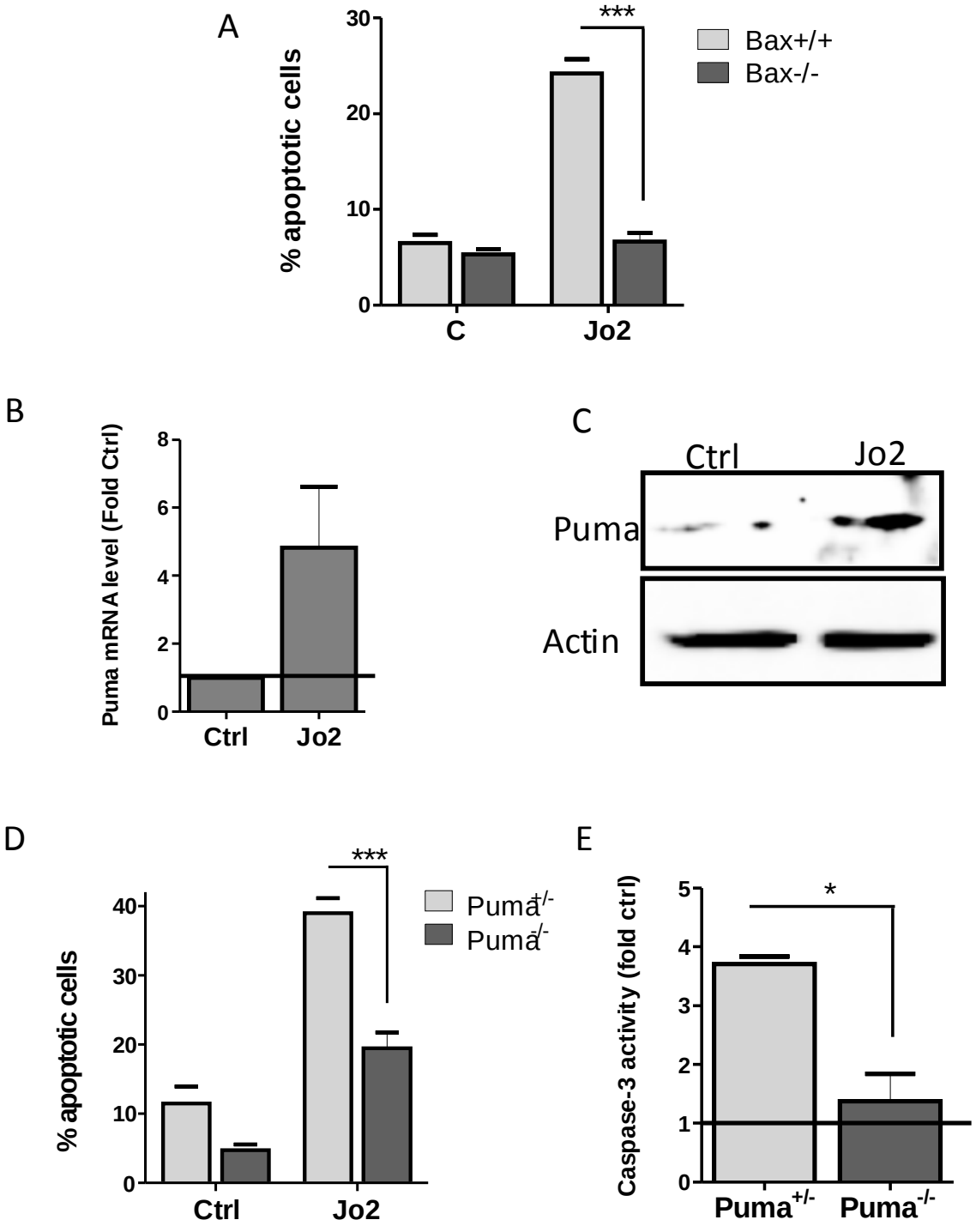
Binding of ligands to Fas leads the recruitment and activation of adaptor molecules and procaspase-8 to form a death inducing signaling complex (DISC). Activation of caspase-8 by self-cleavage can induce apoptosis through either a mitochondrial-dependent or mitochondrial-independent pathway, classifying cells as either type I or type II cells (Kaufman et al., 2012). Following formation of the DISC, type I cells undergo apoptosis through a Bcl-2 family and mitochondrial-independent pathway, involving direct activation of downstream effector caspases (3, 6, 7) by caspase-8. In contrast, Type II cells show a reduced DISC formation and caspase-8 activation, placing more importance on events at the mitochondria that are regulated by the Bcl-2 protein family (Brint et al., 2013). We wanted to determine which pathway of apoptosis was activated by Fas in NPCs. The pro-apoptotic Bcl-2 protein Bax is a known regulator of mitochondrial-mediated apoptosis. Therefore to determine whether Fas induces apoptosis via a mitochondrial pathway, we examined the survival of NPCs derived from Bax-deficient mice and wild-type littermate controls following exposure to Jo2. As shown in figure 4.5A, the fraction of apoptotic cells is significantly reduced in Bax-deficient NPCs treated with Jo2 relative to wild-type cultures. Thus we conclude that Jo2 is inducing apoptosis predominantly through a mitochondrial pathway, regulated by the Bcl-2 family.

Pro-apoptotic BH3-only proteins regulate Bax activation and apoptosis in a stimulus and cell type- specific manner. Interestingly, the BH3-only protein Bid has previously been implicated in death receptor-mediated apoptotic pathways, such that activation of death receptors leads to activation of caspase-8 which is known to cleave Bid into its active, truncated form t-Bid (Kaufman et al., 2012). However, we do not observe an appreciable level of activated/truncated Bid (tBid) in NPCs following treatment with either rTNFα or Jo2 (data not shown). We have however previously shown that the BH3-only family member Puma is a dominant regulator of Bax activation and apoptosis in NPCs following treatment with recombinant TNFα (Guadagno et al., 2013). Therefore, we examined whether Puma may also be required for Fas-mediated apoptosis in NPCs. Intrestingly, we

observed a marked increase in Puma mRNA levels, and a corresponding increase in Puma protein levels in NPCs treated with Jo2 (figure 4.5B and C). We next examined whether Puma is required for Jo2-induced apoptosis in NPCs. To address this, we compared apoptotic measures in Puma-deficient NPCs to wild-type littermate controls. The fraction of apoptotic cells was significantly reduced in $Puma^{-/-}$ NPCs relative to wild-type cultures as assessed by Hoechst 33342 staining (Figure 4.5D). Consistent with this, we observed a significant decrease in caspase-3 activity in Puma-deficient NPCs treated with Jo2 as compared to Jo2-treated wild-type NPCs (figure 4.5E). Taken together these data demonstrate that Fas induces NPC death via a Puma- and Bax-dependent and mitochondrial apoptotic pathway.

