EFFECTS OF ANDROGRAPHOLIDE ON ASTROCYTE-MEDIATED INFLAMMATORY RESPONSE: POTENTIAL FOR ANTI-NEUROINFLAMMATORY THERAPY

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NATIONAL UNIVERSITY OF SINGAPORE

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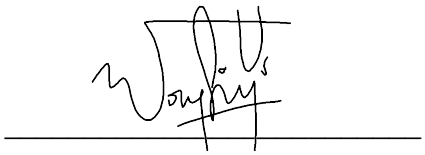
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Neuroinflammation is implicated in the pathogenesis of various neurological disorders. Astrocytes not only actively maintain brain homeostasis but also capable of responding to various brain insults. Upon stimulation, activated astrocytes secrete various proinflammatory mediators like cytokines and chemokines that initiate and maintain inflammatory responses such as leukocyte infiltration. With increasing evidence of astrocytes' role in mediating inflammation in brain injury and neurodegenerative disease, attenuating inflammatory responses elicited by astrocytes might potentially alleviate disease outcomes.

Andrographolide is the main constituent isolated from *Andrographis paniculata*, a native plant in Southeast Asian countries with anti-inflammatory medicinal uses and is known to be brain-penetrant. Thus, I would like to investigate its potential in attenuating inflammatory responses elicited by astrocytes.

IL-1β and TNF-α are important regulators and common cytokines released during inflammation. Both cytokines induced NF-κB activation and CCL-5 chemokine expression in astrocyte cultures. Andrographolide was shown to inhibit p65 and IκBα phosphorylation and CCL-5 secretion with better efficacy and lesser toxicity compared to artesunate and plumbagin when tested in astrocyte. Moreover, andrographolide downregulated expression of GFAP, a marker for astrogliosis.

Lipopolysaccharide (LPS), a constituent of the bacterial cell wall, effectively elicits inflammatory responses during bacterial infections. LPS-induced activation of NF-κB and JNK as well as mRNA expression of various cytokines/chemokines including IL-1β, TNF-α, CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-5, CXCL-10, and CX3CL-1 was attenuated by andrographolide. Treatment with TPCK (NF-κB inhibitor) and SP600126 (JNK inhibitor) prevented elevation of cytokines/chemokines. Thus, andrographolide was proposed to attenuate cytokines/chemokines expression through NF-κB and JNK pathways. Moreover, oral administration of andrographolide in animal model of bacterial infection-related neuroinflammation (LPS injection) attenuated various chemokines (CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-9)

in mice brains. This implies that peripherally administered andrographolide could exert anti-neuroinflammatory effects.

Andrographolide mediates antioxidantive effects by activating Nuclear factor (erythroid-derived 2)-like 2 (Nrf2); a transcription factor that regulates expression of various antioxidant protein including heme-oxygenase 1 (HO-1) and NAD(P)H dehydrogenase quinone 1 (Nqo-1). In primary astrocytes, andrographolide induced mRNA expression of Nrf2, HO-1, and Nqo-1. Instead of inducing *de novo* synthesis of Nrf2, andrographolide enhanced Nrf2 protein stability. Andrographolide activated ERK and p38 also play a role in regulating Nrf2.

In conclusion, andrographolide effectively attenuated activation of NF-κB and JNK signaling pathways and expression of various cytokines/chemokines in astrocytes in response to proinflammatory cytokines and bacterial components. Its antioxidant properties were mediated by activation of Nrf2 signaling through Nrf2 protein stabilization and ERK/p38 activation. Moreover, its ability to cross BBB and mitigate inflammatory responses in the brain suggest its potential use as an anti-neuroinflammation therapeutic.

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxylnonenal
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Andro	Andrographolide
APP	Amyloid precursor protein
ARE	Antioxidant response element
Aβ	Amyloid beta
BACE1	β-secretase 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BVR	Biliverdin reductase
$C_{20}H_{30}O_5$	Andrographolide
CaMKII	Calcium/calmodulin-dependent proteinkinase II
CBR	Carbonyl reductase
CCl_4	Carbon tetrachloride
C-FLIP	Cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein
ChABC	Chondroitinase ABC
CHX	Cycloheximide
CNS	Central nervous system
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CRIDs	Cytokine release inhibitory drugs
CSF	Cerebrospinal fluid
CSPGs	Chondroitin sulfate proteoglycans
CTNF	Ciliaryneurotrophic factor
Cul3	Cullin-3
Cyto	Cytoplasmic
DAMP	Damage-associated molecular pattern
DAPI	6-diamidino-2-phenylindole
DISC	Death-inducing signaling complex
DMEM/F12	Dulbecco's Minimal Essential Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulphoxide
EAE	Experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor
ELR	Glutamic acid-leucine-arginine
EMT	Epithelial mesenchymal transition
eNMDARs	Extrasynaptic NMDA-type glutamate receptors
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FBS	Fetal bovine serum

Fe^{2+}	Ferrous iron
GAG	Glycosaminoglycan
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate type I transporter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gpx2	Glutathione peroxidase 2
GSK-3β	Glycogen synthase kinase-3β
Gsr	Glutathione reductase
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IKK	IκB kinase
IKKβ	IκB kinase β
IL-1R AcP	IL-1R accessory protein
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated kinase
IVR	Intervening region
JAKs	Janus kinases
JNK	C-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
Maf	Masculoaponeuroticfibrosarcoma
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MAPK	Mitogen-activated protein kinases
MEF	Murine embryonic fibroblasts
MKKs	Mitogen-activated protein kinase kinases
MLK	Multi-lineage kinase
MMP3	Matrix metalloproteinase 3
MMPs	Matrix metalloproteinases
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor κB
NGF	Nerve growth factor
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	NO synthases
NOX	NADPH oxidases
NQO1	NAD(P)H:quinineoxidoreductase

Nqo1	NAD(P)H quinoneoxidoreductase 1
Nrf2	NF-E2-related factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer saline
PDI	Protein disulfide isomerase
PDTC	Pyrrolidinedithiocarbamate
PEA-15	Phosphoprotein enriched astrocytes-15 kDa
PED	Phosphoprotein enriched in diabetes
PEG 400	Polyethylene glycol 400
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PRRs	Pattern-recognition receptors
Prx1	Peroxiredoxin1
PVDF	Polyvinylidenedifluoride
RAGE	Receptor for advanced glycation end products
Rbx1	Ring-box 1 protein
RHD	Rel homology domain
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SRs	Scavenger receptors
STAT3	Signal Transducers and Activators of Transcription
Tat	Trans-activator of transcription
tBHQ	Tert-Butylhydroquinone
TBP	TATA box binding protein
TGFβ	Transforming growth factor β
TLR4	Toll-like receptor 4
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
tPA	Tissue plasminogen activator
TPCK	N-***p***-Tosyl-L-phenylalanine chloromethyl ketone
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAF6	TNF receptor-associated factor 6
VCAM-1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor

PUBLICATION ARISING FROM THESIS

1. Wong, S. Y., Chan, S. J., Wong, W. S., Wong, P. T., & Lai, M. K. (2014). Andrographolide attenuates interleukin-1beta-stimulated upregulation of chemokine CCL5 and glial fibrillary acidic protein in astrocytes. *Neuroreport*, **25**(12), 881-886.

2. "Effects of andrographolide in LPS-mediated neuroinflammation" under revision in Journal of Neuroinflammation

3. "Andrographolide in asttrocytes: new insights on Nrf2 regulation" in submission

CHAPTER 1: INTRODUCTION

1.1 The immune-privileged CNS

The central nervous system (CNS) has previously been regarded as an "immune-privileged" organ which is immunologically inert. This belief is largely due to lack to of resident antigen-presenting dendritic cells and draining lymphatic system in CNS, immune-incompetence of CNS immune cells, and separation of the CNS from peripheral immune system by blood brain barrier (BBB) (Carson *et al.*, 2006a; Ransohoff *et al.*, 2012). The BBB forms a highly selective permeability barrier separating the brain extracellular fluid from the peripheral circulation that prevents bacterial infections and limits neuroinflammation by restraining peripheral innate and adaptive immune cells, presumably to protect the vulnerable non-regenerating neurons (Ransohoff *et al.*, 2012). Therefore, one might easily have construed that CNS is immune-privileged but this view has recently been vigorously revised.

1.2 Mediators of neuroinflammation

In healthy individuals, low or near undetectable levels of inflammatory factors are found in the CNS. However, immune responses could be activated by traumatic brain injury, stroke, autoimmune disease, peripheral infection, and neurodegenerative diseases (Rivest, 2009; Wohleb *et al.*, 2013). In comparison to the periphery, the CNS has more conservative immune response with modest recruitment of leukocytes as a strategy to protect neurons that largely do not regenerate (Jensen *et al.*, 2013; Lucas *et al.*, 2006). Mediators of neuroinflammation are divided into two components; the cellular and molecular components. Although many cells in the CNS including neurons, oligodendrocytes, and endothelia are capable of responding to inflammatory

stimuli, microglia and astrocytes are the main cellular components that mediate neuroinflammation.

1.2.1 Microglia

1.2.1.1 Origin and physiological functions

Microglia are the resident macrophage in the CNS and contribute to approximately 12% of the cell population in the brain (Block *et al.*, 2007). During CNS development, microglia play a pivotal role in synaptic clearance and reorganization of neuronal connections by actively engulfing synaptic materials and removing apoptotic neurons (Napoli *et al.*, 2009; Paolicelli *et al.*, 2011). Under normal physiological condition, microglia constantly survey for pathogens or cellular damage in the brain parenchyma. Hence, they are usually the first to detect brain injury and the first line of defense against invading pathogens (Kraft *et al.*, 2011).

1.2.1.2 Microglia activation

Microglia express various pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs), mannose receptors, and scavenger receptors that recognize both exogenous pathogen-associated molecular pattern (PAMP) and endogenous altered molecules damage-associated molecular pattern (DAMP). (Husemann *et al.*, 2002; Shastri *et al.*, 2013). Moreover, they also recognize and respond to cytokines and complement proteins (Crehan *et al.*, 2012; Doens *et al.*, 2014) (Table 1.1). Microglia are readily activated when encounter with inflammatory stimuli or cellular debris. TLR agonists, tumour necrosis factor (TNF), and interferon (IFN)-γ induce classically activated phenotype (M1

state), whereby microglia response by upregulating expression of antigen presenting molecules; a response tending towards pathogen clearance (Cherry *et al.*, 2014; Saijo *et al.*, 2011). On the other hand, alternatively activated phenotype (M2 state) is induced by cytokines like IL-4 and IL-13 which promotes wound healing. (Cherry *et al.*, 2014; Lyman *et al.*, 2014; Saijo *et al.*, 2011). The M1 state is often associated with tissue destruction and up-regulation of inflammatory responses by generating cytokines, chemokines and reactive oxygen species (ROS) (Gordon *et al.*, 2010). Microglia express TLR 1-9 and TLR4 is typically used to induce M1 state (Shastri *et al.*, 2013). Recognition of lipopolysaccharide (LPS), an endotoxin secreted by Gram-negative bacteria by TLR4 receptor will activate NF-κB and multiple MAPK signaling pathway (Figure 1.1). This results in activation of transcription factors that upregulates expression of genes involved in mediating inflammatory responses (Guha *et al.*, 2001; Kaminska, 2005).

1.2.1.3 Implication of microglia activation in neurological diseases

Though activation of microglial is essential for pathogen elimination and neuronal survival, uncontrolled activation often leads to detrimental or neurotoxic effects. Microglial activation is implicated in Alzheimer's disease progression and cognitive decline (Xiang *et al.*, 2006). Reactive microglia are found clustered around amyloid plaque, a hallmark of Alzheimer's disease in human post-mortem brain (Mcgeer *et al.*, 1987). Several lines of evidence showed that microglia could phagocytose amyloid deposits but the process is slow and inefficient (Frautschy *et al.*, 1992; Weldon *et al.*, 1998). Qin *et al.* showed that low concentration of Aβ induced significant neuronal death in neuron-microglia co-culture but not in neuron-enriched culture. This clearly

indicates that microglia play a role in potentiating neurotoxicity of Aβ (Qin *et al.*, 2002). Microglia also induce neurotoxicity through production of ROS by upregulating expression of inducible nitric oxide synthase (iNOS) (Qin *et al.*, 2002; Weldon *et al.*, 1998). Moreover, the importance of reactive microglia in mediating neurological disorder is observed in amyotrophic lateral sclerosis (ALS) mouse model with dominant mutation of superoxide dismutase (SOD). Selective deletion of mutant SOD gene in microglia considerably extend the lifespan of the animal, indicating a role of microglia in mediating disease progression (Boillee *et al.*, 2006). In Parkinson's disease, matrix metalloproteinase 3 (MMP3) and α-synuclein are released from the degenerating dopaminergic neurons. MMPs proteinase stimulates microglia resulting in NF-κB activation and secretion of proinflammatory TNF-α (Kim *et al.*, 2005). Attempts to clear α-synuclein by phagocytosis leads to activation of microglia which subsequently enhance NADPH oxidase (NOX) activity and exacerbate neurodegeneration (Zhang *et al.*, 2005b). Hence, it is crucial that microglia activation is remained tightly regulated as they could release various pro-inflammatory mediators and induce oxidative stress that potentially cause neuronal damages and aggravate neurological disease conditions.

Receptor	Descriptions and Functions
TLR	- Membrane bound PPR that recognise PAMPs (bacteria, virus, fungi, and parasites) and DAMPs (DNA, ATP, uric acid and heat shock proteins) (Shastri *et al.*, 2013) -Twelve TLR has been identified in mammals. Other than TLR3, all TLRs activate the MyD88-dependent signaling pathways which leads to NF-κB and MAPK activation (Guha *et al.*, 2001). - The most well studied TLR 4 is known to be activated by LPS and amyloid beta. This leads to IL-1β, TNF-α, IL-6 and NO production (Shastri *et al.*, 2013).
NLR	- Cytoplasmic PPR that detects PAMPs and DAMPs

	- Consist of 4 subfamilies: NLRA (A for acidic transactivating domain), NLRB (B for BIR domain), NLRC (C for CARD domain), NLRP (P for pyrin domain) (Rosenstiel *et al.*, 2009) - Binding to agonist activates NF-κB and MAPKs signaling pathways and expression cytokines/chemokines (Shastri *et al.*, 2013). - NLR could form a multiprotein complexes termed inflammasome that activates caspase-1 which subsequently induce production of IL-1β and IL-18 (Ransohoff *et al.*, 2012). - Phagocytosis of Aβ by microglia activates NLRP3 inflammasome and causes inflammation and tissue damage in Alzheimer's disease (Halle *et al.*, 2008).
Scavenger	- Transmembrane glycoprotein PPR that binds LPS, lipoteichoic acids, *Escherichia coli*, *Streptococcus pneumonia*, apoptotic cells, and modified lipoprotein (Shastri *et al.*, 2013). - Family of scavenger receptors (SR) includes: SR-A, SR-B, and receptor for advanced glycation end products (RAGE) (Doens *et al.*, 2014). - Activation of SR results in ligand endocytosis and production of superoxide (Block *et al.*, 2007). - SR-A promotes cerebral ischemic injury by pivoting microglia polarization to M1 state. SR-A deficient mice displayed reduced infarct size as well as IL-1β and TNF-α expression (Xu *et al.*, 2012)
Complement	- Membrane bound receptors - Complement receptor types: CR1 binds C3b, C4b, and C1q whereas CR2, CR3 and CR4 are relatively specific for iC3b (Crehan *et al.*, 2012) - Activities initiated by complement receptors activation includes: chemotaxis, phagocytosis, leukocyte migration, ROS and NO production (Shastri *et al.*, 2013). - CR3 is implicated in Aβ activation of microglia. Use of CR3 antagonist attenuates proinflammatory mediators and free radicals production from microglia (Doens *et al.*, 2014).
Cytokine	- Receptors that bind to cytokines and chemokines - Classified based on structures and activities: Type I cytokine receptors, Type II cytokine receptors, chemokine receptor, TNF receptor superfamily, TGFβ receptors, Immunoglobulin superfamily (Foxwell *et al.*, 1992). - Activation of IL-1R and TNFR usually induce proinflammatory responses while IL-10R and TGFR generally elicit anti-inflammatory responses. CCR5 and CXCR4 activate chemotaxis (Shastri *et al.*, 2013). - CX3CR1 is only expressed by microglia in the CNS. It was found to play a role in regulating amyloid deposition and tau pathologies (Bhaskar *et al.*, 2010; Lee *et al.*, 2010; Nash *et al.*, 2013).

Table 1.1 Immune receptors expressed in microglia

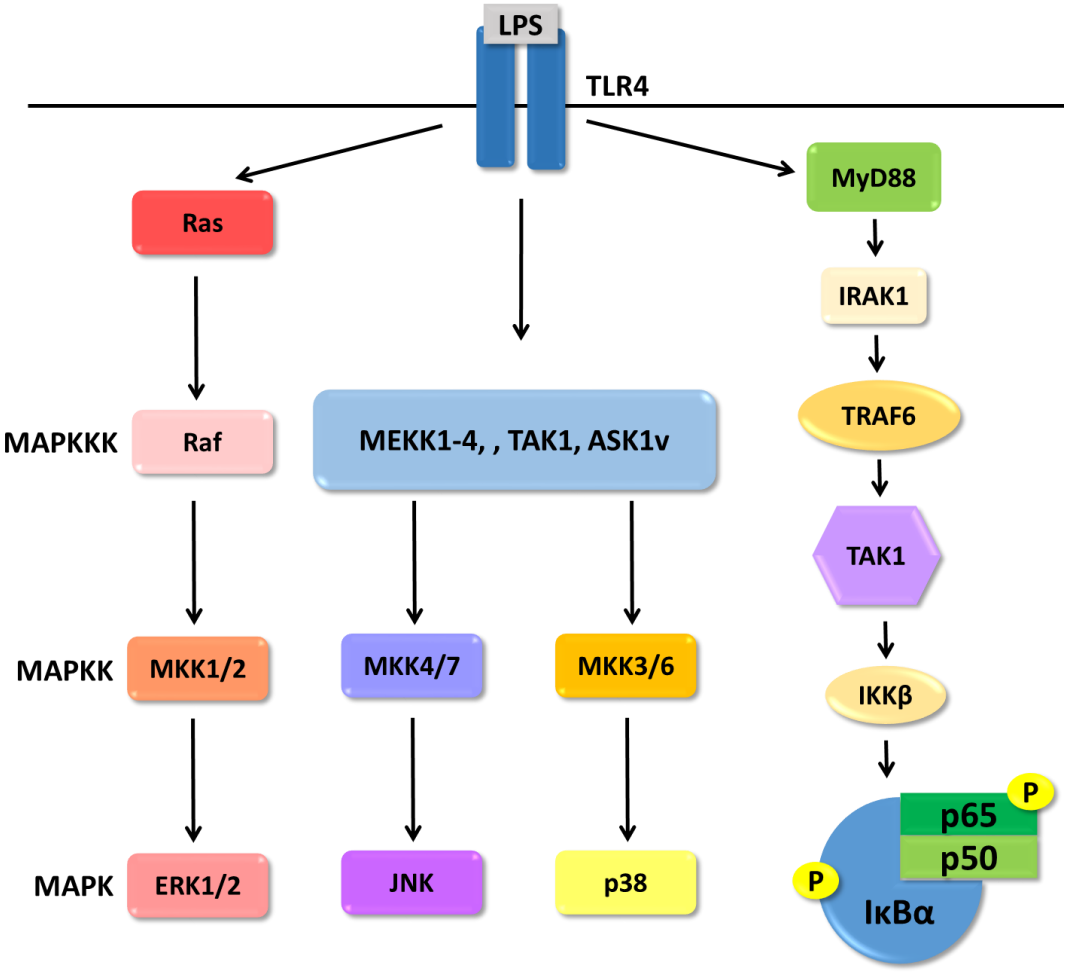


Figure 1.1 Binding of LPS to TLR4 triggers NF-κB and MAPK signaling.

1.2.2 Astrocyte

1.2.2.1 Origin and physiological functions

Similar to microglia, astrocyte is a type of neuroglia that is found in all regions of the CNS. They are the most abundant cell type in the CNS, constituting approximately 35% of the total cell population (Carson *et al.*, 2006b). Astrocytes were once regarded as merely supporting cells in the CNS, providing structural support and maintaining an optimal microenvironment for neuronal functions and survival (Brambilla *et al.*, 2013). However, this viewpoint has dramatically changed in the past decade as growing interest in the study of biology and pathology of astrocytes slowly reveals its functions and roles in healthy and diseased CNS.

During development, astrocytes guide migration of developing neurites by forming boundaries with extracellular matrices and assist in neuronal synapse refinement (Bialas *et al.*, 2013; Powell *et al.*, 1999). In the adult brain, astrocyte play essential roles in maintaining brain health and functions. Astrocytes help to maintain ion, fluid, pH and neurotransmitter homeostasis of the synaptic interstitial fluid (Sofroniew *et al.*, 2010). Furthermore, astrocytes express high levels of glutamate transporters to quickly remove glutamate released to the synaptic cleft upon action potential to prevent over-excitation of glutamate receptors which potentially leads to excitotoxicity (Sattler *et al.*, 2006).

Astrocyte end-feet encircles endothelial cells, helping to maintain the tight junctions of blood brain barrier (BBB); a highly selective permeability barrier that limits passage of molecules into brain parenchyma (Abbott, 2002; Rossi, 2015). Astrocytes also take part in CNS blood flow regulation (Gordon

et al., 2007) and provide supply metabolic support to adjacent neurons (Sofroniew *et al.*, 2010). Moreover, astrocytes are capable of releasing a variety of gliotransmitters including glutamate, GABA, D-serine, purines into the synaptic cleft; modulating potentiation and learning by depressing or reinforcing pre-and post-synaptic membrane (Jensen *et al.*, 2013; Nedergaard *et al.*, 2003; Perea *et al.*, 2009).

1.2.2.2 Reactive astrogliosis

Due to its high resemblance with peripheral macrophage, microglia is often being regarded as the primary immune cells of the CNS. Nevertheless, astrocytes are able to respond to CNS insults ranging from subtle cellular perturbation to severe tissue injuries (Sofroniew, 2009). Thus, astrocytes should also be considered as another key immune cell in the CNS and given equal attention as microglia. In fact, there is evidence of crosstalk between microglia and astrocytes resulting in enhanced inflammatory responses. Co-cultures of microglia and astrocytes stimulated with LPS induces greater neurotoxicity than either cell type alone and hence supports role of astrocyte in mediating inflammatory responses (Saijo *et al.*, 2011).

In addition, astrocytes also share a comparable number of PRRs with microglia which is crucial for recognition of endogenous damage signals and infectious agents. TLR3 are known to be highly expressed in astrocytes (Park *et al.*, 2006) while TLR2, TLR4, TLR5, and TLR9 are found expressed at basal level (Farina *et al.*, 2007). However, expression of TLR3 and TLR4 is known to be upregulated during inflammation (Bsibsi *et al.*, 2002). Astrocytes also express a number of NOD-like receptors (NLRs) including NLRC such as NOD1 and NOD2 (Farina *et al.*, 2007; Sterka *et al.*, 2006) and NLRP like

NLRP2 (Minkiewicz *et al.*, 2013). NLRP2 complexes with adaptors proteins and caspase 1 to form inflammasome which activates caspase leading to processing and secretion of IL-1β upon ATP stimulation (Minkiewicz *et al.*, 2013). Astrocytes also endocytose native or pathologically modified molecules through expression of scavenger receptors (SRs) like SR-A, SR-B1, and RAGE (Godoy *et al.*, 2012; Husemann *et al.*, 2001). Together with expression of various complement and cytokine receptors, PPRs allow astrocytes to rapidly initiate inflammatory response to counteract with infection and cellular damages (Farina *et al.*, 2007; Shastri *et al.*, 2013).

Many molecules released during infection or CNS injury such as LPS, cytokines, glutamate, ATP, NO, ROS, hypoxia, and glucose deprivation, misfolded proteins like Aβ are known to activate astrocytes (Sofroniew *et al.*, 2010). Recognition by related PPRs triggers morphological and molecular changes in astrocytes, a phenomenon commonly knowns as reactive astrogliosis (Rossi, 2015). Reactive astrogliosis is a finely gradated continuum of progressive alterations in molecular expression (Sofroniew, 2009; Sofroniew *et al.*, 2010). When activated, astrocytes will undergo changes including upregulation of intermediate filaments, proliferation, and hypertrophy of cell bodies and processes. In severe cases, reactive astrogliosis form dense and compact glial scars with extracellular matrices (Sofroniew, 2009). One of the hallmarks of reactive astrogliosis observed in human pathology and animal experimental models is upregulation of glial fibrillary acidic protein (GFAP) intermediate filament and GFAP level is known to correspond to the extent of astrogliosis (Eng *et al.*, 1994). Thus, GFAP has become a prototypical marker

for reactive astrogliosis and often used as a marker for astroglial activation (Eng *et al.*, 2000).

1.2.2.3 Protective and detrimental effects of reactive astrogliosis

Activation of astrocytes serves several beneficial functions in protecting CNS by production of glutathione to protect against oxidative stress (Chen *et al.*, 2001), buffering extracellular K^+ and H^+ to prevent anoxic depolarization and cell swelling (Chen *et al.*, 2003), mediating blood brain barrier repair (Sofroniew *et al.*, 2010), clearing and degrading of Aβ peptides (Koistinaho *et al.*, 2004), and production of growth factors such as brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CTNF). In addition, astrocytes express glutamate transporters like GLAST and GLT-1 to uptake extracellular glutamate which potentially induce excitotoxicity in the vulnerable neurons (Chen *et al.*, 2003; Rothstein *et al.*, 1996). Glial scar formation also serves as a physical barrier to restrict the spread of microbial agents or inflammatory cells (Bush *et al.*, 1999; Drogemuller *et al.*, 2008).

Nonetheless, growing evidence reveals numerous undesired effects of reactive astrogliosis especially when astrogliosis persist and do not get resolved after injury. Dysfunction of reactive astrogliosis becomes instrumental contributor of many neurological diseases through either loss of essential functions or gain of detrimental functions (Sofroniew *et al.*, 2010). Activated astrocytes release proinflammatory cytokines like IL-1β, TNF-α, IL-6 that activate surrounding microglia and astrocytes which in turn release more pro-inflammatory cytokines, forming a positive-feedback loop that exacerbate neuroinflammation (Farina *et al.*, 2007). Reactive astrogliosis increases infiltration of peripheral leukocytes into the brain parenchyma by secreting

chemokines such as CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-5, CXCL-12, and CX3CL-1 (Babcock *et al.*, 2003; Farina *et al.*, 2007). Moreover, infiltration of leukocytes is also enhanced by astrocytic production of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1). VEGF increases BBB permeability (Argaw *et al.*, 2012), MMP2 and MMP9 induce BBB breakdown by degradation of extracellular matrix (Rosenberg *et al.*, 2001) while ICAM-1 and VCAM-1 adhere to leukocytes and facilitate their migration into CNS parenchyma (Carpentier *et al.*, 2005; Gimenez *et al.*, 2004). Activated astrocytes also lead to cellular dysfunction with ROS/RNS production that damage lipid, DNA, and protein (Sheng *et al.*, 2013; Swanson *et al.*, 2004). Moreover, expression of cyclooxygenase-2 (COX-2) as well as its main metabolite, prostaglandin E2 (PGE_2, a strong inducer of inflammation), are both elevated during astrogliosis, (Brambilla *et al.*, 1999). Glutamate uptake disruption or efflux of glutamate increases extracellular concentration of glutamate and potentiate excitotoxicity (Chen *et al.*, 2003). In addition, failure to maintain water balance potentially leads to CNS edema (Manley *et al.*, 2000).

Compelling evidence demonstrates that reactive astrogliosis is the fundamental factor impeding axon regeneration after brain trauma (Cho *et al.*, 2005). Mouse models devoid of GFAP and vimentin exhibit limited astroglial reactivity and hypertrophy of cell processes (Wilhelmsson *et al.*, 2004). $GFAP^{-/-}$ $Vim^{-/-}$ mice also showed significant synaptic regeneration and locomotor function recovery after entorhinal cortex and spinal cord lesion respectively (Menet *et al.*, 2003; Wilhelmsson *et al.*, 2004). Furthermore, attenuation of reactive astrogliosis with natural compound also promotes axon regeneration

and gains functional recovery after traumatic spinal cord injury, denoting a role of reactive astrogliosis in impeding axon regeneration (Su *et al.*, 2010). Several studies on spinal cord injury also revealed that removing glycosaminoglycan (GAG) chains from chondroitin sulfate proteoglycans CSPGs (a major component of glial scar) with chondroitinase ABC (ChABC) increased axons regeneration, improved locomotor functions, and proprioceptive behaviors (Bradbury *et al.*, 2002; Yick *et al.*, 2000). These findings highlight the significance of reactive astrogliosis and glial scar in forming a microenvironment adverse to CNS regeneration.

1.2.2.4 Implication of reactive astrogliosis in neurological diseases

Although reactive astrogliosis may serve some beneficial function with production of neurotrophic factors and demarcate healthy brain parenchyma from infected or damaged tissue through glial scar formation, it is often being viewed in a negative light due to its potential pathogenic role in several neurological diseases.

Reactive astrogliosis is a prominent feature of CNS trauma and plays an instrumental role in determining clinical outcome (Sofroniew *et al.*, 2010). It has been reported that human astrocytes expressed NLRP2 inflammasome when activated by ATP during trauma (Minkiewicz *et al.*, 2013). Activation of NLRP2 markedly enhances processing of caspase-1 and the proinflammatory cytokine IL-1β (Minkiewicz *et al.*, 2013). Due to its high abundance as well as its close proximity with BBB, astrocytes are exclusively positioned to create chemotactic gradient to recruit microglia and circulating leukocytes into site of injury (Rossi, 2015). Activation of astrocytes in response to spinal cord injury aggravate inflammation by promoting influx of leukocytes into lesion site and

cause secondary tissue loss by producing proinflammatory cytokines and chemokines such as CCL-2, CXCL-1, and CXCL-2 (Pineau *et al.*, 2010).

ALS is a progressive neurodegenerative disease characterized by neuronal loss in the brain and spinal cord. While most ALS cases appear to be sporadic, SOD1 mutation is associated with about 20% of familial cases (Rossi, 2015). Few studies have addressed the relative contribution of astrocyte in motor neuron loss in ALS. Attenuating expression of $hSOD1^{G37R}$ mutant protein in astrocytes in transgenic mice slowed down ALS progression (Yamanaka *et al.*, 2008). A more recent study also reported delayed disease onset and lifespan extension when expression of $hSOD1^{G85R}$ protein in astrocytes was reduced (Wang *et al.*, 2011). In addition, significant reductions in glutamate transport in ALS patient suggest glutamate-induced excitotoxicity in motor neuron (Rothstein *et al.*, 1992). Ablating glutamate type I transporter (GLT-1) expression in SOD1 mutant mice decreases glutamate uptake resulting in motor neuron loss as a consequence of excitotoxicity (Pardo *et al.*, 2006). Expression of ion pump sodium/potassium ATPase α2 subunit (α2-Na/K ATPase) is found upregulated in $SOD1^{G93A}$ astrocytes and associated with motor neurons degeneration. Interesting, α2-Na/K ATPase also induces expression of a battery of proinflammatory cytokines and chemokines which mediate and perpetuate neuroinflammation in ALS (Gallardo *et al.*, 2014).

Synaptic loss and dysfunction caused by age-dependent accumulation of $A\beta_{1-42}$ is proposed to underlie cognitive decline in AD. High level of presenilins, the catalytic subunits of the γ-secretase are detected in sporadic Alzheimer brains and prominently found in the reactive astrocytes (Walker *et al.*, 2006; Weggen *et al.*, 1998). Neurons express higher level of β-secretase 1 (BACE1)

than glia cells thus is considered the major contributor of amyloid plaque (Laird *et al.*, 2005). That being said, it should be noted that astrocytes outnumber neurons by more than five-fold in the brain, and thus may contribute to the Aβ load in the brain and exacerbate AD pathology as disease progresses (Sofroniew *et al.*, 2010). Cytokines combination (TNF-α and IFN-γ) or $A\beta_{1-42}$ have been documented to elevate BACE1 and APP level and induce amyloidogenic APP processing in astrocytes, suggesting Aβ and cytokine-driven feed-forward mechanism that stimulate Aβ production in astrocyte (Zhao *et al.*, 2011). Another hallmark of AD is neurofibrillary tangles formed by aggregation of hyperphosphorylated tau protein. As the degree of neurofibrillary pathology advances (as indicated by Braak staging), level of reactive astrogliosis increases with concurrent reduction in astrocytic glutamate transported, potentiating excitotoxicity in neurons (Simpson *et al.*, 2010). Recently, $A\beta_{1–42}$ has been demonstrated to induce release of glutamate from astrocytes through stimulation of α7 nicotinic receptors (α7nAChRs). Excessive release of glutamate in turn activates extrasynaptic NMDA-type glutamate receptors (eNMDARs) followed by decrease in mEPSC frequency, increase tau hyperphosphorylation, NO production, and caspase-3 activation, resulting in synaptic dysfunction and cognitive decline (Talantova *et al.*, 2013). Moreover, reactive astrocytes aberrantly produce high levels of inhibitory gliotransmitter GABA through monoamine oxidase-B, impairing synaptic plasticity and memory function in AD mice (Jo *et al.*, 2014).

Due to high energetic cost of the ATP-dependent glutamate reuptake, dramatic increase in extracellular concentration of glutamate is unavoidable during energy failure conditions like ischemic (Chen *et al.*, 2003). During

ischemic damage, disruption of glutamate uptake and elevation of glutamate efflux have both been reported. ATP depletion-induced glutamate release is substantially attenuated by astrocytic glutamate transporter GLT-1 inhibitor, dihydrokinate (DHK), indicating that reversal of glutamate transporter is a potential route of glutamate efflux from astrocytes (Longuemare *et al.*, 1995; Seki *et al.*, 1999). ATP released from damaged cells coupled with purinergic receptor such as $P2Y_1$ (Domercq *et al.*, 2006) and $P2X_7$ (Duan *et al.*, 2003) receptor and evoked Ca^{2+}-dependent glutamate release from astrocytes, suggesting an alternative mechanism astrocyte mediate excitotoxicity. High expression of AQP4 in astrocyte pericapillary end-feet processes implies a role in maintaining brain water balance (Chen *et al.*, 2003). Cerebral edema is markedly decreased in AQP4 deficient mice subjected to ischemic stroke compared to control and has better neurological outcome and survival (Manley *et al.*, 2000). Similarly, elevation of AQP9 expression in astrocytes is also detected at the peri-infarct area upon transient ischemia suggesting a role of astrocytic AQP9 in regulating water homeostasis (Badaut *et al.*, 2001). Together, these findings indicate involvement of astrocyte activation in mediating CNS diseases and neuroinflammation through multiple mechanisms and attenuating reactive astrogliosis may bring beneficial outcomes.

Neuroinflammation is a complex integration of responses from CNS resident cells as well as the molecular immune components released. These molecular components include signaling molecules involved in inflammatory signaling pathways, chemical messengers like cytokines and chemokines, and reactive oxygen and nitrogen species. Most of these molecules work jointly to initiate, enhance, and propagate neuroinflammatory response.

1.2.3 Inflammatory signaling pathways

1.2.3.1 Nuclear Factor-κB (NF-κB)

NF-κB has long been regarded an archetypical proinflammatory signaling pathway which is rapidly activated in response to infection, injury and inflammation. It is a transcription factor that regulates cell proliferation, differentiation, survival and well-known for its role as a central mediator of inflammatory response (Viatour *et al.*, 2005). Understanding of NF-κB signaling primarily come from studying activation of IL-1β and TNF-α receptors as well as TLRs (Lawrence, 2009). All members of NF-κB share a Rel homology domain (RHD), which is crucial for DNA binding and protein dimerization. RelA/p65, RelB, and cRel contain a transactivation domain in their C-termini necessary for transcriptional activation whereas the other remaining two members, p50 and p52 lack intrinsic ability to activate transcription. p50 and p52 are synthesized as large precursor; p105 and p100 respectively which undergo proteasome processing to generate mature proteins. Though formation of several homodimers or heterodimers of NF-κB family subunits have been described, the two main signaling pathways identified are the canonical and the alternative NF-κB pathways (Fischer *et al.*, 2015).

Canonical NF-κB pathway

NF-κB's role in mediating inflammatory responses is mediated primarily, though not exclusively, through activation of the canonical pathway via p65/p50 dimers (Fischer *et al.*, 2015). Under activating conditions, NF-κB is sequestered in the cytoplasm by IκBα. Activation of TLRs by PAMPs and DAMPs, or proinflammatory cytokines (IL-1β and TNF-α) stimulation triggers

a cascade of signaling molecules leading to recruitment and activation of IκB kinase (IKK) complex which comprises of IKKα, IKKβ, and IKKγ (also known as NEMO). Once activated, the IKK complex phosphorylates IκBα at Ser32 and Ser36. Subsequently, IκBα is ubiquitinated and degraded by 26S proteasome (Figure 1.2). Degradation of IκBα release inhibition on NF-κB, allowing its translocation into the nucleus where it binds to specific κB sites and transcribe numerous target genes (Viatour *et al.*, 2005).

NF-κB activation in neurological disorders

NF-κB activation is implicated in several neuroninflammatory and neurodegenerative diseases. NF-κB activation is detected in neuron, microglia, and astrocytes at the close vicinity of early amyloid plaque from patients with AD (Ferrer *et al.*, 1998; Kaltschmidt *et al.*, 1997). Immunoreactivity of NF-κB is also significantly elevated in dopaminergic neurons of PD patients (Hunot *et al.*, 1997). Moreover, prolonged activation of NF-κB is found in the same regions undergoing progressive atrophy, suggesting NF-κB role in mediating chronic inflammatory processes following CNS trauma (Nonaka *et al.*, 1999). The neuroprotective properties of NF-κB in neurodegenerative diseases have been proposed by several studies which demonstrated greater neuronal loss when NF-κB is inhibited in HD and ischemic stroke animal model (Hill *et al.*, 2001; Yu *et al.*, 2000). However, there are also compelling evidences supporting a detrimental role of NF-κB activation in neurological disease. For example, attenuating NF-κB activation with pyrrolidine dithiocarbamate (PDTC) remarkably reduced infarct size (Nurmi *et al.*, 2004). In line with previous data, neuronal deletion of IKKβ resulted in remarkably smaller

ischemic infarct size while constitutive IKKβ activation had the reversed effects (Herrmann *et al.*, 2005).

The pathological function of NF-κB may be attributed to its ability to regulate a vast number of proinflammatory mediators. NF-κB induces secretion of cytokines such as IL-1β, TNF-α, IFN-γ and chemokines like CCL-2, CCL-3, CCL-5, CCL-7, CXCL-9, and CXCL-10 (Brambilla *et al.*, 2005; Van Loo *et al.*, 2006; Werts *et al.*, 2007). Induction of ICAM-1 and VCAM-1 expression by NF-κB, together with upregulation of chemokines, promotes recruitment of peripheral leukocytes into the CNS that further contribute to the inflammatory process (Zhou *et al.*, 2007). Notably, NF-κB is a strong inducer of pro-oxidative enzymes like inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) that generates ROS/RNS. Increasing oxidative/nitrosative stresses promote protein oxidation/nitration which leads to neuronal death (Mir *et al.*, 2009). Furthermore, NF-κB activation upregulates cyclooxygenase-2 (COX-2) that converts arachidonic acid to prostaglandin H2 which produces superoxide during the second step of the reaction (Morgan *et al.*, 2011). NF-κB induction of proinflammatory cytokine/chemokine, leukocyte infiltration, and oxidative/nitrosative stresses enhances tissue damage and exacerbates neuroinflammation therefore NF-κB may be a potential therapeutic target for neurological disorders.

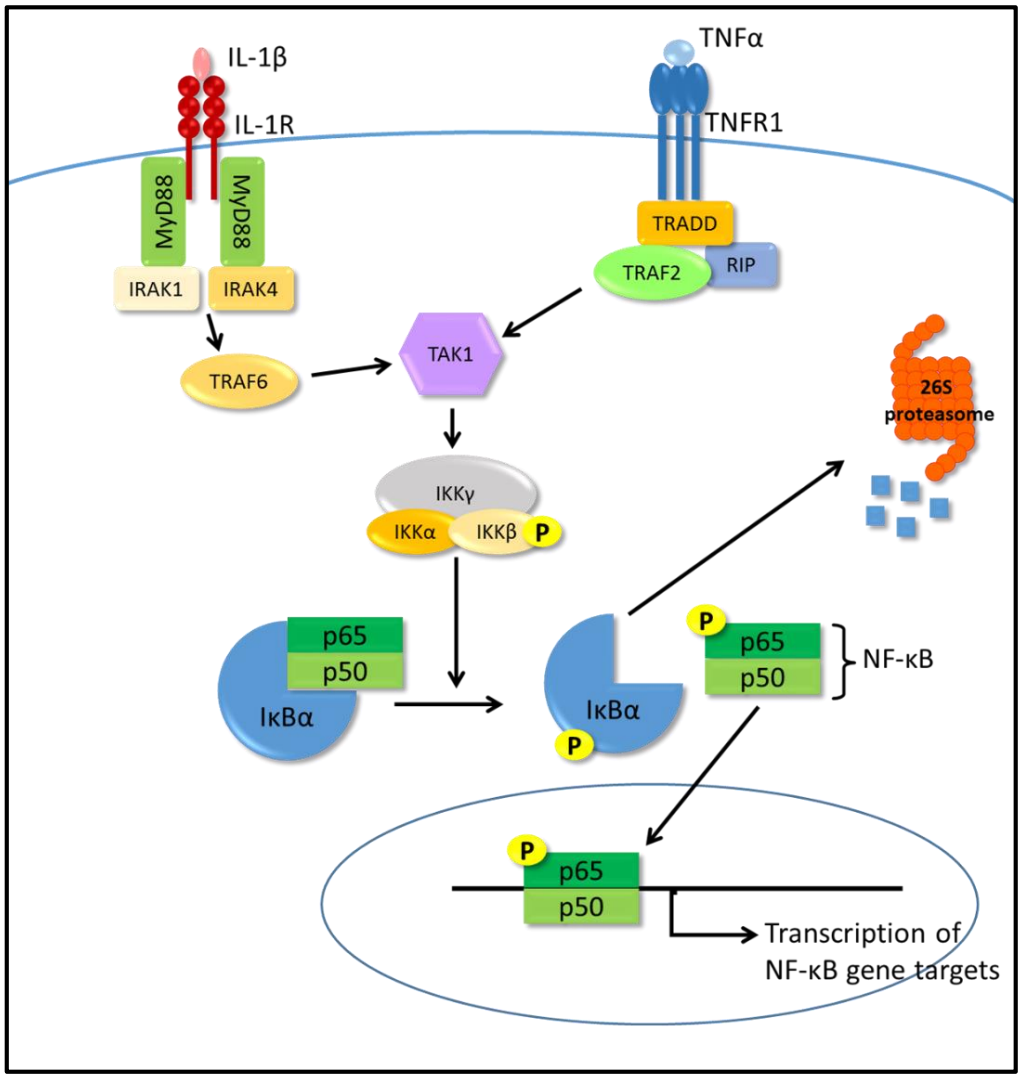


Figure 1.2 Activation of NF-kB in response to IL-1β and TNF-α.

1.2.3.2 MAPKs

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases that regulate a range of cellular activities such as cell survival, death, proliferation, differentiation, and transformation (Kim *et al.*, 2010b). The mammalian MAPK family is comprised of p38, and c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). The signaling axis of MAPK has at least 3 components; a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. Upon activation by extracellular or intracellular stimuli, MAP3K is activated by MAP4Ks or GTPases. Subsequently, MAP3K will phosphorylate and initiate MAP2Ks which will in turn phosphorylate MAPKs. Activated MAPK phosphorylate various substrate proteins like transcription factors and other kinases (Kim *et al.*, 2010b).