Figure 4.5: Fas induces NPC apoptosis via a Puma- and Bax-depdendent mitochondrial pathway.

(A) NPCs derived from Bax-/- and +/+ embryos were treated with Jo2 (5uM) or vehicle control. Apoptosis was assessed at 72H with Hoechst staining (n=3, ***p<0.001). (B) NPCs were treated with Jo2 or vehicle control and RNA was harvested after 24 hours. mRNA levels of Puma were assessed by qRT-PCR, and levels are reported as fold increase over control NPCs (n=4). (C) Puma protein levels were assessed by western blot analysis following treatment with Jo2 (5μM) for 48 hours. A representative blot from three independent experiments is shown. (D) NPCs derived from $Puma^{+/+}$ and $Puma^{-/-}$ were treated with Jo2 and after 72 hours NPCs were stained with Hoechst 33342 and apoptosis was quantified by examining nuclear morphology (n=4; ***P<0.001). (E) Protein extracts from $Puma^{+/-}$ and $Puma^{-/-}$ NPCs were harvested at 72 hours after Jo2 (5μM) treatment and assayed for caspase-3-like activity. Caspase -3 activity is reported as fold increase over vehicle control (n=3, *p<0.05).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Bax-/-
C
Jo2
B
Puma mRNA level (Fold Ctrl)
8
6
4
2
0
Ctrl
Jo2
C
Ctrl
Jo2
Puma
Actin
D
% apoptotic cells
40
30
20
10
0
***
Puma+/-
Puma-/-
Ctrl
Jo2
E
Caspase-3 activity (fold ctrl)
5
4
3
2
1
0
*
Puma+/-
Puma-/-

Figure 4.5

4.1 Discussion

Inflammatory processes are a prominent feature of many pathological conditions as well maintaining a homeostatic environment in the CNS and periphery. The brain is often considered an immune privileged site, as it contains low levels of systemic immune/inflammatory cell types and products. However, the CNS is guarded by its own resident immune macrophages, known as microglia, which survey the CNS microenvironment and mount inflammatory reactions upon injury, pathogen invasion, or neurodegenerative diseases. Importantly, microglia release soluble anti- and pro-inflammatory factors that can both affect cells in the CNS as well as lead to the exacerbation of inflammation through the recruitment of peripheral immune cells (Hickey et al., 1999; Whitney et al., 2009). Fas ligand (FasL) is a pro-inflammatory factor that is upregulated on T cells and macrophages, whereas its receptor, Fas may be expressed by several target cells, whereby interaction between FasL and Fas can induce apoptosis of the specific target (Frigerio et al., 2000). There is evidence that FasL is constitutively expressed at low levels on microglial cells in the CNS to aid in immunological response (Frigerio et al., 2000). In multiple sclerosis (MS) lesions, FasL is upregulated on the surface of microglial cells, suggesting that Fas signaling could contribute to the pathogenesis of the disease (Frigerio et al., 2000). Furthermore, studies have shown that FasL can also be upregulated on microglia activated by pathogens, injury such as TBI and SCI, and neurodegenerative diseases including Alzheimer's disease (Ethell & Buhler, 2003; Demjen et al., 2004; Yu et al., 2008; Frigerio et al., 2000).

Here we demonstrate an upregulation of FasL on microglia *in vitro* following activation by LPS/γIFN. Importantly, we did not detect significantly increased levels of sFasL between unactivated and LPS-activated microglia, which is consistent with the findings of others that the majority of FasL exists as a membrane bound form. The membrane-bound form (mFasL) has been reported to have a more potent apoptotic effect than its soluble counterpart (sFasL) (Weinlich et al., 2010; Brint et al., 2013). Microglia, and other cells expressing FasL can induce apoptosis of target cells that express the receptor Fas. Interestingly, astrocytes, a dominant cell type in the CNS can also upregulate the expression of Fas and FasL during inflammatory conditions. We and others illustrate that

murine neural precursor cells express Fas, which could render this cell type responsive to FasL/Fas signaling, however the role of Fas signaling in NPCs remains unclear. Using the activating anti-Fas antibody Jo2 we demonstrate that treatment of NPCs with a Fas agonist induced apoptosis in NPCs, and this was specific to Fas signaling as $Fas^{lpr/lpr}$ NPCs were resistant to Jo2-induced death. Jo2 was chosen to investigate Fas signaling in NPCs as we did not see significant release of FasL into microglia conditioned media, however we did observe Fas upregulation, suggesting this pathway could be important in NPCs.

Previous studies using Jo2 have shown that the antibody Jo2 can induce mitochondrial membrane permeabilization and apoptosis in spinal cord cultures, as well as apoptosis of neural progenitor cultures expressing Fas (Yu et al., 2008; Semont et al., 2004). In contrast to our findings in NPCs, others have suggested that Fas can mediate neuroregeneration by inducing dendrite branching neurons and by activating adult neurogenesis (Corsini et al., 2009). Suppression of Fas-mediated neurogenesis in the hippocampus results in reduced spatial learning and decreased integration of transplanted NSCs (Beier et al., 2009). Knight et al., (2010) show that murine adult NPCs exhibit increased cell survival in response to via the upregulation of Birc3; a member of the inhibitor of apoptosis (IAP) family (Knight et al., 2010). The discrepancy between the role of Fas may be a result of the cell type used (adult vs embryonic NPCs), or more likely, a result of treatment conditions as Knight and colleagues used a recombinant FasL and observed the effects of Fas on NPCs that had been deprived of growth factors (Knight et al., 2010).