JNK and p38 signaling pathways

JNK and p38 are stress-induced kinases which are activated by cellular stresses such as osmotic shock, inflammatory cytokines, pathogenic stimuli, UV radiation, and oxidative stress (Matsuzawa *et al.*, 2005). Activation of JNK and p38 are important for the regulation of various cellular activities like cell differentiation, apoptosis, and inflammatory cytokines (Hommes *et al.*, 2003; Kim *et al.*, 2010b). p38 and JNK are readily activated by IL-1β, TNF-α, and LPS which results in production of inflammatory mediators like cytokines, chemokines, COX-2, and iNOS (Kaminska, 2005; Oltmanns *et al.*, 2003). ROS also triggers JNK and p38 activation leading to cell apoptosis by promoting cleavage and activation of pro-apoptotic BH3 interacting-domain death agonist (Bid) protein. Bid interacts with Bax and destabilizes mitochondria membrane, followed by release of cytochrome c or Smac/DIABLO from mitochondria and

eventually apoptosis (Deng *et al.*, 2003; Zhuang *et al.*, 2000). Persistent activation of JNK and p38 has also been implicated in neurological diseases. Inactivation of JNK and p38 signaling pathway with multi-lineage kinase (MLK) inhibitor; CEP-1347 attenuated MPTP-mediated loss of dopaminergic neurons in PD mouse model (Saporito *et al.*, 1999) and abated TNF-α, IL-6, and CCL-2 expression in microglia upon stimulation with various endotoxins or the plaque forming peptide $A\beta_{1-40}$ (Lund *et al.*, 2005). Furthermore, amyloidogenic $A\beta_{1-42}$ induces ROS production which triggers JNK and p38 activation and ultimately lead to neuronal death (Marques *et al.*, 2003; Zhu *et al.*, 2002). Attenuating JNK signaling with AS601245 inhibitor or with antisense RNA after induction of white matter injury mitigates against microgliosis, astrogliosis, TNF-α expression, BBB leakage, and apoptosis of oligodendrocyte progenitor, indicating role of JNK in mediating various neuroinflammatory responses (Wang *et al.*, 2012).

ERK signaling pathway

On the other hand, growth factors and cytokines are the strong activators of ERK signaling which mainly involved in proliferation, differentiation and development (Balmanno *et al.*, 2009). ERK signaling pathway is closely associated with cancer development where its activation has been shown to promote tumor survival and migration (Balmanno *et al.*, 2009; Huang *et al.*, 2004). Constitutive activation of ERK1/2 in murine fibroblast cell line NIH 3T3 is pro-survival and anti-apoptotic following H_2O_2 toxicity, indicating role of ERK in cell survival (Guyton *et al.*, 1996). Indeed, there is much evidence supporting neuroprotective function of ERK signaling. ERK1/2 signaling has been demonstrated to mediate the neuroprotective activity of trophic factors like

nerve growth factor (NGF) (Xia *et al.*, 1995). Attenuating ERK 1/2 signaling markedly enhanced neuronal apoptosis induced by cisplatin (Gozdz *et al.*, 2003). In parallel, ERK also mediates protective effects of BDNF on cortical neurons against apoptosis induced by camptothecin, an alkaloid that induces DNA damage. Moreover, ERK1/2 is known to exert its neuroprotective functions via regulation of pro- and anti-apoptotic molecules from the Bcl-2 family (Hetman *et al.*, 2004). Conversely, ERK signaling is demonstrated to mediate inflammatory response induced by LPS or ischemic stroke through production of proinflammatory mediators such as IL-1β, TNF-α, IL-6, and iNOS (Carter *et al.*, 1999; Maddahi *et al.*, 2010). Several findings showed that inhibiting ERK1/2 signaling confers protection to neurons in brain trauma, cerebral ischemia stroke, and epilepsy (Chu *et al.*, 2004). Such contradictory findings are not yet fully resolved but it has been proposed that ERK1 and ERK2 may play distinct roles in CNS pathophysiology (Yu, 2012). More studies need to be done to elucidate the role of ERK in CNS diseases.

1.2.4 Cytokines

Cytokines are a class of small proteins produced by a variety of immune cells that act as intercellular signaling molecules to modulate inflammation and regulate cellular activities like survival, growth, proliferation, and differentiation (Ramesh *et al.*, 2013; Rubio-Perez *et al.*, 2012). Many cytokines are themselves pleiotropic, exerting multiple actions and majority of them have overlapping actions (redundancy) (Leonard, 1994; Paul, 1989). In the CNS, cytokines serve to maintain immune surveillance, facilitate leukocyte recruitment and mediate innate and adaptive immune response (Takeshita *et al.*, 2012). Cytokines in the brain play pivotal role in mediating a number of CNS

pathologies and they are primarily generated by microglia and astrocytes. In response to pathogen invasion or injury, a repertoire of cytokines/chemokines are released to initiate an appropriate immune response (Jensen *et al.*, 2013). Cytokines are generally classified as anti-inflammatory and pro-inflammatory molecules which jointly regulate inflammation. Proinflammatory cytokines known to exacerbate neuroinflammation such as IL-1β, TNFα, and IL-6 (Basu *et al.*, 2004; Kiguchi *et al.*, 2012). These cytokines are acute phase cytokine which appear essential for the induction of other cytokines/chemokines and inflammatory mediators (Kaminska, 2005; Shimada *et al.*, 2002; Van Miert, 1995).

1.2.4.1 IL-1β

IL-1β is involved in the pathophysiology of various CNS disorders including infections, stroke and neurodegeneration diseases (Basu *et al.*, 2004). Actions of IL-1β is primarily mediated by type I IL-1β receptor (IL-1RI) as IL-1RII is a decoy receptor (Shaftel *et al.*, 2008). Engaging to IL-1RI results in association with IL-1R accessory protein (IL-1R AcP) and recruitment of MyD88 adapter protein which is also shared by TLR signaling. This leads to IL-1R-associated kinase (IRAK) recruitment and activation of TNF receptor-associated factor 6 (TRAF6) (Risbud *et al.*, 2014; Rothwell *et al.*, 2000). Subsequently, TRAF6 binds to transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) and induces TAK1 activation which in turn phosphorylates and activates IκB kinase β (IKKβ) to initiate NF-κB signaling and mitogen-activated protein kinase kinases (MKKs) to instigate MAPKs (JNK, ERK, and p38) signaling pathways (Moriguchi *et al.*, 1996; Ninomiya-Tsuji *et al.*, 1999; Shirakabe *et al.*, 1997).

Exposure to IL-1β induces robust activation of microglia and astrocytes (Herx *et al.,* 2000). Growing evidence shows that IL-1β mediate pathology of various neurological diseases through immunological and apoptosis pathway. It is known to drive production of other cytokines (TNF-α IL-6) and chemokines (CXCL-1 and CXCL-2) to exacerbate neuroinflammation (Mrak *et al.*, 1995; Shaftel *et al.*, 2008). Moreover, IL-1β has been shown to induce leukocytes infiltration by upregulating CCL-2 and intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells (Proescholdt *et al.*, 2002). Intraparenchymal injection of IL-1β changes cerebral blood volume accompanied with increased diffusion coefficient of brain water indicating role of IL-1β in enhancing BBB permeability (Blamire *et al.*, 2000). Implication of IL-1β in mediating neuronal damage has been demonstrated in mice deficient of IL-1β which develop smaller infarct size compared to controls when subjected ischemic stroke (Boutin *et al.*, 2001; Schielke *et al.*, 1998). In addition, IL-1β potentiates excitotoxicity by upregulating expression of neuronal glutaminase that convert glutamine to glutamate. This in turn leads to elevation of intracellular and extracellular glutamate level, promoting neuronal cell death (Ye *et al.*, 2013).

Elevated expression of IL-1β is correlated with severity of injury and undesirable clinical outcome and therefore abrogating IL-1β signaling would likely improve patients' condition. Interleukin-1 receptor antagonist (IL-1ra) binds to IL-1R but does not induce any intracellular response therefore effectively attenuates IL-1β signaling. Due to its protective effects, IL-1ra has now been licensed for use in rheumatoid arthritis (Basu *et al.*, 2004). Furthermore, when it is intracerebroventricularly injected into mice, it

attenuates ischemic and excitotoxic neuronal damage (Loddick *et al.*, 1996; Relton *et al.*, 1992). Unfortunately, IL-1ra is a large protein with limited access to CNS due to BBB and thus therapeutic delivery into the brain would be a challenge (Basu *et al.*, 2004). Laliberta *et al*. also identified a series of cytokine release inhibitory drugs (CRIDs) that abated IL-1β-induced cell death by preventing ATP-induced pro-IL-1 processing by caspase-1, potentially reversing the adverse effects of elevated IL-1β in CNS disorders (Laliberte *et al.*, 2003).

1.2.4.2 TNF-α

Although TNF-α and IL-1β have structurally different receptors, they share considerable overlap in functional and post-receptor intracellular signaling cascades (Ziebell *et al.*, 2010). TNF-α often acts synergistically with IL-1β leading to amplification of inflammatory response (Gouwy *et al.*, 2005). TNF-α binds with TNF receptor type 1 (TNFR1) which is widely expressed in most tissue and TNF receptor type 2 (TNFR2) that is only found in immune cells (Grell *et al.*, 1995). The majority of biological responses classically attributed to TNF-α are mediated by TNFR1 (Wajant *et al.*, 2003). Interaction with TNFR1 induces receptor trimerization and recruits TNF receptor-associated death domain (TRADD) that acts as an adapter protein for Fas-associated death domain (FADD) and TNF receptor-associated factor 2 (TRAF2). FADD mediates activation of caspase 8 and caspase 10 which initiate a protease cascade resulting in apoptosis (Olmos *et al.*, 2014). On the other hand, TRAF2 acts similarly to TRAF6 in IL-1β signaling. It recruits TAK1 and promotes TAK1 activation which in turn leads to activation of NF-κB and MAPKs signaling pathways (Verstrepen *et al.*, 2008). TNF-α robustly activates

the stress-induced protein kinases like JNK and p38 but only induce moderate ERK activation and may even absent sometimes (Wajant *et al.*, 2003).

Dysregulated TNF-α signaling has been implicated in the pathophysiology of a number of CNS disorders through several mechanism. TNF-α is a prototypic proinflammatory cytokine that stimulate expression of other inflammatory mediators like cytokines (IL-1α, IL-1β, and IL-6) (Kozawa *et al.*, 1997; Turner *et al.*, 2007) and chemokines (CCL-2, CCL-3, and CXCL-2) (Barna *et al.*, 1994; Czermak *et al.*, 1999). Moreover, activation of TNFR1 induced ICAM-1 and VCAM-1 expression on endothelial cells (Zhou *et al.*, 2007) and enhanced BBB permeability (Kim *et al.*, 1992). Together, these processes promote recruitment of leukocytes into the brain parenchyma which further mediate inflammatory responses and potentially leads to more tissue damage. TNF-α also elevates superoxide (O_2^-) and nitric oxide (NO) production by inducing iNOS and NOX expression (Brandes *et al.*, 2014; Mir *et al.*, 2009; Mir *et al.*, 2008). NO and ROS promote protein oxidation and nitration that promote cellular dysfunction and induce neuronal death (Mir *et al.*, 2009). Another mechanism contributing to neuronal death is through the induction of excitotoxicity via elevation of extracellular glutamate level. TNF-α upregulates neuronal glutaminase (Ye *et al.*, 2013) and downregulates glutamate transporter-1 (GLT-1) thus reducing glutamate uptake by astrocyte (Sitcheran *et al.*, 2005).

TNF-α elevation in CNS is evident in numerous neurological diseases like brain trauma, stroke, AD, and PD (Helmy *et al.*, 2011). Several preclinical and clinical studies in variety of disease models suggest that targeting TNF-α signaling may be an appealing strategy to attenuate or slow disease progression

(Mccoy *et al.*, 2008). Several FDA-approved humanized IgG antibodies including infliximab, adalimumab, and etanercept has also been employed to attenuate TNF-α activity. As reported by Selmaj *et al.*, administration of anti-TNF antibody effectively attenuated leukocyte infiltration and demyelination in EAE, the mouse model of MS (Selmaj *et al.*, 1991). However, inconsistent findings have been reported. Intravenous infusions of a humanized mouse monoclonal anti-TNF antibody (cA2) into MS patient did not observe any clinically significant neurologic changes (Van Oosten *et al.*, 1996). While some studies found TNF receptor knockout to be protective against MPTP-induced striatal damage (Sriram *et al.*, 2002, 2006), others reported that TNF receptor knockdown has little effects on striatal damage or dopaminergic neuron loss (Leng *et al.*, 2005; Rousselet *et al.*, 2002). Cytokine pleiotropy and redundancy may partly explain these discrepancies. TNF-α and IL-1β are known to share similar array of downstream effectors (Allan *et al.*, 2005) and compensatory IL-1β upregulation had also been documented when TNF-α expression was abrogated (Gowing *et al.*, 2006).

1.2.4.3 IL-6

IL-6 is 26 kDa glycoprotein that has an important function in mediating acute phase response and fever. IL-6 receptor is consist of gp130 homodimer and a ligand-binding IL-6Rα chain (Helmy *et al.*, 2011). Binding of IL-6 leads to dimerization of gp130 results in activation of Janus kinases (JAKs) that subsequently activates Signal Transducers and Activators of Transcription (STAT3) transcription factor. Alternatively, IL-6 initiates MAPKs through a second pathway involving G-protein Ras (Gadient et al., 1997). Other than IL-1β and TNF-α, bacterial pathogens like LPS are known to exert a strong

inducing signal for IL-6 in astrocytes (Benveniste *et al.*, 1990; Lee *et al.*, 1993; Norris *et al.*, 1994). IL-6 plays an essential role in orchestrating inflammatory response in the periphery by inducing production of acute phase proteins, development of effector T-cell, and stimulate antibody production (Tanaka *et al.*, 2014). In the CNS, IL-6 induces production of cytokines, cell adhesion molecules, and promote leukocyte infiltration into brain parenchyma (Lemoli *et al.*, 1996; Prudhomme *et al.*, 1996). Pathogenic role of IL-6 has been shown in transgenic mice overexpressing IL-6. Prominent activation of microglia and astrocytes activation is found in these animals along with neurodegeneration and development of severe neurologic disease (Campbell *et al.*, 1993; Chiang *et al.*, 1994; Fattori *et al.*, 1995).

Although IL-6 elevation is often correlated with stroke and neurodegenerative diseases such as AD and PD, there is as yet any clear evidence of IL-6 involvement in the pathologic process of these diseases and IL-6 could be a reflection of the ongoing inflammation caused by the progressive neuronal damage (Blum-Degena *et al.*, 1995; Mogi *et al.*, 1994; Suzuki *et al.*, 2009). Growing evidence suggests that IL-6 exhibit both proinflammatory and anti-inflammatory / neuroprotective roles in the CNS. IL-6 as a neurotrophic factor is first described by Hama and colleagues in year 1989 and 1991. They showed that IL-6 promotes survival of cultured cholinergic neurons isolated from basal forebrain and septum as well as catecholaminergic neurons from mesencephalon (Hama *et al.*, 1991; Hama *et al.*, 1989). Subsequent study by Maeda *et al.* demonstrated that incubation with conditioned medium from astrocytes exposed to hypoxia/reoxygenation promoted survival of hypoxia/reoxygenation treated PC12 cells and the

beneficial effect was attenuated by IL-6 neutralizing antibody (Maeda *et al.*, 1994). Moreover, continuous injection of recombinant IL-6 into lateral ventricle of gerbils subjected to transient ischemia delayed neurodegeneration and prevented learning disabilities (Matsuda *et al.*, 1996). In line with previous data, IL-6 deficient mice develop larger ischemic infarct volume, have poorer neurologic status, and higher mortality rate compared to controls (Herrmann *et al.*, 2003).

IL-6 protective effects may be mediated by preventing oxidative stress and cell apoptosis. Mice with IL-6 deficiency produce lower granulocyte-macrophage colony-stimulating factor (GM-CSF) and metallothionein, an antioxidant protein compared to controls after cryolesion to fronto-parietal cortex (Penkowa *et al.*, 1999). Follow up study with GFAP-IL-6 transgenic mice also revealed that increasing astrocytic IL-6 expression substantially decreased cell apoptosis and oxidative stress after cryolesion by inducing antioxidant proteins like metallothionein (Penkowa *et al.*, 2003). Induction of vascular endothelial growth factor (VEGF) by IL-6 also encourages brain repair via vascular remodeling (Helmy *et al.*, 2011). Together, these studies provide convincing evidences that IL-6 can have dual effects; proinflammatory and anti-inflammatory/neuroprotective. It is therefore interesting to examine how prospective anti-inflammatory compounds modulate IL-6 expression and its role in neuroinflammation.

Chemokine subfamilies	No. of residues between cysteine residues near N-terminus	Type of recruited leukocyte	Examples
CC	0	Mononuclear cells, eosinophils, basophils, dendritic cells, NK cells	CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CCL-11
CXC	1	ELR domain: neutrophils	CXCL-1, CXCL-2, CXCL-5, CXCL-8
		No ELR domain: monocytes, T cells, NK cells	CXCL-9, CXCL-10, CXCL-11
CX3C	3	Monocytes, T cells, NK cells	CX3CL-1
C	Has one cysteine residue	T cells, NK cells	XCL-1, XCL-2

Table 1.2 Chemokine classification (modified from Jaerve *et al*., 2012; Ubogu *et al.*, 2006).

1.2.5 Chemokines and leukocytes infiltration

Chemokines are small chemotactic cytokines ranging from 8-12 kDa that is produced by various inflammatory cells to facilitate leukocyte recruitment (Helmy *et al.*, 2011). While IL-1β and TNF-α are key cytokines that initiate inflammatory response, chemokines are important mediators that perpetuate and sustain inflammation. Chemokines contain at least four cysteine residues that form two disulfide bonds and they are classified based on the position of two conserved cysteine residues in the N-terminus. Chemokines are divided into four subfamilies; namely CC, CXC, CX3C and C (De Haas *et al.*, 2007; Jaerve *et al.*, 2012). Chemokine receptors are seven-transmembrane G-protein coupled receptors that bind to various G-proteins and activate a variety of intracellular signaling pathways. Administration of pertussis toxin attenuated

most of the chemokine-induced response suggesting the role of $G_{\alpha i}$ protein in mediating effects of chemokines (Murphy, 1996). Notably, promiscuity and redundancy are key characteristics of chemokines. This implies that a chemokine ligand could stimulate more than one receptors, and multiple chemokines could act on a single receptor (Ubogu *et al.*, 2006b). Nonetheless, these relationships are generally restricted to within chemokine subfamilies (Ubogu *et al.*, 2006b).

1.2.5.1 CC-chemokines

This is the largest group among the chemokine subfamilies (Kiguchi *et al.*, 2012). Most members from this subfamily exhibit chemotactic to mononuclear cells (monocytes, macrophage, and lymphocytes), eosinophils, basophils, NK cells (Jaerve *et al.*, 2012). CCL-2 and CCL-5 are very well characterized in this subfamily. While neuroprotective role of CCL-2 and CCL-5 has been suggested (Chiu *et al.*, 2010; Tripathy *et al.*, 2010), compelling evidences point towards their involvement in exacerbating neurological diseases such as multiple sclerosis (MS). CCR2 has been has been reported to play a necessary and non-redundant role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE); an animal model of MS characterized by substantial infiltration of mononuclear cells (Izikson *et al.*, 2000). Although CCL-2, CCL-7 and CCL-8 can activate signal transduction pathways through CCR2, CCL-2 is believed to be most potent inducer of leukocyte transmigration (Sozzani *et al.*, 1994). On the other hand, CCL-5 is found robustly elevated among the cytokines and chemokines measured in EAE (Brambilla *et al.*, 2009). Treatment with antibody against CCL-2 or CCL-5 in EAE inhibited leukocyte

adhesion to the brain microvasculature (Dos Santos *et al.*, 2005). Furthermore, mice with $CCR2^{-/-}$ failed to upregulate chemokines CCL-5, CCL-2, CXCL-10, chemokine receptors CCR1, CCR2, and CCR5 expression and had no evident influx of mononuclear cells, indicating the importance of chemokines and chemokines receptors in mediating leukocyte influx and EAE development (Izikson *et al.*, 2000). Chemokine expression level also has implication in the disease course as CCL-5 403*Gly polymorphism that produces lower amount of CCL-5 reduces risk of severe axonal damage while high-producer allele CCL-5 403*Ala is associated with poorer clinical outcomes (Van Veen *et al.*, 2007).

In addition to the chemoattraction of leukocytes, chemokines also potentially exacerbate neuroinflammation by promoting chemotactic migration of microglia, activating microglia and astrocytes, and inducing expression of proinflammatory mediators. Several studies showed that CCL-2 and CCL-5 overexpression induced chemotactic migration of microglia to amyloid plaque (Huang *et al.*, 2009; Huang *et al.*, 2010; Yamamoto *et al.*, 2005). Interestingly, microglia accumulation at the site of amyloid plaque did not promote Aβ clearance, instead it increased fibrillary Aβ deposit (Yamamoto *et al.*, 2005). Role of chemokines in mediating microglia and astrocytes activation has also been implicated in $CCL3^{-/-}$ and $CCR5^{-/-}$ mice. These animals exhibited decreased reactive astrogliosis and microgliosis in the hippocampus upon $A\beta_{1-40}$ intracerebroventricular injection that is associated with reduced NF-κB activation, Cox-2 and iNOS expression, and macrophage chemotaxis. Cognitive deficit and synaptic dysfunction are also attenuated in these animals (Passos *et al.*, 2009). Furthermore, chemokines are potent inducer of other inflammatory

mediators. For instance, CCL-5 could elevate expression of cytokines/chemokines such as CXCL-1, CXCL-2, CCL-2, CCL3, TNF-α, IL-6 and adhesion molecule ICAM-1 in astrocytes (Luo *et al.*, 2002).

1.2.5.2 CXC-chemokines

CXC chemokines are further divided into ELR-positive or ELR-negative depending on the presence of an glutamic acid-leucine-arginine (ELR) motif at the N-terminus of the chemokine (Ambrosini *et al.*, 2004). ELR-CXC chemokine like CXCL-1 (KC), CXCL-2 (MIP-2), CXCL-5 (lix), and CXCL-8 (IL-8) are potent angiogenic factors (Strieter *et al.*, 1995). They act explicitly on neutrophils and bind to CXCR1 and CXCR2 receptors (Rossi *et al.*, 2000). Primary injury after stroke, brain trauma or spinal cord lesion is followed by infiltration of leukocytes that mediates secondary degeneration events, causing further damage to the brain (Jaerve *et al.*, 2012). CXCL-1 and CXCL-2 have been implicated in aggravating CNS injury by promoting transmigration of monocytes and neutrophils into the CNS. Spinal cord injury rapidly induced CXCL-1 and CXCL-2 secretion from astrocytes distributed throughout the spinal cord and attenuation of their expression remarkably reduced leukocyte infiltration which is associated with reduced neuronal death and improved motor function recovery (Kang *et al.*, 2011; Pineau *et al.*, 2010). Role of CXCL-5 in CNS is less studied but it is found upregulated in cerebrospinal fluid (CSF) of bacterial meningitis as well as ischemic stroke patients where CXCL-5 is involved in neutrophil recruitment and associated with brain infract size (Zaremba *et al.*, 2006; Zwijnenburg *et al.*, 2003).

Non-ELR-CXC chemokines bind to CXCR3, CXCR4, and CXCR5 and majority exhibit angiostatic properties with the exception of CXCL-12 (SDF-1)

(Rossi *et al.*, 2000; Strieter *et al.*, 1995). CXCL-9 (MIG), CXCL-10 (IP-10), and CXCL-11 (I-TAC) share the common receptor CXCR3 receptor and recruit monocytes, T cells, and NK cells. These chemokines are strong angiostatic factors, inhibiting endothelial cell chemotaxis and neovascularization (Strieter *et al.*, 1995). Injection of CXCL-9 and CXCL-10 into tumour resulted in tumour regression associated with increased tumour cell necrosis, T cell recruitment and angiogenesis inhibition (Angiolillo *et al.*, 1995; Sgadari *et al.*, 1996; Sgadari *et al.*, 1997). Consistent with these findings, Glaser *et al*. (2004) also reported active role of CXCL-10 in vasculature remodeling and its upregulation after spinal cord injuries inhibited angiogenesis and reduced blood flow to the injury site (Glaser *et al.*, 2004). Besides that, CXCL-10 promoted apoptosis and prevented corticospinal axon sprouting (Glaser *et al.*, 2006). This was probably mediated through intracellular Ca^{2+} elevation that released cytochrome C from mitochondria which subsequently activated apoptosis (Sui *et al.*, 2006). While elevation of CXCL-9 and CXCL-10 may diminish tumour growth, upregulation of these chemokines upon CNS lesion may be detrimental as they could exacerbate neuroinflammation by recruiting leukocytes into CNS, induce neuronal death and also prevent tissue repair by inhibiting angiogenesis and neovascularization (Keeley *et al.*, 2011).

1.2.5.3 CX3CL and C chemokines

CX3C chemokine and C chemokine are two small chemokine subfamilies, having only one and two members respectively. CX3CL-1, also commonly known as fractalkine, is the only member identified in the CX3C subfamily so far. Unlike other chemokine receptors, CX3CL-1 is the only known ligand for CX3CR1 (Jaerve *et al.*, 2012). In the CNS, CX3CL-1 is

constitutively expressed in neurons while its receptor is preferentially expressed in microglia, suggesting instrumental role of CX3CL-1 in regulating microglial activity (Briones *et al.*, 2014). Neuroprotective properties have been demonstrated in ischemic stroke and inhibition of CX3CL-1/CX3CR1 signaling in hippocampus aggravates microglia activation, pro-inflammatory cytokines release, and cognitive impairment (Briones *et al.*, 2014). In addition, CX3CR1 receptor knockdown enhanced tau phosphorylation and aggregation upon LPS stimulation (Bhaskar *et al.*, 2010). Meanwhile, several contradictory findings were reported, pointing towards detrimental role of CX3CL-1. CX3CR1 deficiency in AD transgenic mice model was associated with reduced neuronal loss, microglial and astrocytes activation, cytokines expression (TNF-α and CCL-2), enhanced Aβ uptake and decreased amyloid deposits (Fuhrmann *et al.*, 2010; Lee *et al.*, 2010; Liu *et al.*, 2010). In an ischemia stroke model however, attenuating CX3CL-1 signaling by CX3CL-1 or CX3CR1 knockdown both did not result in microglia neurotoxicity but rather substantially reduced infarct size, cytokines expression (IL-1β and TNF-α), and leukocyte infiltration (Dénes *et al.*, 2008; Soriano *et al.*, 2002). Detrimental effects of CX3CL1 on microglia activation are evident as attenuation of CX3CL-1/CX3CR1 signaling with antibodies or gabapentin; a recommended first-line treatment for multiple neuropathic conditions, inhibited microglia activation arise from epileptic seizure or monoarthritis respectively (Ali *et al.*, 2015; Yang *et al.*, 2012).

C chemokines subfamily is consists of XCL-1 and XCL-2. They have only one cysteine in the N-terminus and induce chemoattractant activities on T cell and NK cells (Ubogu *et al.*, 2006b). XCL-1 can be produced by astrocytes, microglia, and monocytes. HIV-1 trans-activator of transcription (Tat) protein

has been reported to upregulate XCL-1 expression, consequently increased T cell infiltration into the brain, suggesting pathogenic function of XCL-1 in the development of HIV-induced neurological disorders (Kim *et al.*, 2004a). Nevertheless, little is known about the role of this subfamily and further studies are required.

Collectively, it is evident that chemokines play a critical function in pathogenesis of various neurological disorders by orchestrating leukocytes transmigration. Notably, chemokines are versatile proteins with the ability to induce expression of other proinflammatory cytokines/chemokines, adhesion molecules, mediate gliosis, promote neuronal death, inhibit angiogenesis, and facilitate microglia migration in addition to chemoattractant activities on peripheral leukocytes. Several chemokine receptor blockers have been designed and are currently being examined *in vitro*, in animal models, and in early clinical trials. BX471, a novel CCR1 antagonist is a potent, selective, orally available agent that is safe in Phase I clinical trials for MS. Unfortunately, it failed the larger Phase II clinical trials (Ubogu *et al.*, 2006b). TAK779 is a quaternary ammonium salt that antagonizes the binding of chemokine ligands to CCR5 and CXCR3 receptors, making it a potential therapeutic for MS (Gao *et al.*, 2003). However, it is limited by its poor oral absorption and rapid elimination (Ubogu *et al.*, 2006b). On the other hand, a broad spectrum inhibitor of CC and CXC chemokines, NR58–3.14.3, has been documented to successfully reduce infarct size and improve neurological functions in animal model of ischemic stroke (Beech *et al.*, 2001). As dysregulation of cytokines and chemokines is a key feature in the development of neuroinflammation and neurodegeneration (Glass *et al.*, 2010; Smith *et al.*, 2012), molecules that target cytokines and chemokines

signaling may appear to be potential therapeutic for a variety of neurological diseases.

1.2.6 Reactive oxygen/nitrogen species

In neuroinflammatory diseases, proinflammatory mediators may cause a redox imbalance with production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS that predominantly responsible for oxidative stress are superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-) while RNS includes nitric oxide (NO). Reaction between O_2^- and NO generates peroxynitrite ($ONOO^-$), a strong oxidant that is highly cytotoxic (Fischer *et al.*, 2015). While oxidative stress at low level is important for brain homeostasis and elimination of invading pathogens, high level of ROS/RNS is implicated is the pathogenesis of various neurological disorders such as neurodegenerative diseases and stroke (Kraft *et al.*, 2011). Despite its relatively small size, representing only 2% of the body weight, the human brain accounts for 20% of the oxygen and thus energy consumption by the body. Due to high oxygen consumption and abundance of polyunsaturated fatty acids in neuronal lipid membrane which are susceptible to free radical attack, CNS is especially vulnerable to excessive generation of ROS/RNS (Kraft *et al.*, 2011).

1.2.6.1 Source of ROS/RNS

O_2^- is produced in by complex I and complex III in the electron transport chain during mitochondria activity and O_2^- can be converted by SOD to produce H_2O_2 which can be a source of the highly reactive HO^-. Thus, mitochondria dysfunction in the pathological conditions could release copious amount of ROS, causing oxidative damage in the CNS (Hroudová *et al.*, 2014). Alternative source of ROS/RNS comes from NADPH oxidases (NOX) and NO synthases

(NOS), particularly NOX2 and iNOS. Activated microglia and astrocytes induce expression of NOX2 and iNOS, resulting in high level of O_2^- and NO respectively (Fischer *et al.*, 2015). NOX is a multi-protein complex that catalyzes production of O_2^- from oxygen and plays an instrumental role in host defense against pathogens by mediating rapid O_2^- respiratory burst (Block *et al.*, 2007). Recent research supports emerging role of NOX2 in neurodegeneration. Activation of NOX2 is found in the patients' brain with AD. Moreover, elevation of NOX2 induces neuronal cell death while NOX-2 deficient diminishes ischemic stroke injury (Infanger *et al.*, 2006; Zekry *et al.*, 2003). Elevation of extracellular glutamate can trigger excessive NMDA receptor activity that induces Ca^{2+} influx which in turn activates NOS resulting in overproduction of NO (Lipton *et al.*, 2007).

1.2.6.2 ROS/RNS damaging effects on CNS

ROS/RNS causes damage to many cellular components including lipids, proteins, and DNA. Lipid peroxidation disrupts membrane fluidity and produces reactive aldehydes like 4-hydroxylnonenal (4-HNE) that is highly neurotoxic (Ran *et al.*, 2006). Free radicals also triggers protein misfolding and contributes to neuronal injury and death. For instance, NO induces S-nitrosylation of protein disulfide isomerase (PDI), an enzyme in endoplasmic reticulum that catalyzes proper protein folding, consequently leads to protein misfolding and accumulation (Lipton *et al.*, 2007). Dysfunction of the ubiquitin-proteasome system induced by ROS/RNS abolishes protein degradation and further aggravates accumulation of misfolded protein *(Gao et al., 2012)*. As a consequence of oxidative stress, disrupted mitochondria integrity causes reduction in ATP production and enhances more ROS release from

mitochondria *(Gao et al., 2012)*. Besides that, ROS is deleterious as it impedes tissue repair and regeneration by inhibiting neurogenesis *(Taupin, 2010)*. Furthermore, oxidative stress induces sustained activation of NF-κB and MAPK signaling pathways that promote neuronal cell apoptosis and propagate neuroinflammation through cytokines/chemokines production (Deng *et al.*, 2003; Kaltschmidt *et al.*, 1997; Zhuang *et al.*, 2000). It is worth noting that neuroinflammation and oxidative stress are often featured in various neurological disorders. Both are known to work hand-in-hand to exacerbate neuroinflammation and neurodegeneration. Thus, therapeutic approaches that attenuate both processes will be potential treatments for various CNS diseases characterized by dysregulated neuroinflammation.

1.2.7 Nrf2 as the key mediator of antioxidant response

1.2.7.1 Nrf2 activation mechanism

NF-E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that plays a fundamental role in the cellular defense against oxidative stress. Nrf2 expression is maintained at low level under basal condition by interacting with Kelch-like ECH-associated protein 1 (Keap1) homodimer. In the absence of oxidative stress, Keap1 sequesters Nrf2 at the cytosol and acts as an adaptor for cullin-3 (Cul3) E3 ubiquitin ligase. Binding of Nrf2 to Keap1 is mediated by two distinctive binding motifs located at Neh2 domain of Nrf2; the ETGE motif that binds with high affinity and DLG with low affinity. These two binding sites hold Nrf2 into place to facilitate poly-ubiquitination at the Neh2 domain by Cul3. The ubiquitinated Nrf2 is subsequently degraded by 26S proteasome, ensuring low expression level of Nrf2 (Bryan *et al.*, 2013). In the presence of electrophiles, certain cysteine thiol groups in Keap1 react with

oxidants resulting in formation of disulfide bonds and thus conformational changes. This in turn causes release of Nrf2 from the low affinity binding site and impede ubiquitination of Nrf2. Nrf2 escapes from the Keap1-dependent degradation and translocate into the nucleus. It heterodimerizes with small masculoaponeurotic fibrosarcoma (Maf) proteins that facilitates Nrf2 binding to antioxidant response element (ARE) at the promoter of Nrf2-regulated gene and results in cytoprotective genes transcription (Bryan *et al.*, 2013). Nrf2 contains high percentage of serine, threonine, and tyrosine residues which allow several signaling kinases to act upon (Rojo *et al.*, 2012). Protein kinase C (PKC) has been shown to phosphorylate Nrf2 at Ser40 in the Neh2 domain, disrupting the Keap1/Nrf2 interaction and promotes nuclear accumulation of Nrf2 (Huang *et al.*, 2002; Niture *et al.*, 2009). Nonetheless, Bloom and colleagues reported that although Ser40 phosphorylation promotes Nrf2 release from Keap1, it is not required for Nrf2 nuclear localization or transcriptional activities (Bloom *et al.*, 2003). Thus, it is still debatable if phosphorylation of Ser40 is essential for Nrf2 activation and more investigation is required to elucidate its role in Nrf2 activation.

1.2.7.2 Nrf2-regulated genes

Nrf2 activates a battery of detoxifying enzymes and antioxidant proteins including heme oxygenase-1 (HO-1), NAD(P)H:quinine oxidoreductase (NQO1), glutathione S-transferase (GST), and peroxiredoxin1 (Prx1) (Hun Lee *et al.*, 2013). High expression of heme oxygenase in the CNS, a system that does not actively engage in red blood cell metabolism, proposes instrumental role of this enzyme in the CNS. CNS conditions that is accompanied with oxidative stress strongly elevates expression level of the inducible HO-1 (Jazwa *et al.*,

2010). HO-1 is involved in the first step of heme metabolism, deterring heme-mediated free radical production especially during conditions like hemorrhagic stroke and trauma that release hemoglobin into the CNS (Chen *et al.*, 2003). HO-1 catalyzes degradation of heme to yield CO, ferrous iron (Fe^{2+}), and biliverdin which is then converted to bilirubin by biliverdin reductase. Endogenously generated CO has important biologic activities like neuronal transmission and maintenance of cerebral vasculature (Kraft *et al.*, 2011). Bilirubin is a potent antioxidant which is able to inhibit lipid peroxidation better than antioxidants like vitamin E (α-tocopherol).

NQO1 catalyzes two-electron reduction of quinones to hydroquinones, preventing the deleterious one-electron reduction of quinones by other reductase that generate ROS as a result of redox cycling of reactive semiquinone (Vasiliou *et al.*, 2006). Interestingly, induction of NQO1 expression reduced the glutamate toxicity in neuronal cell line that leads to glutathione depletion and oxidative stress (Murphy *et al.*, 1991). As ROS/RNS is strongly implicated in the pathology of neuroinflammatory diseases, activating Nrf2 signaling and upregulating antioxidant proteins can be neuroprotective and protect against oxidative damage.

1.3 Implication of inflammation in neurological disorders

1.3.1 Neurodegenerative diseases

Neurodegeneration usually progresses with a chronic neuropathy characterized by selective loss of neurons. The most common neurodegenerative disease is Alzheimer's disease (AD). AD patients develop memory deficit and cognitive decline due to neurodegeneration that occurs predominantly at hippocampus and neocortex. Parkinson's disease on the other

hand is a degenerative disorder that affects the dopaminergic neurons in the substantia nigra which causes manifestation of the cardinal motor symptoms (Glass *et al.*, 2010). Protein aggregation is the disease hallmark shared by both disorders. AD is characterized formation of amyloid beta (Aβ) aggregates produced by proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase. Neurofibrillary tangles generated from aggregates of hyperphosphorylated tau protein are also generally found in the neurons of AD patients. In PD, α-synuclein accumulates and forms protein aggregates known as Lewy bodies (Glass *et al.*, 2010).

Neuroinflammation is implicated in the disease initiation and propagation of AD/PD. Both Aβ and α-synuclein are known to engage and activate TLRs, particularly TLR2 in microglia and astrocytes and induce production of inflammatory mediators (Fischer *et al.*, 2015). Activation of TLRs initiates NF-κB signaling which leads to secretion of cytokines/chemokine and adhesion molecules that facilitate leukocyte recruitment to the CNS. NF-κB induction of COX-2 expression and prostaglandins production also further enhance leukocyte infiltration (Fischer *et al.*, 2015). Moreover, NF-κB immunoreactivity is found to be significantly elevated in the diffuse Aβ plaques in AD patients (Ferrer *et al.*, 1998; Kaltschmidt *et al.*, 1997) as well as in the nuclei of mesencephalic dopaminergic neurons of PD patients (Camandola *et al.*, 2007; Hunot *et al.*, 1997), supporting the role of NF-κB activation in the pathophysiology of neurodegenerative diseases.

Expression of proinflammatory cytokines and chemokines is evident in patients with AD/PD pathology. Studies showed that IL-1β and TNF-α can

induce γ-secretase activity and increase production of Aβ (Liao *et al.*, 2004). High level of TNF-α also substantially increases Ca^{2+}-dependent release of glutamate from astrocyte which potentially leads to neuronal excitotoxicity (Rossi, 2015). Several reports demonstrated that CCL-2 and CCL-5 overexpression promotes chemotactic migration of microglia to amyloid plaque (Huang *et al.*, 2009; Huang *et al.*, 2010; Yamamoto *et al.*, 2005). Nonetheless, microglia accumulation at the site of amyloid plaque did not enhance Aβ clearance, instead increased fibrillary Aβ deposit (Yamamoto *et al.*, 2005). Interestingly, neutralizing CCL-5 with antibodies abrogated Aβ deposit enlargement (Huang *et al.*, 2009). Role of CCL-5 in PD has also been described in hemiparkinsonian monkeys, enhancing dopaminergic neuronal loss by mediating T cell infiltration to the substantia nigra (Roy *et al.*, 2015). Furthermore, attenuating CX3CL-1 signaling in AD transgenic mice model conferred protection to neurons, reduced glia activation, cytokines expression, increased Aβ uptake and decreased amyloid deposits, indicating importance of chemokines in mediating disease pathology (Fuhrmann *et al.*, 2010; Lee *et al.*, 2010; Liu *et al.*, 2010).

Oxidative stress also appears to be a major determinant of neurodegenerative disease pathogenesis and progression. Activation of NF-κB promotes oxidative stress by inducing NOX2 and iNOS expression that result in generation of O_2^- and NO by microglia as well as astrocytes (Fischer *et al.*, 2015). Aβ and mutated α-synuclein proteins can cause mitochondria dysfunction and release large amount of ROS, disrupting redox balance in the CNS (Hroudová *et al.*, 2014). Oxidized α-synuclein in turn induces chronic activation of glia cells, creating a feedforward state that aggravates oxidative

damage and thus progressive neuronal loss (Fischer *et al.*, 2015). Neurotoxic product of lipid peroxidation like 4-HNE has been reported to upregulate β-secretase expression through JNK and p38 activation, thereby promoting Aβ generation (Tamagno *et al.*, 2005). Furthermore, persistent activation of JNK has been associated with tau hyperphosphorylation and Aβ oligomerization, underscoring the role of stress-induced kinases in mediating disease development and progression (Ploia *et al.*, 2011; Sclip *et al.*, 2011).

1.3.2 CNS trauma

CNS trauma encompasses stroke, brain trauma and spinal cord injury. Stroke is one of the leading cause of death in industrialized countries while brain trauma is a common cause of disability and death among children and young adults (Bramlett *et al.*, 2004). Stroke is a cerebrovascular accident that occurs when blood supply to the brain is disrupted or severely reduced, leading to brain cells death due to oxygen and nutrients deprivation, whereas external mechanical force causes injury to the brain or spinal cord. In CNS trauma, both the initial insult and the subsequent secondary degenerative processes magnify the initial tissue damage resulting in neurological dysfunction (Jaerve *et al.*, 2012).

Activation of NF-κB is evident in stroke and CNS trauma. Importance of NF-κB activation in potentiating neuroinflammation and neuronal damage is depicted in IKKβ knockout animal studies. Attenuating NF-κB signaling reduced infarct size of ischemic stroke (Herrmann *et al.*, 2005) and attenuated neuronal damage and motor activity deficits in traumatic spinal cord injury (Kang *et al.*, 2011). Elevated TNF-α and IL-1β cytokines also promote

astrocytes and microglia activation and induce release of excitotoxic glutamate from astrocytes (Basu *et al.*, 2002; Ye *et al.*, 2013).

Upregulation of various CC and CXC chemokines were reported in stroke, brain and spinal cord injury. This is particularly crucial as post-traumatic infiltration of leukocytes has been associated with secondary tissue damage and neuronal death (Jaerve *et al.*, 2012). Indeed, elevation of CXCL-5 in the CSF of ischemic stroke patients increases neutrophil recruitment which correlates with infarct size (Zaremba *et al.*, 2006). NR58–3.14.3 peptide, a broad spectrum inhibitor of CC and CXC chemokines decreases neuroinflammation and infarct volume of cerebral ischemia (Beech *et al.*, 2001). Deleterious effects of chemokines have also been reported in spinal cord lesion studies. Gonzalez *et al*. demonstrated that CXCL-10 attenuation reduced T-cell infiltration, prevented secondary tissue damage and improved motor function (Gonzalez *et al.*, 2003). Treatment with reparixin, a recently identified inhibitor that blocks CXCR1 and CXCR2 receptors substantially abolished neutrophil influx, astrocyte proliferation, and expression of cytokines (IL-1β, TNF-α, IL-6, and CXCL-2) after spinal cord injury (Gorio *et al.*, 2007).

Absence of functional NOX has been reported to improve ischemic stroke outcome indicating ROS in mediating neuronal damage (Block *et al.*, 2007). In addition, elevation of iNOS activity and NO level has been reported in cerebral ischemia model (Del Zoppo *et al.*, 2000). ROS like superoxide can react with NO to form the highly reactive peroxynitrite and cause further cellular damage. Upregulation of adhesion molecules like P-selectin and ICAM-1 in ischemic stroke sustained for up to 24 hours after reperfusion, supporting

the role of inflammatory cells infiltration in mediating secondary tissue damage (Del Zoppo *et al.,* 2000).

1.3.3 Brain infection- bacterial meningitis

Bacterial meningitis is caused by peripheral bacterial infection that invades into meninges and causes inflammation in the arachnoid membrane and subarachnoid space. In many cases, the inflammatory process will spread from meninges to other brain parenchyma, the ventricles, and spinal cord (Kastenbauer *et al.*, 2001; Swartz, 1984). The most common pathogens of bacterial meningitis in both children and adults are *Streptococcus pneumoniae, Neisseria meningitides, and Hemophilus influenza* (Ramesh *et al.*, 2013). Bacterial meningitis requires immediate medical attention and treatment as CNS infection could be life-threatening causing memory deficits, edema, hearing loss, cerebral palsy, and seizures (Nelson Jr, 2006). Patients surviving bacterial meningitis showed cognitive slowness, poor performance in execution and linguistic functions as well as learning/memory tests even if they were clinically well recovered (Schmidt *et al.,* 2006; Van De Beek *et al.*, 2002).