Importantly, using Jo2 we have established a novel connection between Fas activation and critical mediators of the intrinsic apoptotic pathway. In the majority of cell types, ligation of FasL with Fas, results in activation of caspase-8, and cleavage of Bid to its truncated form tBid, which can translocate to the mitochondria and through its interactions with Bcl-2 family proteins can induce mitochondrial membrane permeabilization, release of cytochrome c and downstream caspase activation (Brint et al., 2013). Here we show that Jo2 induces NPC apoptosis through a Bax-dependent, mitochondrial pathway. The pro-apoptotic Bcl-2 protein Bax can be activated by BH3-only proteins such as Bid, Puma, and Bim to induce mitochondrial outer membrane permeabilization and apoptosis. Yu et al., (2008) illustrated that in a model of SCI *in vivo*, Fas^{lpr} mice exhibit reduced production of

truncated Bid protein (tBid) as well as in increase in the expression of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL compared to wild-type mice, suggesting that Fas-mediated apoptosis is augmented by the intrinsic apoptotic pathway (Yu et al., 2008). Here we show that Jo2 induces the expression of the pro-apoptotic BH3-only Bcl-2 family member Puma in NPCs. We and others have previously identified Puma as a critical regulator of NPC apoptosis in response to genotoxic stress, or inflammatory stimuli (Akhtar et al., 2006; Guadagno et al., 2013). While Fas-induced apoptosis is classically thought to occur via tBid translocation and activation of Bax, we do not observe detectable levels of tBId following Jo2 treatment. Interestingly, a study by Leonard and colleagues, suggested that Bid deficiency had no effect on neural precursor cell death either in vivo or in vitro, implying that other BH3-only proteins can compensate for Bid and that Bid is dispensable for NPC apoptosis (Leonard et al., 2001; Akhtar et al., 2004). We note that Puma-deficient NPCs are resistant to Jo2-induced apoptosis; identifying Puma as a regulator of Fas-induced apoptosis in NPCs. though we cannot conclusively rule out a potential role for Bid.

Increases in Fas expression can be induced under inflammatory conditions by soluble factors such as gamma-interferon (γ-IFN), reactive oxygen species, TNFα, or IL-1β (Curtin & Cotter, 2003; Huang et al., 2003; Elzey et al., 2001; Ouaaz et al., 1999; Darville & Eizirik, 2001). In a study of type I diabetes it was noted that inflamed pancreatic tissue from diabetic patients exhibited increased levels of Fas expression on β-cells, and it was suggested that this could be due to the release of cytokines by infiltrating immune cells and macrophages (Moriwaki et al., 1999; Darville & Eizirik, 2001). In this study we demonstrate that neutralization of TNFα in MCM was not sufficient to mitigate Fas upregulation in NPCs. However, we show that neutralization of IL-1β in MCM significantly reduced Fas mRNA and protein in NPCs. Furthermore, we are the first to show that rIL-1β could also induce upregulation of Fas, indicating that IL-1β can regulate Fas expression in cultured embryonic NPCs. Consistent with our finding that IL-1β can regulate Fas expression, a study on pancreatic islet cells demonstrated that Fas could be induced by IL-1β, and that the cells were sensitive to apoptosis when exposed to a Fas monoclonal antibody (Darville & Eizirik, 2001). A variety of signaling pathways and transcription factors have been associated with Fas upregulation. These include the JNK

pathway, STAT-1, and p53, each of which can increase transcription of Fas in a cell-type and stimulus specific manner. Specifically, in neural progenitor cells, p53 was found to upregulate Fas in response to ionizing radiation (Semont et al., 2004). We investigated a potential role for p53, as it has previously been identified as a regulator of neural precursor cell apoptosis and neurogenesis (Fatt et al., 2014; Meletis et al., 2006). Our evidence suggests that p53 is not involved in the regulation of Fas in NPCs in response to MCM or rIL-1β, as p53-deficiency did not mitigate the upregulation of Fas following treatment. Ouaaz et al., (1999) suggested that NF-κB activation was required for maximal Fas induction, as p65-deficient mouse embryonic fibroblasts (MEFs) exhibit decreased levels of Fas following treatment with TNFα or lipopolysaccharide (LPS) (Ouaaz et al., 1999; Darville & Eizirik, 2001). Furthermore, analysis of the Fas promoter indicates putative NF-κB binding sites. Therefore we investigated the potential role for NF-κB in the regulation of Fas using the pharmacological inhibitor of IκB-α phosphorylation, BAY-117082. We show that inhibition of NF-κB using BAY-117082 attenuated Fas-upregulation in both MCM and rIL-1β-treated NPCs. Furthermore, as a more direct approach knock-down of the NF-κB subunit p65 by shRNA, confirmed our findings, identifying NF-κB as a regulator of Fas expression in NPCs. Previous findings suggest NF-κB as a transcriptional regulator of Fas in various cell types including tumour cells, and pancreatic islet cells (Ouazz et al., 1999; Darville & Eizirik, 2001; Liu et al., 2012). Specifically, our results are consistent with a previous report which found that a mutation in one of the NF-κB binding sites in the Fas promoter mitigated IL-1β-induced Fas expression, and that p65 homodimers are the main activators of Fas transcription in pancreatic islet cells (Darville & Eizirik, 2001).

Taken together, this study identifies a novel signaling pathway in NPCs, whereby microglial-derived and recombinant IL-1β can induce the p65(NF-κB)-dependent upregulation of Fas expression. Furthermore, we identify Puma as an effector of Fas-mediated apoptosis in NPCs.

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Chapter 5

5 Summary and Discussion

The overall objective of this study was to examine the effects of neuroinflammation on neural precursor cells with the goal of identifying potential targets to improve regenerative therapies employing NPCs. To do this, we used a conditioned media model whereby microglia, the resident CNS immune cells were activated to a pro-inflammatory phenotype to minic neuroinflammation conditions in the CNS that occur during neurodegenerative disease and acute injury. Throughout this study we have examined the effects of microglial-derived soluble factors on neural precursor cell proliferation, and survival. We demonstrate that the microglial-derived cytokine TNFα induces apoptosis of NPCs through the NF-κB-mediated upregulation of Puma *in vitro*. We also establish a role for the pro-inflammatory cytokine IL-1β in the regulation of both proliferation and survival of NPCs. Furthermore, we have identified a novel pathway whereby IL-1β activates p53, which has a dual role in the induction of cell cycle arrest, and apoptosis. Importantly, transplantation of Puma-deficient NPCs into the injured mouse spinal cord revealed a protective effect of Puma-deletion in an *in vivo* model of inflammation. Lastly, we have uncovered the mechanism of Fas upregulation in NPCs and examined the contribution of Fas signaling to neural precursor cell death. Together, this study provides insight into the mechanisms by which neuroinflammatory conditions can have detrimental effects on transplanted or endogenous neural precursor cells.

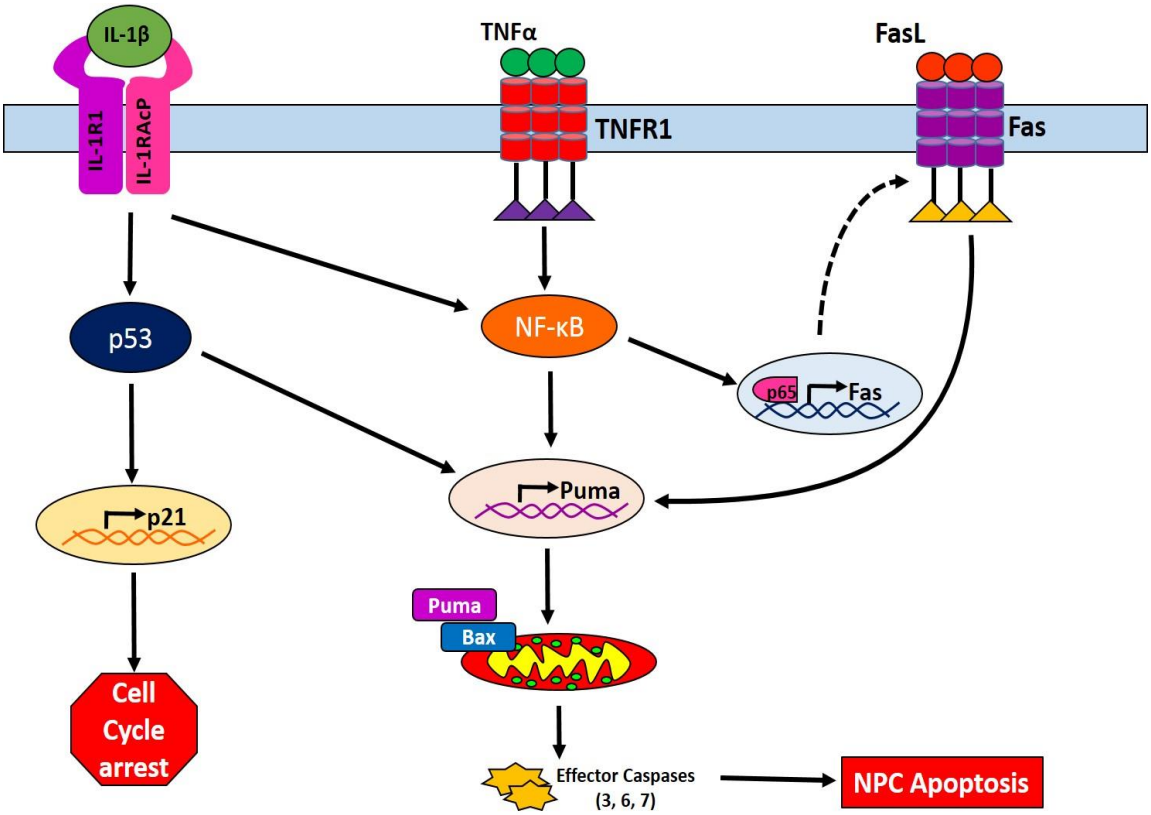

Figure 5.1

Figure 5.1: Summary of findings

Signaling through IL-1β, and TNFα result in activation of transcription factors NF-κB, and p53. Activation of p53 leads to upregulation of p21 and Puma, and a corresponding induction of cell cycle arrest and apoptosis, respectively. Activation of NF-κB by TNFα also leads to upregulation of Puma. Activation of NF-κB by IL-1β induces expression of Fas. Activation of Fas signaling culminates in Puma activation and apoptosis. Together our studies have identified Puma as a convergence point for cytokine induced apoptosis of NPCs.

5.1 Effect of TNFα on neural precursor cells

Tumour necrosis factor- alpha (TNFα) is a well characterized pro-inflammatory cytokine that is released by immune cells such as macrophages or microglia following injury or during disease (Czeh et al., 2011; Graeber & Streit, 2010). In our study we have identified TNFα as a key effector of NPC apoptosis induced by pro-inflammatory microglia and have delineated a novel mechanism by which TNFα induces NPC apoptosis. Specifically, we demonstrate that conditioned media from activated microglia induced an upregulation of TNF Receptor-1 (TNFR1) in NPCs which may have sensitized NPCs to soluble TNFα contained in conditioned media. The mechanism of TNFR1 upregulation in our model remains unclear and elicits further study. Furthermore, this study demonstrates that microglial-derived and exogenous TNFα induce the expression of the BH3-only Bcl-2 family member Puma (Galehdar et al., 2010; Akhtar et al., 2006; Steckley et al., 2007). We have proposed a novel mechanism whereby the transcription factor NF-κB is activated by TNFα and acts as a transcriptional activator of Puma in NPCs. Puma has been characterized as a potent regulator of Bax activation for the subsequent induction of apoptosis under many cellular stress conditions. Moreover, our study has demonstrated a novel and essential role for Puma in the induction of NPC apoptosis both *in vitro*, as well as in an *in vivo* model of inflammation. Injury to the spinal cord results in a rapid inflammatory response that is associated with the infiltration of microglia, and peripheral immune cells as well as elevated levels of pro-inflammatory factors, including TNFα (Pineau et al., 2007). To test our findings of Puma's importance in an *in vivo* model of inflammation, we transplanted Puma-deficient NPCs into the lesion site of the injured mouse spinal cord. Our findings revealed a robust difference in the survival of transplanted wild-type NPCs versus Puma-deficient NPCs in that very few wild-type NPCs were detected in the injured spinal cord three weeks following transplantation, suggesting that these cells had died. In contrast, transplantation of Puma-deficient NPCs resulted in a greater number (~13-fold) of engrafted cells at three weeks post-transplant, suggesting that Puma-deficiency confers protection from the harsh inflammatory environment of the injured spinal cord. This result identifies Puma as a potential therapeutic target to increase the survival of transplanted NPCs used for regeneration following injury. However, it is important to note that while we have increased survival of NPCs, it is not known whether