Bacterial invasion and release of PAMPs elicit a strong immune response, promoting inflammation, leukocyte recruitment and glia cells activation. Neuroinflammatory response in meningitis represent a "double-edged sword". While it is crucial to eliminate invading pathogens, excessive release of proinflammatory mediators can contribute to tissue damage (Braun *et al.*, 2001). Activated microglia and astrocytes release cytokines/chemokines, free radicals, excitatory glutamate and proteases that mediates inflammatory processes, ultimately leads to neuronal death (Gerber *et al.*, 2010). Bacteria and endotoxin like LPS activates microglia and astrocytes to rapidly produce IL-1β

and TNF-α which in turn initiate a cascade of secondary cytokines and chemokines to propagate inflammatory response (Van Miert, 1995).

Bacterial meningitis is characterized by the infiltration of peripheral leukocytes into the subarachnoid space and their subsequent activation (Ramesh *et al.*, 2013). Various chemokines including CCL-2, CCL-3, CCL-5, CXCL-1, and CXCL-2, CXCL-5 are upregulated during meningitis (Hanisch *et al.*, 2001; Rock *et al.*, 2004a; Zwijnenburg *et al.*, 2003). High levels of CXCL-5 was detected in the CSF of patients with bacterial meningitis, and CXCL-5 was shown to facilitate neutrophils transmigration to the CNS (Zwijnenburg *et al.*, 2003). Antibodies neutralizing CXCL-2 and CCL-3 mitigate neutrophil recruitment while CCL-2 neutralization attenuated macrophage infiltration, suggesting chemokine as a potential target for therapeutic intervention to inhibit leukocyte-mediated neuroinflammation in bacterial meningitis (Diab *et al.*, 1999).

Moreover, MMPs and ROS expression are upregulated as a host defense response during bacterial meningitis. CSF concentrations of MMP-8 and MMP-9 are remarkably higher in children with bacterial meningitis that develop neurological sequealae compared to those who have gained full recovery (Meli *et al.*, 2003). MMPs produced by the resident brains cells have been suggested to mediate neuroinflammation and BBB dysfunction, and neuronal death (Meli *et al.*, 2003). Hsieh *et al*. showed that lipoteichoic acids (a main constituent of Gram-positive bacteria) upregulated MMP-9 expression in astrocytes through a NF-κB-dependent pathway and promoted cell migration of astrocytes. In addition, various cytokines, ROS, and endotoxins have been demonstrated to

upregulate MMP-9 in astrocytes, indicating participation of astrocytes in mediating meningitis pathology through MMP-9.

1.4 Rationale for novel therapeutics against neurological diseases

Development of effective treatments for neurological disease has been challenging and with low success rate. This is mainly ascribed to the complexity of the CNS; a tendency for CNS drug to cause CNS-mediated side effects such as dizziness, nausea, and seizure, and tight-regulation of BBB; where most drugs failed to penetrate into the brain or reach a sufficient concentration to elicit desired effects (Pangalos *et al.*, 2007). Nonetheless, several therapeutics are available to treat of neurodegenerative diseases, stroke, and bacterial meningitis. Their benefits and limitations are described here.

1.4.1 Alzheimer's Disease

Currently available treatments for AD are acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonist, memantine. Memantine also seems to benefit PD patient as reported in a double-blind, placebo-controlled, multicenter trial (Aarsland *et al.*, 2009). Although acetylcholinesterase inhibitors and memantine, used alone or in combination improves disease symptoms and cognition test, very often their effectiveness decreases as AD progresses (Potter, 2010). This leads to the search for new AD therapeutics and anti-inflammatory agents appear as a potential new approach due to the increasing evidence implicating neuroinflammatory processes in pathogenesis of AD. One of such approaches include inhibition of inflammatory response with the use nonsteroidal anti-inflammatory drugs (NSAIDs) which is a cyclooxygenase (COX) inhibitors. NSAIDs inhibition on COX attenuates the

formation of proinflammatory mediator, prostaglandins from arachidonic acids (Krause *et al.*, 2010). Prospective cohort study with 6989 subjects revealed that long-term use to NSAIDs protect against AD (In T' Veld *et al.*, 2001). Another study performed by Szekely *et al.* reported similar but mainly in individuals with ApoE4 (Szekely *et al.*, 2008). Nonetheless, not all trials demonstrated positive outcomes. NSAIDs like celecoxib and ibuprofen did not show significant improvement in cognitive function (Potter, 2010). Moreover, use of NSAIDs increase risk of gastrointestinal and cardiovascular complications. The side effect is mainly due to its non-selective inhibition on COX-1 and therefore depletion of COX-1-derived prostaglandins that serves protective role in stomach mucosa (Sostres *et al.*, 2010).

Another prospective approach to treat AD is to target TNF-α signaling using etanercept, a fusion protein that function as decoy receptor for TNF. A clinical pilot study involving individuals with mild-to-severe AD who received perispinal administration of etanercept (25-50 mg) once weekly for a period of six months claimed detectable cognitive improvement compared to individuals that received placebo (Tobinick *et al.*, 2006). However, discrepant findings were reported in recent clinical trials. In a double-blind study, patients with mild to moderate AD were recruited and treated with subcutaneous etanercept (50 mg) once a week over a period of 6 months. Although etanercept was well tolerated by the participants, no significant changes in cognitive function and behavior were detected (Butchart *et al.*, 2015). Thus, etanercept need to be examined in a bigger and more heterogeneous group to determine its efficacy in treating AD.

1.4.2 Ischemic stroke

Tissue plasminogen activator (tPA) is the only FDA approved treatment for ischemic stroke. It is a serine protease that improves blood flow to the affected area by dissolving blood clots. tPA may increase the chances of recovery if given within 3 hours (or sometimes up to 4.5 hours) after stroke event. Due to its side effects like bleeding and short therapeutic time window, and also low efficacy of other therapies like heparin, aspirin, and dipyridamole, new therapeutic approaches are critically needed (Turley *et al.*, 2005). Interestingly, medical approaches targeting inflammatory processes seem promising. Minocycline, an antibiotic that exerts anti-inflammatory properties protects neuron against ischemic insult. Oral administration of minocycline 6 to 24 hours after acute stroke for 5 days is associated with better clinical outcome (Plane *et al.*, 2010). It is believed that minocycline mediates neuroprotective effects by inhibiting glial activation, ROS/RNS production, caspases, and MAPKs (Cai *et al.*, 2010). Minocycline administration in spinal cord injury promoted recovery as reported in a pilot study. Nonetheless, sample size of the study was small, and conclusions on protective properties may be premature (Plane *et al.*, 2010). Furthermore, an observational study involving 629 patients with chronic stroke and traumatic brain injury treated with perispinal etanercept showed a substantial improvements in cognitive, motor, and behavioral function with reduced spasticity and sensory impairment (Tobinick *et al.*, 2012). Together, these studies suggest that attenuating neuroinflammation may be a promising therapeutic approach to produce clinical improvements in patients with CNS trauma.

1.4.3 Bacterial meningitis

Rapid treatment with antibiotics is mandatory in patients suffering from bacterial meningitis. Mortality rate of patients with acute bacterial meningitis is high and neurologic sequelae among the individuals that survived is common (Schmidt *et al.*, 2006; Van De Beek *et al.*, 2002). CSF cultures showed that ineffectiveness of antibiotic therapy is unlikely the cause of these undesirable neurologic outcomes. Animal studies revealed that antibiotic-induced bacterial lysis mediates inflammatory response in the subarachnoid space which may explain the unfavorable outcomes (De Gans *et al.*, 2002). Therefore, adjuvant treatment with anti-inflammatory agents like dexamethasone, a corticosteroid was proposed to reduce inflammation in both CSF and neurologic sequelae (Gerber *et al.*, 2010). Clinical studies performed on children (Tunkel *et al.*, 2002) and adults (De Gans *et al.*, 2002) with bacterial meningitis showed that early dexamethasone administration attenuated inflammation in subarachnoid space and reduced risk of undesirable outcome. Nevertheless, conflicting results were reported in other clinical trials. A more recent meta-analysis of patient data from five clinical trials did not find significant benefits in adjunctive treatment with dexamethasone, thus benefits of adjunctive dexamethasone in bacterial meningitis remain debatable.

Taken together, therapeutics currently available for treating neurological disorders are limited. Drugs like acetylcholinesterase inhibitors and memantine only provide temporary symptomatic relieves while use of tPA is only applicable to ischemic stroke but not hemorrhagic stroke and has a short therapeutic window. Moreover, antibiotics are the only treatment for bacterial meningitis which unable to prevent neurologic sequelae that follows after

recovery. Due to these reasons, there is an urge for new therapeutic approaches to improve disease outcomes. Established research demonstrating the central role of neuroinflammation underlying the pathological features of many neurological disorder, thus making targeting of inflammatory processes in the CNS a promising therapeutic approach. Indeed, some of the new interventions such as NSAIDs, etanercept, minocycline, and dexamethasone target on inflammatory processes. Nonetheless, their use as a neuroprotectant in CNS diseases remains ambivalent as conflicting results has been reported. This prompted us to screen for anti-neuroinflammatory compounds that may potentially ameliorate neuroinflammatory diseases. Several natural bioactive compounds were investigated in the initial study and subsequent findings lead us to focus on andrographolide, a lipid soluble labdane diterpenoid that penetrates BBB (Bera *et al.*, 2014b; Zheng, 1982)

1.5 Andrographolide as a potential anti-neuroinflammatory therapeutic

Andrographolide is a labdane diterpenoid compound derived from the herbaceous plant *Andrographis paniculata* belonging to the Family Acanthaceae. *A. paniculata* is widely known as 'king of bitters' and can be found throughout tropical and subtropical Asia, south-east Asia and India. The herb is also known by various vernacular names. It is known as Kalmegh in India*;* Chuan-Xin-Lian in China*;* Senshinren in Japan; Fah Tha Lai in Thailand*;* and Hempedu bumi in Malaysia (Lim *et al.*, 2012a). The herb contains diterpenoids, flavonoids and polyphenols as the main bioactive components and andrographolide ($C_{20}H_{30}O_5$) is the major diterpenoid found in *A. paniculata* (Chao *et al.*, 2010b). *A. paniculata* is traditionally used to treat a variety of ailments, including fever, cough, tuberculosis, snake bites, respiratory tract, and

urinary tract infections (Panossian *et al.*, 2002). A double-blind human study with oral administration of *A. paniculata* dried extract relieved cold symptoms and improved recovery. In this study, participants were given 1,200 mg/day of herb extract for 5 days and no side effects were detected when they were monitored for changes in blood counts, liver, heart and kidney functions together with other laboratory tests (Hancke *et al.*, 1995). This shows that *A. paniculata* extract has low toxicity and effective against respiratory illnesses like flue and cold. These beneficial effects are found mainly attributed to andrographolide and its related derivatives (Panossian *et al.*, 2002). In parallel with the human study, oral gavage of andrographolide to mice twice daily for a period of 7 days revealed that andrographolide has very low toxicity with LD_{50} >4000 mg/kg/day (Chen *et al.*, 2009a).

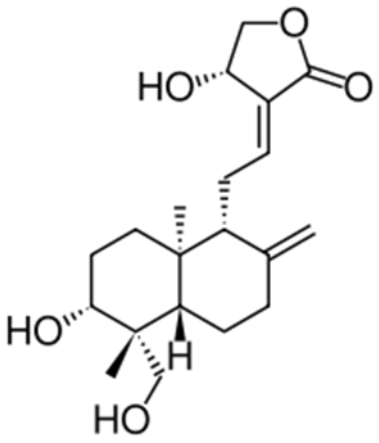


Figure 1.3 Chemical structure of andrographolide.

Pharmacologically, andrographolide has been found to have anticancer, anti-bacterial, anti-inflammatory, and antioxidative effects (Arifullah *et al.*, 2013; Lim *et al.*, 2012a; Sheeja *et al.*, 2006). However, there is a dearth of studies on the effects of andrographolide in the CNS. Here, I summarized the study of andrographolide in the CNS in Table 1.3. All studies reported beneficial effects of andrographolide with one exception. Yen *et al.* (Yen *et al.*, 2013) found that andrographolide induced cerebral endothelial cell apoptosis and enhanced infarct volume in transient middle cerebral artery occlusion (tMCAO) mouse model by disrupting the BBB integrity. Nonetheless, Chern *et al*. and Chan *et al*. reported the opposite with significantly smaller infarct size observed in transient MCAO and permanent MCAO animal model respectively (Chan *et al.*, 2010; Chern *et al.*, 2011). While andrographolide is well-known for its powerful anti-inflammatory activities in the peripheral tissues (Lim *et al.*, 2012a), few studies were done to address its potential effects on neuroinflammation. Of the few studies reported, study focus had been on the role of andrographolide in regulating microglial inflammatory response, and its effects on astrocyte-mediated neuroinflammation are largely unknown. As mentioned earlier, astrocytes are critically involved in mediating neuroinflammation. They also express various PRRs that enable them to respond to endogenous damage signals and infectious agent by releasing proinflammatory mediators like cytokines and chemokines (Bsibsi *et al.*, 2002; Farina *et al.*, 2007). Tzeng *et al*. showed that andrographolide reduced cytokines (IL-1β and IL-6) secretion and oxidative stress in TNF-α stimulated primary astrocytes (Tzeng *et al.*, 2012). However, it is unclear whether the effects of andrographolide extend to astrocytic responses such as chemokine production

and astrogliosis. As growing evidence reveals numerous undesired effects of reactive astrogliosis (Sofroniew *et al.*, 2010) and role of chemokines in perpetuating and sustaining neuroinflammation (Ramesh *et al.*, 2013; Ubogu *et al.*, 2006b), it is particularly interesting to examine andrographolide's effects on astrocytes-mediated chemokines expression. Furthermore, andrographolide administered peripherally has been shown to penetrate the blood brain barrier (BBB) due to its high lipid solubility (Bera *et al.*, 2014b; Zheng, 1982), indicating its use as a potential therapeutic for neurological disorders where detrimental role of astrocytes has been implicated such as brain trauma (Minkiewicz *et al.*, 2013), amyotrophic lateral sclerosis (Yamanaka *et al.*, 2008), AD (Jo *et al.*, 2014; Walker *et al.*, 2006), stroke (Longuemare *et al.*, 1995; Seki *et al.*, 1999), and cerebral edema (Manley *et al.*, 2000). Therefore in my study, I investigated effects of andrographolide on astrocyte activation and proinflammatory mediators' expression with special emphasis on chemokine expression in addition to cytokines.

Antioxidant properties of andrographolide have been proposed in the peripheral tissues especially in the liver. Andrographolide was shown to attenuate carbon tetrachloride (CCl_4)-induced hepatic injury by reducing lipid peroxidation, increasing GSH content and HO-1 (Ye *et al.*, 2011). In order to establish the genes expression profile regulated by andrographolide, Chatuphonprasert *et al*. performed a microarray analysis on hepatocytes treated with andrographolide. Among the 28,853 genes analyzed, 18 genes were upregulated and most are detoxifying enzymes and antioxidant protein such as glutathione S-transferase (GST), carbonyl reductase (CBR), biliverdin reductase (BVR), Nqo1, glutathione peroxidase 2 (Gpx2) and glutathione

reductase (Gsr) (Chatuphonprasert *et al.*, 2009). Ability of andrographolide in attenuating oxidative stress in the CNS has also been demonstrated in several studies (Chern *et al.*, 2011; Das *et al.*, 2009; Zhang *et al.*, 2014). Although the antioxidant properties of andrographolide are well-recognized, the underlying molecular mechanisms involved are yet unequivocally determined. Nevertheless, andrographolide has been proposed to upregulate antioxidant enzymes by activating Nrf2 signaling pathway. In fact, many detoxifying enzymes upregulated by andrographolide in hepatocytes microarray analysis are primarily Nrf2 gene targets. Moreover, Guan *et al*. also showed that andrographolide protected against cigarette smoke-induced oxidative lung injury by promoting Nrf2 transcriptional activity of ARE-regulated gene (Guan *et al.*, 2013). Notably, of all the 54 natural compounds examined, andrographolide had the highest efficacy in activating Nrf2 signaling, underscoring its potential as an effective antioxidant compound (Wu *et al.*, 2014b). While Nrf2 signaling activation is implicated in andrographolide-mediated antioxidant effects in peripheral tissues like lung (Guan *et al.*, 2013), liver (Chen *et al.*, 2014a), macrophage (Zhang *et al.*, 2013), and endothelial (Lu *et al.*, 2014), the underlying mechanism of antioxidant effects in the CNS mediated by andrographolide is yet elucidated. Hence, in order to examine if andrographolide modulates oxidative stress in CNS through Nrf2 signaling, astrocytes which is a resident cell in the CNS was used in my study and the potential mechanisms involved were investigated.

Author	Cell/ Animal model	Stimul-ant	Anti-inflammatory effects	Other effects	Clinical implicati-on
(Serrano *et al.*, 2014)	AβPPswe/ PS-1 mice	Endogenous Aβ deposits	-	- ↓ Aβ depositions and phos-tau - ↓ GSK-3β - ↑ spatial memory functions	AD
(Tapia-Rojas *et al.*, 2015)	Primary rat hippocam-pal neurons	-	-	- ↑ Wnt/β-catenin signaling - ↓ GSK-3β	Neurodegenerative diseases
(Yang *et al.*, 2014)	C6 glioblastoma	-	-	- ↑ apoptosis	Glioma cancer
	ICR mice	C6 cells s.c injection	-	- ↓ tumour growth	
(Zhang *et al.*, 2014)	SH-SY5Y and primary CGNs	MPP^+	- ↓ ROS, RNS - ↓ NF-κB activation	- ↓ neuronal death	PD
	MPTP mouse model of PD	MPTP	- ↓ phos-p65 - ↓ NO and SOD	- ↓ SNpc dopaminergic neuron loss - ↓ locomotion deficit	
(Yen *et al.*, 2013)	Cerebral endothelial cells	-	-	- ↑ apoptosis	BBB integrity in stroke
	Ischemic stroke mouse model	tMCAO	-	- ↑ infarct size	
(Li *et al.*, 2012b)	U251 & U87 glioblastoma	-	-	- ↑ G2/M arrest - ↓ PI3K-Akt	Glioma cancer
(Tzeng *et al.*, 2012)	Primary astrocytes	TNFα	- ↓ IL-1β, IL-6 secretions - ↓ oxidative stress	- ↓ chondroitin sulfate proteoglycan	Astrocyte-induced inflammation
	PC12 cell line	H_2O_2	-	- ↓ cell death	Oxidative stress
(Chern *et al.*, 2011)	Ischemic stroke mouse model	tMCAO	- ↓ nuclear translocation of p65 - ↓ NO and ROS - ↓ NOX2, iNOS - ↓ CD11b staining	- ↓ infarct size	Ischemic stroke

	Mouse BV-2 microglial	Oxygen-glucose deprivation	-↓ PI3K/AKT-dependent degradation of IκBα - ↓ NO and ROS	-	
(Chan *et al.*, 2010)	Ischemic stroke rat model	pMCAO	- ↓ p65 nuclear translocation - ↓ IL-1β, TNFα, PGE_2 - ↓ microglia activation	- ↓ infarct size - ↓ neurological deficits	Ischemic stroke
(Das *et al.*, 2009)	Wistar rats	Nicotine	- ↓ NO - ↓ oxidative stress markers - ↑ antioxidant enzymes	- ↑ activity of mitochondrial complexes (I–III)	Nicotine-induced disorders
(Wang *et al.*, 2004)	Mesencephalic neuron-glia culture	LPS	- ↓ microglia activation - moderately ↓ TNFα and PGE_2 - attenuate O_2^-, NO	- ↓ shortening of neurites - ↓ dopaminergic neuron degeneration	PD
	Mouse BV-2 microglia	LPS	- ↓ COX2 - ↓ iNOS	-	

Table 1.3 Andrographolide study in the CNS.

1.6 Aim and hypothesis:

Neuroninflammation is a prominent feature in various neurological disorders, and there is increasing awareness of the pathogenic role played by dysregulated inflammatory processes in CNS disorders, leading to the advancement of treatment approaches based on immunomodulatory NSAIDs, neutralizing antibodies, and corticosteroids. Though some appeared to be beneficial, they are often hampered by adverse effects and have variable treatment efficacies, thus prompting the need to develop novel, safe and effective anti-neuroinflammatory drugs. Andrographolide has emerged as a potential candidate due to its low toxicity, ability to cross the blood-brain barrier, and more importantly, its inhibitory effects on inflammation has been reported in non-neural tissues like lung (Lim *et al.*, 2012a). Much research focus on CNS inflammatory processes has been focused on microglia; in contrast, the potential role of astrocytes in regulating neuroinflammation is less well-studied. In this thesis, I investigated the potential use of andrographolide as an anti-neuroinflammatory therapeutic in astrocyte-mediated inflammatory responses. I hypothesized that andrographolide is a powerful anti-neuroinflammatory compound that attenuate multiple inflammatory signaling pathways as well as the upregulation of proinflammatory cytokines and chemokines in activated astrocytes.

Therefore, the first aim of this project is to examine andrographolide's efficacy in attenuating NF-κB activation and the expression of its target gene, CCL-5 in astrocytes stimulated with IL-1β and TNF-α, in comparison with artesunate and plumbagin that has previously demonstrated anti-inflammatory effects (Chapter 3). The second aim is to examine andrographolide in more

disease relevant conditions like bacterial infection with the use of LPS. Its effects on LPS-activated signaling pathways and cytokine/chemokine profile will be examined. The potential of orally administered andrographolide in attenuating neuroinflammation arise from peripheral bacterial infection will also be explored in mouse model with peripheral LPS injection (Chapter 4). Lastly, as oxidative stress is an integral part of inflammation, I aim to investigate andrographolide's role in regulating Nrf2 activation, a key transcription factor of antioxidant proteins (Chapter 5).

CHAPTER 2: MATERIAL AND METHODS

2.1 Chemicals and biologics

Chemicals and biologics used are listed in Table 2.1. Plumbagin was a generous gift from A/P Gautam Sethi, Dept Pharmacology, NUS and artesunate was kindly provided by A/P Fred Wong Wai-Shiu, Dept Pharmacology, NUS.

Reagents	**Source**
Andrographolide, 98%	Sigma, MO, USA
Polyethylene glycol 400 (PEG 400)	Sigma, MO, USA
Human IL-1β	Cell Signaling, MA, USA
Human TNF-α	Cell Signaling, MA, USA
Rat IL-1β	Abcam, Cambridge, UK
Rat TNF-α	Abcam, Cambridge, UK
Lipopolysaccharide (LPS)	Sigma MO, USA
TPCK (N-*p*-Tosyl-L-phenylalanine chloromethyl ketone)	Sigma MO, USA
SP 600125 (Anthra[1-9-*cd*]pyrazol-6(2*H*)-one)	Tocris, BS, UK
SB 202190 (4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol)	Tocris, BS, UK
PD 98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)	Tocris, BS, UK
Cycloheximide	Sigma MO, USA

Table 2.1 Chemicals and biologics used in the study.

2.2 Preparation of Andrographolide

For in vitro treatment:

Andrographolide was dissolved in DMSO and concentration of DMSO for cell treatment was no more than 0.1%.

For in vivo mouse treatment:

Andrographolide was prepared in PEG 400, mixed well before oral administration.

2.3 Cell cultures

2.3.1 Mammalian cell line:

Materials:

- **Human glioblastoma U373 cell line** (A kind gift from Dr Michelle Tan, Dept Clinical Research, Singapore General Hospital)

- **U373 cell culture media**

  - Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, CA, USA)
  - Fetal bovine serum (FBS) (Invitrogen, CA, USA)
  - 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, CA, USA)

- **1X 0.25% Trypsin-EDTA with phenol red** (Invitrogen, CA, USA)

- **Freezing media**

  - 80% DMEM (Invitrogen, CA, USA)
  - 10% Dimethyl sulphoxide (DMSO) (Sigma, MO, USA)
  - 10% FBS (Invitrogen, CA, USA)

Methods:

Human glioblastoma U373 cell line were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Sub-culturing was performed by passaging confluent cell culture flask with 1X trypsin. Excess cells were frozen in freezing media and stored in -80˚C freezer. Cell treatment was performed with 10% FBS supplemented media unless otherwise stated.

2.3.2 Rat primary astrocytes:

Materials:

- Primary astrocyte culture media

- Dulbecco's Minimal Essential Medium: Nutrient Mixture F-12 (DMEM/F12)
- Fetal Bovine Serum (FBS)
- 100 IU/ml penicillin and 100 µg/ml streptomycin

- 1X Phosphate buffer saline (PBS)

- 1X 0.25% Trypsin-EDTA with phenol red (Invitrogen, CA, USA)

- 40 µm nylon cell strainer (BD Falcon, NJ, USA)

Methods:

Primary astrocytes cultures were prepared from cortical tissues of postnatal day 1–3 Sprague Dawley rats using protocols adapted from *A dissection and tissue culture manual of the nervous system* (Cole *et al.*, 1989). This is an established procedure producing primary culture with purity more than 98% (Mcleod *et al.*, 1995; Strange *et al.*, 1994). Briefly, isolated cortices were separated from the meninges in 1X PBS. 1X PBS was drained out and tissues were disassociated with 0.25% trypsin-EDTA and neutralized with three parts of culturing media. Disassociated tissues were then filtered through 40 µm nylon cell strainer. The resultant filtrate was centrifuged at 200 x g and resuspended in DMEM-F12 media supplemented with 10% heat-inactivated FBS, 100U/ml penicillin, and 100 µg/ml streptomycin. After 7-9 days of culture in a humidified atmosphere with 5% CO_2 at 37 °C, astrocytes were enriched by vigorously shaking the cell culture flasks at 350 rpm for 2 hours to remove non-adherent microglial cells. Shaking resume after overnight incubation with 350

rpm for another 12 hours to remove oligodendrocytes. The purified astrocyte culture was allowed to grow till confluent before plating for cell treatment. Cell treatment was performed with 10% FBS supplemented media unless otherwise stated.

2.4 Mouse treatment regime:

Materials:

- **Andrographolide** (Sigma, MO, USA)
- **Lipopolysaccharide (LPS)** (Sigma, MO, USA)
- **Polyethylene glycol 400 (PEG 400)** (Sigma, MO, USA)
- **1X Phosphate buffer saline (PBS)**

Methods:

Upon arrival at NUS animal holding unit, ICR mice (male, 20g ± 3g) were allowed to acclimatize for 3 days. The animals were subjected to 3 intraperitoneal injection of LPS (3 mg/kg) and followed by oral gavage of andrographolide (25mg/kg or 50mg/kg) 1 hour after each injection. Three LPS injection regimen was adopted from Erickson *et al*. (2011) which has shown to elicit robust cytokines and chemokines expression. The time interval of each treatment is illustrated in Figure 2.1. Injection and oral administration for each treatment groups are indicated in Table 2.2.

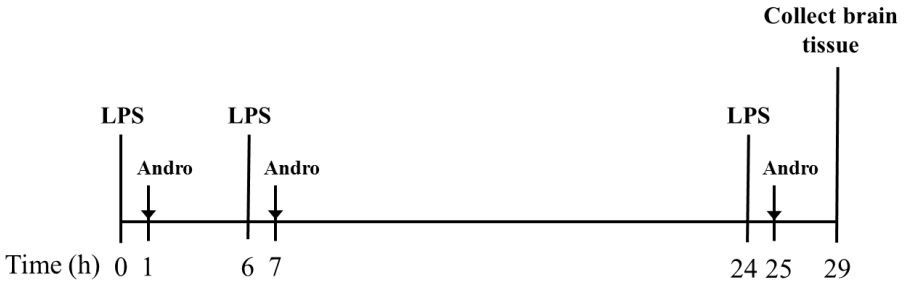


Figure 2.1 Mouse treatment regime.

Treatment group	Injection	Gavage
Control	PBS	PEG 400
LPS	3 mg/kg LPS in PBS	PEG 400
LPS + Andrographolide 25 mg/kg	3 mg/kg LPS in PBS	25 mg/kg Andrographolide
LPS + Andrographolide 50 mg/kg	3 mg/kg LPS in PBS	50 mg/kg Andrographolide

Table 2.2 Mouse treatment groups.

2.5 Preparation of mouse brain tissues

Materials:

- IKA® ULTRA-TURRAX® homogenizer (Sigma-Aldrich, MI, USA)

- Homogenizing buffer

- 50 mM Tris-HCl (Invitrogen, CA, USA)
- 120 mM NaCl (Millipore, MA, USA)
- 5 mM KCl (Sigma, MO, USA)
- 2 μg/mL Pepstatin A (Sigma, MO, USA)
- cOmplete ULTRA Tablets, Mini, EDTA free protease inhibitor (Roche Diagnostics, USA)
- PhosSTOP *EASY*pack phosphatase inhibitor (Roche Diagnostics, USA)

Methods:

Frozen mouse brain tissues were homogenized with ice cold homogenizing buffer at a tissue concentration of 50 mg/ml. Homogenates were aliquoted and stored at -80 °C until use. In the preparation for immunoblotting, brain homogenates were added with 1:1 ration of boiling laemmli sample buffer and heated up to 95˚C for 5 minutes. For Luminex sample preparation, 200 μl aliquot of homogenates were agitated at 800 rpm for 40 min on ice and

subsequently centrifuged at 6,000 x g for 20 minutes at 4 °C. The supernatant was aliquoted and stored at -80 °C until use.

2.6 Subcellular fractionation

Materials:

- **Nuclear Extract Kit** (Active Motif, Tokyo, Japan)

Methods:

Subcellular fractionation was performed using Nuclear extract kit according to manufacturer's instructions (Active Motif, Tokyo, Japan). Briefly, media was aspirated out of 10 cm culture dish and rinsed with 5 ml ice cold PBS/phosphatase inhibitors. Primary cells were harvested and transferred to a pre-chilled conical tube. Cell suspension was then centrifuged at 200 x g for 5 minutes at 4 °C and supernatant was discarded. Cells were resuspended with 1X Hypotonic buffer and allowed swelling for 15 minutes on ice. Subsequently, 25 µl of Detergent was added and followed by vigorous vortex for 10 seconds. Upon checking under microscope to ensure complete cell lysis, cell suspension was centrifuged at 14,000 g for 30 seconds at 4 °C. Cytoplasmic fraction (supernatant) was transferred into a new tube and nuclear pellet was resuspended in 50 µl Complete lysis buffer. Samples were incubated for 30 minutes on a rocking platform set at 150 rpm at 4 °C before centrifugation at 14,000 g for 10 minutes at 4 °C. Lastly, supernatant (nuclear fraction) was transferred to a new tube and stored at -20 °C until use.

2.7 Immunoblotting

. **Materials:**

- Lysis buffers

- **Laemmli sample buffer**

  Laemmli sample buffer (Bio-Rad, CA, USA) added with 5% β-mercaptoethanol (BDH, PA, USA)

- **RIPA buffer**

  RIPA buffer (Santa Cruz Biotechnology, CA, USA) added with cOmplete ULTRA Tablets, Mini, EDTA free protease inhibitor (Roche Diagnostics, USA) and phosSTOP *EASY*pack phosphatase inhibitor (Roche Diagnostics, USA) (one tablet each for every 10 ml of RIPA buffer)

- Protein quantification assay

- Coomasie Plus reagent (Thermo Pierce, MA, USA)
- Bovine serum albumin (BSA) (Sigma, MO, USA)

- Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

- Precision Plus Protein™ Dual Color Standards (Biorad, CA, USA)
- Running buffer (Biorad, CA, USA)
- Stacking gel
- Resolving gel
- Nitrocellulose membrane

  iBlot gel transfer stacks nitrocellulose (Invitrogen, CA, USA)

- Polyvinylidene difluoride (PVDF) membrane

  iBlot gel transfer stacks PVDF (Invitrogen, CA, USA)

- HRP substrate (Detection solution)

  Luminata™ Forte (Merck Milipore, MA, USA)

Luminata™ Crescendo (Merck Milipore, MA, USA)

- Blocking buffer

- 5% skim milk in PBST (0.1% Tween 20 (Sigma, MO, USA) in 1X PBST)

- Antibody incubation solutions

- **Primary antibody incubation solution**

  5% Bovine serum albumin (BSA) (Sigma, MO, USA) in PBST

- **Secondary antibody incubation solution**

  5% skim milk in PBST

- Stripping buffer

- 10X Reblot Plus Strong Solution (Merck Milipore, MA, USA) diluted into 1X solution

- Primary Antibodies:

Name	**Source**	**Application (dilution)**
Anti-GFAP rabbit pAb	Sigma, MO, USA	WB (1:10,000) ICC (1:200)
Anti-phospho-NFκB p65 (Ser536) rabbit mAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-NFκB p65 rabbit mAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-phospho-IκBα (Ser32) rabbit mAb	Cell Signaling, MA, USA	WB (1:500)
Anti-IκBα mouse mAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-phospho-JNK (Thr183/Tyr185) (81E11) rabbit mAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-JNK rabbit pAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-phospho-Erk1/2 (Thr202/Tyr204) rabbit pAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-Erk1/2 rabbit pAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-phospho-p38 (Thr180/Tyr182) (D3F9) rabbit mAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-p38 rabbit pAb	Cell Signaling, MA, USA	WB (1:1,000)

Anti-phospho-Nrf2 (Ser40) rabbit mAb	Abcam, Cambridge, UK	WB (1:1,000)
Anti-Nrf2 (C-20) rabbit pAb	Santa Cruz Biotechnology, CA, USA	WB (1:1,000)
Anti-NQO1 (C19) goat pAb	Santa Cruz Biotechnology, CA, USA	WB (1:1,000)
Anti-HO1 rabbit pAb	Abcam, Cambridge, UK	WB (1:1,000) ICC (1:200)
Anti-Keap1(E-20) goat pAb	Santa Cruz Biotechnology, CA, USA	WB (1:1,000)
Anti-EGFR rabbit pAb	Cell Signaling, MA, USA	WB (1:1,000)
Anti-Lamin B1 rabbit Ab	Abcam, Cambridge, UK	WB (1:10,000)
Anti-TBP mouse mAb	Abcam, Cambridge, UK	WB (1:5,000)
Anti-GAPDH mouse mAb	Millipore, MA, USA	WB (1:5,000)
Anti-β-actin mouse mAb	Sigma, MO, USA	WB (1:5,000)

Table 2.3 List of primary antibodies.

- Secondary Antibodies:

Name	**Source**	**Application (dilution)**
Anti-rabbit IgG, HRP-linked goat Ab	Jackson ImmunoResearch, PA, USA	WB (1:5,000)
Anti-mouse IgG, HRP-linked goat Ab	Jackson ImmunoResearch, PA, USA	WB (1:10,000)
Anti-goat IgG, HRP-linked donkey Ab	Jackson ImmunoResearch, PA, USA	WB (1:5,000)

Table 2.4 List of secondary antibodies.

WB- Western Blot; ICC- Immunocytochemistry; IHC- Immunohistochemistry

Methods:

- **Soluble cell lysate**

Cells were lysed either by addition of boiling laemmli sample buffer and heated up to 95˚C for 5 minutes. Alternatively, cells were lysed with ice-cold RIPA buffer followed by scrapping with a cell scrapper. RIPA lysates were further clarified by centrifugation at 16,000 x g for 20 minutes and collection of supernatant for protein concentration determination. Lysates were stored in -20˚C until further use.

- **Protein Quantification**

Each standard (ranging from 1.41 to 42.3 µg/ml of BSA) and test samples were added with 1:1 ratio of Coomasie Plus reagent, mix well and loaded into 96-well assay plate. The intensity of blue colour developed was read at 595 nm using microplate reader (Biotek Flx800). Concentration of the test samples were then calculated based on the standard curve generated.

- **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Samples were electrophoretically separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Invitrogen). Blocking of non-specific binding of primary antibodies was achieved by incubating the membrane in blocking buffer (5% milk in PBST) at room temperature for an hour. Membranes were then probed with primary antibody (listed in Table 2.3) diluted in PBST with 5% bovine serum albumin (BSA) overnight at 4 °C. Following incubation, blots were washed in PBST 3 times, 10 minutes each. Membranes were then incubated with respective horse radish peroxidase conjugated secondary antibodies (listed in Table 2.4) for one hour at room temperature and washed 3 times, 10 minutes each. Immunoblots were visualized using HRP substrate (Luminata™ Forte or Crescendo) and quantified by image analyser (UVItec Ltd., Cambridge, UK). Some membranes were also stripped and re-blotted with a different primary antibody. For detection of housekeeping genes (β-actin and GAPDH), membranes were incubated with primary antibody for one hour at room temperature.

2.8 Immunofluorescence staining

Materials:

- Fixative

- 4% paraformaldehyde (Sigma, MO, USA) in 1X phosphate buffered saline (PBS)

- 1X Phosphate buffer saline (PBS)

- Permeabilizing buffer

- 0.1% Triton-X-100 (Sigma, MO, USA) in 1X phosphate buffered saline (PBS)

- Blocking solution

- 5% BSA (Sigma, MO, USA) in permeabilizing buffer

- Antibody incubation solution

- **Primary antibody incubation solution**

  5% BSA (Sigma, MO, USA) in permeabilizing buffer

- **Secondary antibody solution**

  5% BSA (Sigma, MO, USA) in permeabilizing buffer

- Mounting media

- Vectashield mounting medium with nucleus-staining 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA)

- Primary antibodies (refer to Table 2.3)

- Secondary antibodies

Anti-mouse IgG Alexa Flour® 488 (Cell Signaling, MA, USA)

Method:

Treated primary astrocytes plated on glass coverslips were fixed with 4% paraformaldehyde/1X PBS for 15 minutes at room temperature, washed

trice with 1X PBS, then permeabilized with permeabilizing buffer for 5 minutes in room temperature. The cells were then blocked in blocking solution (5% BSA in permeabilizing buffer) for an hour. Subsequently, cells were incubated overnight with primary antibodies against GFAP (1:200) or HO-1 (1:200) (Table 2.3) in blocking solution at 4 °C. Subsequently, the cell were washed 3 times with 1X PBS and incubated with secondary antibody (dilution 1:400) for one hour in room temperature. Cells were washed 3 times with 1X PBS before mounting the coverslips onto glass slides using Vectashield mounting medium with DAPI nucleus-staining. Confocal images were taken with Axioplot microscope which was equipped with Carl Zeiss 510 confocal imaging scan-head and software (Carl Zeiss MicroImaging, NY, USA).

2.9 Cell viability and cytotoxicity assays

Materials:

- **CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)** (Promega, WI, USA)
- **CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH)** (Promega, WI, USA)
- **Muse® Count &Viability Assay** (Millipore, MA, USA)

Methods:

MTS assay is a colorimetric method to determine the number of viable cells in cytotoxicity assays. The MTS tetrazolium compound is reduced by NAD(P)H-dependent dehydrogenase into a soluble colored formazan product in metabolically active cells. First, cells were plated onto 24-well-tissue culture plates at a density of $1x10^5$ cells per well in 500µl media supplemented with

0.5% FBS. 100µl of MTS reagent was added per culture well and incubated for 3 hours at 37˚C. Cell viability was then assessed by measuring the absorbance of the formazan product at 490nm using a microplate reader (Biotek Flx800). Readings from treatment groups were normalized against control group.

Cell membrane integrity is assessed using LDH assay which quantitatively measures a stable cytosolic enzyme, lactate dehydrogenase that was released into the culture supernatant upon cell lysis. Cells were seeded in 96-well-tissue culture plates at a density of $1x10^4$ cells per well and 100µl of 0.5% FBS supplemented media was used for treatments. Upon completion of treatment, culture supernatant was collected, centrifuged at 16090 x g for 15 minutes and plated in a new 96-well enzymatic assay plate. On the other hand, the adhesive cells were lysed with 50µl lysis buffer per culture well for 45 minutes in 37˚C. Subsequently, 50µl of Substrate Mix were added into both cell lysate and culture supernatant, incubated in 37˚C for 30 minutes. Lastly, 50µl Stop Solution was added to stop the reaction and absorbance of the red formazan product were read at 490nm using a microplate reader. Readings from treatment groups were normalized against control group.

Cell concentration and viability was determined with Muse Count and Viability using Muse™ Cell Analyzer (Millipore, Massachusetts, USA). The assay uses dual fluorescent probes that identify nucleated cells from debris and live cells from dead or dying therefore providing more accurate cell concentration and viability results. The assay was performed according to manufacturer's instructions. $1x10^5$ cells were plated in 24-well plate for treatment. Upon treatment, cells were trypsinized and neutralized with culturing media. 10µl of cell suspension was mixed with 490µl of Muse Count and

Viability reagent, allowed 5 minutes incubation at room temperature before reading. Percentage of viable cells were recorded.

2.10 Luminex multiplex platform – Measurement of secreted cytokines

Material:

- Milliplex MAP kit

- Rat Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, MA, USA)
- Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, MA, USA)

Methods:

Measurement of secreted cytokines were performed using Luminex xMAP® based assays according to manufacturer's instructions (Merck Millipore, MA, USA). Briefly, the 0.5% FBS supplemented media of U373 cells and rat astrocytes collected after 24hrs of stimulation was centrifuged at 16,090 x g for 20 minutes at 4˚C. Cell supernatant or mouse brain homogenates were added into 96-well-plate together with Assay Buffer and fluorescent-coded magnetic beads. Samples were incubated 16-18 hours at 4˚C before removing the contents and wash 2X with Wash Buffer. After incubation with biotinylated Detection Antibody for 1 hour at room temperature, well contents were removed and washed 2 times with Wash Buffer. Lastly, Sheath fluid was added into the plate and plate were ran on Luminex 200^{TM} (Merk Millipore, MA, USA).Concentrations of cytokines were calculated based on standard curve generated.

2.11 Real-Time PCR (RT-PCR)

Material:

- Trizol (Ambion, CA, USA)

- Chloroform-isoamyl alcohol (Sigma, MO, USA)

- 70% ethanol

- 70ml of 100% ethanol with 30ml of water

- NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany)

- High capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA)

- GoTaq® qPCR Master Mix (Promega, WI, USA)

- Rat Primers:

Gene	Forward Primer	Reverse Primer
CCL-2	5'-ATGCAGTTAATGCCCCACTC-3'	5'-TTCCTTATTGGGGTCAGCAC-3'
CCL-5	5'-CCTTGCAGTCGTCTTTGTCA-3'	5'-ATCCCCAGCTGGTTAGGACT-3'
CXCL-1	5'-GCGGAGAGATGAGAGTCTGG-3'	5'-AGGCATTGTGCCCTACAAAC-3'
CXCL-2	5'-TCAATGCCTGACGACCCTA-3'	5'-GGACGATCCTCTGAACCAAG-3'
CXCL-5	5'-CGCTAATTTGGAGGTGATCC-3'	5'-AGTGCATTCCGCTTTGTTTT-3'
CXCL-10	5'-GCTTATTGAAAGCGGTGAGC-3'	5'-GGTCAGGAGAAACAGGGACA-3'
CX3CL-1	5'-CGCTCTGAATAGCTCCAACC-3'	5'-CTGCTCCTCAGGCCTACAAC-3'
IL-1β	5'-AGGCTTCCTTGTGCAAGTGT-3'	5'-TGAGTGACACTGCCTTCCTG-3'

TNF-α	5’-CCCAGACCCTCACACTCAGAT-3’	5’-TTGTCCCTTGAAGAGAACCTG-3’
IL-6	5’-TGAGAAAAGAGTTGTGCAATGG-3’	5’-AACGGAACTCCAGAAGACCA-3’
Nrf2	5’-CAGTCTTCACCACCCCTGAT-3’	5’-CAGTGAGGGGATCGATGAGT-3’
HO-1	5’-GGCTCTCTTTTCTTGGGCCT-3’	5’-GCCTCTACCGACCACAGTTC-3’
Nqo1	5’-GCGAGCGGGGAAAATACTCT-3’	5’-CCTCCTGCCCTAAACCACAG-3’
β-actin	5’-ACCCGCGAGTACAACCTTCT-3’	5’-TTCTGACCCATACCCACCAT-3’
GAPDH	5’-CTCATGACCACAGTCCATGC-3’	5’-TTCTGACCCATACCCACCAT-3’

Table 2.5 List of rat primers for RT-PCR.

-Mouse Primers:

Gene	**Forward Primer**	**Reverse Primer**
GFAP	5’-AATCCGTGTCAGAAGGCCAC-3’	5’-ACACCTCACATCACCACGTC-3’
β-actin	5’-TGTACCCAGGCATTGCTGAC-3’	5’-AACGCAGCTCAGTAACAGTCC-3’
GAPDH	5’-TGCGACTTCAACAGCAACTC-3’	5’-GCCTCTCTTGCTCAGTGTCC-3’

Table 2.6 List of mouse primers for RT-PCR.

Methods:

RNA extractions were performed using NucleoSpin RNA kit according to manufacturer’s protocol. In brief, cells lysed with Trizol or mouse brain homogenized in Trizol were added wtih 200 µl chloroform, sample was vortexed for 1 minute and centrifuged at 16,090 xg for 10 minutes at 4˚C. The aqueous phase was collected, mixed with equal volume of 70% ethanol and

added into NucleoSpin RNA column. Salt was removed by washing with Membrane Desalting Buffer and DNA was digested with DNase reaction mixture provided in the kit. Subsequently, RAW2 Buffer was added to inactivate DNase and washed with RA3 Buffer before eluting RNA with RNAse-free water. The concentration and purity of RNA were assessed by absorbance at 260 and 280 nm using using Nanodrop (Spectrophotometer, ND 1000 Biofrontier Technology). cDNA was synthesized from RNA samples using high capacity cDNA reverse transcriptase kit and quantitative real-time PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystem, CA, USA). Genes of interest were normalized against geometric mean of GAPDH and β-actin. Relative gene expression were expressed as fold change computed using formula 2^(-[delta][delta]Ct). Whereby,

[delta][delta]Ct = [delta]Ct,sample - [delta]Ct,reference

2.12 Immunoprecipitation

Materials:

- 1X PBS

- RIPA lysis buffer

- RIPA buffer (Santa Cruz Biotechnology, CA, USA) added with cOmplete ULTRA Tablets, Mini, EDTA free protease inhibitor (Roche Diagnostics, USA) and phosSTOP *EASY*pack phosphatase inhibitor (Roche Diagnostics, USA) (one tablet each for every 10 ml of RIPA buffer)

- Laemmli sample buffer

- Laemmli sample buffer (Bio-Rad, CA, USA) added with 5% β-mercaptoethanol (BDH, PA, USA)

- **Anti-Ub (P4D1), agarose conjugated mouse IgG1 mAb** (Santa Cruz Biotechnology, CA, USA)

- **Immunoprecipitation buffer**

- 20 mM Tris-HCl pH 8 (Invitrogen, CA, USA)
- 140 mM NaCl (Millipore, MA, USA)
- 1% Triton X-100 (Sigma, MO, USA)
- 2 mM EDTA (Thermo Fisher Scientific, MA, USA)

Methods:

Primary astrocytes were plated and treated in 10 cm culture dish. Upon treatment, culture media was aspirated and cells were rinsed with 8 ml ice cold 1X PBS. Primary cells were harvested with RIPA added with protease and phosphatase inhibitor (one tablet each for every 10 ml of RIPA buffer). Lysates were transferred to a pre-chilled eppendorf tube and incubated at 4 °C for 30 minutes with rotation. Lysate was sonicated and followed by centrifugation at 14,000 x g for 10 minutes at 4 °C. Supernatant was collected and subjected to protein assay for concentration determination. In the preparation for input sample, 55 µg protein was added with 1:1 ratio of boiling laemmli sample buffer and heated up to 95˚C for 5 minutes. For immunoprecipitation, 1 mg of protein were added with 30 µl of anti-Ub (P4D1), agarose beads conjugated mouse antibody and incubated in 4 °C for 3 hours with rotation. The agarose beads were pelleted and washed 4 times with cold immunoprecipitation buffer and the precipitates were subjected to immunoblot analysis. Immunoprecipitates and input samples were resolved on 8% and 10% SDS-PAGE respectively. Both

were immunoblotted for Nrf2 with anti-Nrf2 (C-20) rabbit pAb (Santa Cruz Biotechnology, CA, USA).

2.13 Statistical analysis

All statistical analyses were performed with the SPSS 13.0 for Windows software (SPSS Inc. USA). Data was reported as mean ± S.E.M of three (or more) independent experiments unless otherwise stated. Time course experiments and dose effects of andrographolide and LPS were compared with controls (DMEM/F12 with 0.1% DMSO for andrographolide experiments and DMEM/F12 for LPS experiments) using one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* tests, while other multiple pair-wise comparisons were performed using ANOVA followed by Bonferroni's *post-hoc* tests, with p values <0.05 considered to be statistically significant.*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

CHAPTER 3: ANTI-NEUROINFLAMMATORY EFFECTS OF ANDROGRAPHOLIDE IN CYTOKINES (IL1-β AND TNF-α) MEDIATED INFLAMMATION

3.1 Results

3.1.1 IL1-β and TNF-α activated NF-κB and induced CCL-5 secretion in U373 glioblastoma

As detailed in Chapter 1, IL-1β and TNF-α are the main initiators of inflammation. Therefore, these cytokines were selected to induce inflammatory responses in an astrocyte cell line (U373). U373 was chosen in the initial study because it has been widely used as a model for inflammatory responses in the CNS and TNF-α strongly activated NF-κB pathway in U373 as demonstrated by DNA microarray (Schwamborn et al., 2003). Upon stimulation with human IL-1β (hIL-1β) or human TNF-α (hTNF-α) for 24 hours, conditioned media of U373 cells was collected and assayed for secreted CCL-5 using the Luminex xMAP® based assays. Results showed that hIL-1β and hTNF dose-dependently induced secretion of CCL-5 (Figure 3.1). From this assay, 1 ng/ml IL-1β and 10 ng/ml TNF-α were chosen as the optimal stimulation concentrations used for subsequent experiments. Previous studies had shown that IL-1β and TNF-α can activate the NF-κB pathway (Jiang *et al.*, 2003; Sakurai *et al.*, 1999). Therefore, I incubated U373 cells with hIL-1β or hTNF-α, and collected cell lysates at various time-points for immunoblotting, and found that maximal phosphorylation of p65 at Ser536 residue (a marker for p65 transactivation, (Sasaki *et al.*, 2005)) occurred at 7 minutes for both cytokines (Figure 3.2 A & C). Furthermore, maximal IκB phosphorylation was observed between 2 to 7 minutes incubation while total IκB reached maximal degradation at around 30 minutes for hIL-1β and around 10 to 20 minutes for hTNF-α (Figure 3.2 B & D). Therefore, 7 minutes stimulation time was selected for subsequent experiments.

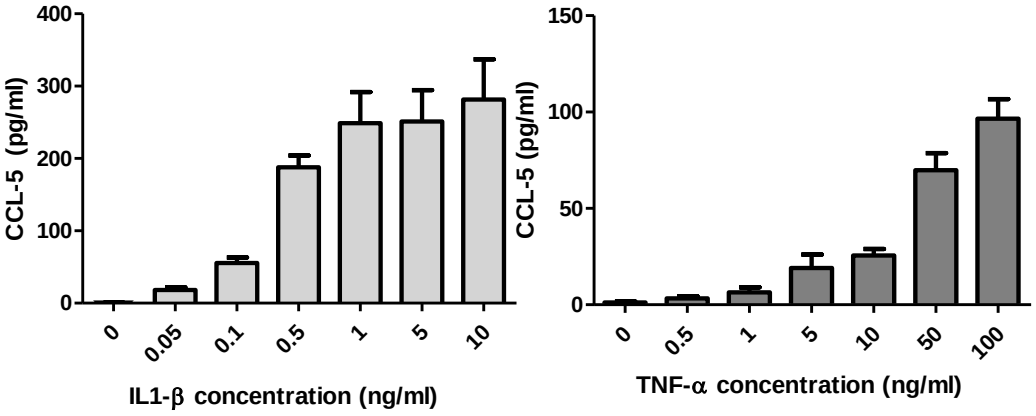
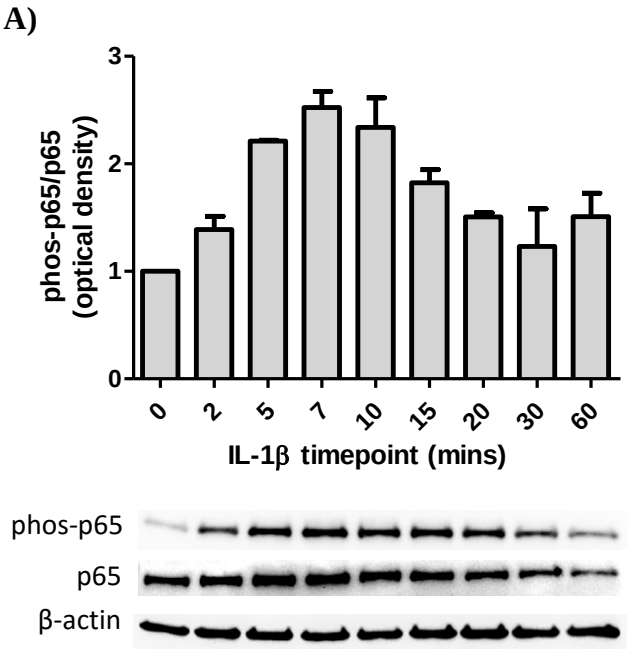
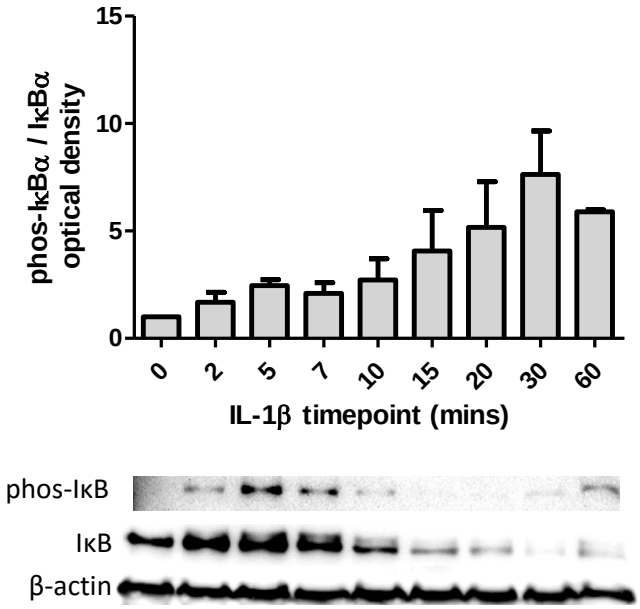


Figure 3.1 hIL1-β and hTNF-α dose dependently induced CCL-5 secretion in U373 glioblastoma. CCL-5 secretion in U373 cells was determined using Luminex assays after 24 hours of hIL-1β or hTNF-α stimulation. Sample size n = 3. Data shown are mean ± S.E.M.



B)


15
10
5
0
phos-IκBα / IκBα optical density
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IL-1β timepoint (mins)
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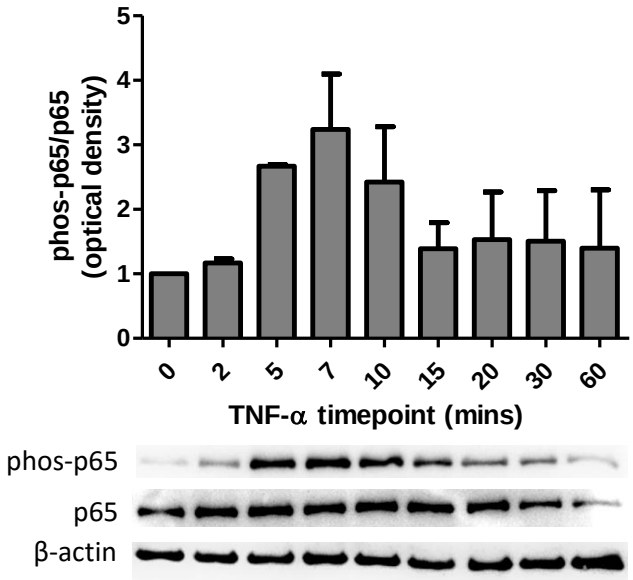
C)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3
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phos-p65/p65 (optical density)
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β-actin

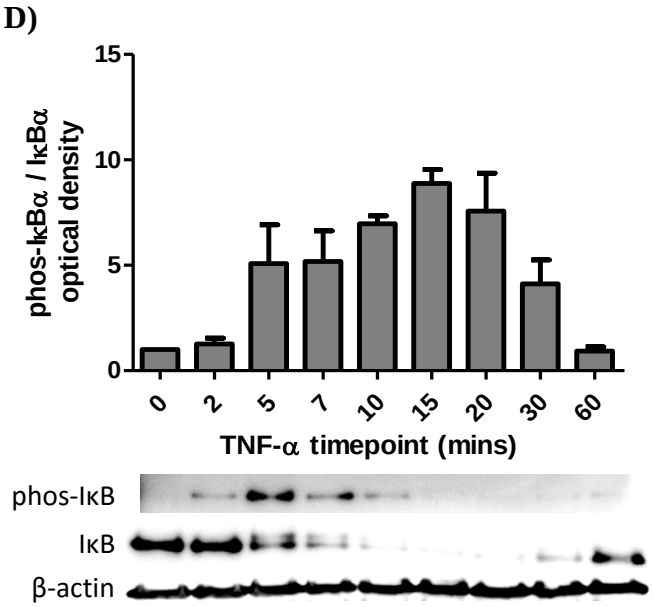


Figure 3.2 hIL1-β and hTNF-α induced NF-κB activation in U373 cells. U373 were incubated with 1 ng/ml IL-1β **(A, B)** or 10 ng/ml TNF-α **(C, D)** for various indicated time. Phosphorylation levels of p65 and IκBα were analyzed with immunoblotting. Data shown are mean ± S.E.M of two separate experiments.

3.1.2 Investigating potential anti-neuroinflammatory compounds in U373 glioblastoma

A few natural compounds including andrographolide (Andro), artesunate, and plumbagin had previously been found to exhibit anti-inflammatory effects (Wang *et al.*, 2014; Xia *et al.*, 2004; Xu *et al.*, 2007). In order to identify a potentially efficacious anti-neuroinflammation compound, I screened these three compounds for their anti-neuroinflammatory properties.

3.1.2.1 Cytotoxicity and anti-neuroinflammatory effects of plumbagin

Preliminary toxicity study of andrographolide, artesunate, and plumbagin suggested that 24 hours incubation of plumbagin reduced U373 cell viability at 10 μM (Appendix 1). Based on the effective concentration reported to exhibit anti-inflammaotory effects in other system, a single concentration

from each compound was chosen to treat U373 to investigate their ability to reduce CCL-5 secretion. U373 cells were pretreated with 10 μM artesunate, 30 μM andrographolide, or 5 μM plumbagin for 16 hours followed by 24 hours of hIL-1β or hTNF-α stimulation with the presence of the compounds. Secreted CCL-5 was then measured with Luminex assay. Results demonstrated that only andrographolide significantly abated CCL-5 secretion induced by hIL-1β and hTNF-α (Figure 3.3). Study of plumbagin was then discontinued due to its toxicity and inability to attenuate CCL-5 cytokine secretion.

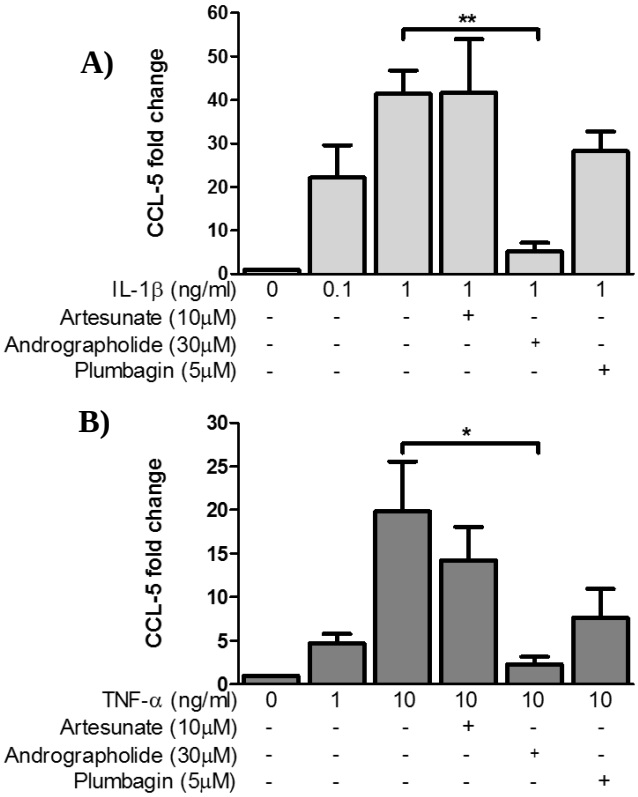
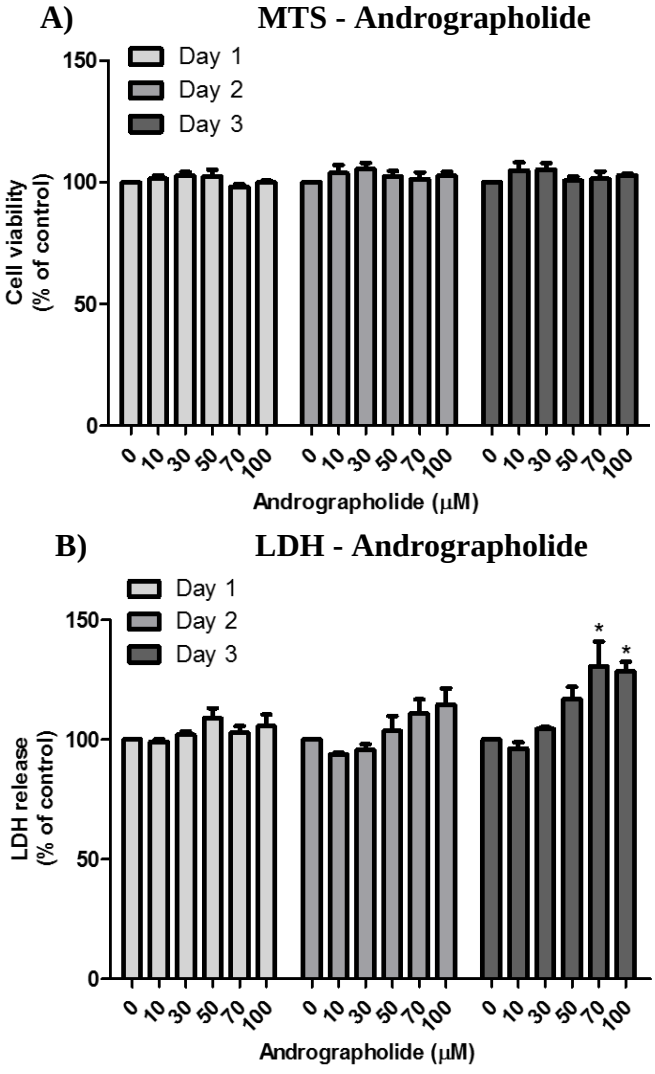


Figure 3.3 Andrographolide abated CCL-5 secretion induced by hIL-1β and hTNF-α in U373 cells. U373 cells were pretreated with 10 μM artesunate, 30 μM andrographolide, or 5 μM plumbagin for 16 hours followed by 24 hours of hIL-1β **(A)** or hTNF-α **(B)** stimulation with the presence of the test compounds. CCL-5 secretion was measured using Luminex assay. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*p < 0.05 and **p < 0.01)

3.1.2.2 Cytotoxicity and anti-neuroinflammatory effects of andrographolide and artesunate

More detailed study was then performed on andrographolide and artesunate. Effects of andrographolide and artesunate on U373 cell viability and cell cytotoxicity were examined using MTS and LDH assays respectively (see Chapter 2). U373 cells were incubated in 0.5% FBS supplemented media for up to three days with various concentrations of andrographolide or artesunate, MTS and LDH measurements were taken every 24 hours. Though no difference was observed in cell viability, both compounds significantly enhanced cell cytotoxicity at high concentration (Figure 3.4). Thus, compound treatment time was kept within two days in the subsequent experiments.



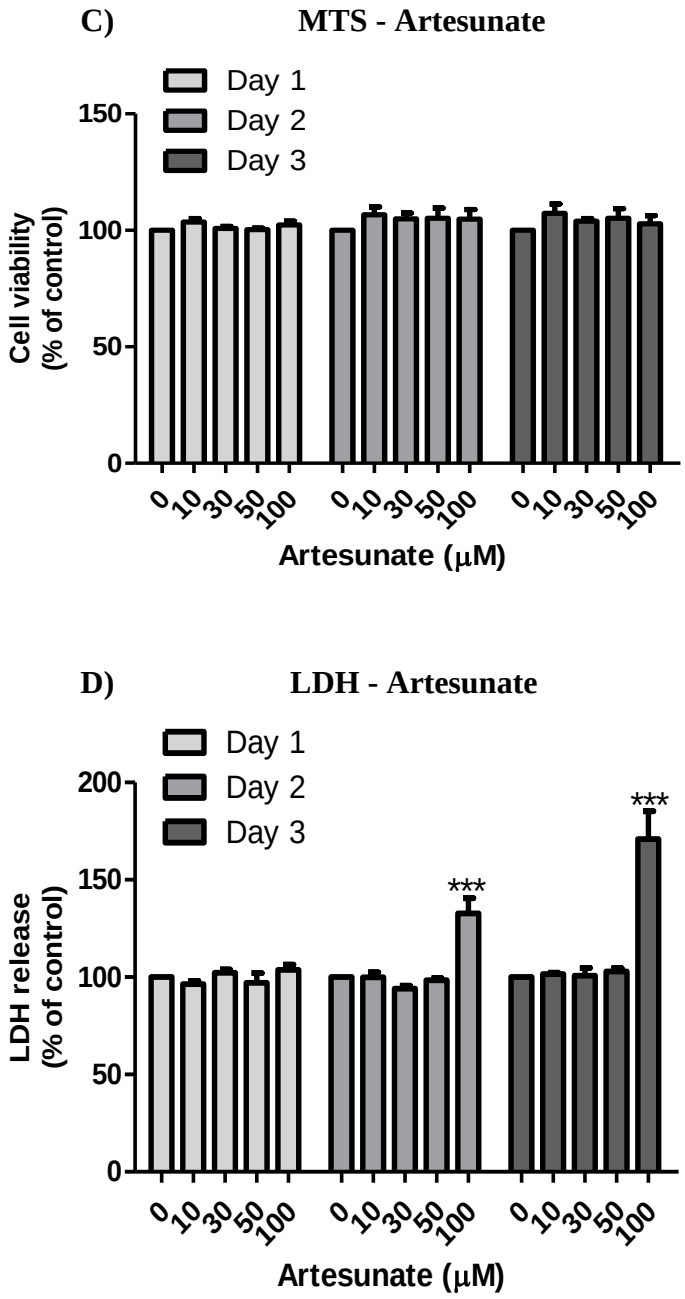


Figure 3.4 Andrographolide and artesunate induced cell cytotoxicity in U373 cells at high concentration. MTS **(A)** and LDH **(B)** assay of U373 cells treated with various concentrations of andrographolide for up to three days. Similarly, U373 cells incubated with various artesunate concentrations for up to three days were subjected to MTS **(C)** and LDH **(D)** assays. Sample size n = 3. Data shown are mean ± S.E.M. Test compound dose effects were compared with controls (0.1% DMSO) with one way ANOVA followed by Dunnett's *post-hoc* tests (*$p < 0.05$ and ***$p < 0.001$)

In order to compare the efficiency of andrographolide and artesunate in reducing neuroinflammatory responses, both compounds were tested side by side. U373 cells were pretreated with 16 hours (according to protocols used in (Chao *et al.*, 2011)) of andrographolide or artesunate (3μM to 100μM) followed by 24 hours of hIL-1β or hTNF-α stimulation in the presence of the test compounds (in 0.5% FBS supplemented media). Luminex assay results suggested that andrographolide reduced CCL-5 secretion to a greater extend compared to artesunate regardless of the cytokine administered (Figure 3.5). Moreover, immunoblots revealed that U373 cells pretreated with andrographolide abrogated hIL-1β or hTNF-α induced phosphorylation of p65 and IκBα (Figure 3.6 and Figure 3.7). However, equivalent doses of artesunate could not significantly reduce p65 and IκBα phosphorylation. Together, these results demonstrated that andrographolide may be a more efficacious anti-neuroinflammatory compound which led to my decision to focus on andrographolide.

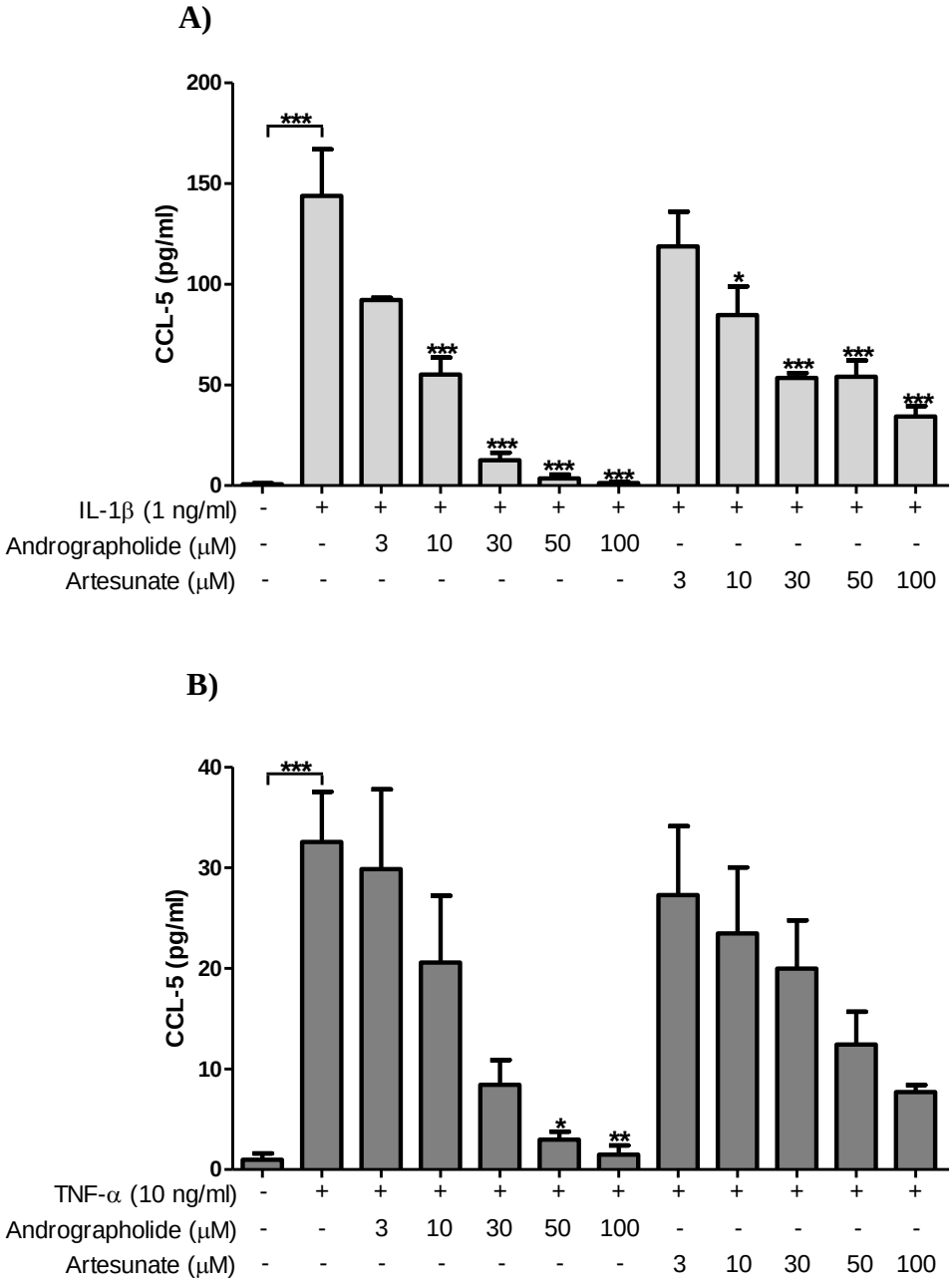


Figure 3.5 Andrographolide reduces CCL-5 secretion to a greater extend compared to artesunate. CCL-5 secretion was determined by Luminex assay. U373 cells were pretreated for 16 hours with andrographolide or artesuante followed by 24 hours of hIL-1β **(A)** or hTNF-α **(B)** stimulation with the presence of the test compounds. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons with hIL-1β/hTNF-α stimulation were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$ and **$p < 0.01$); ns = not significant ($p > 0.05$)

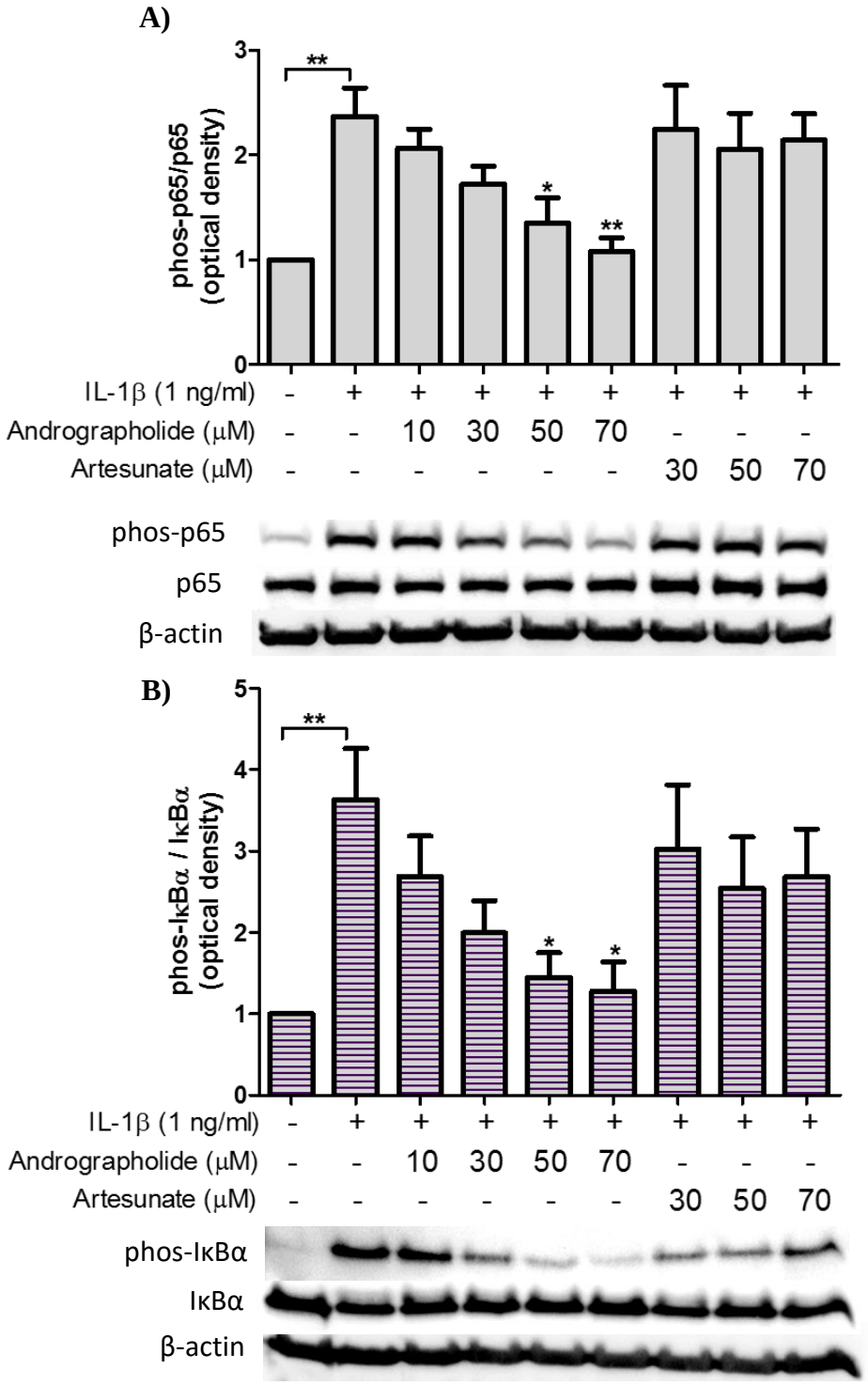


Figure 3.6 Andrographolide but not artesunate abrogated hIL-1β induced NF-κB activation. U373 cells were first pretreated with andrographolide or artesunate for 16 hours followed by 7 minutes of stimulation with hIL-1β (with presence of test compounds). Whole cell lysate were immunoblotted with phos-p65 and p65 antibodies **(A)** or phos-IκBα and IκBα antibodies **(B).** β-actin was used as a loading control. Sample size n = 4. Data shown are mean ± S.E.M. Multiple pair-wise comparisons with hIL-1β stimulation were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$ and **$p < 0.01$); ns = not significant ($p > 0.05$)

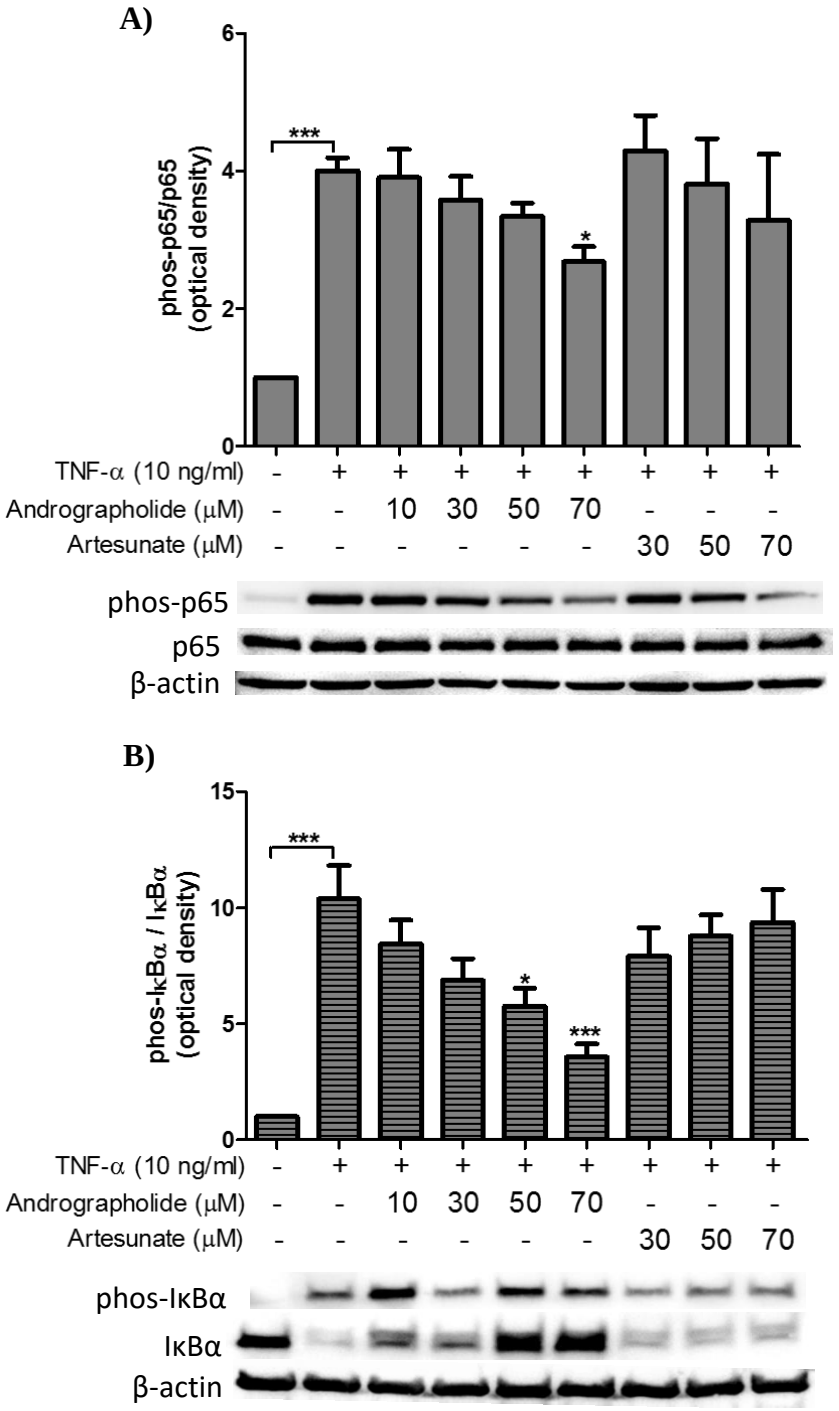


Figure 3.7 Andrographolide but not artesunate abrogated hTNF-α induced NF-κB activation. U373 cells were first pretreated with andrographolide or artesunate for 16 hours followed by 7 minutes of stimulation with hTNF-α (with presence of test compounds). Whole cell lysate were immunoblotted with phos-p65 and p65 antibodies **(A)** or phos-IκBα and IκBα antibodies **(B).** β-actin was used as a loading control. Sample size n = 4. Data shown are mean ± S.E.M. Multiple pair-wise comparisons with hTNF-α stimulation were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$ and ***$p < 0.001$)); ns = not significant ($p > 0.05$)

3.1.3 Andrographolide did not affect primary astrocytes cell viability

Cancer derived cell lines (e.g., U373) may exhibit abnormal metabolic functions and proliferative properties, resulting in phenotypes which may be different from normal cells in many important aspects. Primary cell cultures, on the other hand, retains many if not all cellular properties functions and phenotypes *in vitro*. Therefore, in order to increase the biological relevance of cell-based studies, I followed up with rat primary astrocyte cultures. MUSE® cell count and viability assays revealed that andrographolide induced no or low toxicity to rat primary astrocytes (Figure 3.8). Specifically, no obvious change in cell viability was observed when incubated with andrographolide for up to two day with concentrations up to 100 μM (in 10% FBS supplemented media).

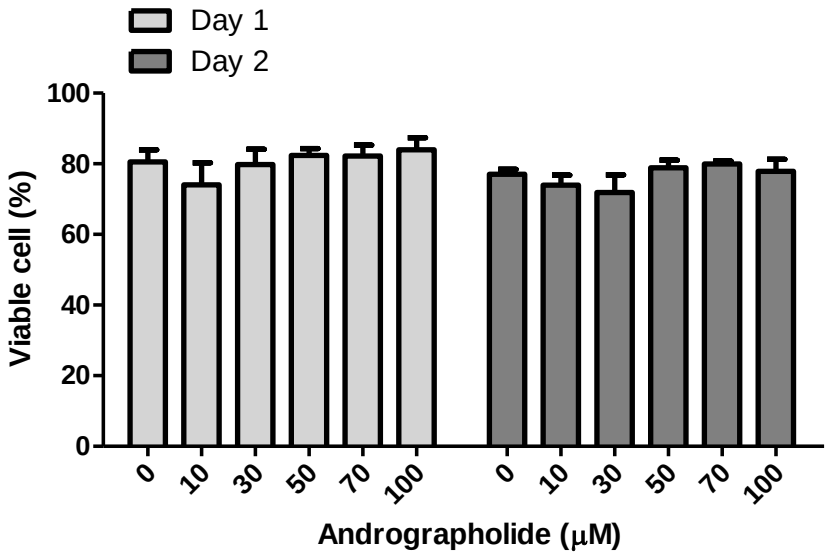


Figure 3.8 Andrographolide did not reduce cell viability in rat primary astrocytes. Upon incubation with andrographolide for the indicated time, cell viability was determined with Muse Count and Viability assay. Sample size n = 3. Data shown are mean ± S.E.M. Test compound dose effects were compared with controls (0.1% DMSO) with one way ANOVA followed by Dunnett's *post-hoc* tests.

3.1.4 Andrographolide pretreatment inhibited CCL-5 secretion and NF-κB activation induced by cytokines (rIL-1β and rTNF-α) in primary astrocytes

In corroboration with findings in U373, andrographolide attenuates rat IL-1β (rIL-1β) and rat TNF-α (rTNF-α) activated NF-κB pathway and CCL-5 secretion in rat primary astrocytes. Upon 16 hours of peincubation with andrographolide, primary astrocytes were either stimulated with rIL-1β 30 minutes or rTNF-α 10 minutes. Whole-cell lysates were collected and immunoblotted for phos-p65 and p65 (Figure 3.9 A and Figure 3.10 A) or phos-IκBα and IκBα (Figure 3.9 B and Figure 3.10 B). Andrographolide significantly inhibited phosphorylation of p65 and IκBα in a dose-dependent manner. Conditioned media collected from primary astrocytes pretreated 16 hours with andrographolide and stimulated 24 hours were subjected to Luminex assay. Results revealed that andrographolide remarkably lowered CCL-5 secretion induced by rIL-1β or rTNF-α (Figure 3.9 C and Figure 3.10 C).

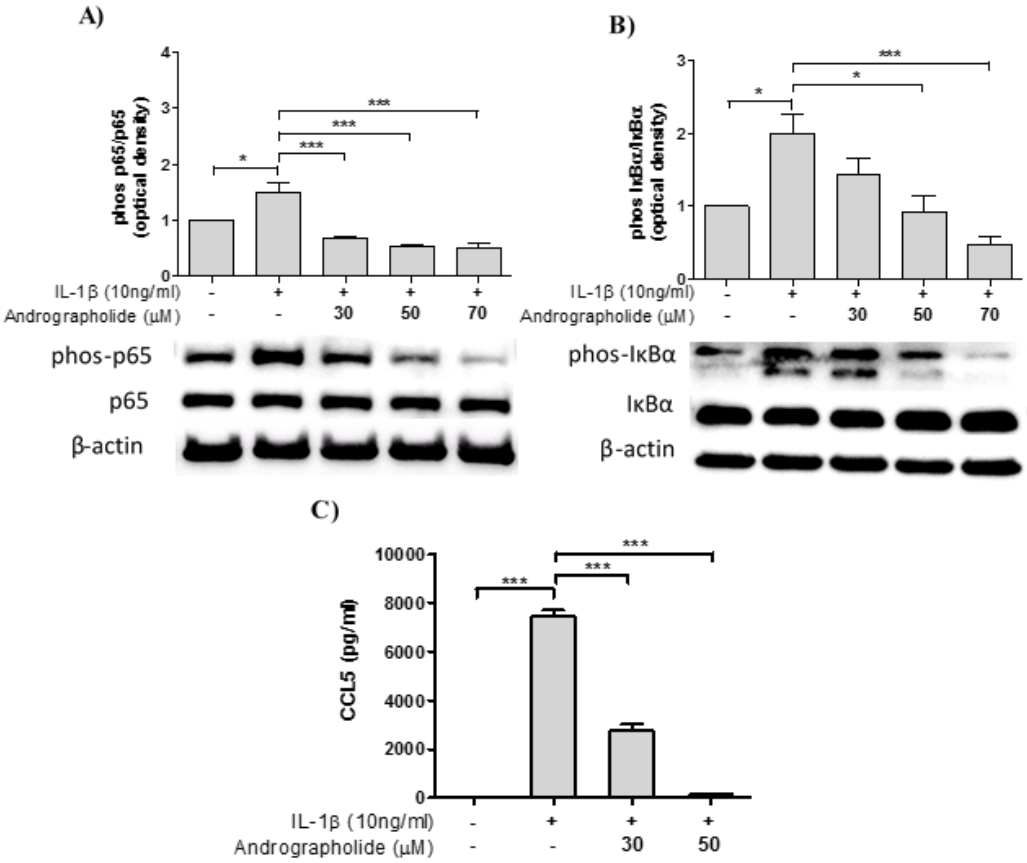


Figure 3.9 Andrographolide attenuated rIL-1β induced NF-κB activation and CCL-5 secretion in primary astrocytes. Primary astrocytes were pretreated for 16 hours with andrographolide before rIL-1β stimulation in the presence of test compound. Whole-cell lysates collected after 30 minutes of stimulation were probed for phos-p65 and p65 **(A)** or phos-IκBα and IκBα **(B)**. β-actin was used as a loading control. **(C)** Media collected from primary astrocytes stimulated for 24 hours were subjected to Luminex assay where CCL-5 secretion was measured. Sample size n = 4 for (A) and (B), n = 3 for (C). Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*p < 0.05 and ***p < 0.001)

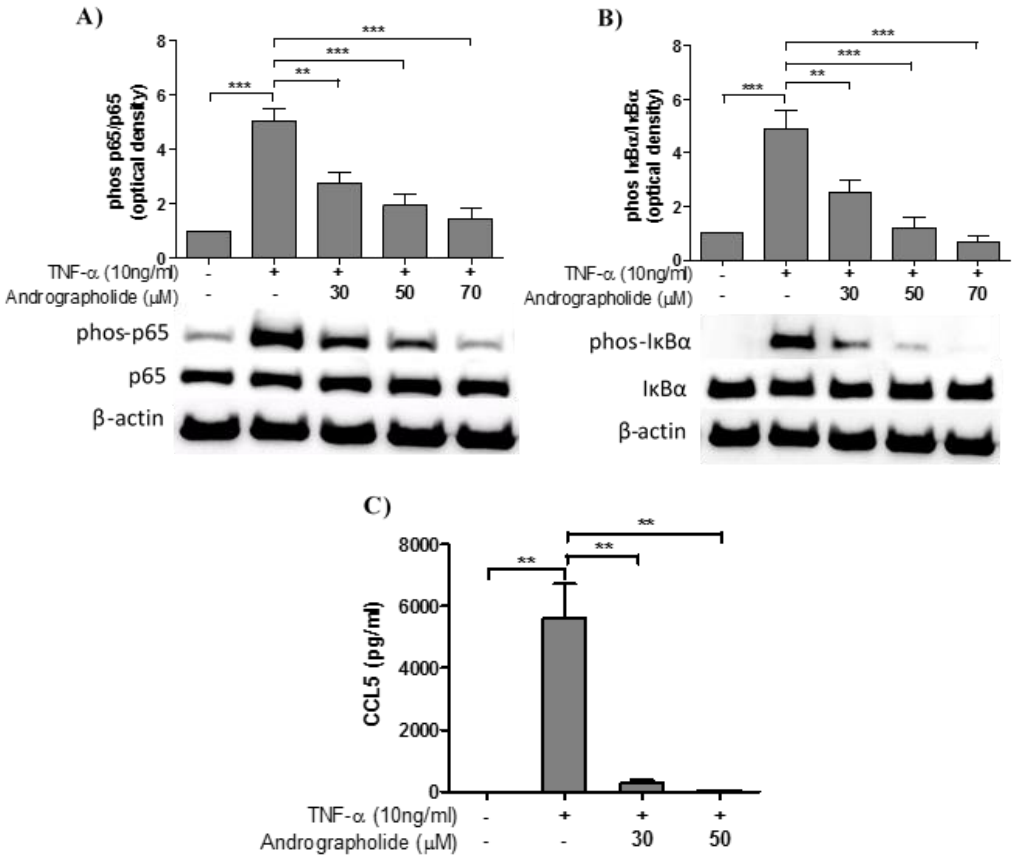


Figure 3.10 Andrographolide pretreatment attenuated rTNF-α induced NF-κB activation and CCL-5 secretion in primary astrocytes. Primary astrocytes were pretreated for 16 hours with andrographolide before rTNF-α stimulation in the presence of test compound. Whole-cell lysates collected after 10 minutes of stimulation were probed for phos-p65 and p65 **(A)** or phos-IκBα and IκBα **(B)**. β-actin was used as a loading control. **(C)** Media collected from primary astrocytes stimulated for 24 hours were subjected to Luminex assay where CCL-5 secretion was measured. Sample size n = 4 for (A) and (B), n = 3 for (C). Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (**p < 0.01 and ***p < 0.001)

3.1.5 Andrographolide administration following cytokines (rIL-1β and rTNF-α) stimulation also attenuated CCL-5 secretion in primary astrocytes

The preceding studies were conducted with pretreatment of andrographolide before cytokine stimulation. Hence, it is interesting to examine if andrographolide could also abolish CCL-5 release when administered after IL-1β and TNF-α stimulations. Rat primary astrocytes were stimulated with rat rIL-1β (Figure 3.11 A) or rTNFα (Figure 3.11 B) followed by addition of andrographolide 1, 4, or 8 hours later. Cytokine stimulation was maintained for a total of 24 hours in 0.5% FBS supplemented media. CCL-5 release was measured using Luminex assay. Results showed that andrographolide could reduce CCL-5 secretion even when administered up to 4 hours after rIL-1β or TNF-α stimulation. This implied that andrographolide did not require pre-incubation in order to exert its anti-neuroinflammatory effects. Nonetheless, andrographolide had a therapeutic time window and no CCL-5 reduction was detected when it was given 8 hours after stimulation.