the NPCs have undergone differentiation and/or integrated into the circuitry of the spinal cord, or what the functional outcome of this transplantation would be. Studies have suggested that the restorative potential of endogenous or transplanted NPCs may be limited, however NPCs can elicit a positive role in the repair process through the release of trophic factors and modulation of innate and adaptive immune responses; a concept termed the 'bystander effect' (Martino & Plucchino, 2006; Hermann et al., 2014). Future studies would examine the role of transplanted Puma-deficient NPCs in the restoration and regeneration of the injured tissue, as well as examining the behavioural outcome at various time points following transplantation to determine whether transplantation results in improvement of motor skills in injured mice. Taken together, this study identifies TNFα as a stimulus for Puma-dependent apoptosis of neural precursor cells.

5.2 The role of IL-1β in neural precursor cells

Interleukin-1β is a pro-inflammatory cytokine that is processed and released from microglia and can elicit effects on proliferation, neurogenesis and lineage fate of NPCs. IL-1β has been shown to limit neurogenesis and proliferation of NPCs in various neuropathologic conditions as well as well as in response to chronic and acute stress (Gabay et al., 2010; Mathieu et al., 2010). The goal of this part of our study was to identify the effects of IL-1β on NPCs, and the pathways downstream of IL-1R1 activation. This study has uncovered a novel relationship between IL-1β and p53 in the regulation of NPC processes *in vitro*. We show that IL-1β reduced proliferation and increases NPC apoptosis via a p53-dependent mechanism. P53 is known to be activated by oxidative stress (Liu et al. 2008) and IL-1β has been reported to induce ROS production in retinal epithelial cells through NADPH-oxidase activation (Yang et al. 2007) and in pancreatic β-cells via induction of iNOS (Gurzov et al. 2009). Using N-Acetylcysteine (NAC), a precursor of the antioxidant glutathione, we suggest that inhibition of reactive oxygen species reduces P53 expression in NPCs. This suggests a model whereby IL-1β may lead to production of intracellular reactive oxygen species in NPCs which leads to the observed increase in p53 expression. However, we cannot rule out a contribution from other signaling pathways downstream of IL-1R1 activation such as JNK, MAPK, and NF-κB. NF-κB specifically is

a known downstream target of IL-1β signaling and can co-operate with p53 in the induction of apoptosis by the pro-inflammatory cytokine TNFα (Ryan et al., 2000). This is interesting as we have previously identified NF-κB as an activator of Puma in response to TNFα signaling. It is also possible that activation of redox sensitive kinases may lead to p53 activation. Apoptosis signal-regulating kinase (Ask1) is inhibited by thioredoxin in unstressed cells, however this inhibition is attenuated in the presence of ROS. Activated Ask1 is implicated in the activation of MK3 and downstream activation of p38 and JNK which results in phosphorylation of their respective targets including ATF2, c-Jun, and p53 (Adler et al., 1999). Future studies would also investigate the relationship between the DNA damage response and ROS in the context of p53 activation. Specifically to determine if DNA damage activates ROS production; or whether ROS production activates the DNA damage response leading to activation of p53 (Kang et al., 2012).

Activation of p53 by IL-1β results in transcriptional upregulation of the CDK inhibitor p21, leading to cell cycle arrest in NPCs. Furthermore, p53 also induced the expression of the pro-apoptotic BH3-only protein Puma, which we identify as a critical step in the apoptosis of NPCs. We propose that p53 induces the expression of p21 which arrests the NPCs in the G1-S phase of the cell cycle, leading to cellular senescence and irreversible cell cycle arrest as the stress to the NPCs persists. Simultaneously, p53 induces the expression of Puma which functions to remove arrested cells by apoptosis. We cannot however reconcile whether Puma activation is occurring in parallel to, or is a consequence of the induction of cellular senescence, or perhaps whether senescence sensitizes NPCs to apoptosis, as has been shown in other models (Vjetrovic et al., 2014; Childs et al., 2014). Further studies could be carried out using p21-deficient NPCs to determine the hierarchy of this pathway.

The role of p53 family members in the regulation of NPC processes has been previously illustrated. In these studies, p53 has been identified as a negative regulator of NPC survival and self-renewal processes (Meletis et al., 2006; Fatt et al., 2014). Our results are consistent with the duality of p53, and furthermore we are the first to implicate p53 as a negative regulator of NPC proliferation and survival in response to IL-1β. The implications of our findings are wide-spread. Increases in IL-1β are reported in many neurodegenerative conditions including Alzheimer's disease, and stroke as well as acute conditions such as

spinal cord injury. In each of these cases, an increase in p53 expression is also observed, and has been suggested to negatively affect cells in the injured/diseased CNS (Buizza et al., 2012; Ohyagi et al., 2005; Floriddia et al., 2012; Luo et al., 2009). Importantly, p53 also plays an important role in neuronal survival during injury, therefore activation of p53 could be detrimental by both causing neuronal loss and by decreasing the regenerative capacity in the CNS (Cregan et al., 2004). This provides a pathway to target and manipulate when using endogenous or transplanted NPCs as regenerative therapies for many diseases and conditions.

5.3 Fas signaling in neural precursor cells

Fas is a well-studied death receptor and member of the TNF family of receptors, which possess an intracellular death domain through which it can activate signaling cascades that lead to apoptosis. Fas receptor expression can be increased on cells during inflammatory conditions, likely as a means to target the cells for death (Curtin & Cotter, 2003; Brint et al., 2013). Previous studies have suggested a role for pro-inflammatory cytokines as the signal for Fas upregulation, however, the mechanism of Fas upregulation in NPCs was unclear. Our study implicates the pro-inflammatory cytokine IL-1β as an important factor in Fas upregulation in NPCs. We demonstrate that release of IL-1β from LPS-activated microglia leads to the activation of the NF-κB subunit p65, which functions as a transcriptional activator of Fas. Furthermore, inhibition of NF-κB signaling mitigates Fas expression as well as the expression of the pro-apoptotic BH3-only protein Puma.