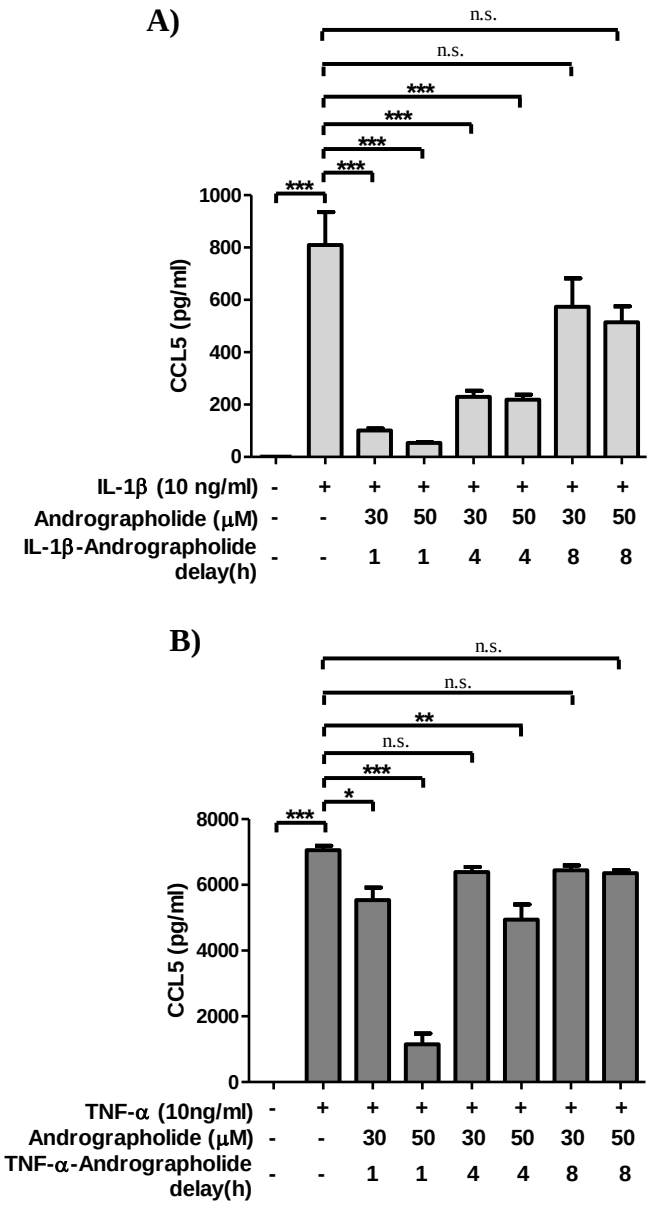


Figure 3.11 Andrographolide attenuated CCL-5 release in primary astrocyte when administered after cytokines (rIL-1β and rTNF-α) stimulation. Rat primary astrocytes were stimulated with rIL-1β (A) or TNF-α (B) followed by addition of andrographolide 1,4 , or 8 hours later, as indicated on the graph by the IL-1β-Andrographolide delay or TNFα-Andrographolide delay. Cytokine stimulation was maintained for a total of 24 hours and media collected were subjected to Luminex assay for CCL-5 measurement. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's post-hoc tests. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$); ns = not significant ($p > 0.05$)

3.1.6 Inhibition of NF-κB by TPCK decreased CCL-5 secretion induced by IL-1β and TNF-α

3.1.6.1 TPCK inhibited phosphorylation of p65

Thus far I have found that andrographolide efficiently attenuated CCL-5 secretion in astrocytes. Nevertheless, the mechanisms by which andrographolide regulated CCL-5 was unclear. I proposed that andrographolide attenuates CCL-5 secretion through inhibition of NF-κB. Therefore, a known NF-κB inhibitor was employed to study the causal relationship between NF-κB inhibition and CCL-5 reduction. N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a serine/cysteine protease inhibitor. It modifies thiol groups (-C-SH) on Cys-179 of IKKβ and Cys-38 of p65 to tosylphenylalanylmethyl group and inhibits p65 binding to DNA (Ha *et al.*, 2009). Primary astrocytes was preincubated with TPCK for an hour followed by rIL-1β or rTNF-α stimulation. Immunoblot results revealed that TPCK prevented p65 activation (Figure 3.12), confirming its action in deactivating p65.

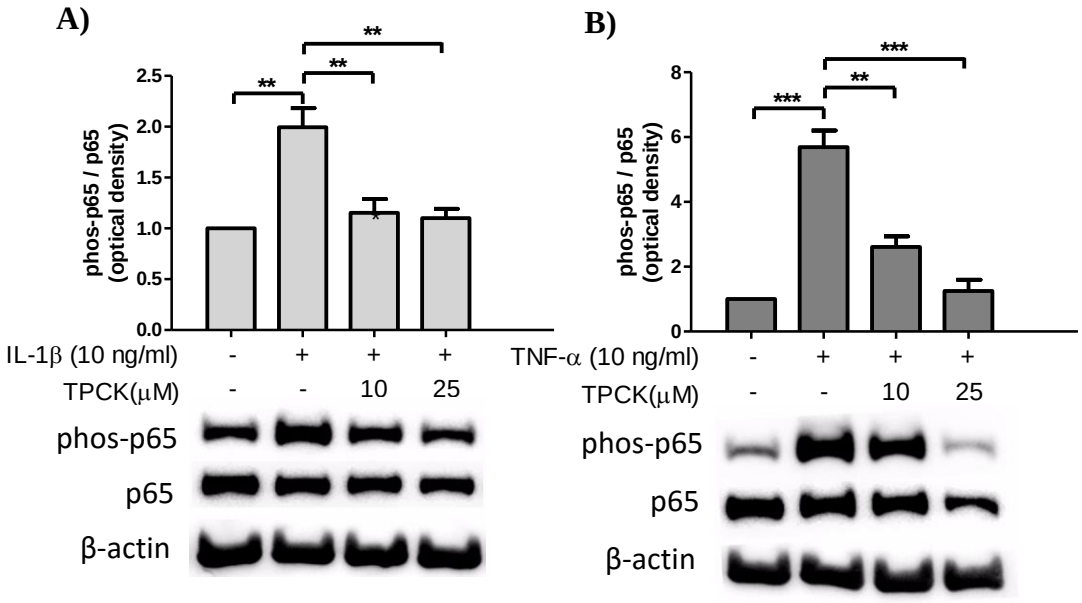


Figure 3.12 TPCK inhibited p65 phosphorylation. Primary astrocytes was preincubated with TPCK for an hour followed by rIL-1β or rTNF-α stimulation. Whole-cell lysate was probed for phos-p65 and p65. β-actin was used as loading control. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (**p < 0.01 and ***p < 0.001)

3.1.6.2 TPCK decreased CCL-5 secretion induced by cytokines

Effects of TPCK on CCL-5 was then examined by preincubating primary astrocytes with TPCK for an hour followed by 24 hours of rIL-1β or rTNF-α stimulation in 0.5% FBS supplemented media. Concentration of secreted CCL-5 was later determined by Luminex assay. Similar to andrographolide, TPCK also dramatically reduced CCL-5 release (Figure 3.13). This suggested that andrographolide could possibly attenuated CCL-5 release via NF-κB inhibition.

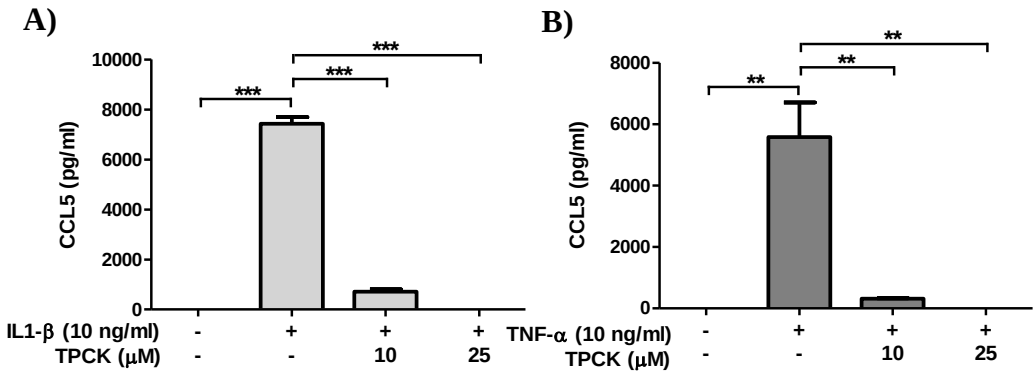


Figure 3.13 TPCK decreased CCL-5 release induced by cytokines. Primary astrocytes was preincubated with TPCK for an hour followed by 24 hours of rIL-1β or rTNF-α stimulation. Concentration of secreted CCL-5 was determined by Luminex assay. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (**p < 0.01 and ***p < 0.001)

3.1.7 Andrographolide abated GFAP expression elevated by rIL-1β in primary astrocytes

Any form of CNS insults ranging from subtle cellular perturbation to severe brain injury could cause astrocytes to undergo molecular and morphological changes termed reactive astrogliosis (Sofroniew, 2009). Detrimental effects of astrogliosis include production of cytokines/chemokines that exacerbate neuroinflammation, generation of reactive oxygen species, compromise of the blood brain barrier, and release of glutamate that potentially leads to cell excitotoxicity (Sofroniew, 2009). Glial fibrillary acidic protein (GFAP) is intermediate filament specifically expressed by astroglial cells in the CNS (Pekny *et al.*, 2004). It is widely used as a marker for astrogliosis. To access andrographolide's capability in moderating GFAP expression, primary astrocytes were pretreated with andrographolide for 16 hours followed by 24 hours of rIL-1β and GFAP level were visualized with immunofluorescence

staining or quantified with immunoblotting (Figure 3.14). Andrographolide treatment successfully lowered GFAP immunofluorescence staining and protein level induced by rIL-1β.

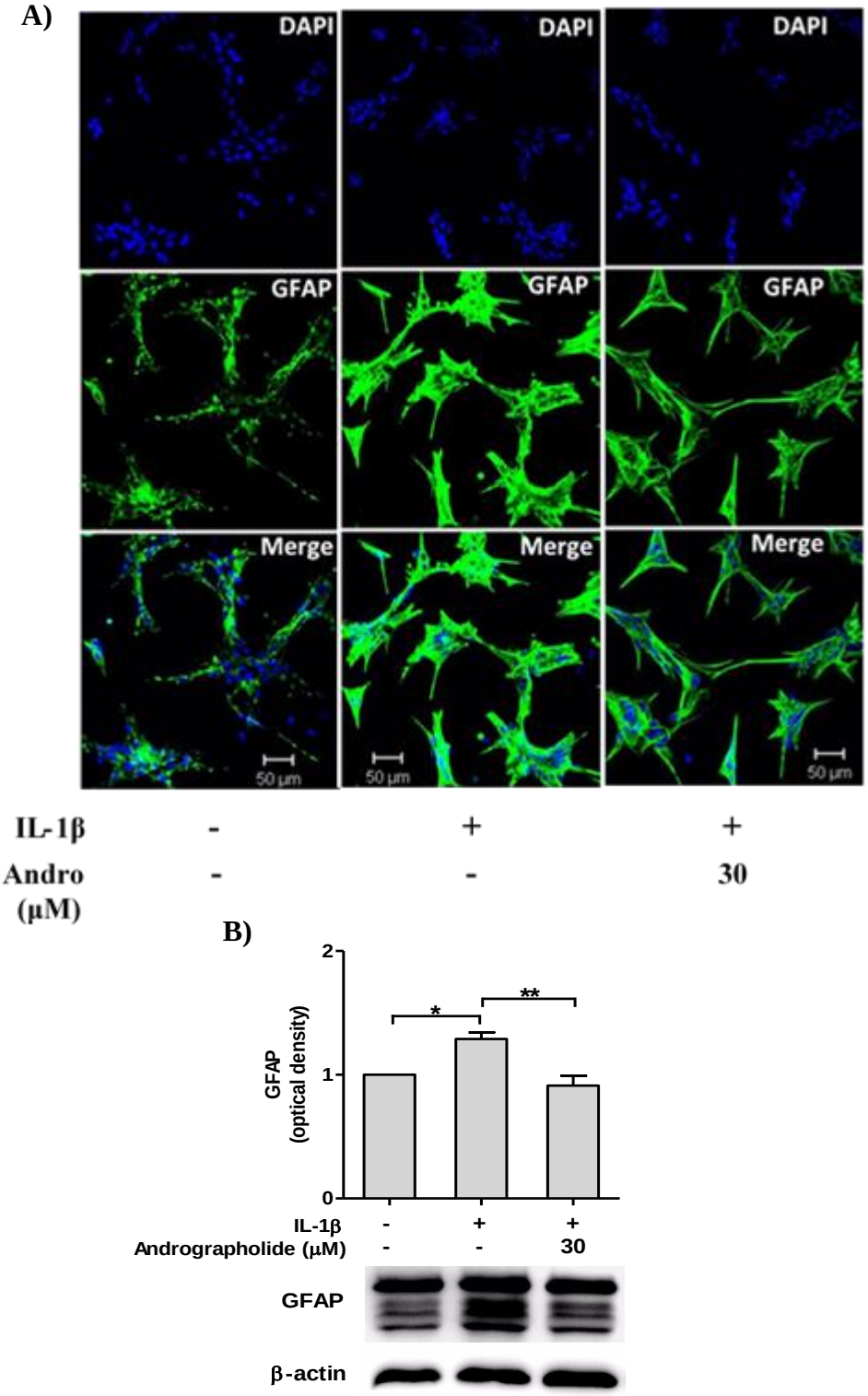


Figure 3.14 Andrographolide abated GFAP expression elevated by rIL-1β in primary astrocytes. Primary astrocytes were pretreated with andrographolide for 16 hours and stimulated with rIL-1β (using 10 ng/ml for immunoblotting, 1 ng/ml for immunofluorescence) for a further 24 hours before processing for immunofluorescence staining **(A)** or immunoblotting with GFAP antibody **(B)**. Scale bar denotes 50 µm. β-actin was used as a loading control. Sample size n = 4. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$ and **$p < 0.01$)

3.2 Discussion

Neuroinflammation is implicated in the pathogenesis of ischemic stroke, traumatic brain injury, multiple sclerosis, meningitis, and neurodegenerative diseases (Rivest, 2009; Wohleb *et al.*, 2013). Being the most abundant cell type in the CNS, astrocytes, once thought to merely serve in supportive roles, have recently emerged as a crucial participant in brain development, function and disease. Activation of astrocyte initiates inflammatory signaling pathways, induces release of proinflammatory cytokines/chemokines and reactive oxygen species (ROS) that exacerbate various neurological diseases (Sofroniew *et al.*, 2010). Therefore, in my study, I used astrocytic culture as cell model to investigate natural bioactive compounds for potential anti-neuroinflammation properties.

IL-1β and TNF-α are elevated in many neuroinflammatory conditions like injuries, infections, and neurodegeneration (Basu *et al.*, 2004; Olmos *et al.*, 2014). They are early mediators released during inflammatory events (Van Miert, 1995) which act to promote a cascade of secondary cytokines production (Mrak *et al.*, 1995). Moreover, they are known to activate NF-κB; the key regulator of immune response through well-established mechanism (Beg *et al.*, 1993). Indeed, I showed that IL-1β and TNF-α induced phosphorylation of p65 subunit of NF-κB at Ser536 which enhances its transactivation potential (Jiang *et al.*, 2003; Sakurai *et al.*, 1999). They also promote degradation of IκBα that sequester NF-κB in the cytoplasm through ubiquitin/proteasome-mediated pathway by inducing phosphorylation IκBα at Ser32 residue (Chen *et al.*, 1996; Zandi *et al.*, 1997). My study suggested that IL-1β and TNF-α cytokines are both effective inducer of neuroinflammation.

In the first part of my study, CCL-5 was used as the readout of NF-κB and inflammatory activation. This is mainly because CCL-5 is a recognized gene target of the NF-κB transcription factor (Werts *et al.*, 2007). Not only is CCL-5 found in the neurons and microglia, astrocytes also highly express it in the CNS (Kim *et al.*, 2004b; Rock *et al.*, 2004b). It is a chemotactic cytokine (a.k.a. chemokine) that facilitates leukocyte adhesion to brain microvessels and migration into the CNS (Dos Santos *et al.*, 2005; Ubogu *et al.*, 2006a). Other than recruiting peripheral leukocytes into brain parenchyma to mediate inflammatory responses, CCL-5 is also a potent mediator that induce secretions of other cytokines/chemokines to further perpetuate neuroinflammation (Luo *et al.*, 2002; Škuljec *et al.*, 2011). It is robustly elevated among the cytokines and chemokines measured in experimental autoimmune encephalomyelitis (EAE) indicating its role in mediating neurological diseases (Brambilla *et al.*, 2009). Moreover, growing body of evidence supported the pathological role of CCL-5 in multiple sclerosis and Alzheimer's disease (Huang *et al.*, 2010; Van Veen *et al.*, 2007). Thus, CCL-5 expression may be a pertinent indicator of inflammation in CNS.

Andrographolide is the most potent anti-neuroinflammatory compound among three bioactive compounds tested in U373 glioblastoma

Many plants produce a vast number of bioactive compounds that protect them against microbial infection and herbivory. Some of these bioactive compounds also help defend against abiotic stresses like UV exposure (Padhye *et al.*, 2012). Many of these compounds have desired biological properties such

as anti-bacterial, anti-inflammatory, and anti-cancer making them prospective candidates for further development as therapeutics for human diseases. I started the study by examining three natural compounds; andrographolide, artesunate, and plumbagin which have been previously found to exhibit anti-inflammatory effects (Wang *et al.*, 2014; Xia *et al.*, 2004; Xu *et al.*, 2007). Andrographolide and plumbagin are both natural compounds extracted from plants. Andrographolide is a labdane diterpenoid derived from the herbaceous kalmegh plant, *Andrographis paniculata*. It is traditionally used in India, China, and other parts of Asia to treat a variety of ailments including tuberculosis, snake bites, respiratory tract, and urinary tract infections (Panossian *et al.*, 2002). Plumbagin is a simple hydroxy-naphthoquinone found in the root of *Plumbago zeylanica* with therapeutic properties against dyspepsia, diarrhea, and skin diseases (Sandur *et al.*, 2006). On the other hand, artesunate is a semi-synthetic derivative of artemisinin isolated from *Artemisia annua* (sweet wormwood) (Xu *et al.*, 2007). Artemisinin and its derivatives are considered a safe antimalarial agent with higher efficacies against parasites compared to chloroquine and pyrimethamine (Liu *et al.*, 2011). Therefore, World Health Organization has recommended it to be the first line treatment of severe malaria (Noubiap, 2014).

Preliminary toxicity study of andrographolide, artesunate, and plumbagin on U373 glioblastoma suggested that plumbagin had the greatest anticancer effect among the three compounds tested, substantially reducing U373 cell viability at 10 μM (Appendix 1). This is not surprising as anticancer properties of plumbagin had been well-described in various cancer cell lines (Gomathinayagam *et al.*, 2008; Sandur *et al.*, 2006). It also inhibited intestinal tumorigenesis in animal study (Sugie *et al.*, 1998). Mechanisms proposed on

how plumbagin exert its anticancer activities include NF-κB pathway inhibition (Ahmad *et al.*, 2008; Sandur *et al.*, 2006), ROS generation (Aziz *et al.*, 2008), and S-G2/M cell cycle arrest (Wang *et al.*, 2008a). Plumbagin was eventually excluded for further study when potentially cytotoxic concentrations (5 μM) failed to attenuate CCL-5 secretion. However, my data points to plumbagin as a potential anti-glioma agent which should be further studied.

High concentration and long incubation of andrographolide and artesunate could also induce cell cytotoxicity in U373 glioblastoma as demonstrated by LDH assay. Evident release of LDH was observed after 2 days of artesunate incubation, followed by andrographolide on the third day of incubation (Figure 3.4). Consistent with previous findings, both compounds exhibited anticancer properties (Liu *et al.*, 2011; Rajagopal *et al.*, 2003). Hence, treatment time of andrographolide and artesunate in U373 cells were kept within 2 days to prevent non-specific effects induced by cell cytotoxicity. When andrographolide and artesunate were compared side by side for their ability to attenuate NF-κB activation and CCL-5 secretion induced by hIL-1β and hTNF-α, andrographolide abrogated p65 phosphorylation and CCL-5 release to a greater extent than artesunate. This suggests that andrographolide is a more efficacious anti-neuroinflammatory compound compared to artesunate. While my group had recently demonstrated neuroprotective effects of andrographolide in rodent stroke model (Chan *et al.*, 2010), several studies found neurotoxic effects of artemisinins. *In vitro* studies reported neuronal cells susceptibility to artesiminins-induced toxicity but not in glioma cell (Wesche *et al.*, 1994). In addition, Smith *et al.* reported that neurite outgrowth is prohibited by artesunate in differentiating NB2a neuroblastoma (Smith *et al.*, 1997). *In vivo* studies

further confirmed neurotoxic properties of artesunate. Mice (Nontprasert *et al.*, 2000) and rats (Genovese *et al.*, 2000) administered with artesunate developed abnormalities in balance and coordination. Based on these evidences, andrographolide was therefore selected for further evaluation as an anti-neuroinflammatory agent.

Andrographolide abrogated cytokines (hIL-1β and hTNF-α) induced inflammatory responses in primary astrocytes

I supplemented U373 studies with rat primary astrocytes prepared by an adaptation of *A dissection and tissue culture manual of the nervous system* (Cole *et al.*, 1989). This is an established procedure producing primary culture with purity more than 98% (Mcleod *et al.*, 1995; Strange *et al.*, 1994). Viability assay confirmed that andrographolide did not affect primary astrocyte viability even up to 2 days of treatment. Primary astrocytes were maintained in 10% FBS supplemented media. As high concentration of protein, albumin, and growth factors present in FBS may interfere with antigen-antibody binding in ELISA for detection of secreted CCL-5, it was replaced with 0.5% FBS supplemented media during drug/cytokine treatment. Cell viability assay was also performed to ensure lowering FBS in culture media does not affect cell viability of primary astrocytes (Appendix 2). Moreover, I verified that andrographolide treatment alone did not induce CCL-5 secretion (Appendix 3).

Andrographolide pretreatment significantly inhibited CCL-5 secretion and NF-κB activation induced by IL-1β and TNF-α in a dose dependent manner. Interestingly, andrographolide treatment attenuated CCL-5 release even when

it was administered 4 hours after exposure to cytokine stimulants. This implies that andrographolide does not require preincubation in order to observe its anti-neuroinflammatory effects. However, it should be noted that andrographolide has a therapeutic time window and no CCL-5 reduction was detected when it was given 8 hours after stimulation. Nonetheless, when an inflammatory response is unresolved after initiation, inflammatory mediators like IL-1β and TNF-α are continuously produced/present in the brain parenchyma and will continue to induce cytokine release that further perpetuates inflammatory responses. Thus, administration of andrographolide may be beneficial even hours after initiation of neuroinflammation due to brain injury or infection.

Molecular mechanisms underlying andrographolide's anti-inflammatory action

Andrographolide remarkably abated CCL-5 secretion stimulated by cytokines but the underlying mechanisms remain unclear. Since andrographolide effectively attenuated activation of NF-κB and CCL-5 is a known gene target of NF-κB, I proposed that inhibition of NF-κB leads to reduction of CCL-5 release. I then used TPCK, a known NF-κB inhibitor in parallel experiments to show the cytokine-induced CCL-5 secretion can indeed be ameliorated by p65 dephosphorylation/deactivation. This suggests that andrographolide's inhibitory effects on CCL-5 secretion in astrocytes may at least be partially mediated through NF-κB inactivation (Lim *et al.*, 2012b). However, the detailed molecular mechanisms have yet been fully elucidated and may be controversial. For instance, Xia et al. (2004) reported that

andrographolide formed a covalent adduct with p50, but did not suppress either the degradation of inhibitory IκBα or the nuclear translocation of p50/p65; processes initiated by phosphorylation of IκBα and p65 which lead to activation of NF-κB-mediated transcriptional activities (Jiang *et al.*, 2003; Traenckner *et al.*, 1995). In contrast, my group previously demonstrated that andrographolide suppressed p65 nuclear translocation in rat brain (Chan et al., 2010). I also demonstrated in this study that andrographolide reduced phosphorylation of both IκBα and p65, in agreement with Zhu *et al.* (Zhu *et al.*, 2013). In my study, IκBα phosphorylation was only occasionally accompanied by detectable degradation of IκBα (Fig. 3.7B), which mainly due to differences in the time course of IL-1β- versus TNF-α-mediated signaling events although both cytokines activate NF-κB through a relatively similar pathway (Figure 1.2). TNF-α mediated IκBα degradation was evident after 10 minutes of incubation while IL-1β activated IκBα degradation was detected slightly later (Figure 3.2 B & D). As IκBα degradation occurs after its phosphorylation and IL-1β/TNF-α stimulation time was decided based on detection of p65 and IκBα phosphorylation rather than IκBα degradation, a relatively short stimulation time was usually chosen. Thus, reduction in total IκBα level may or may not be observed in such case and inhibitory effects of andrographolide on IκBα degradation could not be reliably assessed. Nonetheless, I showed that phospho-IκBα/IκBα ratios were consistently decreased by andrographolide pre-treatment and on the occasions where cytokine-stimulated IκBα degradation was detectable, andrographolide was able to reverse this process (Figure 3.7 B). Conversely, Hsieh *et al.* showed in rat vascular smooth muscle cells that andrographolide reduced p65 phosphorylation in a protein phosphatase 2A-

dependent manner, but did not affect IκBα phosphorylation and degradation (Hsieh *et al.*, 2011). Taken together, these results suggested that andrographolide's anti-inflammatory effects likely involve multiple actions on NF-κB which may vary depending on cell-types and stimuli used.

Andrographolide attenuates reactive astrogliosis

Neurodegenerative diseases, traumatic brain injury, infection, and ischemia stroke release molecules like misfolded protein Aβ, proinflammatory cytokines, endotoxins, glutamate, and ROS which are known to result in astrogliosis (Rossi, 2015; Sofroniew, 2009). Although reactive astrogliosis may serve some beneficial functions, it is often being viewed in a negative light due to its deleterious role in mediating various neurological diseases with production of proinflammatory cytokines, promote leukocyte infiltration, induce oxidative stress, release of excitotoxic glutamate, and induce BBB breakdown (Chen *et al.*, 2003; Farina *et al.*, 2007; Rosenberg *et al.*, 2001; Sheng *et al.*, 2013). One of the hallmarks of reactive astrogliosis observed in human pathology and animal experimental models is upregulation of GFAP intermediate filament and the elevation of GFAP is known to correspond to the extent of astrogliosis (Eng *et al.*, 1994). Thus, GFAP has become a prototypical marker for reactive astrogliosis and often used to detect astrocyte activation (Eng *et al.*, 2000). In my study, exposure to IL-1β increased GFAP expression which is attenuated with pretreatment of andrographolide as shown in immunofluorescence and immunoblots. This suggests andrographolide’s capability to abrogate reactive astrogliosis.

When using rabbit polyclonal antibody (from Sigma) for GFAP immunoblots, multiple bands were observed with a prominent band detected at 50 kDa. This is likely due to the detection of various GFAP isoforms by the polyclonal antibody. To date, a number of GFAP isoforms has been identified. The transcript level of various isoforms in mouse brain has been determined; with the well-established GFAP-α being the most abundant isoform expressed (100%) followed by GFAP-δ (7.9%), GFAP-ζ (4.5%), GFAP-κ (1.0%), GFAP-γ (0.3%), and GFAP-β (0.008%) (Kamphuis *et al.*, 2012). GFAP-α has nine exon which encodes for a 50 kDa protein that appear as the most prominent band in immunoblots while GFAP-β has an alternative upstream transcriptional start site in the 5'UTR (Kamphuis *et al.*, 2012). GFAP-δ and GFAPκ isoforms are encoded by alternative splice variant of the GFAP gene (Blechingberg *et al.*, 2007). Interestingly, GFAP-δ is found highly expressed in subventricular zone and rostral migratory stream in both human and mice brain and probably play a role in modulating intermediate filament cytoskeleton properties (Roelofs *et al.*, 2005). However, little is known about the role of the other isoforms as they are only beginning to be investigated.

Astrocyte isolation and primary cultures have been used for decades to study astrocyte's roles and functions in healthy or diseased brain. However, these isolated astrocytes maintained in the conventional two-dimensional culture systems could lose some features found in the *in vivo* cells. These include reduced morphological complexity and undesired baseline reactivity of astrocytes upon *in vitro* culture (Lange *et al.*, 2012; Puschmann *et al.*, 2013). Consistent with these observations, primary astrocyte immunofluorescence staining of GFAP did not exhibit complex morphology of astrocytes with long

cellular processes commonly observed *in vivo*. Moreover, relatively high basal expression of GFAP was detected in the untreated cell. This undesired baseline reactivity of astrocytes could potentially affect the study of induced-astrogliosis. Therefore, effects of andrographolide on astrogliosis was further evaluated in animal model of neuroinflammation which will be discussed in the next chapter.

CHAPTER 4: EFFECTS OF ANDROGRAPHOLIDE IN LPS-MEDIATED NEUROINFLAMMATION

4.1 Results

4.1.1 LPS induced neuroinflammation in primary astrocytes

Earlier, I used cytokines (IL-1β and TNF-α) to model conditions where cytokines are released to initiate and perpetuate neuroinflammation. Here, I aimed to expand my investigations into andrographolide's ability to regulate neuroinflammation under bacterial/meningeal infection by using lipopolysaccharide (LPS), a polyglycan found in the outer membrane of gram-negative bacteria. It is a prototypical endotoxin used to elicit strong immune responses in mammalian cells.

4.1.1.1 LPS caused little or no effects on primary astrocyte cell viability

Cytotoxic dosage of LPS was first determined before subsequent experiments. Primary astrocytes were incubated with LPS for up to two days and cell viability was measured with MUSE® viability assays. Figure 4.1 showed that LPS caused little or no decreases in primary astrocytes cell viability at doses up to 1 μg/ml.

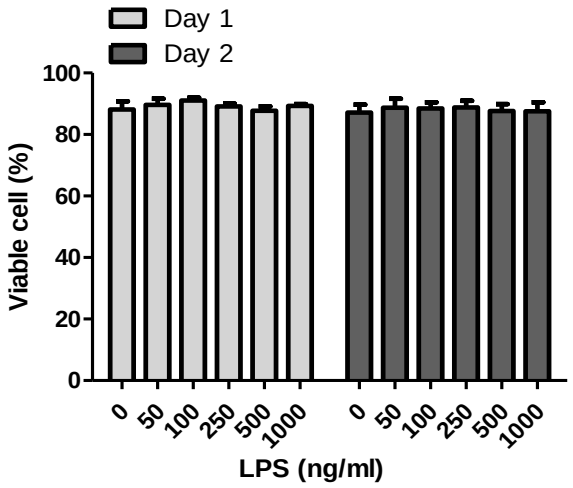
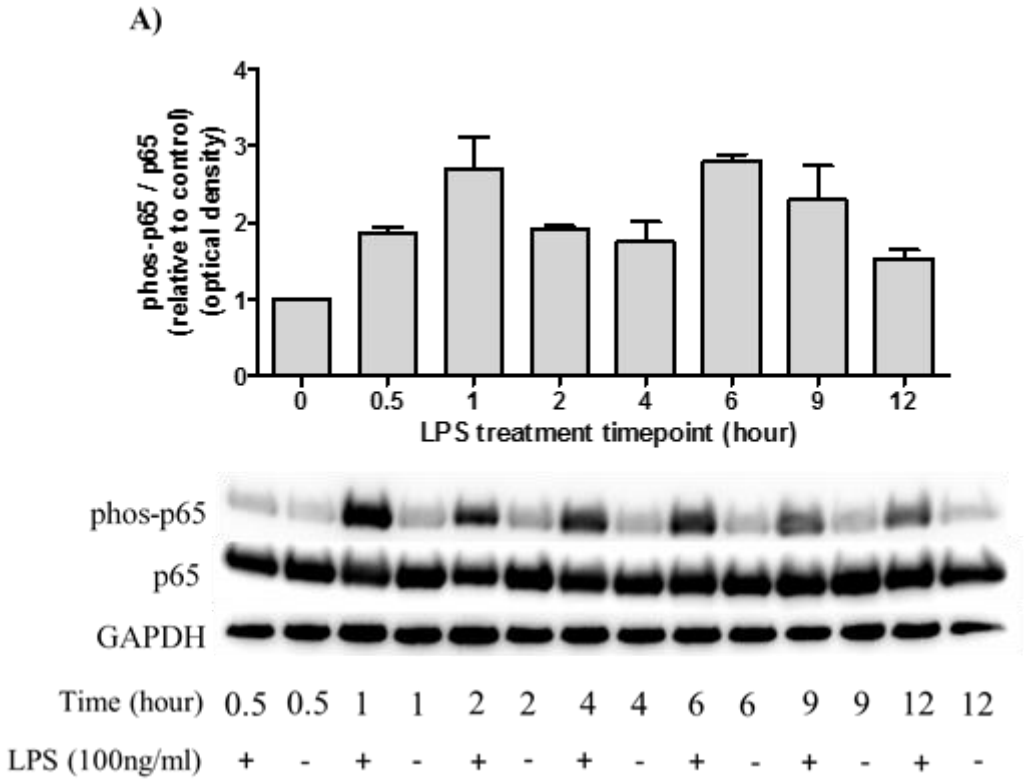


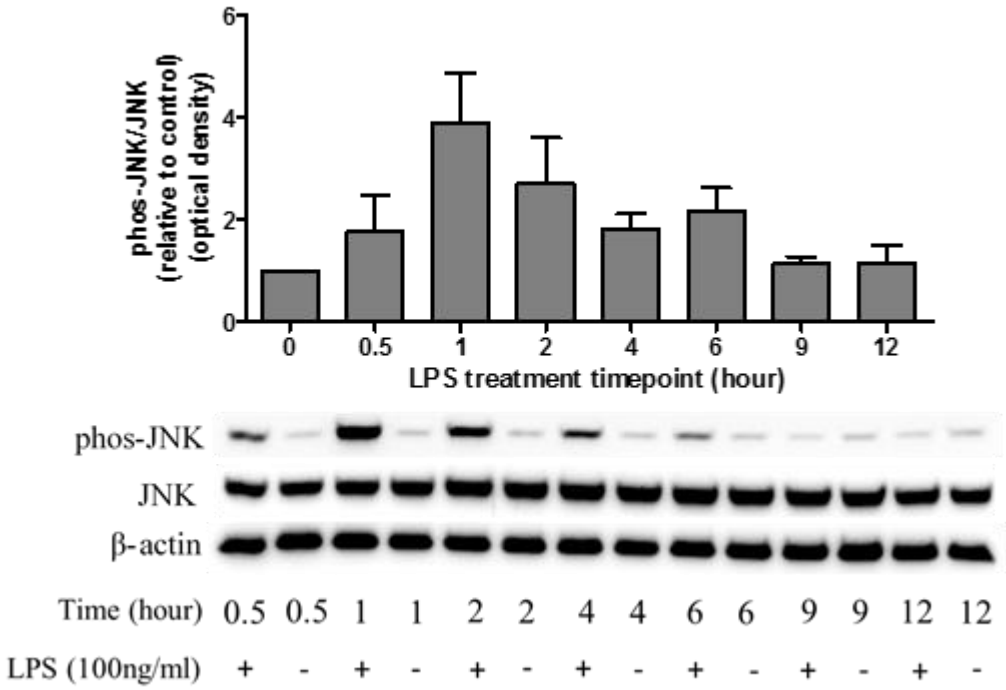
Figure 4.1 LPS effects on primary astrocytes cell viability. Data shown are mean ± S.E.M. Sample size n = 4. LPS dose effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests.

4.1.1.2 LPS activated NF-κB, JNK, ERK, and p38 pathways in primary astrocytes

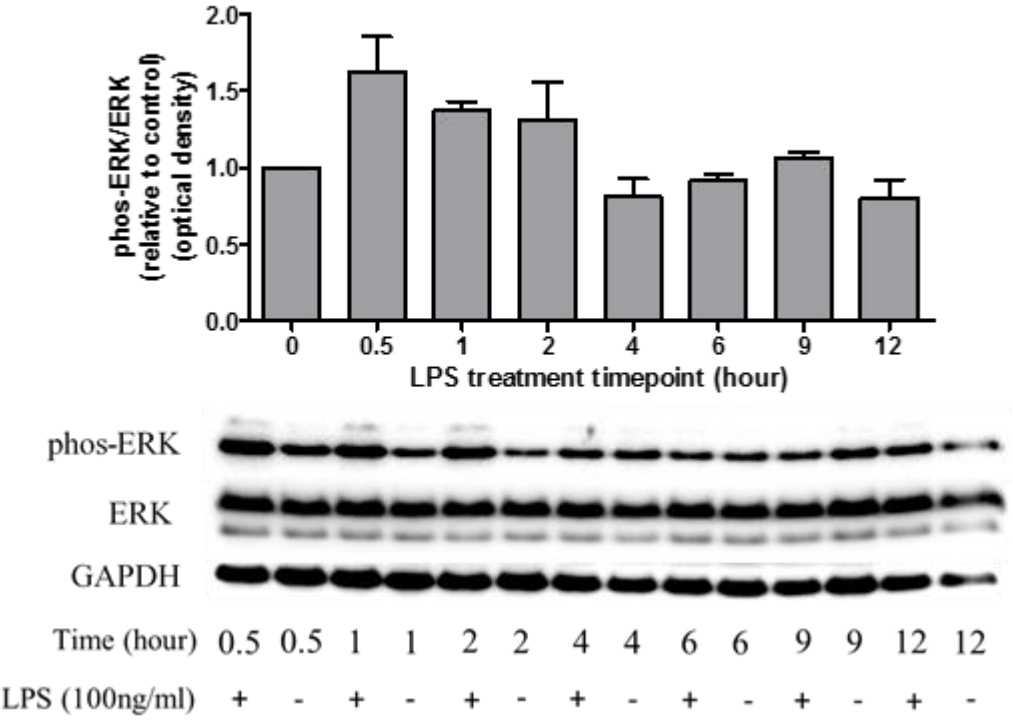
LPS is known to activate many signaling pathways including NF-κB and mitogen-activated protein kinases (MAPK) whose family comprises of c-Jun N-terminal kinases (JNKs), extracellular-signal-regulated kinases (ERKs), and p38 MAPK. Treatment of primary astrocytes with LPS at various time-points indicated that optimal activation of NF-κB, JNK, and p38 pathways occur mostly at 1 hour of stimulation while ERK activation occurs within half an hour stimulation (Figure 4.2). Furthermore, LPS stimulation for 12 hours in primary astrocytes induced mRNA expression of several cytokines/chemokines (Appendix 5).



B)


phos-JNK/JNK
(relative to control)
(optical density)
0
2
4
6
0
0.5
1
2
4
6
9
12
LPS treatment timepoint (hour)
phos-JNK
JNK
β-actin
Time (hour) 0.5 0.5 1 1 2 2 4 4 6 6 9 9 12 12
LPS (100ng/ml) + - + - + - + - + - + - + -

C)


phos-ERK/ERK
(relative to control)
(optical density)
0.0
0.5
1.0
1.5
2.0
0
0.5
1
2
4
6
9
12
LPS treatment timepoint (hour)
phos-ERK
ERK
GAPDH
Time (hour) 0.5 0.5 1 1 2 2 4 4 6 6 9 9 12 12
LPS (100ng/ml) + - + - + - + - + - + - + -

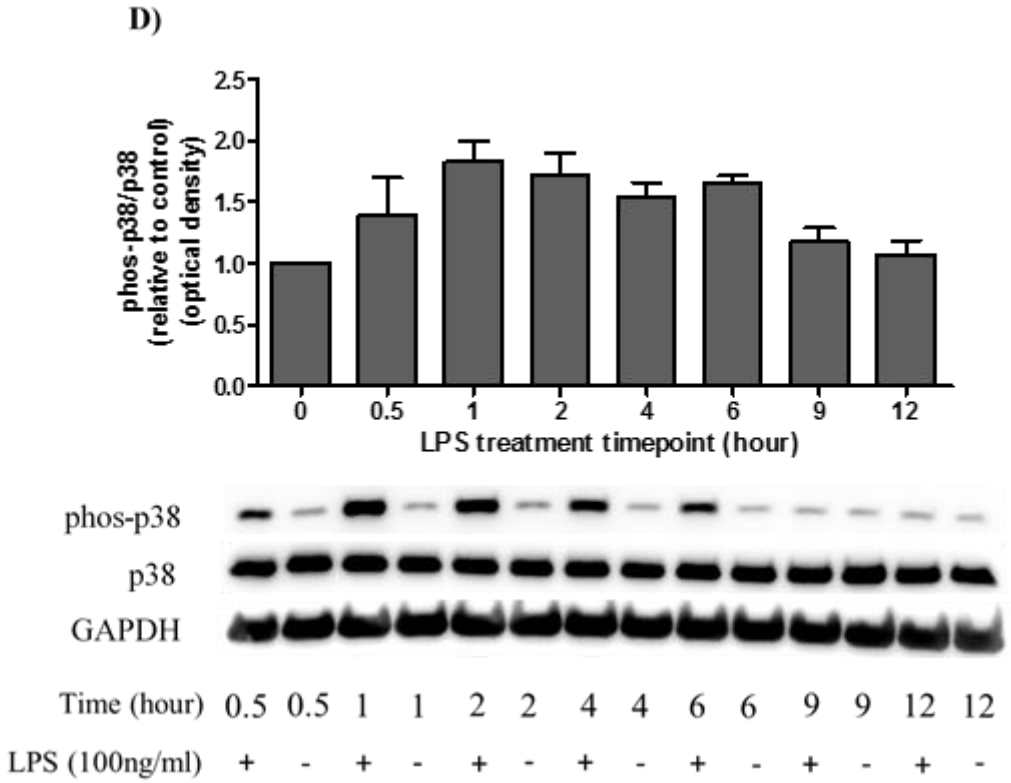
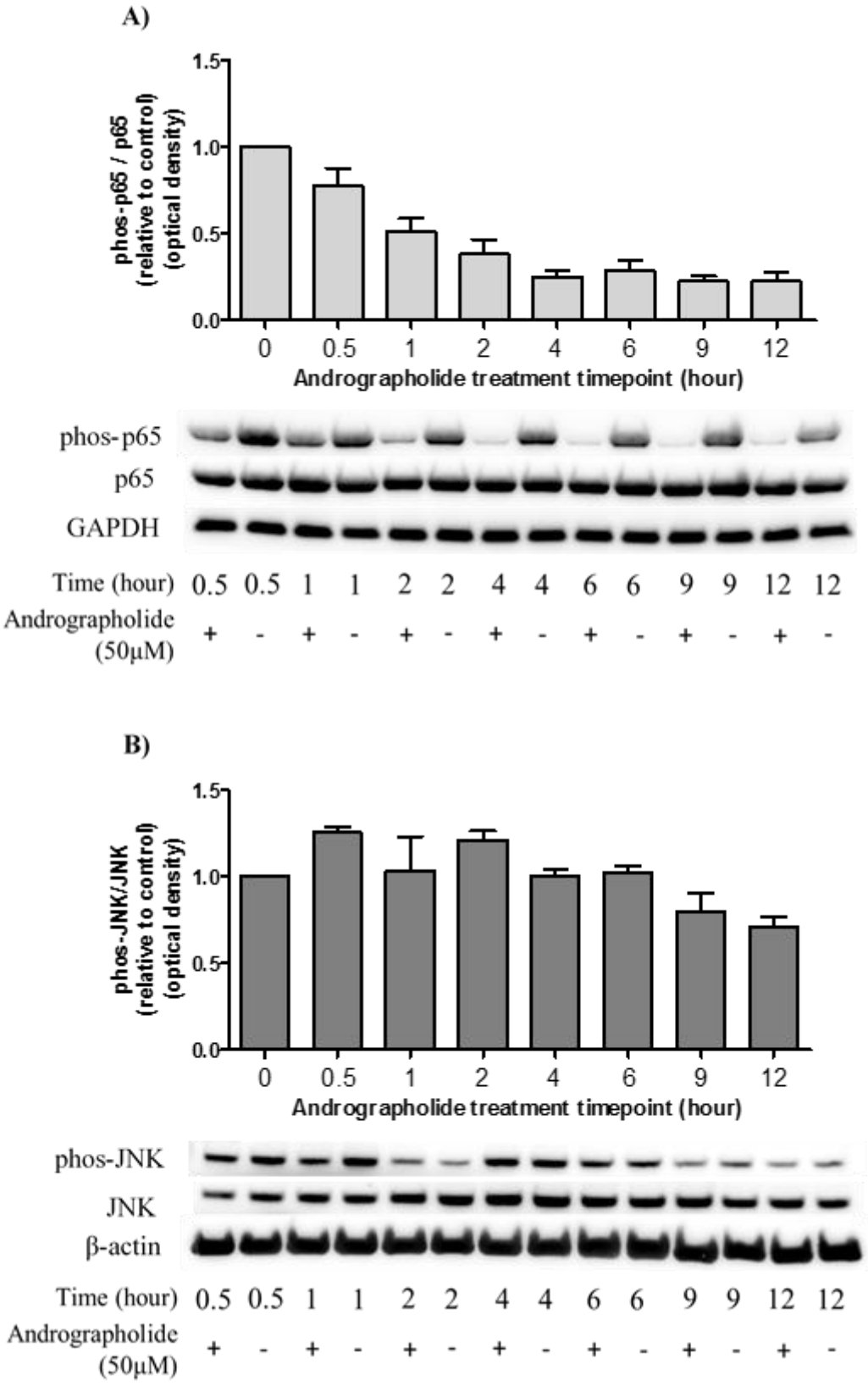


Figure 4.2 LPS activated NF-κB, JNK, ERK and p38 pathways. Primary astrocytes were treated with or without LPS for indicated timepoints. Whole-cell lysate collected were immunoblotted for phos-p65 and p65 **(A)**, phos-JNK and JNK **(B)**, phos-ERK and ERK **(C)**, or phos-p38 and p38 **(D)**. Sample size n = 3. Data shown are mean ± S.E.M.

4.1.2 Effects of andrographolide on NF-κB and MAPKs activation

Incubation of primary astrocytes with 50 μM andrographolide for various time-points revealed that andrographolide lowered the level of phosphorylated p65 while JNK phosphorylation remained unchanged. Interestingly, it enhanced phosphorylation of ERK and p38 (Figure 4.3). Dose-dependent activation of ERK and p38 by andrographolide became more apparent when primary astrocytes were treated with increasing concentration of andrographolide for 6 hours (Figure 4.4).


A)
phos-p65 / p65
(relative to control)
(optical density)
1.5
1.0
0.5
0.0
0
0.5
1
2
4
6
9
12
Andrographolide treatment timepoint (hour)
phos-p65
p65
GAPDH
Time (hour) 0.5 0.5 1 1 2 2 4 4 6 6 9 9 12 12
Andrographolide (50μM) + - + - + - + - + - + - + -
B)
phos-JNK/JNK
(relative to control)
(optical density)
1.5
1.0
0.5
0.0
0
0.5
1
2
4
6
9
12
Andrographolide treatment timepoint (hour)
phos-JNK
JNK
β-actin
Time (hour) 0.5 0.5 1 1 2 2 4 4 6 6 9 9 12 12
Andrographolide (50μM) + - + - + - + - + - + - + -

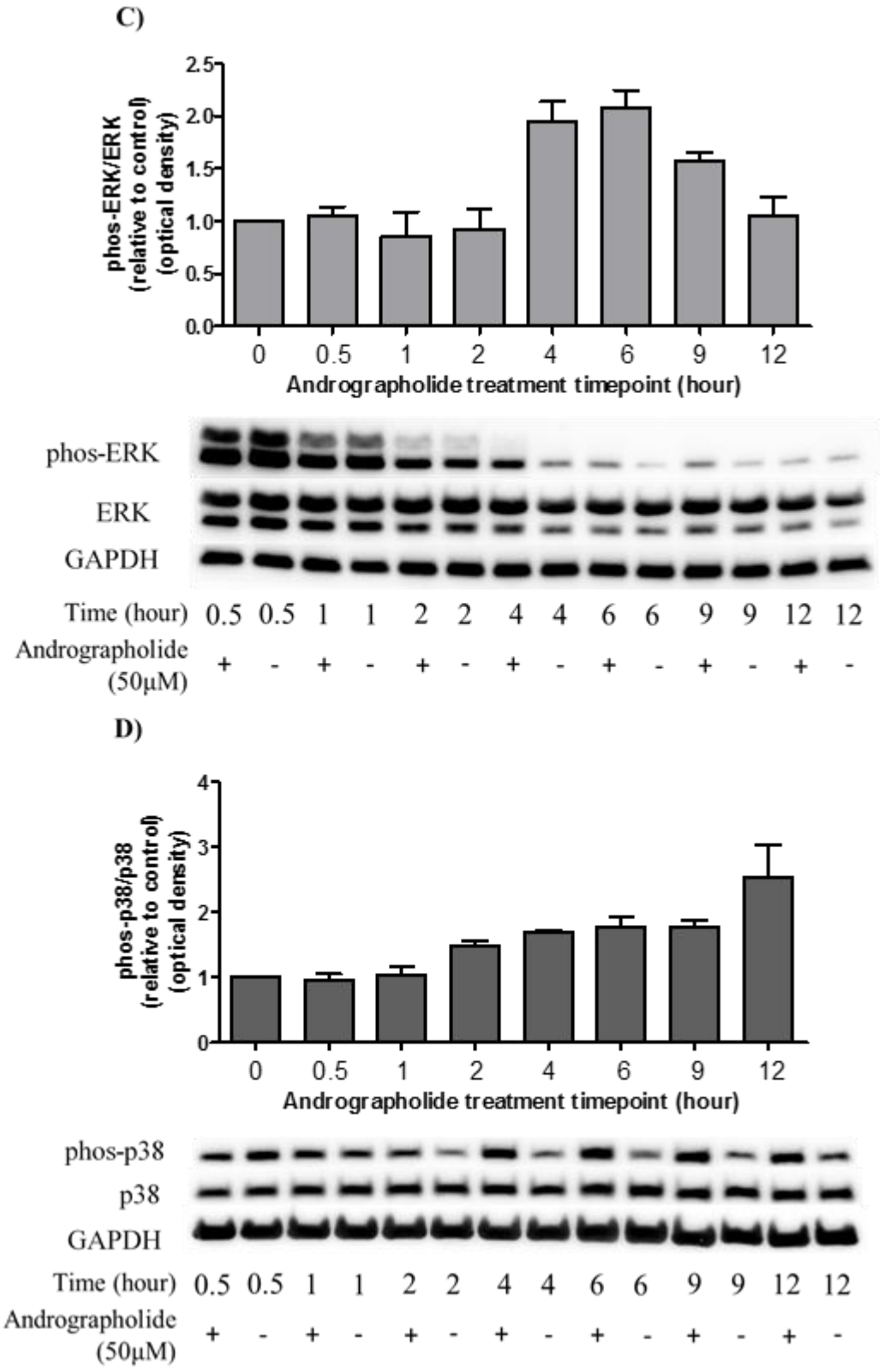


Figure 4.3 Incubation of andrographolide for various timepoints. Primary astrocytes were treated with or without andrographolide for indicated timepoints. Whole-cell lysate collected were immunoblotted for phos-p65 and p65 **(A)**, phos-JNK and JNK **(B)**, phos-ERK and ERK **(C)**, or phos-p38 and p38 **(D)**. Sample size n = 3. Data shown are mean ± S.E.M.

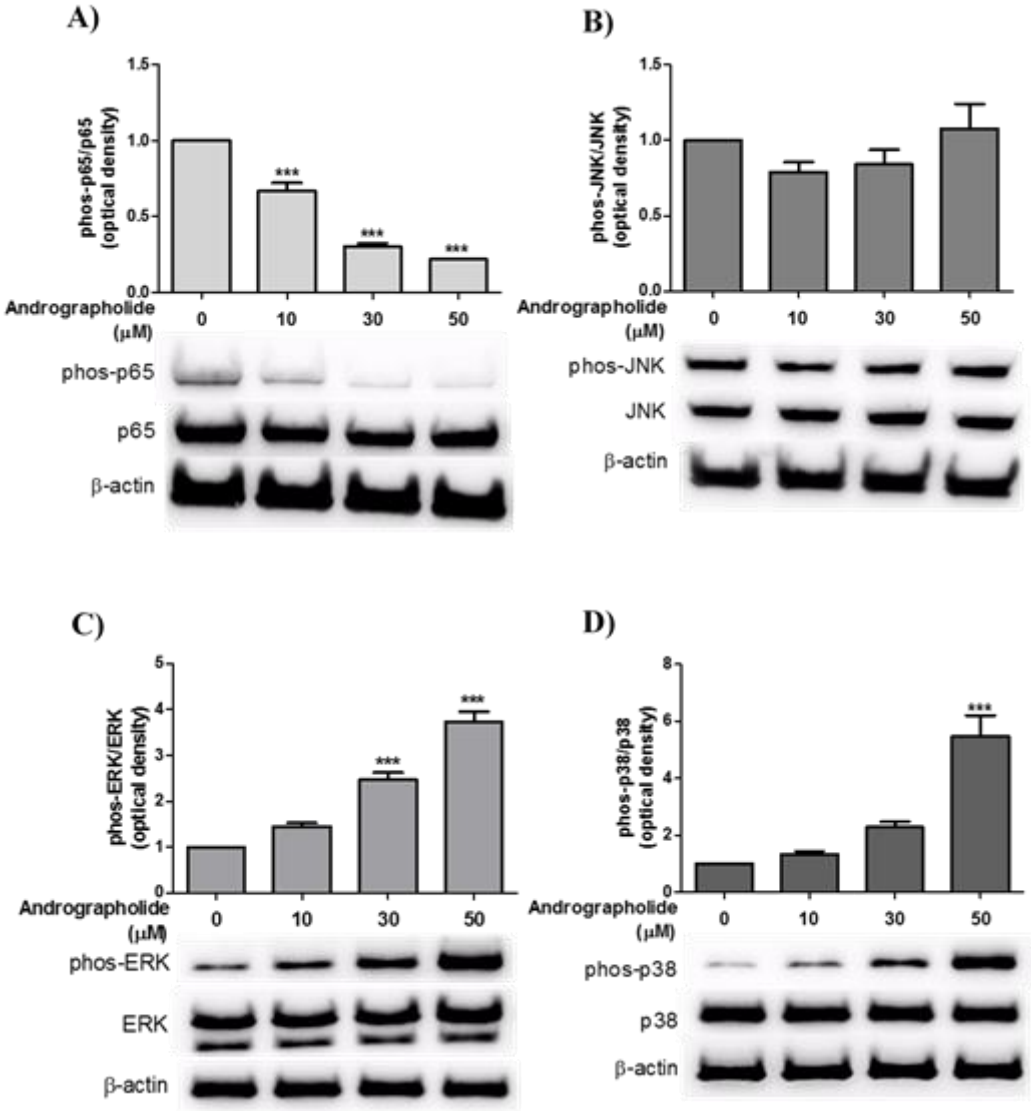


Figure 4.4 Dose effects of andrographolide on p65 and MAPKs activation. Primary astrocytes were treated with increasing concentration of andrographolide for 6 hours. Whole-cell lysate collected were immunoblotted for phos-p65 and p65 **(A)**, phos-JNK and JNK **(B)**, phos-ERK and ERK **(C)**, or phos-p38 and p38 **(D)**. Sample size n = 4. Data shown are mean ± S.E.M. Andrographolide dose effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (**p < 0.01 and ***p < 0.001)

4.1.3 Andrographolide attenuated LPS-activated NF-κB and JNK pathways

NF-κB plays an important role in regulationg immune response to infection and activation of JNK pathway promotes pro-inflammatory cytokines production and apoptosis (Kim *et al.*, 2010b; Viatour *et al.*, 2005). In order to assess effects of andrographolide in regulating these pathways, primary astrocytes were pretreated with andrographolide for 4 hours and stimulated with LPS for 1 hour (with presence of andrographolide) before processed for immunoblotting. Dose dependent reduction in phosphorylated phos-65, phos-IκBα and phos-JNK indicates that andrographolide substantially mitigated LPS-induced NF-κB and JNK activation (Figure 4.5).

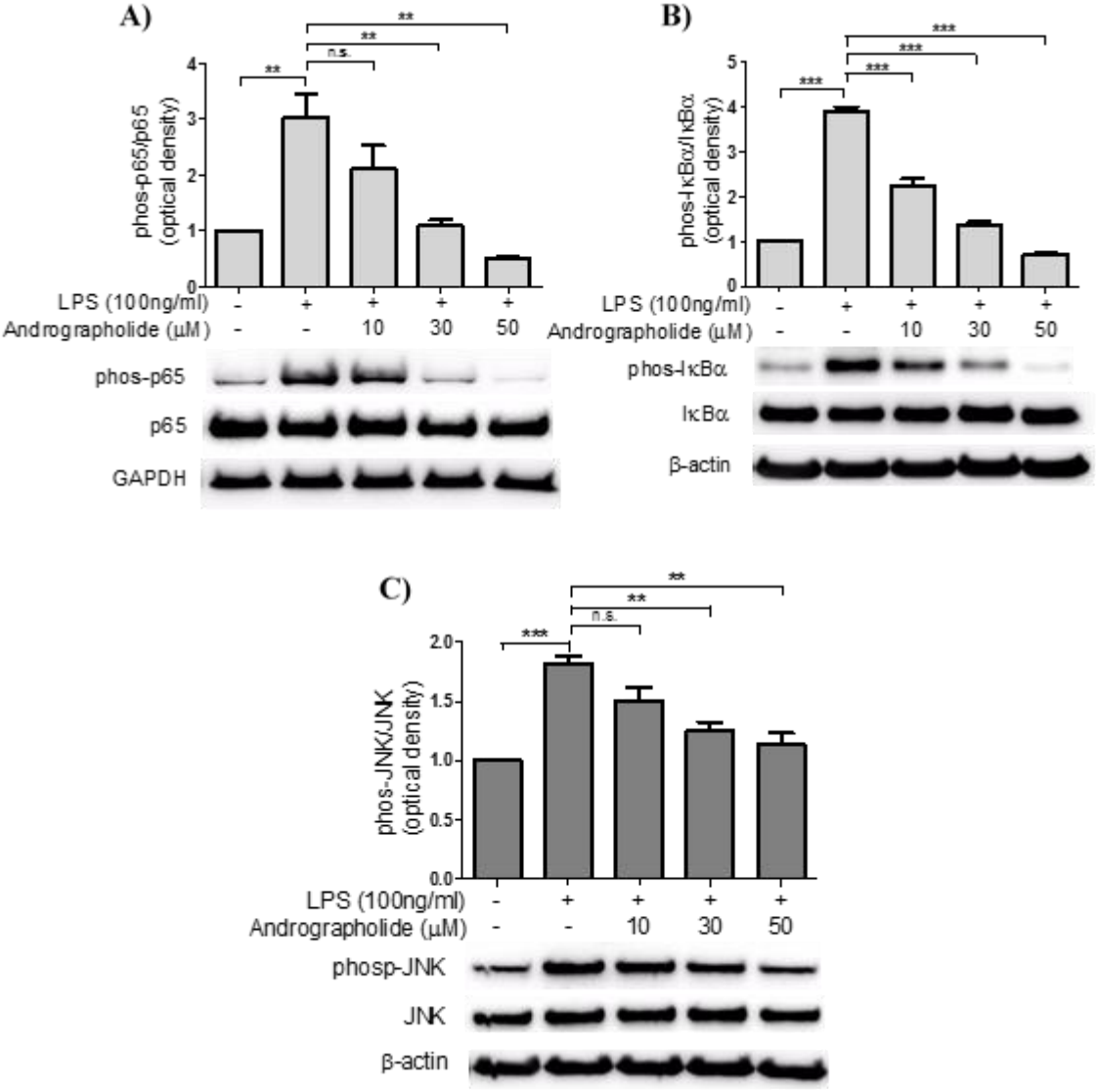


Figure 4.5 Andrographolide attenuated LPS-activated NF-κB and JNK. Primary astrocytes were pretreated with andrographolide for 4 hours followed by 1 hours LPS stimulation with the presence of andrographolide. Whole-cell lysate collected were immunoblotted for phos-p65 and p65 **(A)**, phos-IκBα and IκBα **(B)**, or phos-JNK and JNK **(c)**. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (**p < 0.01 and ***p < 0.001); ns = not significant (p > 0.05)

4.1.4 Andrographolide further enhanced LPS-activated ERK and p38 pathways

Earlier, I showed evidence of andrographolide treatment leading to increased phosphorylation of ERK and p38. Because LPS induces an inflammatory action which by itself could activate these kinases, I immunoblotted whole-cell lysates collected from primary astrocytes pretreated 4 hours with andrographolide followed by 1 hours LPS stimulation (in the presence of andrographolide) and found that andrographolide added to LPS-activated ERK and p38 pathways (Figure 4.6).

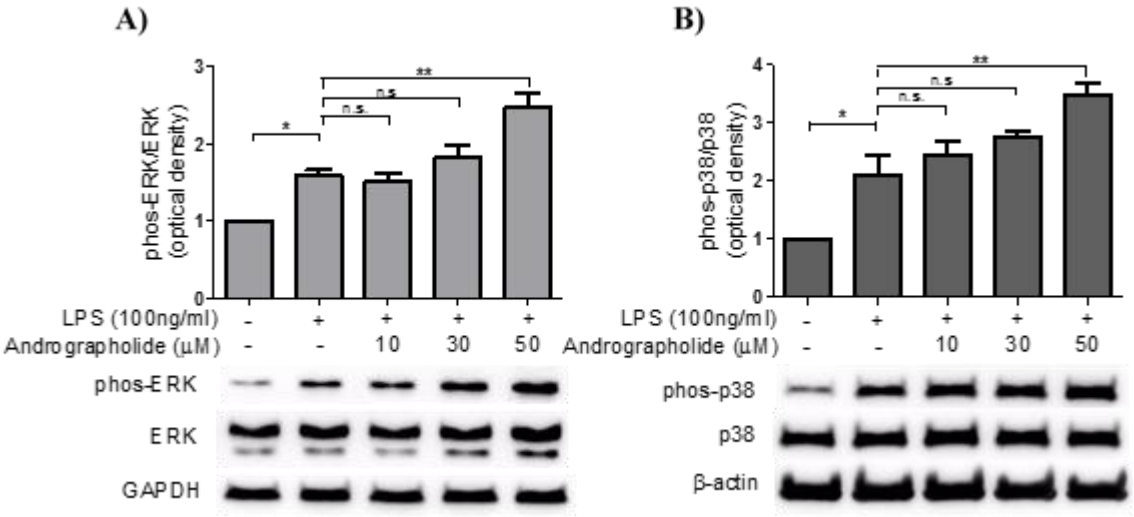
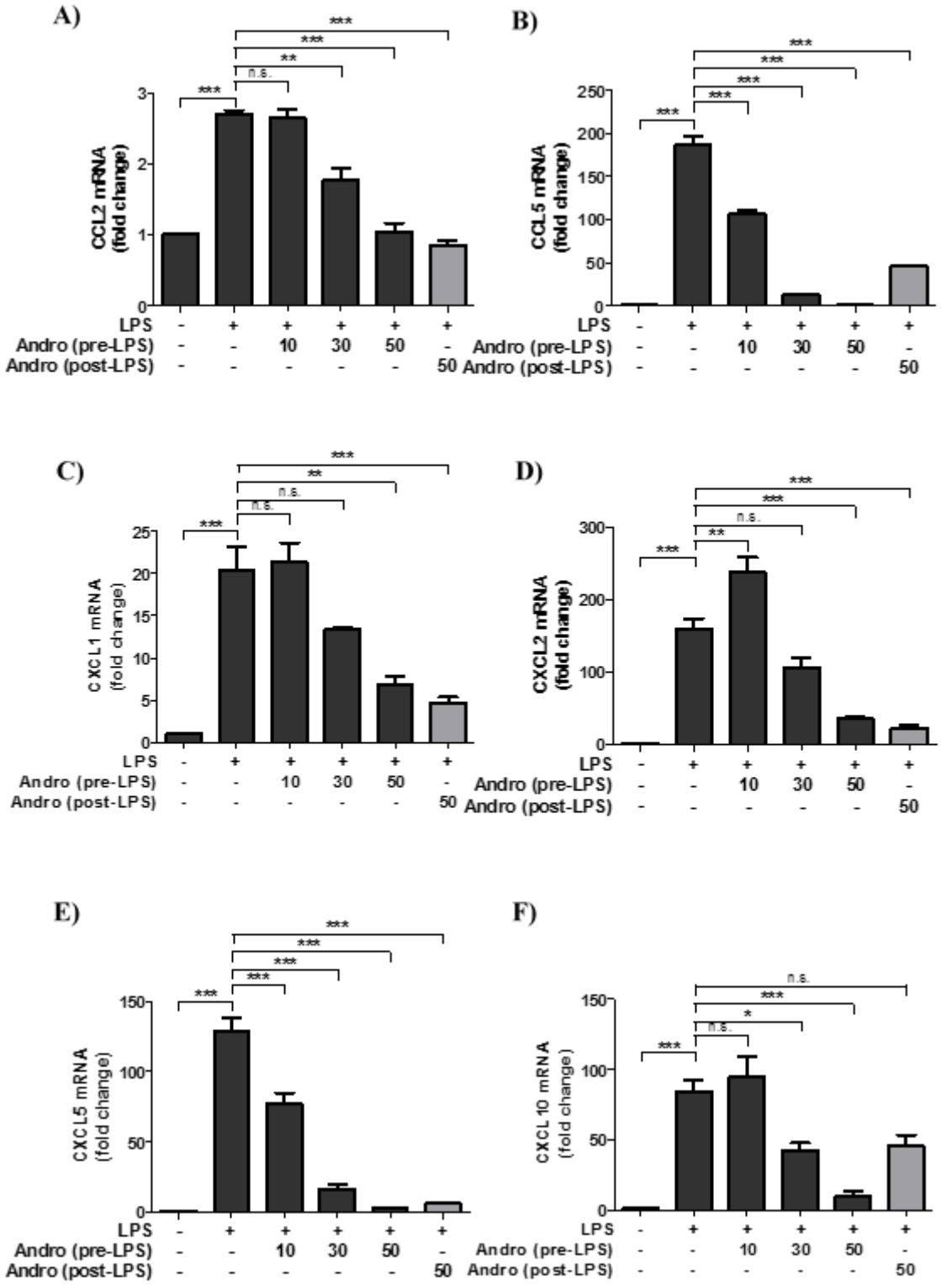


Figure 4.6 Andrographolide further enhanced LPS-activated ERK and p38. Primary astrocytes were pretreated with andrographolide for 4 hours followed by 1 hours LPS stimulation with the presence of andrographolide. Whole-cell lysate collected were immunoblotted for phos-ERK and ERK **(A)**, or phos-p38 and p38 **(B)**. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$ and **$p < 0.01$); ns = not significant ($p > 0.05$)

4.1.5 Andrographolide effects on various cytokines/chemokines expression induced by LPS

Next, given the signaling kinase and transcription factor activation results in upregulation of many inflammatory markers, the effects of andrographolide on several LPS-induced cytokines/chemokines was examined. Andrographolide was administered 4 hours before or after LPS stimulation; treatment was in 0.5% FBS supplemented media. Primary astrocytes were harvested for real-time PCR after 12 hours of LPS stimulation. Elevated mRNA levels of cytokines, IL-1β and TNF-α; CC motif chemokines (CCL-2 and CCL-5); CXC motif chemokines (CXCL-1, CXCL-2, CXCL-5, and CXCL-10); CX3C motif chemokine (CX3CL-1) were dose-dependently attenuated by andrographolide (Figure 4.7). Andrographolide also substantially reduced mRNA levels of all these cytokines/chemokines when it was administered 4 hours after LPS stimulation. Therefore, preincubation with andrographolide is not required in order to observe its anti-neuroinflammatory effects, in line with my previous finding on cytokine-stimulated CCL-5 secretion (see Chapter 3). Unexpectedly, andrographolide further enhanced IL-6 expression induced by LPS in a dose-dependent manner suggesting possible induction of IL-6 by andrographolide (Figure 4.7J). Follow up study with andrographolide incubation for increasing time revealed that indeed andrographolide alone could induce IL-6 expression (Figure 4.8).


A)
CCL2 mRNA (fold change)
B)
CCL5 mRNA (fold change)
C)
CXCL1 mRNA (fold change)
D)
CXCL2 mRNA (fold change)
E)
CXCL5 mRNA (fold change)
F)
CXCL10 mRNA (fold change)
LPS
Andro (pre-LPS)
Andro (post-LPS)
n.s.

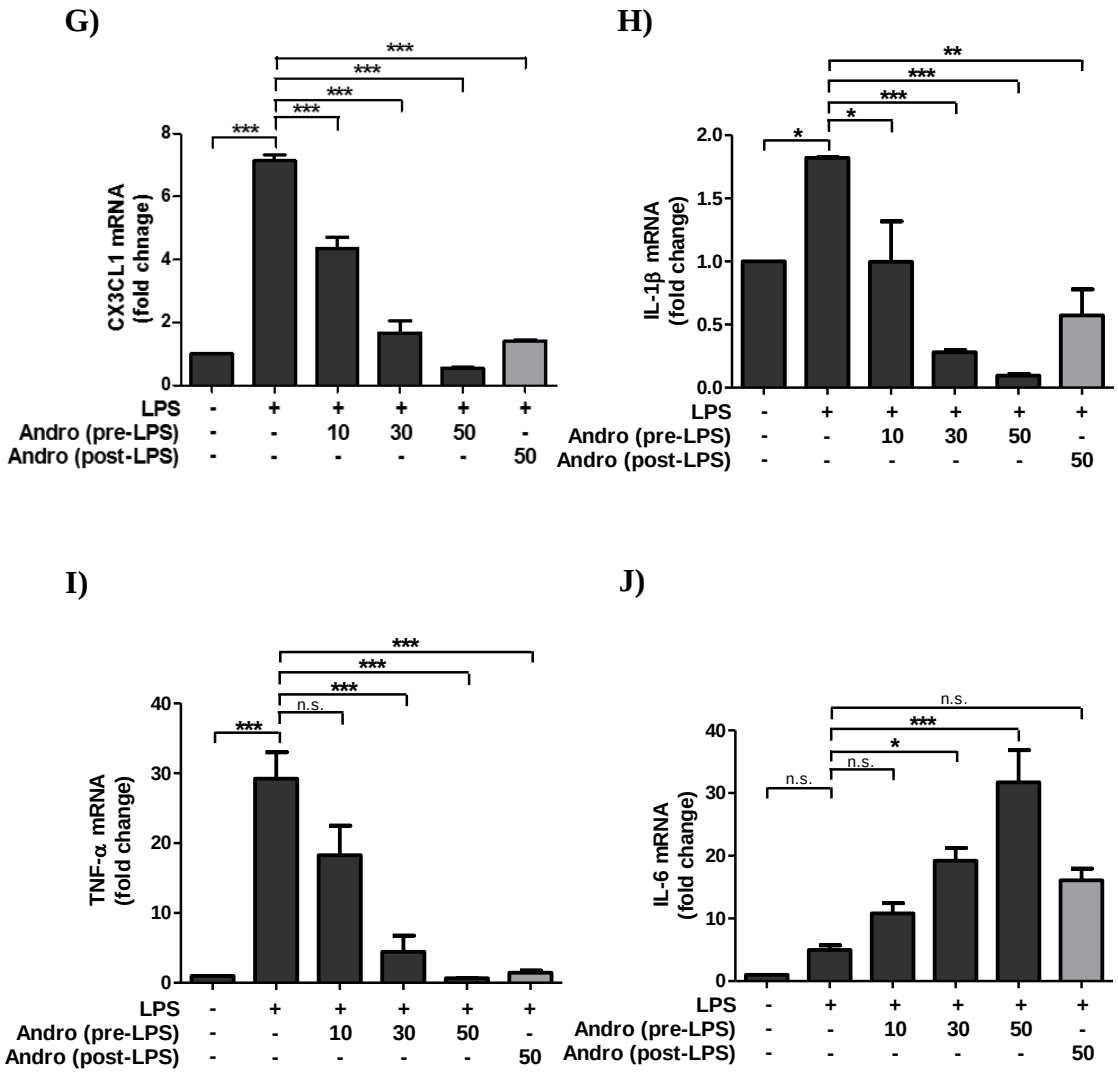


Figure 4.7 Andrographolide effects on LPS-induced cytokines/chemokines mRNA expression. Andrographolide was administered 4 hours before or after LPS stimulation. Primary astrocytes were harvested after 12 hours of 100 ng/ml LPS stimulation. Transcript level of CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-5, CXCL-10, CX3CL-1, IL-1β, TNF-α, and IL-6, were analyzed by real-time PCR. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$); ns = not significant ($p > 0.05$)

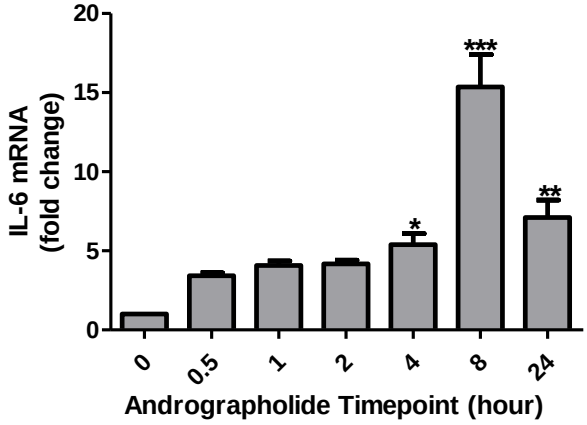
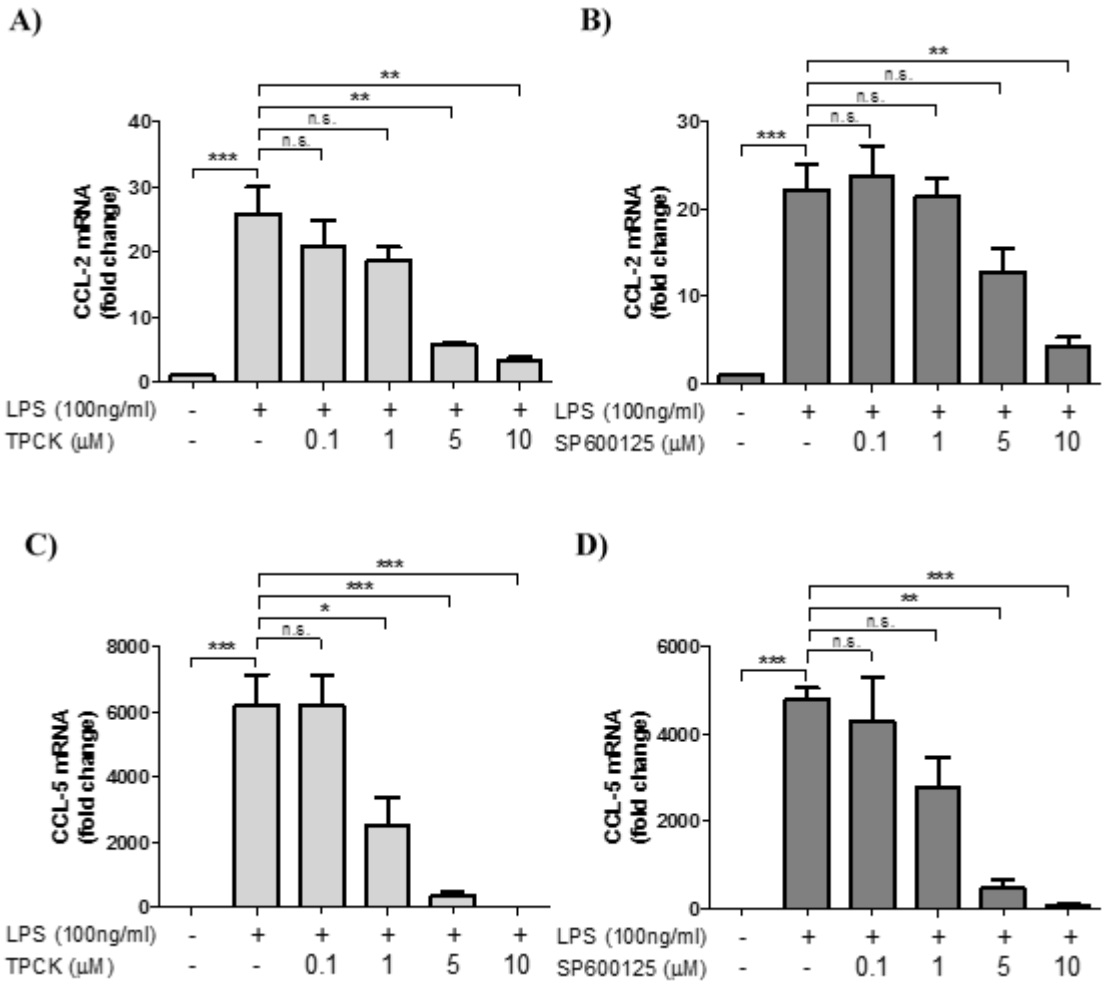


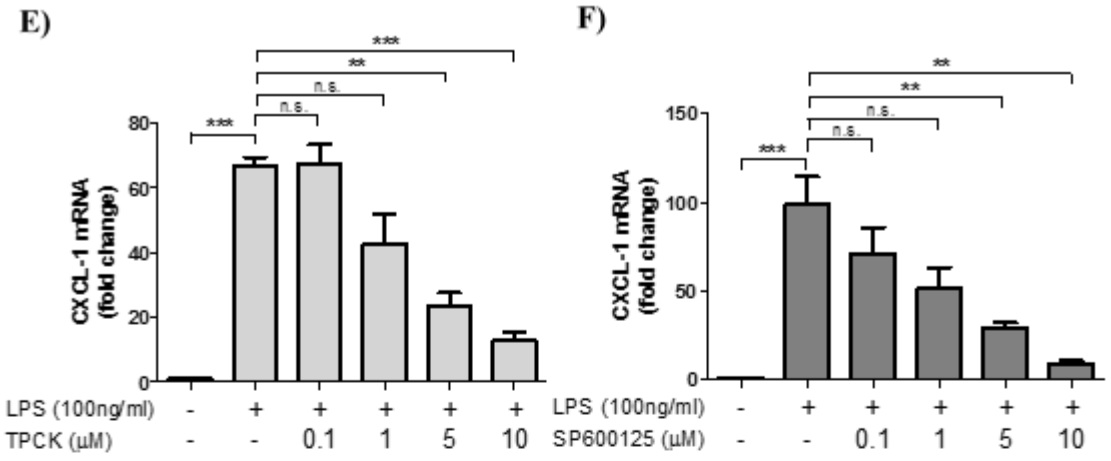
Figure 4.8 Andrographolide elevated IL-6 expressions in primary astrocytes. Primary astrocytes were incubated with andrographolide 50 μM for the indicated timepoints. Transcript level of IL-6 were analyzed by real-time PCR. Sample size n = 3. Data shown are mean ± S.E.M. Andrographolide time effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (*p < 0.5, **p < 0.01, and ***p < 0.001)

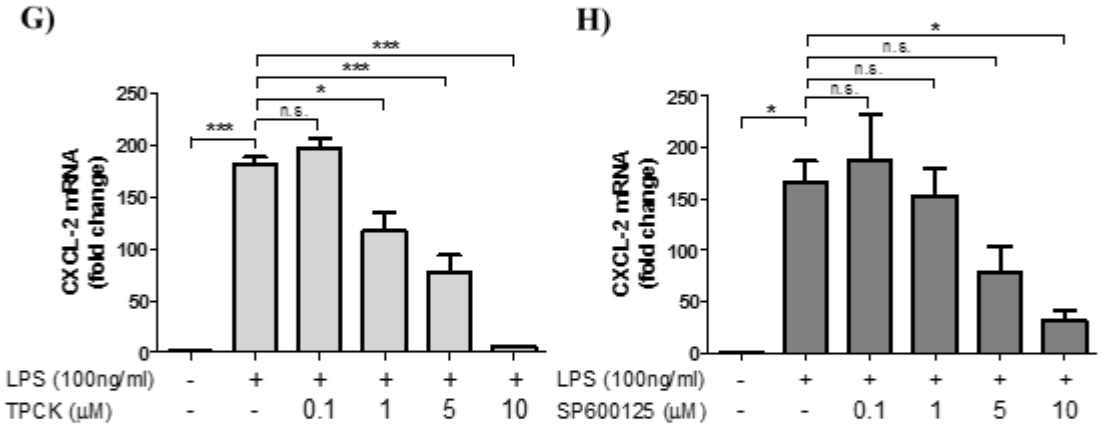
4.1.6 Inhibition of NF-κB and JNK by TPCK and SP600125 respectively decreased cytokines/chemokines expression induced by LPS

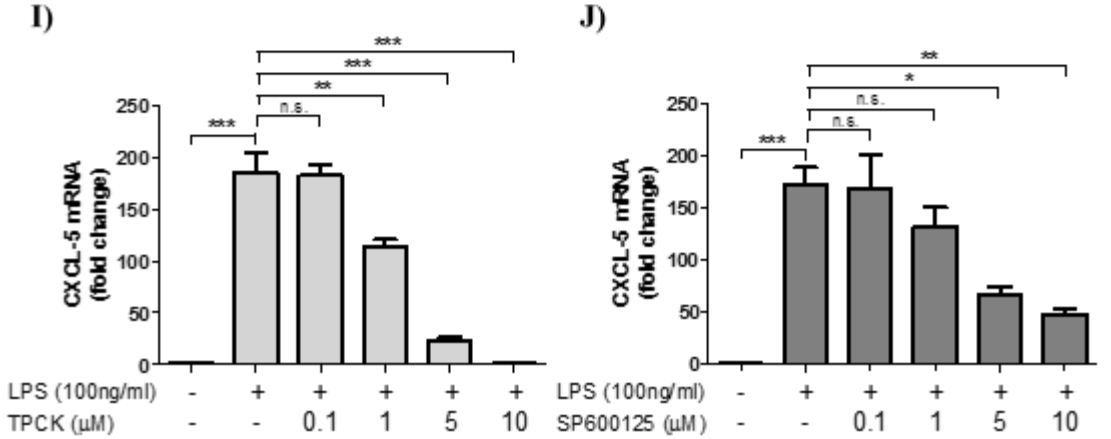
As previously reviewed, NF-κB plays a central role in inflammation through regulating expression of proinflammatory cytokines and mediators. JNK activation was involved in cytokines production including TNF-α and CCL-5 during inflammation (Oltmanns *et al.*, 2003). To understand role of NF-κB and JNK in LPS-induced cytokines/chemokines expressions, TPCK, an established inhibitor of NF-κB or SP600125, a JNK inhibitor with high selectivity for JNK-1/ -2/ -3 (Bennett *et al.*, 2001) were used. Primary astrocytes were incubated 1 hour with TPCK or SP600125, followed by 12 hours of LPS stimulation in 0.5% FBS supplemented media. Trizol®-lysed samples were processed for real-time PCR to quantify transcript levels of

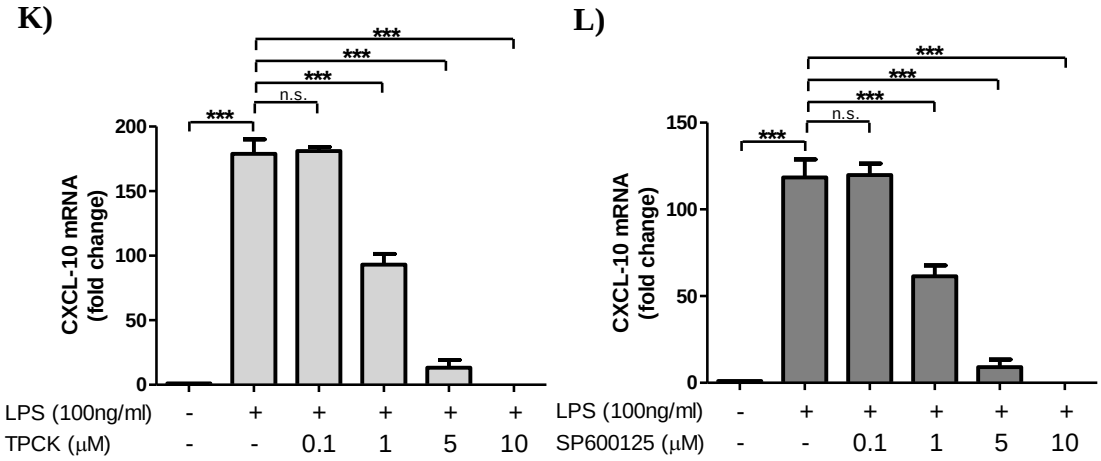
cytokines/chemokines. Majority of LPS-induced cytokines/chemokines were dose dependently diminished by TPCK and SP600125 (Figure 4.9). These may suggest that andrographolide could potentially inhibit NF-κB and JNK concurrently and result in partial if not complete reduction of cytokines expression. Results also demonstrated that NF-κB inhibitor could significantly reduce IL-6 expression level but not JNK inhibitor proposing that the JNK-mediated pathway may not be important player in inducing IL-6 expression (Figure 4.9 S & T). It is worthwhile noting that in contrast to NF-κB inhibitor that suppress IL-6 expression, andrographolide (which also inhibited NF-κB pathway) elevates IL-6 expression. This directs toward the involvement of other signaling pathways in andrographolide-mediated IL-6 expression.