Activation of Fas by its cognate ligand, FasL often serves as a mechanism whereby immune cells such as microglia can induce apoptosis of target cells expressing Fas through upregulation of membrane bound FasL (mFasL) and/or release of soluble FasL (sFasL) (Frigerio et al., 2000; Graeber & Streit, 2010). The outcome of Fas activation may be dependent on which of the two ligands engages the Fas receptor; as mFasL exhibits a more potent apoptotic effect than sFasL, which could be a result of activation of different downstream pathays. Interestingly, we observe an increase in the expression of FasL in microglia following LPS activation, though this did not correspond with an increase in the

levels of sFasL, indicating that the majority of FasL remained membrane bound. For this reason we used a monoclonal anti-Fas antibody to examine Fas signaling in NPCs. Previously, the consequence of Fas signaling in NPCs was unclear, as some had suggested activation of Fas resulted in increased proliferation, and others had implicated Fas as a regulator of programmed cell death pathways (Knight et al., 2010). In our attempt to clarify the role of Fas signaling in NPCs we show that activation of Fas by anti-Fas antibody results in a significant induction of apoptosis in NPCs *in vitro*. Interestingly, our study represents a novel link between Fas activation, and activation of Puma, a pro-apoptotic regulator of the intrinsic apoptotic pathway. In future experiments we would employ a co-culture environment whereby microglia and NPCs could be in with contact one another, allowing us to investigate FasL/Fas signaling in a more physiological manner. In this case we would hypothesize that upon activation, mFasL would be upregulated on the microglial membrane, and this may recruit NPCs. We also hypothesize that this interaction between mFasL on microglia and Fas on NPCs would result in apoptotic death of NPCs via a Puma-dependent mechanism. Fas is classically thought to induce apoptosis through the extrinsic pathway of apoptosis via activation of caspase-8 and cleavage of the BH3-only protein Bid to tBid which can act at the mitochondria to induce mitochondrial membrane permeabilization and subsequent caspase activation (Strasser, 2009; Yin et al., 2006). While we cannot rule out a role for Bid in Fas-mediated NPC apoptosis, our results suggest that Puma is a dominant regulator of this pathway. Future studies using Bid-deficient NPCs would provide greater insight into the relationship between Fas and Bid in NPCs, as well as examine the question of why Puma plays a more critical role than Bid in NPC apoptosis.

5.4 Bcl-2 family in NPC apoptosis

The Bcl-2 family of proteins function to regulate programmed cell death pathways, specifically the intrinsic (mitochondrial) pathway of apoptosis. The intrinsic pathway is activated by a variety of stimuli including developmental cues, or cellular stressors such as genotoxic stress, oxidative stress, endoplasmic reticulum stress, or growth factor withdrawal (Cregan et al., 1999; Steckley et al., 2007; Galehdar et al., 2010). Upon cellular stress stimuli, BH3-only family members which include Puma, Noxa, Bid, and Bim are

activated either transcriptionally or post-translationally in a stimulus and cell type-specific manner. Once activated, BH3-only proteins can activate pro-apoptotic Bcl-2 family members Bax and Bak either directly, or indirectly to target Bax/Bak to the mitochondria where they can induce permeabilization of the outer mitochondrial membrane, releasing soluble proteins such as cytochrome c or AIF into the cytosol. In post-mitotic neurons, Bax is necessary for induction of intrinsic apoptosis, whereas Bak is dispensable. However, it has been suggested that in neural precursor cells Bax and Bak play redundant roles in the regulation of apoptosis (Lindsten et al., 2003). Interestingly, in our studies we have demonstrated that Bax is necessary for NPC apoptosis in response to MCM, TNFα, IL-1β, and Jo2 (FasL). Bax/Bak double-deficient mice are embryonic lethal, therefore we attempted to inviestigate the role of Bax/Bak using $Bax^{flox/flox}Bak^{-/-}$ NPCs and infecting with an adenovirus expressing GFP tagged Cre-recombinase. Importantly, we did not notice any difference between Bax/Bak DKO NPCs compared to Bax-deficiency alone. Evidence of this is demonstrated in Bax-deficient NPCs, which are markedly resistant to apoptosis induced by each of these stimuli, suggesting that Bak cannot compensate to induce apoptosis in the absence of Bax. We hypothesize that this may be a stimulus specific effect, or may be a result of Bax and Bak being activated by different mechanisms.

The activation of Bax and Bak by BH3-proteins remains a widely studied area of research, as how each of the BH3-only proteins 'activates' Bax/Bak is unclear and is likely largely dependent on stimulus and cell type. It has been proposed that the BH3-only protein Bid, Bim and Puma act as direct 'activators' of Bax/Bak whereas other BH3-proteins (eg. Noxa, Bmf, Bad) act as 'sensitizers' by liberating 'activator' BH3 proteins from sequestration by anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-x) (Villunger et al., 2011; Kim et al., 2009). In our first study we observed increased mRNA levels of both Puma and Noxa in response to treatment with conditioned media from LPS-activated microglia (MCM) however upon further investigation using Noxa-deficient and Puma-deficient NPCs, we noted that Puma-deficiency conferred resistance to MCM-induced apoptosis, whereas Noxa-deficiency did not.

Interestingly, we observed cleavage of Bid to its active form tBid, following treatment with MCM, however levels of tBid, compared to total Bid were relatively low. This is an