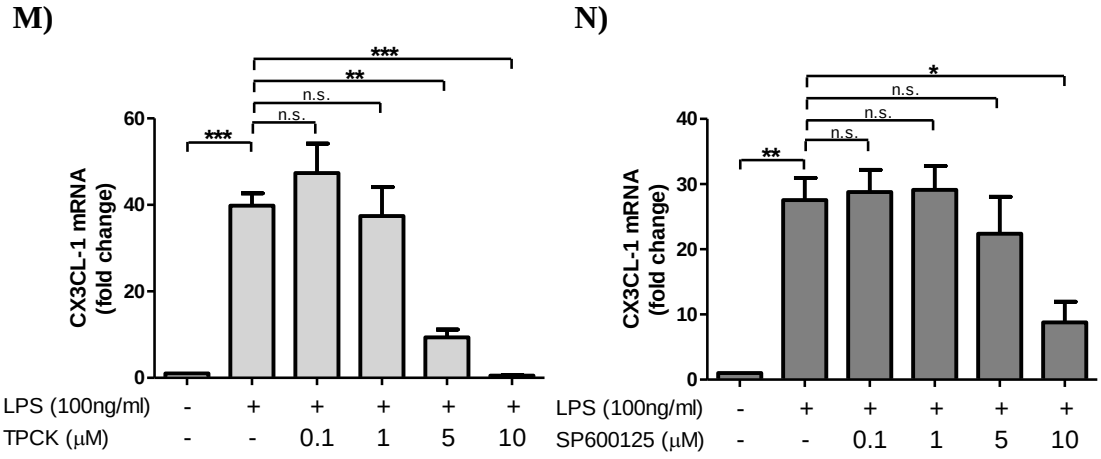


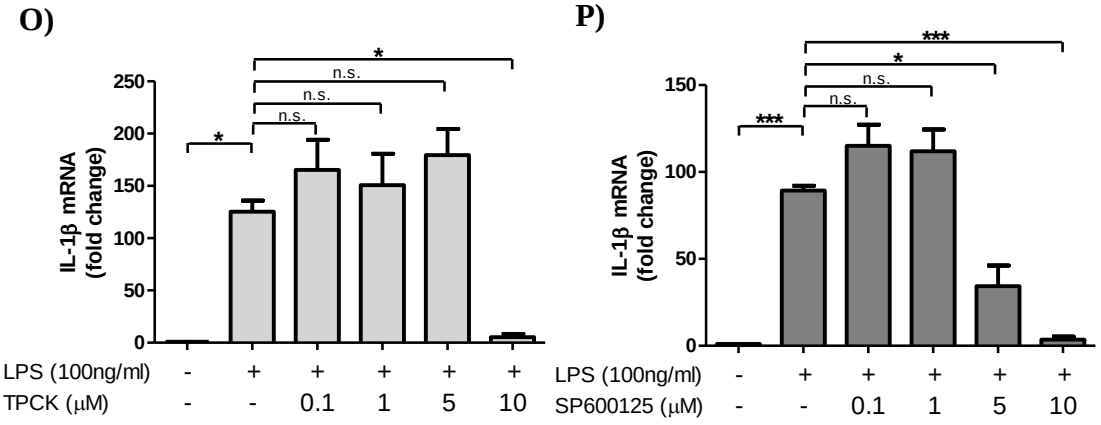

E)
CXCL-1 mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
F)
CXCL-1 mRNA (fold change)
LPS (100ng/ml)
SP600125 (μM)


G)
CXCL-2 mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
H)
CXCL-2 mRNA (fold change)
LPS (100ng/ml)
SP600125 (μM)


I)
CXCL-5 mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
J)
CXCL-5 mRNA (fold change)
LPS (100ng/ml)
SP600125 (μM)


K)
L)
CXCL-10 mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
SP600125 (μM)
n.s.
***


M)
N)
CX3CL-1 mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
SP600125 (μM)
n.s.
*
**
***


O)
P)
IL-1β mRNA (fold change)
LPS (100ng/ml)
TPCK (μM)
SP600125 (μM)
n.s.
*
***

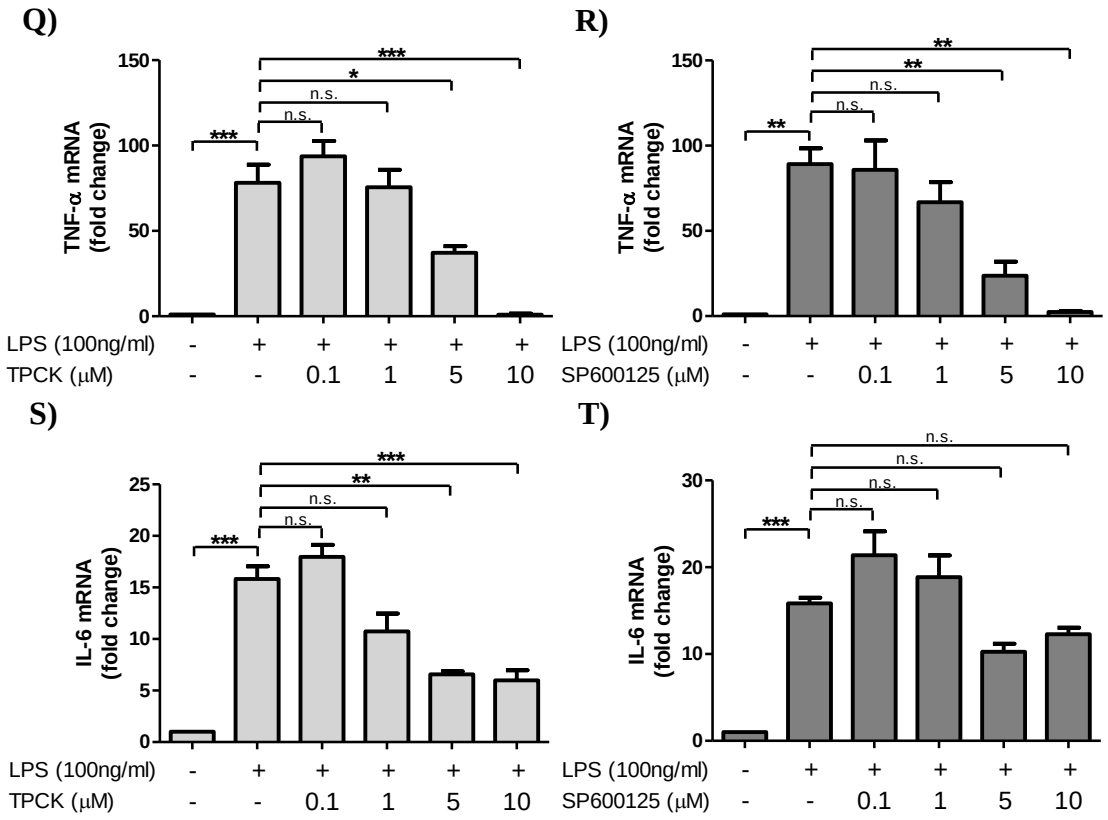


Figure 4.9 Effects of NF-κB and JNK inhibitors on LPS-induced cytokine/chemokines. Primary astrocytes were pretreated with NF-κB inhibitor, TPCK **(A, C, E, G, I, K, M, O, Q, S)** or JNK inhibitor, SP600125 **(B, D, F, H, J, L, N, P, R, T)** for 1 hours before 12 hours stimulation with LPS. Transcript level of CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-5, CXCL-10, CX3CL-1, IL-1β, TNF-α, and IL-6, were analyzed by real-time PCR. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*p < 0.05, **p < 0.01, and ***p < 0.001); ns = not significant (p > 0.05)

4.1.7 Andrographolide inhibited chemokines expression in LPS-mediated neuroinflammation mouse model

As cultured cells lack tissue phenotype and the resulting complex interactions among different cell types, I followed up the primary astrocyte work with a LPS-treated mouse model (IACUC approval S13-6210). Increasing

evidence indicated that infection-induced peripheral inflammation could lead to neuroinflammation and neurodegeneration (Biesmans *et al.*, 2013; Jeong *et al.*, 2010; Qin *et al.*, 2007). To assess potentially beneficial effects of andrographolide on neuroinflammatory responses, ICR mice were subjected to 3 intraperitoneal injection of LPS (3mg/kg) and followed by oral gavage of vehicle or andrographolide (25mg/kg or 50mg/kg) 1 hour after each injection (refer to Material and Methods for detail descriptions on mouse treatment regime). Mice were sacrificed 5 hours after the last injection, and the cortex was dissected, homogenized and processed for Luminex assay or immunoblots. Expression of several chemokines including CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-9, CXCL-10 were elevated in the brains of LPS-injected animals, and all measured cytokines except CXCL-10 were considerably abated by andrographolide (Figure 4.10 A-E). Andrographolide gavage alone (up to 100mg/kg) did not induce any cytokine expression (data not shown). Though a decreasing trend was observed in the expression of CXCL-10, it did not reach statistical significance (Figure 4.10 F). IL-1β and TNF-α expression in the brain were measured but no significant difference was detected among the different treatment groups (data not shown).

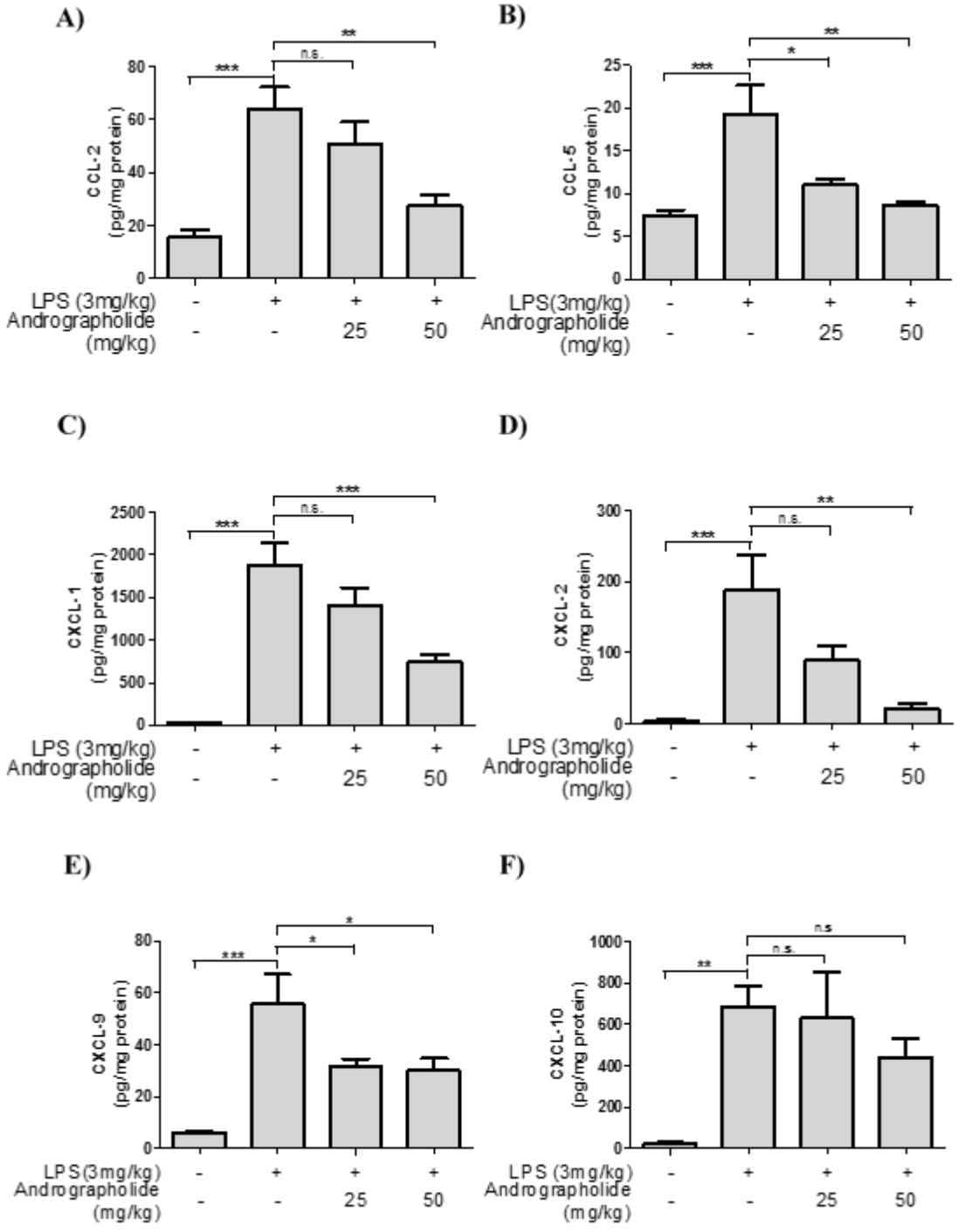


Figure 4.10 Andrographolide effects on LPS-mediated cytokines/chemokines expression in mouse brain. Data shown were derived from Luminex assays and are mean ± S.E.M. (control, n = 9; LPS, n = 7; LPS + andrographolide 25 mg/kg, n = 8; LPS + andrographolide 50 mg/kg, n = 7). Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*p < 0.05, **p < 0.01, and ***p < 0.001); ns = not significant (p > 0.05)

4.1.8 Andrographolide abolished elevated GFAP expression in LPS-mediated neuroinflammation mouse model

Being the most abundant cell in the brain, astrocytes are important in providing structural supports and maintaining brain homeostasis. Recently, more findings point to the roles of astrocytes in mediating neuroinfammation. For example, astrocyte activation resulted in elevated secretions of various proinflammatory cytokines and chemokines leading to infiltration of leukocytes which further perpetuated inflammatory processes. Moreover, astrocytes activation was often accompanied with release of reactive oxygen species that cause neuronal damages (Sheng *et al.*, 2013; Swanson *et al.*, 2004). GFAP elevation is a hallmark of astrogliosis. Therefore, GFAP expression level was investigated in mice treated to LPS / andrographolide (as above) using immunoblotting or RT-PCR to examine effects of andrographolide on astrogliosis. (Figure 4.11). 50 μg of protein was loaded for each sample and each sample was normalized against internal control. Internal control was a collection of brain homogenates from individuals of various treatment groups. mRNA level of GFAP was significantly reduced by andrographolide prophylactic treatment. Though there was a reducing trend in GFAP protein level with andrographolide oral admistration, it did not reach statistical significance.

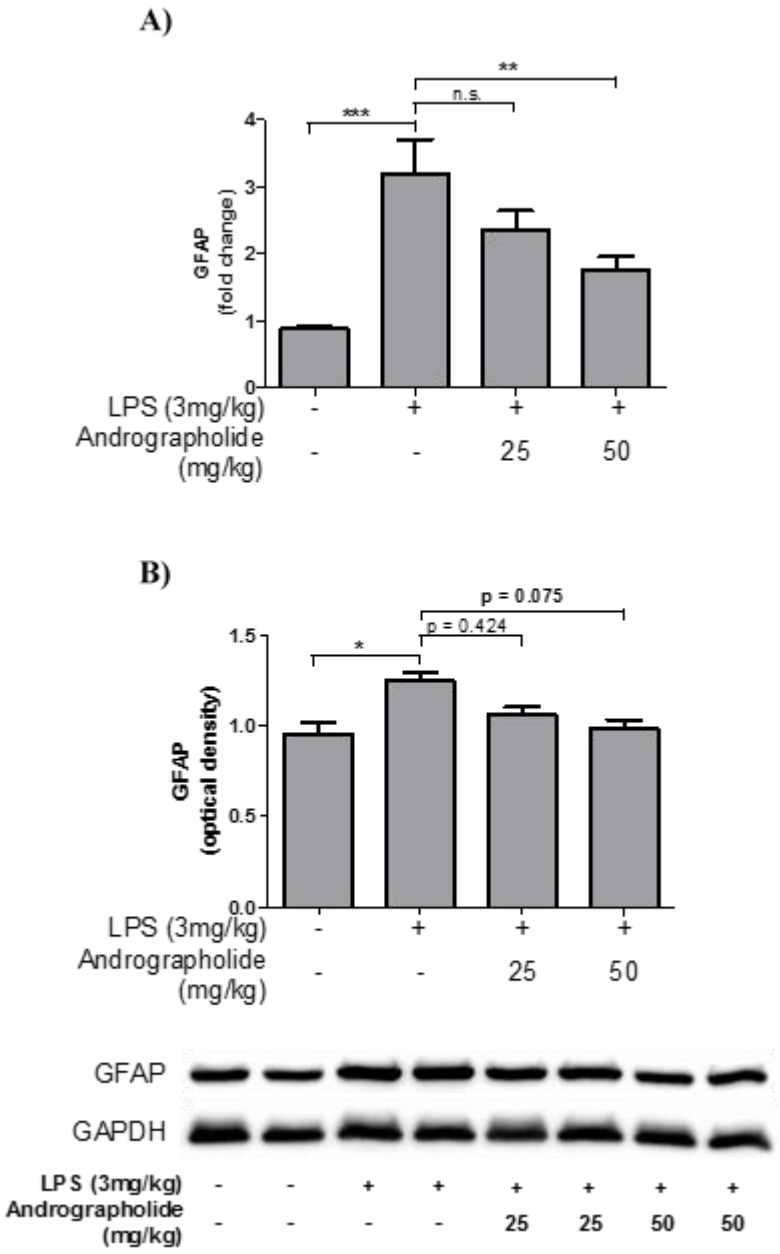


Figure 4.11 Andrographolide abolished elevated GFAP expression in LPS-mediated neuroinflammation mouse model. ICR mice were subjected to 3 intraperitoneal injection of LPS (3mg/kg) and followed by oral gavage of andrographolide (25mg/kg or 50mg/kg) 1 hour after each injection. Brain homogenates were processed for real-time PCR **(A)** or immunoblotting for GFAP **(B)**. 50 μg of protein was loaded for each sample and each sample was normalized against internal control. GAPDH was used as loading control. Data shown are mean ± S.E.M. (control, n = 8; LPS, n = 5, LPS + andrographolide 25 mg/kg, n = 7; LPS + andrographolide 50 mg/kg, n = 7). Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$); ns = not significant ($p > 0.05$)

4.2 Discussion

In Chapter 3, IL-1β and TNF-α were used to induce inflammatory responses in order to address general inflammatory events whereby cytokines are released to mediate and perpetuate neuroinflammation; a typical phenomenon in various forms of brain insults (Basu *et al.*, 2004; Mccoy *et al.*, 2008). After validating effects of andrographolide in cytokine-mediated inflammation, I carry on to examine andrographolide's therapeutic effects in disease condition like CNS infection. Lipopolysaccharide (LPS), a polyglycan found in the cell wall of gram-negative bacteria is a prototypical endotoxin known to illicit robust immune responses. Recognition of LPS by toll-like receptor 4 (TLR4) triggers various signal pathways and lead to an array of cytokines and chemokines production (Block *et al.*, 2007; Guha *et al.*, 2001). Thus, I accessed andrographolide's effect on LPS-mediated inflammatory response in the second part of the study.

Primary astrocytes are resistant to LPS-induced apoptosis

LPS-induced apoptosis is dependent on activation of TNF receptors. Mice with ablated TNF receptor had reduced fibroblastic cell apoptosis when treated with LPS as compared to wild type mice (Alikhani *et al.*, 2003). Upon binding of TNF-α to tumor necrosis factor receptor 1 (TNFR1), TRADD, RIP1, and TRAF2 are rapidly recruited to the receptor to form complex I. Subsequently, RIP1 dissociates and binds to FADD death adaptor and procaspase 8 forming complex II where procaspase undergo autocleavage to form active caspase 8 and resulting in apoptosis (Jin *et al.*, 2006; Micheau *et al.*, 2003). Likewise, cytokines from TNF family such as Fas and TRAIL

ligands bind to their respective receptor and directly recruits FADD to form death-inducing signaling complex (DISC) and mediate apoptosis through similar mechanism (Ashkenazi *et al.*, 1999). Nonetheless, I reported that LPS treatment up to 1 μg/ml did not affect cell viability of primary astrocyte, in agreement with Brahmachari *et al.* (Brahmachari *et al.*, 2006). Interestingly, Song *et al*. (2006) also reported similar whereby FAS/TRAIL ligands was able to trigger cell death in U343 glioblastoma cells in a dose-dependent manner but not in human fetal astrocytes. The constitutively activated calcium/calmodulin-dependent protein kinase II (CaMKII) was found to be the main factor for human astrocyte resistance to Fas/TRAIL-induced apoptosis (Song *et al.*, 2006). CaMKII mediates the phosphorylation of phosphoprotein enriched astrocytes-15 kDa/phosphoprotein enriched in diabetes (PEA-15/PED) and cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein (c-FLIP). This leads to their recruitment to the DISC through DED–DED domain interaction with FADD which inhibits procaspase 8 autocleavage and therefore resistance to apoptosis (Song *et al.*, 2006). Differ from other vulnerable brain cells, astrocytes resistance to death receptor-induced programmed cell death suggest that they are capable of surviving through inflammatory insults and may play fundamental role in regulating inflammation.

LPS activates NF-κB and MAPKs signaling pathways and induces cytokines/chemokines production

LPS recognition triggers TLR4 and initiates a cascade of downstream signaling molecules through MyD88- and TRIF-dependent pathways which also commonly shared by other TLRs when activated by pathogen associated

molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) released during infections or tissue injuries (Lu *et al.*, 2008). Compelling evidence showed that activation of TLRs is involved in the pathogenesis of neurodegeneration. TLR2 knockout or TLR4 mutation reduced brain damage and neurological deficits in animal model of ischemic stroke (Tang *et al.*, 2007). Activation of TLR4 signaling resulted in rapid downstream activation of NF-κB and mitogen-activated protein kinases (MAPKs) indicated by increased phosphorylation of p65, JNK, p38 and ERK within 1 hour LPS exposure (Figure 4.2). Killic *et al*. demonstrated that mice with TLR4 deficiency exhibited reduced infarct size and enhanced survival of striatal neurons after cerebral ischemia through mechanisms involving deactivation of JNK, p38, and ERK, indicating contributory role of MAPKs in neurological diseases (Kilic *et al.*, 2008). JNK and p38 are stress-induced kinases which are activated by cellular stresses including cytokines, pathogenic stimuli, UV radiation, and ROS. Activation of JNK and p38 results in regulation of various cellular activities like cell differentiation, apoptosis, and inflammatory cytokines (Hommes *et al.*, 2003; Kaminska *et al.*, 2009; Kim *et al.*, 2010a). On the other hand, growth factors and cytokines are the strong activators of ERK signaling which mainly involved in proliferation, differentiation and development (Kim *et al.*, 2010a). ERK signaling pathway is closely associated with cancer development where its activation has been shown to promote tumor survival and migration (Balmanno *et al.*, 2009; Huang *et al.*, 2004). Nevertheless, ERK signaling also mediates inflammatory response induced by LPS or ischemic stroke through production of inflammatory mediators such as IL-1β, TNF-α, IL-6, and iNOS (Carter *et al.*, 1999; Maddahi *et al.*, 2010). LPS is a strong inflammatory stimuli

which potentially induced multiple inflammatory cytokines. I showed that stimulation with LPS significantly upregulated transcript level of multiple cytokines/chemokines including CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-5, CXCL-10, CX3CL-1, IL-1β, TNF-α, and IL-6 in primary astrocytes (Figure 4.7). These data suggest that LPS stimulation generates a robust inflammatory response via activation of NF-κB and MAPKs signaling pathways which potentially leads to production of cytokines/chemokines that perpetuate neuroinflammation. Impeding activation of these signaling pathways may therefore be an effective therapeutic strategy for neuroinflammatory diseases.

Andrographolide abrogates LPS-induced NF-κB and JNK activation but initiates ERK and p38 signaling

Previously, I demonstrated that andrographolide attenuated cytokines-induced activation of NF-κB. LPS-induced phosphorylation of p65 and IκBα were also attenuated by pretreatment of andrographolide as anticipated. Likewise, andrographolide mitigated phosphorylation of JNK in dose-dependent manner (Figure 4.5). He *et al.* (2013) reported that andrographolide impaired LPS-induced expression of cytokines, adhesion molecules, and coagulant mediators through inhibition of NF-κB and all family members of MAPKs in rat cardiac microvascular endothelial cells (He *et al.*, 2013). However, pretreatment of andrographolide in primary astrocyte culture did not attenuate ERK or p38 activation; rather, it further enhanced their phosphorylation (Figure 4.6) which suggests cell-type specific responses on the effects of andrographolide. When treated with andrographolide alone, ERK and p38 phosphorylation increased in a dose-dependent manner (Figure 4.4 C & D). Such discrepancies were observed in several *in vitro* studies that investigated

anti-cancer properties of andrographolide. Andrographolide was reported to abrogate expression of matrix metalloproteinases (MMPs), cytokines, and epithelial mesenchymal transition (EMT) markers in several cancer cell types including, colon, breast and prostate cancer. And these anti-cancer activities were mainly accredited to andrographolide inhibition on ERK signaling (Chao *et al.*, 2013; Chao *et al.*, 2010a; Chun *et al.*, 2010; Kayastha *et al.*, 2015). In addition, p38 attenuation by andrographolide treatment promoted vascular smooth muscle cells death that prevented abnormal cell growth; a crucial pathogenic progress of inflammatory vascular disease (Chen *et al.*, 2014b). Immunomodulatory effects of andrographolide were also investigated in non-cancerous cells. Attenuation of ERK signaling abolished LPS-induced cytokines expression and macrophage activation (Qin *et al.*, 2006; Wang *et al.*, 2010). Similarly, andrographolide prevented p38 activation and elevation of TNF-α, IL-1β, IL-6 and iNOS expression in liver from septic mice injected with LPS (Guo *et al.*, 2012).

Although majority of studies reported inhibitory activity of andrographolide on ERK and p38 signalings, Yang *et al.* (Yang *et al.*, 2014) recently showed that andrographolide remarkably increased ERK and p38 phosphorylation and induced C6 glioblastoma cells apoptosis via ERK-mediated activation of tumor suppressor p53. This finding may imply cell-specific effects of andrographolide; activating ERK and p38 signaling in astrocytic culture and promotes apoptosis in glioblastoma while inhibiting ERK/p38-mediated inflammatory response and cancer development in other cell types. It is unclear whether the role of ERK and p38 signaling mediated by andrographolide may play in primary astrocytes, however some antioxidant

compounds have been shown to induce Nrf2 signaling and cytoprotective proteins expression through ERK and p38 activation, suggesting potential protective effects against oxidative stress (Choi *et al.*, 2015; Ci *et al.*, 2015; Lee *et al.*, 2015). Therefore, prospective anti-oxidant properties of andrographolide mediated through ERK and p38 signaling were investigated and discussed in the following chapter.

Andrographolide attenuates NF-kB- and JNK-dependent cytokines/ chemokines expression and its potential therapeutic indications

In the CNS, cytokines and chemokines serve to maintain immune surveillance, facilitate leukocyte recruitment and mediate innate and adaptive immune response (Takeshita *et al.*, 2012). Under physiological conditions, most proinflammatory cytokines/chemokines are maintained at low or undetectable level. In response to pathogen invasion or injury, a repertoire of cytokines/chemokines are then released by resident cells (primarily microglia and astrocytes) in the brain parenchyma (Jensen *et al.*, 2013). While upregulating inflammatory cytokines/chemokines helps to clear harmful stimuli by initiating inflammatory responses and recruiting other immune cells, dysregulation of cytokines/chemokines is the key feature in the development of neuroinflammation and neurodegeneration (Glass *et al.*, 2010; Smith *et al.*, 2012). Due to its high abundance as well as its close proximity with BBB, astrocytes are exclusively positioned to create chemotactic gradient to recruit microglia and circulating leukocytes into site of injury. Thus, I examined effect of andrographolide on astrocyte-secreted cytokines/chemokines and to my delight, LPS-induced transcript level of IL-1β, TNF-α, CC motif, CXC motif, and CX3C motif chemokines were all dose dependently attenuated by

andrographolide (Figure 4.7). Administration of andrographolide after LPS exposure also effectively abated expression of almost all cytokines/chemokines measured.

CCL-2 (MCP-1) and CCL-5 (Rantes) are CC-motif chemokines that promote chemotaxis of a variety of leukocytes including granulocytes, monocytes, T-cell, natural killer (NK) cells and dendritic cells (Jaerve *et al.*, 2012). Pneumococcal meningitis; the most common type of meningitis significantly induced microglial expression of various chemokines including CCL-2, CCL-3, and CCL-5 and remarkably increased leukocytes infiltration to the CSF (Hanisch *et al.*, 2001). While leukocyte transmigration is a strategies to eliminate pathogens, it was proposed that overwhelming inflammatory response caused more tissue damage (Ramesh *et al.*, 2013). Antibodies neutralizing CXCL-2 and CCL-3 mitigate neutrophil recruitment while CCL-2 neutralization attenuated macrophage infiltration, suggesting that chemokine may be a potential target for therapeutic intervention to inhibit leukocyte-mediated neuroinflammation in bacterial meningitis (Diab *et al.*, 1999). In addition to infection, CCL-2 and CCL-5 are also been implicated in several neurological diseases. CCL-2 is believed to be most potent inducer of leukocyte transmigration among the chemokines that activate signal transduction pathways through CCR2 which has been reported to play a non-redundant role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) (Izikson *et al.*, 2000; Sozzani *et al.*, 1994). Moreover, CCL-5 is found robustly elevated among the cytokines and chemokines measured in EAE (Brambilla *et al.*, 2009). Treatment with antibody against CCL-2 or CCL-5 in EAE inhibited leukocyte adhesion to the brain microvasculature (Dos Santos *et al.*, 2005).

Several studies showed that CCL-2 and CCL-5 overexpression induced chemotactic migration of microglia to amyloid plaque (Huang *et al.*, 2009; Huang *et al.*, 2010; Yamamoto *et al.*, 2005). Interestingly, microglia accumulation at the site of amyloid plaque however did not promote Aβ clearance, instead increasing fibrillary Aβ deposit (Yamamoto *et al.*, 2005).

CXC chemokines are further divided into ELR-positive or ELR-negative depending on the presence of an glutamic acid-leucine-arginine (ELR) motif at the N-terminus of the chemokine (Ambrosini *et al.*, 2004). ELR-CXC chemokine like CXCL-1 (KC), CXCL-2 (MIP-2), and CXCL-5 (lix) act explicitly on neutrophils and binds to CXCR1 and CXCR2 receptors (Rossi *et al.*, 2000). Expression of CXCL-1 and CXCL2 was induced in microglial upon stimulation of pneumococcal cell walls, suggesting role in promoting leukocytes migration during bacterial infection like meningitis (Hanisch *et al.*, 2001). Moreover, CXCL-1 and CXCL-2 have been implicated in aggravating CNS injury by promoting transmigration of monocytes in addition to neutrophils into CNS. Spinal cord injury rapidly induce secretion of CXCL-1 and CXCL-2 from astrocytes distributed throughout the spinal cord and attenuation of chemokines expression remarkably reduced neutrophils and monocytes infiltration which is associated with reduced neuronal death and improved motor function recovery (Kang *et al.*, 2011; Pineau *et al.*, 2010). High level of CXCL-5 was detected in the CSF of patients with bacterial meningitis and upregulation of CXCL-5 was shown to facilitate neutrophils transmigration to the CNS (Zwijnenburg *et al.*, 2003). Detrimental effect of CXCL-5 mediated neutrophils has also been demonstrated in ischemic stroke patients where

CXCL-5 elevation in CSF was associated with brain infract size (Zaremba *et al.*, 2006; Zwijnenburg *et al.*, 2003).

On the other hand, CXCL-10 (IP-10) is like CXCL-9 (MIG), are non-ELR-CXC chemokines that bind to CXCR3 receptor and recruit monocytes, T cells, and NK cells (Rossi *et al.*, 2000). Expression of CXCL9 and CXCL10 are strongly induced in the CNS following viral infection. CXCL-10 is mainly expressed by astrocytes and was shown to promote migration of leukocytes such as B cells into the parenchyma (Phares *et al.*, 2013). While elevation of CXCL-10 was thought to confer protective immunity during infection, Bhowmick and colleagues demonstrated that upregulation of CXCL-10 in viral encephalitis enhanced severity of virus infection and induced neuronal injury (Bhowmick *et al.*, 2007). This is in parallel with previous finding that CXCL-10 contributes to enhanced severity in clinical diseases (Liu *et al.*, 2001). ELR-CXC chemokines are strong angiostatic factors that inhibits endothelial cell chemotaxis and neovascularization (Strieter *et al.*, 1995). Injection of CXCL-9 and CXCL-10 into tumour results in tumour regression associated with increased tumour cell necrosis, T cell recruitment and angiogenesis inhibition (Angiolillo *et al.*, 1995; Sgadari *et al.*, 1996; Sgadari *et al.*, 1997). In consistent with these findings, Glaser *et al*. (2004) has also reported active role of CXCL-10 in vasculature remodeling and its upregulation after spinal cord injuries inhibits angiogenesis. Treatment with antibody neutralizing CXCL-10 brought great functional improvements ascribed to improved blood flow and oxygenation at the site of lesion (Glaser *et al.*, 2004).

Another chemoattractant investigated in my study is CX3CL-1 (Fractalkine) that recognized by CX3CR1 and promotes monocytes, T-cells,

and NK cells chemotaxis. In the CNS, CX3CL-1 is constitutively expressed in neurons while its receptor is preferentially expressed in microglia, indicating instrumental role of CX3CL-1 in regulating microglial activity (Briones *et al.*, 2014). Neuroprotective effects have been demonstrated in ischemic stroke and inhibition of CX3CL-1/CX3CR1 signaling in hippocampus aggravated microglia activation, pro-inflammatory cytokines release, and cognitive impairment (Briones *et al.,* 2014). Meanwhile, several contradicting findings were reported, pointing towards detrimental role of CX3CL-1. CX3CR1 deficiency in AD transgenic mice model is associated with reduced neuronal loss, microglial and astrocytes activation, cytokines expression (TNF-α and CCL-2), enhanced Aβ uptake and decreased amyloid deposits (Fuhrmann *et al.*, 2010; Lee *et al.*, 2010; Liu *et al.*, 2010). In ischemia stroke model, attenuating CX3CL-1 signaling by CX3CL-1 or CX3CR1 knockdown both did not result in microglia neurotoxicity but rather substantially reduced infarct size, cytokines expression (IL-1β and TNF-α), and leukocyte infiltration (Dénes *et al.*, 2008; Soriano *et al.*, 2002). Effect of CX3CL1 on microglia activation is evident as attenuation of CX3CL-1/CX3CR1 signaling with antibodies or gabapentin; a recommended first-line treatment for multiple neuropathic conditions inhibited microglia activation arise from epileptic seizure or monoarthritis respectively (Ali *et al.*, 2015; Yang *et al.*, 2012). In my study, I showed that astrocytes could upregulate CX3CL-1 expression upon LPS stimulation implying crosstalk between activated astrocytes and microglia. Growing evidence suggests that crosstalk between astrocytes and microglia can lead to amplification of inflammatory responses. For instance, LPS-activated microglia release pro-inflammatory mediators that induce astrogliosis which in turn release cytokines

and further promotes microglial activation, forming a positive feedback loop. Indeed, co-cultures of microglia and astrocytes stimulated with endotoxins generated more neurotoxic factors than either cell type alone (Saijo *et al.*, 2011). Therefore, andrographolide may interfere with astrocyte-microglia crosstalk and abate microglia activation by attenuating CX3CL-1 expression, though further experiments on microglia will be needed to confirm this.

IL-1β and TNF-α are early injury signals which are pivotal for the induction of other cytokines/chemokines and inflammatory mediators like adhesion molecules, prostaglandin E2, leukotrienes, and NO (Kaminska, 2005; Van Miert, 1995). Bacterial recognition rapidly induce expression of IL-1β and TNF-α which upregulate expression of adhesion molecules in addition to chemokines to facilitate migration of leukocyte (Ramesh *et al.*, 2013). In animal model of pneumococcal meningitis, several cytokines such as TNF-α and IL-6 were upregulated. Attenuation of cytokines expression mitigated leukocyte infiltration to the CSF, indicating importance of inflammatory cytokines in modulating inflammatory response in CNS infection (Hanisch *et al.*, 2001). IL-1β or TNF-α has been reported to enhance neuronal damage through excitotoxicicty potentiation by upregulating expression of glutaminase which result in elevation of intracellular and extracellular glutamate levels in rat and human neuronal cultures (Ye *et al.*, 2013). Abrogating IL-1β signaling through treatment with IL-1β neutralizing antibody or IL-1R1 knockout mice exhibited numerous beneficial effects after stroke event including decreased ischemic infarct volume, leukocyte infiltration and brain edema (Basu *et al.*, 2005; Yamasaki *et al.*, 1995). Importantly, microglia and astrocyte activation were

attenuated together with a number of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (Basu *et al.*, 2002; Basu *et al.*, 2005).

Compelling evidence shows that neuroinflammation is implicated in many neurological disorders including CNS infection, neurodegenerative disease, stroke and trauma. And elevation of various cytokines and chemokines is one of the key features of neuroinflammation (Ramesh *et al.*, 2013). Cytokines play an important role in initiating inflammation while chemokines propagate and sustain inflammatory responses. Notably, though chemokines are well-known for their roles in facilitating leukocyte infiltration into CNS, they are versatile proteins capable of modulating inflammatory responses through various means. Thus, andrographolide that inhibits expression of various cytokines and chemokines as demonstrated in my study, is a potential anti-neuroinflammatory therapeutic suitable for various neurological diseases including but not limited to CNS infections.

In this study, I demonstrated that all cytokines and chemokines examined were dose-dependently attenuated by TPCK and SP600125; which are NF-κB and JNK inhibitor respectively. With exception of IL-1β that did not exhibit dose dependency inhibition and had an abrupt plunge at highest TPCK concentration. Meanwhile SP600125 showed a clear dose response in IL-1β reduction suggest that LPS-induced IL-1β is likely a downstream response of JNK-dependent signaling (Figure 4.9 O & P). The sudden plunge observed at highest TPCK concentration may due to nonspecific activity of TPCK as TPCK is also a known serine/cysteine protease inhibitor (Huang *et al.*, 1999) and recently shown to inhibit caspases too (Frydrych *et al.*, 2008). Even at highest concentration, SP600125 could only partially reduced CX3CL-1 level while

TPCK could lower its expression close to the basal level suggesting that LPS-induced CX3CL-1 is mainly NF-κB dependent (Figure 4.9 M & N). I have shown that andrographolide efficiently abrogated NF-κB and JNK activation as well as cytokines/chemokines upregulation by LPS stimulation. Therefore, andrographolide is proposed to inhibit various cytokines/chemokines by attenuating NF-κB and JNK signaling.

Potential anti-inflammatory role of andrographolide-mediated IL-6 expression

IL-6 is often considered a proinflammatory cytokine where elevated expression level has been reported in serum, plasma, and CSF of ischemic stroke patients. The high level of IL-6 was positively correlated with infarct size, body temperature, early neurological decline, and poor functional outcome (Beamer *et al.*, 1995; Fassbender *et al.*, 1994; Ferrarese *et al.*, 1999; Tarkowski *et al.*, 1995; Vila *et al.*, 2000). Pathogenic role of IL-6 was also shown in transgenic mice overexpressing IL-6. Microglia and astrocytes activation was prominent in these animals, accompanied with neurodegeneration and development of severe neurologic diseases (Campbell *et al.*, 1993; Chiang *et al.*, 1994; Fattori *et al.*, 1995). Although andrographolide abated expression of various LPS-induced cytokines/chemokine, IL-6 was the only one among the examined cytokines/chemokines that was not attenuated. Rather, andrographolide further elevated IL-6 expression level in a dose dependent manner (Figure 4.7 J). IL-1β and TNF-α are recognized inducer of IL-6 (Benveniste *et al.*, 1990; Norris *et al.*, 1994). However, IL-6 was upregulated even when IL-1β and TNF-α were inhibited by andrographolide suggesting that IL-1β and TNF-α did not play a role in IL-6 elevation here. Time course

experiment with andrographolide treatment alone also showed time dependent increase of IL-6 transcript. This confirms that andrographolide treatment indeed induces IL-6 expression in primary astrocyte (Figure 4.8). IL-6 expression is significantly attenuated by NF-κB inhibitor but not JNK inhibitor, suggesting that IL-6 expression is not primarily mediated by JNK signaling (Figure 4.9 S & T). I have demonstrated that andrographolide is an effective blocker of NF-κB, but instead of inhibiting IL-6, it further enhances IL-6 expression implying that other mechanism is involved in andrographolide-mediated IL-6 expression in astrocytes. Interestingly, Brambilla *et al*. found that transgenic mice with astroglial NF-κB inactivation (GFAP-IκBα-dominant negative) exhibit higher IL-6 level than wild type mice and exhibited a remarkable functional improvement after EAE induction (Brambilla *et al.*, 2009). This may indicate that overexpressing IL-6 in astrocytes has neuroprotective effects. Though IL-6 elevation in CSF or parenchyma is often correlated with stroke and neurodegenerative diseases such as AD and PD, there is yet any evidence of IL-6 involvement in the pathologic process of these diseases (Blum-Degena *et al.*, 1995; Mogi *et al.*, 1994; Tarkowski *et al.*, 1995). IL-6 could be a reflection of the ongoing inflammation caused by the progressive neuronal damage and using IL-6 as a clinical marker for disease severity remains controversial (Suzuki *et al.*, 2009). In fact, potential neuroprotective roles of IL-6 have long been proposed. In 1994, Maeda *et al*. demonstrated paracrine neurotrophic effect of IL-6 secreted by astrocytes. Incubation with conditioned medium from astrocytes exposed to hypoxia/reoxygenation promoted survival of hypoxia/reoxygenation treated PC12 cells and the beneficial effect was attenuated by IL-6 neutralizing antibody (Maeda *et al.*, 1994). More recent

study by Herrmann *et al*. supported the protective role of IL-6 against ischemic stroke as IL-6 deficient mice developed larger infarct volume, had poorer neurologic status, and higher mortality rate compared to controls (Herrmann *et al.*, 2003). IL-6 deficient astrocytes also resulted in higher levels of CXCL-1 and CCL-5 upon LPS stimulation, implying autocrine modulatory activities of IL-6 on other cytokines (Bolin *et al.,* 2005). Together, these studies provide convincing evidence that IL-6 can have dual effects; proinflammatory or anti-inflammatory/neuroprotective and IL-6 elevation in astrocytes mediated by andrographolide is likely a neuroprotective response.

Recently, Nrf2 is reported to induce IL-6 expression via an antioxidant response element (ARE) located within IL-6 promoter. Nrf2 knockout mice failed to induce IL-6 expression and suffer from oxidative stress and neuronal death in response to oxidant treatment (Wruck *et al.*, 2011). My earlier study showed that andrographolide activated ERK and p38 MAPKs which had been indicated in Nrf2 activation and cytoprotective proteins expression (Choi *et al.*, 2015; Ci *et al.*, 2015; Lee *et al.,* 2015). Undeniably, there seems to be a link between andrographolide-activated MAPKs, Nrf2 activation, and IL-6 expression. I later revealed that andrographolide is indeed a strong inducer of Nrf2, and MAPKs does play a role in mediating Nrf2 activation (which will be discussed in more details in the next chapter). Thus, I proposed that andrographolide mediates IL-6 expression through Nrf2 activation. Nonetheless, precise mechanism involved in IL-6 production and the exact role of IL-6 elevation in inflammation mediated by andrographolide remain to be elucidated.

Therapeutic effects of oral andrographolide in neuroinflammation mouse model

From primary cell culture, I further the investigation in intact animal model as cultured cell could not provide physiological conditions and lack of complex interactions among different cell types and tissues. In my study, neuroinflammation was induced in mice by systematically administrating LPS. Systemic LPS elicits a rapid and strong inflammatory response. A sharp increase in serum and brain TNF-α level was observed 1 hour after intraperitoneal (i.p.) LPS injection which sustained up to 10 months in the brain (Qin *et al.*, 2007). Elevation of multiple cytokines/chemokines in the CNS and sickness behavior were manifested as early as 2 hours after i.p. LPS (Biesmans *et al.*, 2013). Systemic inflammation also leads to microglia activation, reactive astrogliosis, neutrophil infiltration, upregulation of CNS cytokines/chemokines expression and TLRs; which are indications of neuroinflammation (Biesmans *et al.*, 2013; Jeong *et al.*, 2010; Noh *et al.*, 2014; Qin *et al.*, 2007). Emerging evidences showed that peripheral immune activation is a risk factor of neuroinflammatory and neurodegenerative diseases. Denes *et al*. reported that gut infection substantially exacerbated experimental stroke by upregulating CCL-5 expression (Denes *et al.*, 2010). Moreover Lee *et al*. demonstrated that systemic endotoxin challenge could contribute to AD pathology. Memory impairment was observed in mice with i.p. LPS injection. Repeated LPS injections activated astrocytes and upregulated β- and γ-secretase activities resulted in $A\beta_{1\text{-}42}$ accumulation in cerebral cortex and hippocampus (Lee *et al.*, 2008). Furthermore, intranasal inoculation of *Chlamydia pneumonia* and release of chlamydial products like LPS into the brain stimulates neuroinflammation

which induces Alzheimer-like amyloid plaques in mice brain that increase in density, size, and number as the infection progressed (Itzhaki *et al.*, 2004; Little *et al.*, 2004). These studies indicate that systemic administration of LPS is a relevant model to examine neuroinflammation and neurodegeneration arise from or exacerbated by peripheral infection.