important point as Bid cleavage is a critical step in the activation of extrinsic apoptosis by death receptor ligation (Guicciardi & Gores, 2009). TNF-R1 and Fas are members of the death receptor family which are generally thought to activate the extrinsic pathway to induce apoptosis in cells. Death receptor signaling can be reduced or neutralized by the presence of high levels of two death receptor inhibitory proteins, cFLIP (caspase-8-like inhibitor protein) or PED/PEA-15 which interact with the adaptor protein FADD and compete with caspase 8 in the DISC, thereby preventing caspase 8 activation, and subsequent Bid cleavage. Interestingly, cFLIP is expressed at low levels in NPCs, whereas PED/PEA-15 is strongly expressed, and exposure of NPCs to IFNγ or IL-1β can increase PED/PEA-15 expression. PED/PEA-15 also demonstrated a robust recruitment to the DISC of NPCs after stimulation of the Fas receptor in NPCs (Ricci-Vitiani et al., 2004). Together this suggests that in NPCs effective DISC formation may be impaired due to the expression if PED/PEA-15, forcing cells to execute apoptosis through the intrinsic (mitochondrial) pathway, independent of caspase-8 and Bid. Furthermore, studies using knockout mice indicate that neuronal cell ablation during development occurs through the activation of the intrinsic apoptotic pathway, as mice lacking caspase-9 or the caspase adaptor Apaf-1 have abnormal neuronal growth and severe neurological malformations (Ricci-Vitiani et al., 2004). In contrast, mice lacking caspase 8 or Bid do not have neuronal defects, suggesting that the extrinsic apoptotic pathway is not necessary in developmental apoptosis. In addition, analysis of wild-type and Bid-deficient telencephalic NPCs revealed no difference in the amount of apoptosis observed following treatment with the DNA damaging agent, AraC, suggesting Bid is not important for DNA damage-induced NPC apoptosis (Leonard et al., 2001). A study by Engel et al., (2010) demonstrated that Bid cleavage was observed following status epillepticus in the mouse brain. However, deletion of Bid did not alter the number of degenerating neurons, or AIF release from the mitochondria, indicating that Bid may be functionally redundant and not necessary for neuronal apoptosis in this model (Engel et al., 2010). Taken together, this may reconcile why Puma appears to be the more potent mediator of apoptosis in NPCs. However, future studies using Bid-deficient NPCs would allow for a better understanding of the role of Bid in cytokine-induced NPC apoptosis.

5.5 Clinical relevance of this work

Inflammation accompanies many neurological conditions both acute injuries, and chronic neurdegenerative diseases. Increased levels of pro-inflammatory cytokines are observed following spinal cord injury, traumatic brain injury, stroke, and in patients with Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), and multiple sclerosis. The presence of neural stem cells in the adult brain presents as a useful tool for regeneration following injury. However, inflammation can often limit neurogenesis, and decrease survival of both endogenous and transplanted neural stem cells, therefore limiting the capacity for repair. Numerous studies have attempted to target inflammatory pathways using rodent in vitro, and in vivo experiments. For example, an important study by Ekdahl et al., reported that treatment with the anti-inflammatory drug minocycline could improve neurogenesis in the hippocampus following status epilepticus in the mouse (Ekdahl et al., 2003). A study by Erlandsson et al., reported that immunosuppression by genetic manipulation or with the immunosuppressive drug cyclosporin A resulted in increased migration of endogenous neural precursor cells in the adult mouse brain following ischemic injury as well as improved functional recovery (Erlandsson et al., 2011). While immunosuppression may have some positive effects on neurogenesis, inflammatory cytokines can also have beneficial effects such as by promoting oligodendrocyte proliferation and regeneration, or by promoting tissue repair by astrocytes (Arnett et al., 2003; Whitney et al., 2009). Therefore, targeting Puma, a common downstream mediator of cytokine-induced apoptosis, may be a more effective strategy than targeting individual cytokines.

In addition to endogenous NPCs, the use of transplanted NPCs has also exhibited promising results. Interestingly, many of the beneficial effects were not due to differentiation and incorporation of transplanted NPCs, but to other 'bystander' mechanisms exerted by NPCs. Numerous studies have investigated the most effective strategy for NPC transplantation taking into account the proliferation and survival of NPCs as well as modulation of the CNS microenvironment to promote remodeling and plasticity (Hermann et al., 2014). There is evidence that transplanted NPCs can augment endogenous neurogenesis by inducing an increase in the number of proliferating cells within the

subventricular zone (SVZ), which is associated with an augmented survival, migration and maturation of endogenous neuroblasts in the striatum. This was accompanied by suppression of microglia-driven inflammatory responses (Mine et al., 2013). Injection of NPCs alleviates that clinicopathological features of stroke in murine models by reducing secondary neurodegeneration, scar formation, and promoting endogenous neurogenesis (Doeppner et al., 2012). A meta-analysis was conducted in 2010 investigating preclinical results of intravenous cell delivery for treatment of neurological disorders. From this study it was concluded that NPCs had a positive effect on behavioural and molecular outcomes in neurological disorders. Interestingly this analysis also revealed that inhibition of apoptosis was the most positive change that resulted from cell therapy. Furthermore, it was suggested that transplanted cells may act as scavengers to aid in the toxic waste removal produced by injury or disease, thus promoting survival of endogenous cells (Janowski et al., 2010). While many studies have focused on exogenous sources of NPCs for cell-based therapies, a less invasive approach would be to develop strategies to promote the contribution of endogenous NPCs for repair and regeneration. This premise relies on the ability to activate the endogenous pool of NPCs through the use of various factors such as growth factors, or cytokines that promote proliferation, migration, and differentiation of NPCs (Dibajnia et al., 2013). Furthermore, promoting survival of NPCs would be necessary for the use of NPCs in injury models. Using the knowledge gained from our studies, targeting Puma or p53 in NPCs could be a viable option for improving cell-based therapies.

5.6 Conclusion

Together this work characterizes signaling pathways that regulate neural precursor cells proliferation and survival during inflammatory conditions. Our studies have identified the TNFα, IL-1β and Fas pathways as activators of NPC apoptosis through a common mediator, the BH3-only Bcl-2 family member Puma. This work provides therapeutic targets to improve the efficacy of NPC-based regenerative therapies. Moreover, this could be tailored to specific injuries or conditions which may be accompanied by increases in specific pro-inflammatory cytokines.

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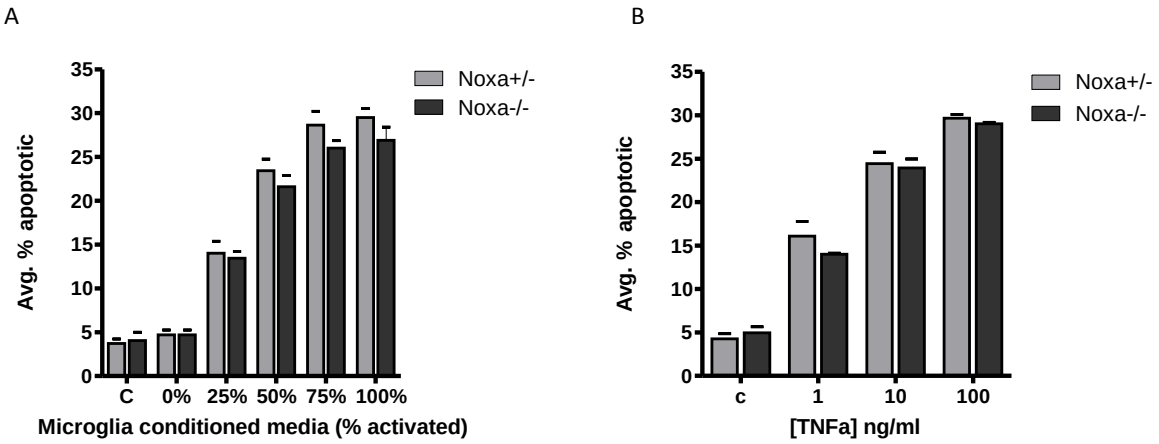
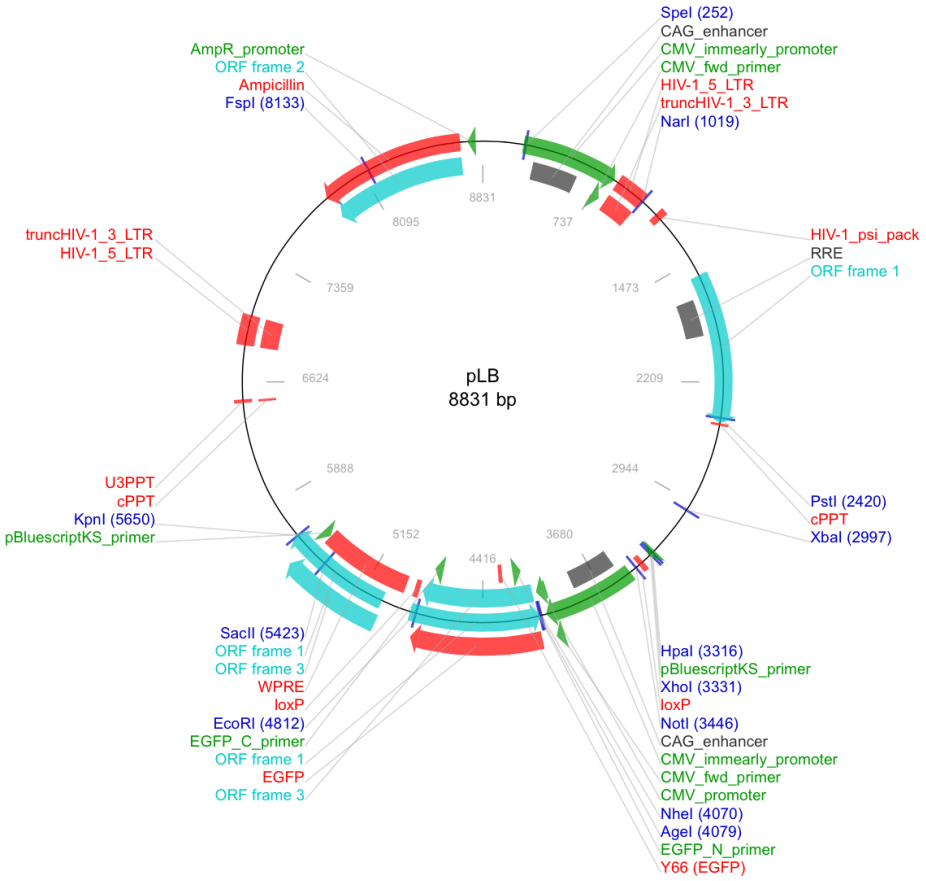
Appendices



Appendix Figure A1.

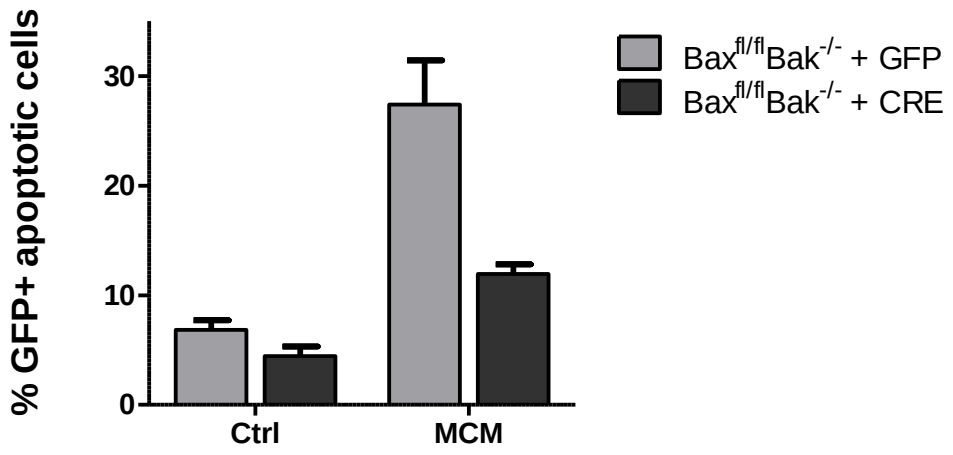
Appendix Figure A1. Noxa is not required for MCM and TNFα-induced apoptosis. (A) NPCs derived from Noxa$^{+/+}$ and Noxa$^{-/-}$ embryos were maintained in unconditioned stem cell media (C) or treated with increasing concentrations of LPS-activated microglia conditioned media (MCM), or increasing doses of rTNFα (1-100ng/ml) and the fraction of apoptotic cells was determined by Hoechst staining at 72 hours (N=4; N.S.).



Appendix Figure A.2

Appendix Figure A.2. Lentivirus plasmid map.

Oligonucleotides designed to target p65, and non-coding sequence. Oligonucleotides were annealed, phosphorylated and cloned into the HpaI site of the pLB plasmid (Addgene).



Appendix Figure A3

Appendix Figure A3: Bak is not required to induce apoptosis in MCM-treated NPCs.

$Bax^{flox/flox}Bak^{-/-}$ NPCs were infected with an adenovirus expressing GFP or GFP-CRE (MOI 50). NPCs were treated with MCM for 72 hours and then stained with Hoechst 33342 (1ug/ml). The GFP+ cells were evaluated for nuclear morphology changes consistent with apoptosis (n=3, N.S)

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