Bacterial meningitis is neuroinflammatory disease caused by peripheral bacterial infection that invade into meninges and causes inflammation in the arachnoid membrane and subarachnoid space. (Kastenbauer *et al.*, 2001; Swartz, 1984). The most common pathogens of bacterial meningitis in children and adults are *Streptococcus pneumoniae, Neisseria meningitides, and Hemophilus influenza* (Ramesh *et al.*, 2013). Bacterial meningitis could be life-threatening causing memory deficits, edema, hearing loss, cerebral palsy, and seizures; thus requires immediate diagnosis and prompt treatment (Nelson Jr, 2006). Patients surviving bacterial meningitis develop cognitive impairment even if they were clinically well recovered (Schmidt *et al.*, 2006; Van De Beek *et al.*, 2002). Peripheral administration of LPS simulate bacterial meningitis where infectious agent could be originated from the peripheral tissue and finally make its way to the brain parenchyma. In my study, I performed three i.p. LPS injections to ICR mice with 6 hours' time interval in between 1st and 2nd injection and 18 hours' time interval between 2nd and 3rd injection, a treatment regime adopted from Erickson *et al.* (2011). Such treatment regime had demonstrated to produce a significantly higher levels of cytokines and chemokines in mice brain as compared to single LPS injection (Erickson *et al.*, 2011). In consistent with previous finding, I found substantial elevation in CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-9, and CXCL-10 expression in mice brain

(Figure 4.10). Leukocytes recruitment into the CSF is a hallmark of bacterial meningitis (Hoffman *et al.*, 2009), therefore chemokines elevation observed here may be the major source of leukocyte infiltration. In fact, both CCL-2 and CCL-5 are potent chemoattractant that recruit a range of leukocytes including granulocytes, monocytes, T-cell, natural killer (NK) cells and dendritic cells (Jaerve *et al.*, 2012). Among the measured chemokines, CXCL-1 was upregulated most, reaching more than 1500 pg/mg brain tissue. This may imply that large number of neutrophils and monocytes were being recruited into the CNS to mediate neuroinflammation. Moreover, upregulation in astrogliosis marker GFAP was also evident, indicating activation of astrocytes. These provide evidences of inflammation process in the CNS upon peripheral LPS administration.

Inflammatory response evoked by meningitis is a protective response to eradicate pathogens but at the same time it could also contribute to inflammatory mediator-induced tissue damage (Braun *et al.*, 2001). In order to access therapeutic effects of orally administered andrographolide on neuroinflammation, andrographolide was given through oral gavage. As LPS elicit a rapid inflammatory response within 1 hour, andrographolide is administered 1 hour after each LPS injection. This treatment regime could resemble episodes of inflammatory responses after bacterial infection and andrographolide treatment is given after occurrence of each inflammatory episodes to control neuroinflammation. Oral administration of andrographolide significantly attenuated all chemokines measured except CXCL-10 though a reducing trend was observed. Besides that, GFAP mRNA level was considerably downregulated. Nonetheless, GFAP protein level did not reach

statistical significance which may be improved by increasing the number of animal. Together, results imply that orally administered andrographolide could exert anti-neuroinflammatory effects. This is further supported by previous findings which demonstrated that andrographolide administered peripherally can penetrate the blood brain barrier (BBB) due to its high lipid solubility (Bera *et al.*, 2014b; Zheng, 1982). In addition, andrographolide's accumulation in the brain may also be enhanced in neuroinflammatory conditions where the BBB is disrupted (Chan *et al.*, 2010). While effective antibiotic therapy is compulsory in patient suffering bacterial meningitis, andrographolide could be used as an adjunctive treatment to ameliorate neuroinflammation to prevent secondary tissue damage. These observations suggest that orally administered andrographolide may be efficacious in CNS diseases like meningitis, stroke and neurodegenerative disease where neuroinflammation is implicated.

CHAPTER 5: ANDROGRAPHOLIDE REGULATION ON NRF2 EXPRESSION; MECHANISM MEDIATING ANTIOXIDATIVE EFFECTS

5.1 Results

5.1.1 LPS induced Nrf2 and HO-1 expression in primary astrocytes

Maladaptive neuroinflammation often comes hand in hand with oxidative stress which generates reactive oxygen species that leads to neuronal death (Hsieh *et al.*, 2013). Being referred as the "master regulator" of antioxidant response, NF-E2-related factor 2 (Nrf2) is a transcription factor that regulates expression of many antioxidant proteins including heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (Nqo1) (Kumar *et al.*, 2014; Niture *et al.*, 2014; Rushworth *et al.*, 2005). First, I showed that primary astrocytes treated with LPS 100 ng/ml time dependently increased Nrf2 mRNA and protein expression (Figure 5.1 A & B). Furthermore, both mRNA and protein level of HO-1 also induced by LPS treatment (Figure 5.1 C & D). This was likely an adaptive mechanism that protects the cells from LPS-induce oxidative damage. Interestingly, coincubation of LPS with andrographolide for 8 hours dramatically enhanced HO-1 mRNA level (Figure 5.2). This result revealed possible antioxidative properties of andrographolide.

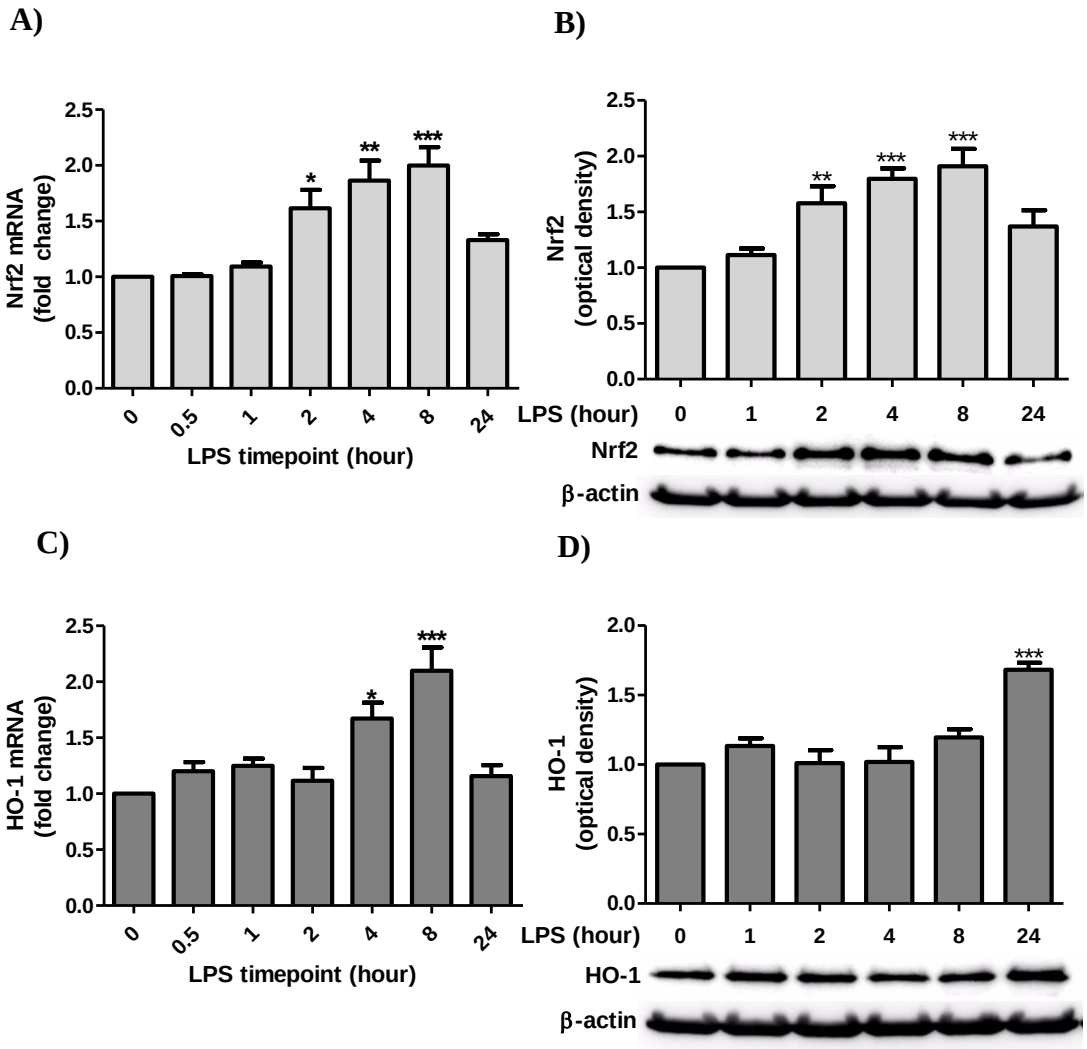


Figure 5.1 LPS induced Nrf2 and HO-1 expression in primary astrocytes. Primary astrocytes were incubated with 100 ng/ml LPS for various timepoints. Transcript level of Nrf2 **(A)** and HO-1 **(C)** were analyzed by real-time PCR. Protein level of Nrf2 and **(B)** and HO-1 **(D)** were quantified by immunoblotting. β-actin was used as loading control. Sample size n = 3 for (A) and (C); n = 4 for (B) and (D). Data shown are mean ± S.E.M. LPS time effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (*p < 0.05, **p < 0.01, and ***p < 0.001)

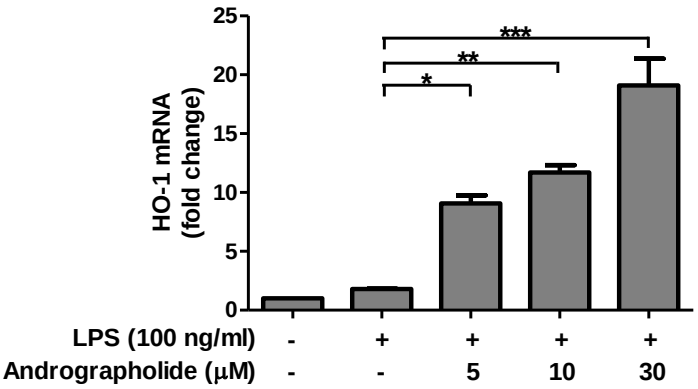


Figure 5.2 Andrographolide further enhanced LPS induced Nrf2 and HO-1 mRNA expression in primary astrocytes. Andrographolide was coincubated with LPS for 8 hours. Transcript level of HO-1 was analyzed by real-time PCR. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

5.1.2 Andrographolide elevated HO-1 and Nqo1 in primary astrocyte

Expression of HO-1 and Nqo1 were then further evaluated to confirm the antioxidative property of andrograpaholide in astrocytes. When treated with 50 μM andrographolide, primary astrocytes showed a time-dependent elevation of HO-1 and Nqo1 in both mRNA and protein levels (Figure 5.3). Immunofluorescence staining of HO-1 also corresponded with the immunoblot result. HO-1 staining intensified with an overall increase in all cells when incubated with andrographolide during a time course experiment (Figure 5.4).

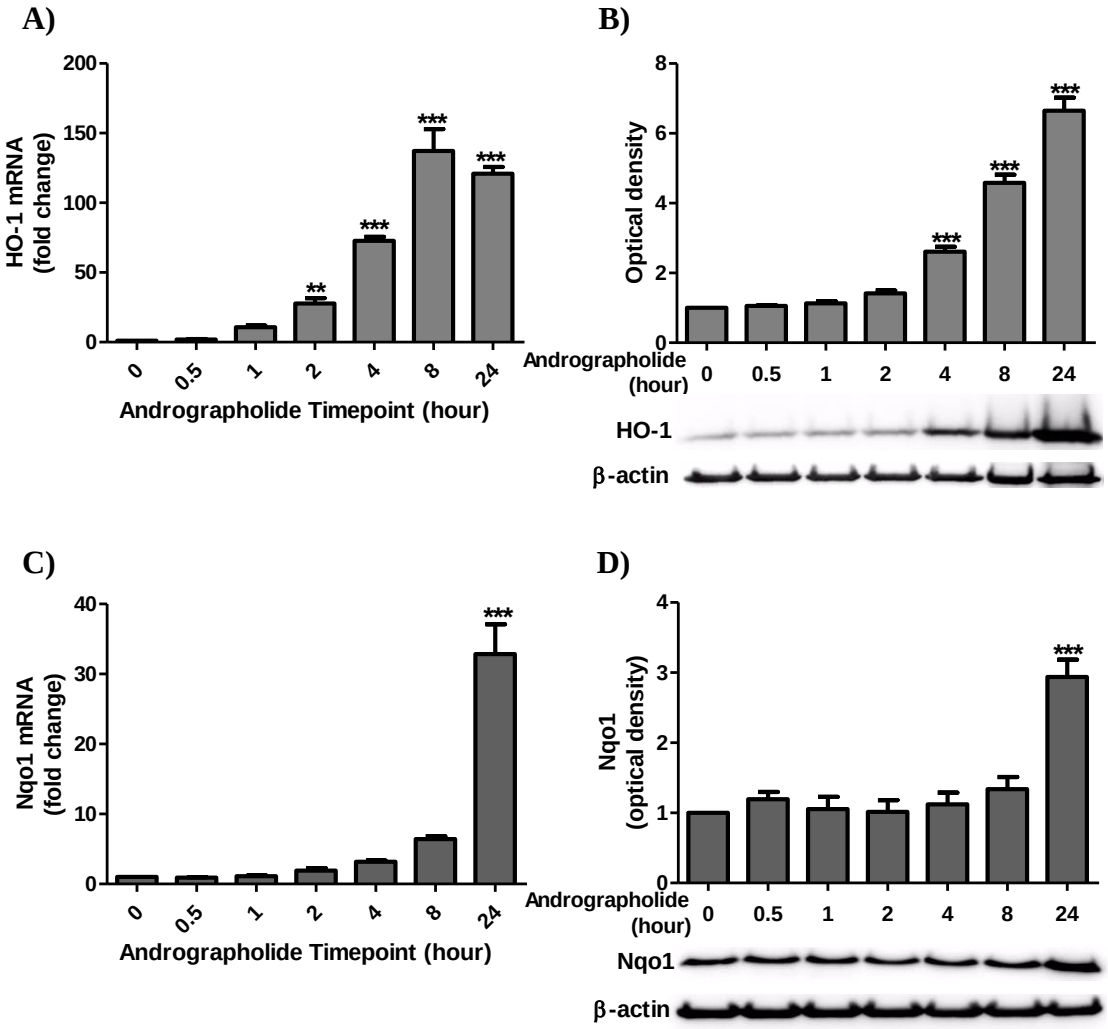


Figure 5.3 Andrographolide elevated HO-1 and Nqo1 expressions in primary astrocytes. Primary astrocytes were incubated with andrographolide 50 μM for the indicated timepoints. Transcript level of HO-1**(A)** and Nqo1 **(C)** were analyzed by real-time PCR. Protein level of HO-1 and **(B)** and Nqo1 **(D)** were quantified by immunoblotting. β-actin was used as loading control. Sample size n = 3. Data shown are mean ± S.E.M. Andrographolide time effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (**p < 0.01 and ***p < 0.001)

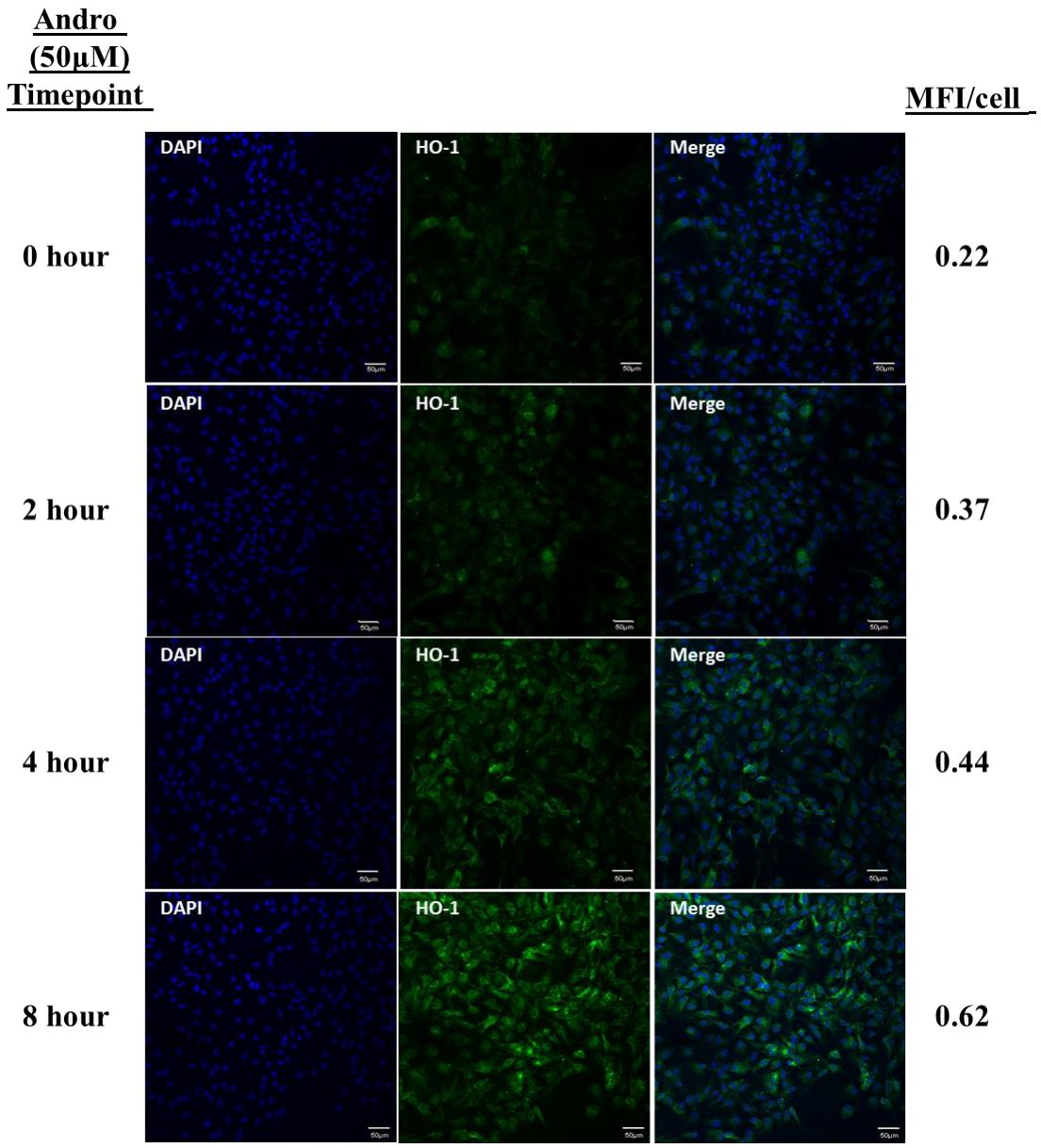


Figure 5.4 Andrographolide time dependently increased HO-1 immunofluorescence staining. Primary astrocytes were incubated with andrographolide 50 μM for the indicated timepoints and processed for immunofluorescence staining. Scale bar denotes 50 μm. Mean fluorescence intensity per cell is computed by dividing area fluorescence intensity with total number of cells.

5.1.3 Andrographolide increased Nrf2 protein level independently of mRNA transcription

As shown in Figure 5.3, transcript levels of HO-1 and Nqo1 were also elevated by andrographolide. As HO-1 and Nqo1 are both target genes of Nrf2,

it was proposed that expression of Nrf2 may be elevated as well. Thus, primary astrocytes were incubated with 50 μM andrographolide for various timepoints and cells were then harvested and processed for real-time PCR or western blotting. Interestingly, mRNA level of Nrf2 did not increase even up to 8 hours of andrographolide incubation and significant increase was only observed at 24 hours of incubation (Figure 5.5 A). On the other hand, protein levels showed a significant increase as early as 30 minutes and the increment sustained until 24 hours which was the longest timepoint tested (Figure 5.5 B). Dose-dependent effects of andrographolide on Nrf2 protein level were also observed (Figure 5.5 C). Apparent elevation of HO-1 mRNA was observed by 2 hours of andrographolide incubation (Figure 5.3 A), much earlier than the elevation of Nrf2 mRNA level. Hence, the results suggested that andrographolide induced elevation of Nrf2 protein level was independent of mRNA level. Moreover, subcellular fractionation revealed that andrographolide promoted Nrf2 accumulation in both nuclear and cytoplasmic compartments (Figure 5.6). Significant nuclear translocation of Nrf2 was observed at early 30 minutes which coincides with early upregulation of HO-1 transcript level. Together, my results implied that andrographolide acutely promoted nuclear accumulation of Nrf2 and induced transcription of HO-1 and Nqo1.

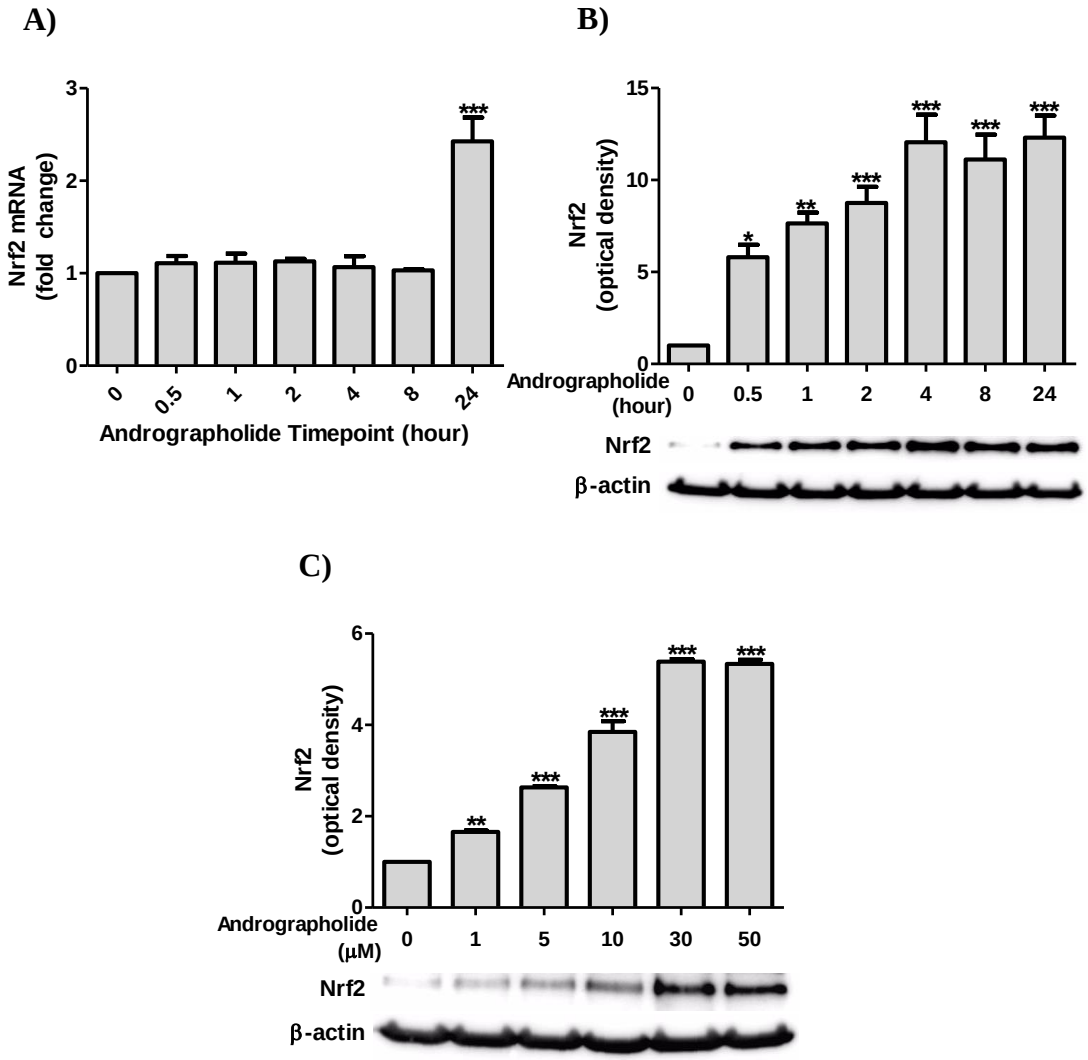


Figure 5.5 Andrographolide elevated Nrf2 protein level independently of mRNA level. (A) Transcript level of Nrf2 was analyzed by real-time PCR while **(B)** protein level of Nrf2 was quantified by immunoblotting after incubation with andrographolide 50 μM for the indicated timepoints. **(C)** Primary astrocytes treated with indicated doses of andrographolide for 1 hour was processed and immunoblotted for Nrf2. β-actin was used as loading control. Sample size n = 4 for (A) and (B); n = 3 for (C). Data shown are mean ± S.E.M. Andrographolide time effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (*p < 0.05, **p < 0.01 and ***p < 0.001)

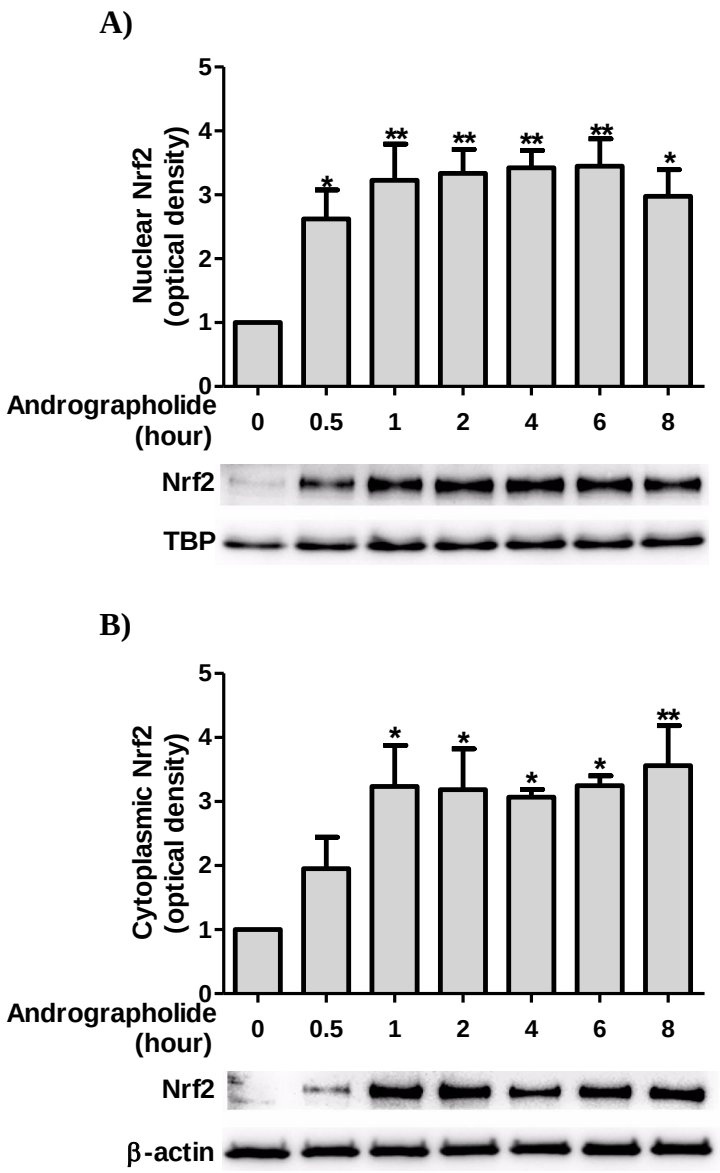


Figure 5.6 Andrographolide increased nuclear and cytoplasmic accumulation of Nrf2. Primary astrocytes were incubated with andrographolide 50 μM for the indicated timepoints and subjected to subcellular fractionation. Nrf level in nuclear compartment **(A)** and cytoplasmic compartment **(B)** were quantified by immunoblotting. TATA box binding protein (TBP) was used as loading control for nuclear protein while β-actin was used as loading control cytoplasmic protein. Sample size n = 3. Data shown are mean ± S.E.M. Andrographolide time effects were compared with controls with one way ANOVA followed by Dunnett's *post-hoc* tests. (*$p < 0.05$ and **$p < 0.01$)

5.1.4 Andrographolide increased Nrf2 level by enhancing Nrf2 protein stability

The observation that andrographolide promoted early accumulation of Nrf2 protein in the absence of increased rate of transcription inferred that turnover rate for Nrf2 may be decreased in response to andrographolide treatment. In order to examine this possibility, primary astrocytes were treated with 10 μg/ml cycloheximide (CHX), a protein synthesis inhibitor (Egorova *et al.*, 2015) in a time-course experiment for 0, 10, 20, 40, 60, and 90 minutes. Whole cell lysate collected were immunoblotted for Nrf2. Result showed that Nrf2 protein level reduced to about 50% within 10 minutes of CHX treatment and only a trace amount of Nrf2 was detected after 40 minutes, indicated that Nrf2 had a high turnover rate. However when primary astrocytes were pretreated with andrographolide 50 μM for 1 hour prior co-incubation with CHX in a similar time-course experiment, Nrf2 protein degradation was delayed. Nrf2 protein level remained high for at least 20 minutes and substantial decrease was only detected after 40 minutes of CHX exposure (Figure 5.7). These findings suggested that andrographolide enhanced protein stability of Nrf2 and therefore decreased Nrf2 turnover rate.

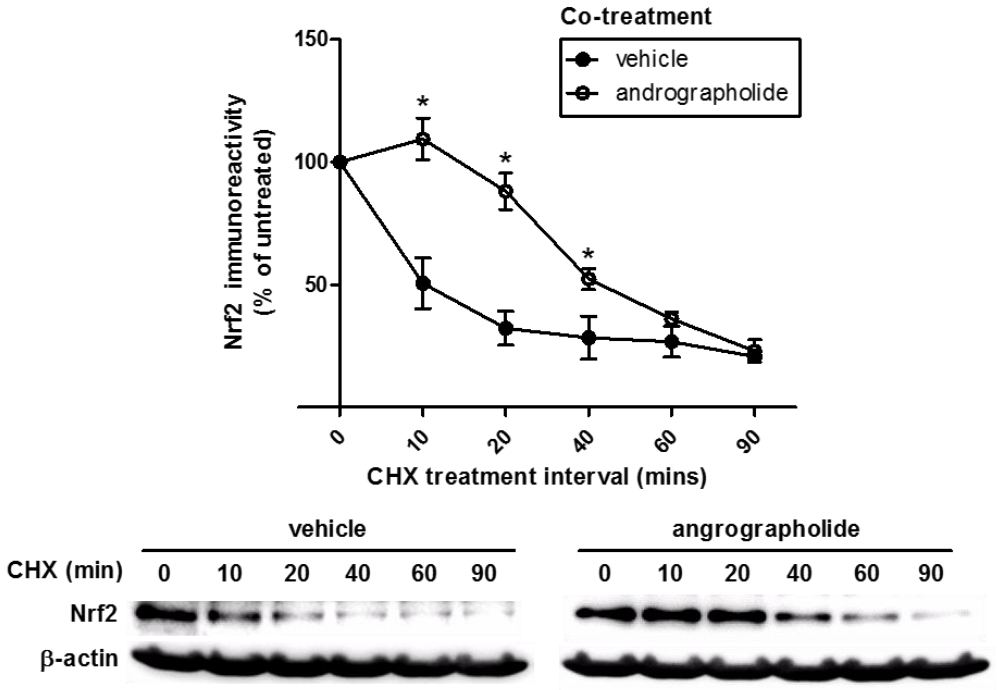


Figure 5.7 Andrographolide enhanced protein stability of Nrf2. Primary astrocytes were incubated in culture media or andrographolide 50 μM for 1 hour prior co-incubation of CHX (10 μg/ml) for the indicated timepoints. Whole-cell lysate collected were immunoblotted for Nrf2. β-actin was used as loading control. Sample size n = 4. Data shown are mean ± S.E.M. Andrographolide coincubated group was compared with vehicle group with Student's *t*-tests. (*p < 0.05)

5.1.5 Andrographolide enhanced Nrf2 protein stability by reducing ubiquitination of Nrf2

Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, which acts as a substrate adapter for E3 ubiquitin ligase complex. Nrf2 was then subjected to ubiquitination and degradation by 26S proteasome (Furukawa *et al.*, 2005). As ubiquitination plays a crucial role in regulating Nrf2 stability, I further examined endogenous level of ubiquitinated Nrf2 in andrographolide treated primary astrocytes. After treatment with 0.1% DMSO or andrographolide 50 μM for 1 hour, cells were lysed and incubated with ubiquitin antibody-conjugated agarose beads followed by Nrf2 immunoblotting.

Immunoprecipitation result revealed that absolute amount of ubiquitinated Nrf2 did not change with andrographolide treatment. In contrast, total Nrf2 level increased in an andrographolide dose-dependent manner (Figure 5.8). Therefore, the proportion of ubiquitinated Nrf2 in andrographolide treated cells were substantially reduced. Thus, I inferred that andrographolide enhanced protein stability by impeding Nrf2 ubiquitination and degradation.

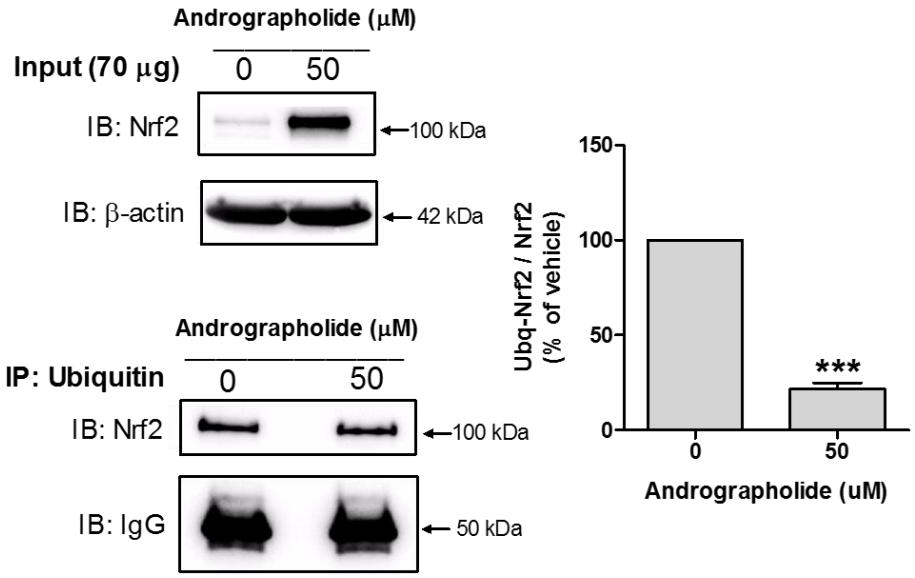


Figure 5.8 Andrographolide decreased ubiquitination of Nrf2. Primary astrocyte were treated with 0 and 50 μM of andrographolide for 1 hours and processed for immunoprecipitation. Lysates were incubated with ubiquitin antibody-conjugated agarose beads for 3 hours in 4 ˚C and subjected to immunoblotting for Nrf2. Heavy chain of IgG was used a loading control for immunoprecipitation. 70 μg input protein (from crude lysate) were immunoblotted for Nrf2 and β-actin was used as a loading control. Sample size n = 4. Data shown are mean ± S.E.M. Andrographolide was compared with control group with Student's *t*-tests. (***$p < 0.001$)

5.1.6 Andrographolide did not decrease Keap-1 expression level

Keap1 is an important regulator of Nrf2, binding of Nrf2 to Keap1 promotes Nrf2 degradation (Furukawa *et al.*, 2005). Thus, disruption of Keap1 expression level may play a part in modulating Nrf2 protein stability. Zhang *et*

al. (2005a) demonstrated that quinone-induced oxidative stress promoted Keap1 ubiquitination and subsequently led to degradation of Keap1 (Zhang *et al.*, 2005a). To examine this possibility, a time-course study was performed with 50 μM andrographolide. Immunoblot result showed no change in Keap1 protein level upon andrographolide time-course treatment, suggesting that andrographolide exerted no effect on Keap1 protein level (Figure 5.9).

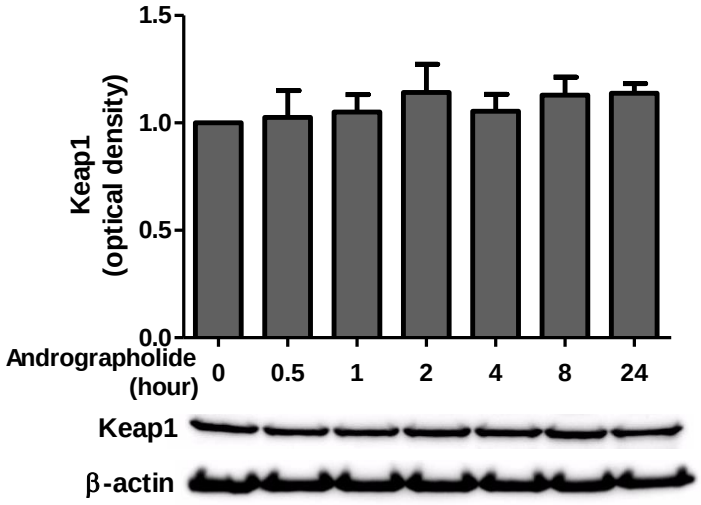


Figure 5.9 Andrographolide effects on Keap1 protein level. Primary astrocytes were exposed to 50 μM andrographolide for the indicated timepoints. Whole-cell lysate collected were immunoblotted for Keap1 and β-actin was used as loading control. Sample size n = 4. Data shown are mean ± S.E.M.

5.1.7 Andrographolide reduced Nrf2 phosphorylation at Ser40 site

Several studies reported that phosphorylation at Ser40 by protein kinase C (PKC) disrupted Nrf2 binding to Keap1, hence induced nuclear accumulation of Nrf2 (Huang *et al.*, 2002; Niture *et al.*, 2009). In order to examine if andrographolide promoted Nrf2 nuclear accumulation through Ser40 phosphorylation, time-course experiments were performed with 50 μM andrographolide. Negligible change was detected in the absolute amount of

phospho-Ser40 but total Nrf2 protein increased along the treatment time course. Thus, the proportion of phospho-Ser40 reduced as incubation time increases. This implied that andrographolide did not enhance Nrf2 protein stability through Ser40 phosphorylation-mediated Nrf2 escape from Keap1.

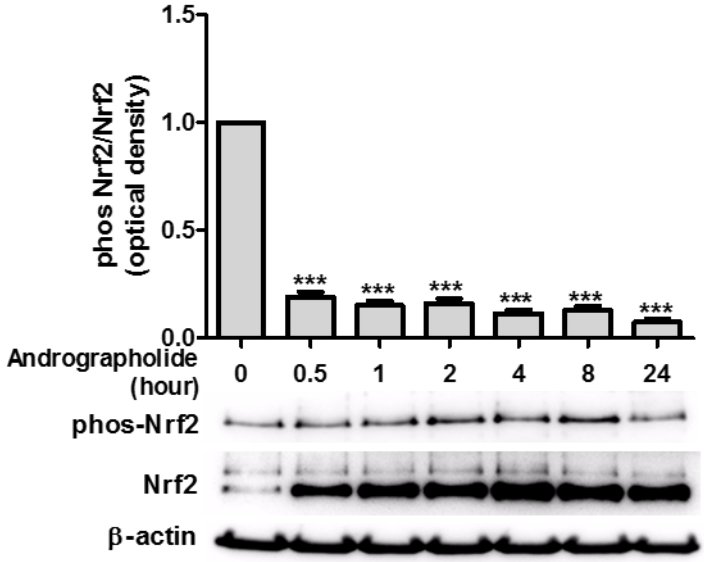


Figure 5.10 Andrographolide reduced Nrf2 phosphorylation at Ser40 residue. Primary astrocytes were incubated with andrographolide 50 μM for the indicated timepoints. Whole cell lysates were collected and probed for phos-Nrf2 Ser40 and Nrf2. β-actin was used as a loading control. Sample size n = 4. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (***$p < 0.001$)

5.1.8 ERK and p38 inhibitors attenuated andrographolide-induced Nrf2 accumulation in both nuclear and cytoplasmic compartment

There are recent reports on MAPKs activating Nrf2 and increasing expression of Nrf2 target genes (Eom *et al.*, 2009; Keum *et al.*, 2006; Nguyen *et al.*, 2003; Zipper *et al.*, 2000). I previous showed that andrographolide activated ERK and p38 kinases (Figure 4.4 C & D). Possible role of ERK and p38 in inducing Nrf2 accumulation was thus investigated. Primary astrocytes were pretreated with ERK inhibitor (PD98059) or p38 inhibitor (SB202190) for an hour followed by another one hour of incubation with 30 μM andrographolide. SB202190 and PD98059 both inhibitors considerably reduced

Nrf2 in nuclear and cytoplasmic compartments in a dose dependent manner (Figure 5.9).

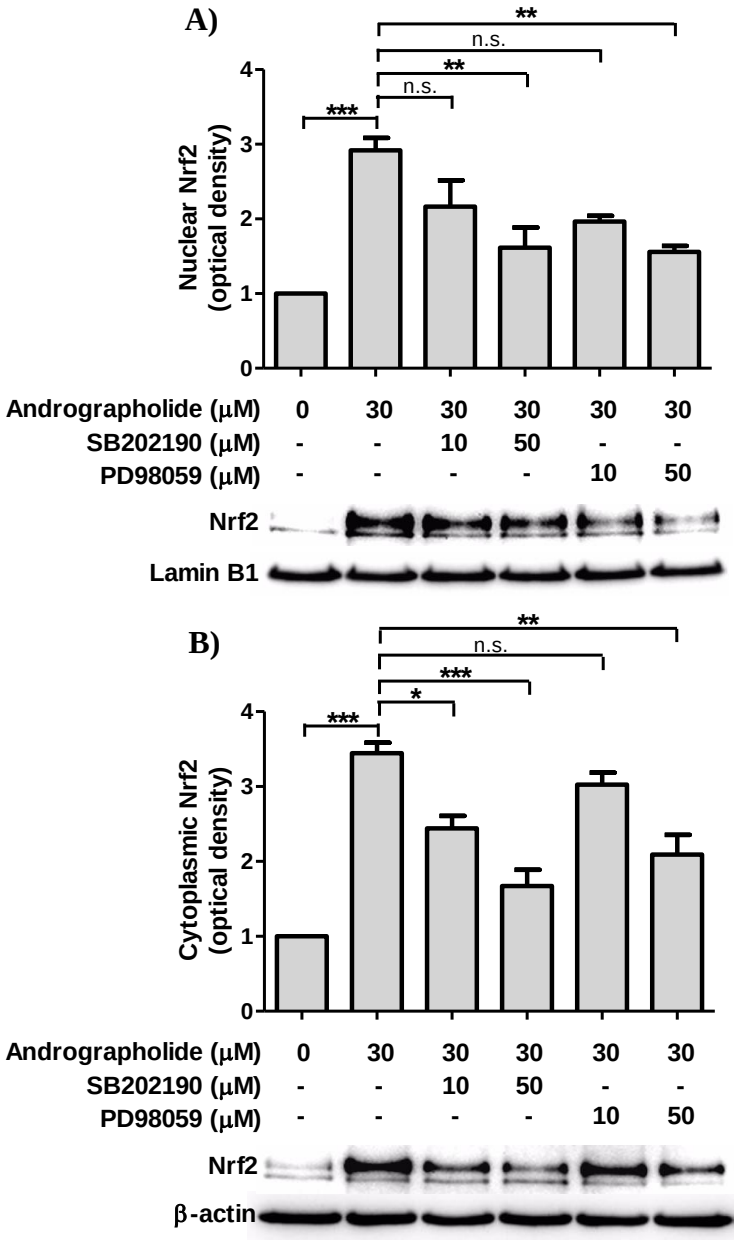


Figure 5.11 ERK and p38 inhibitors attenuated andrographolide-induced Nrf2 accumulation in nuclear and cytoplasmic compartment. Primary astrocytes were pretreated with ERK inhibitor (PD98059) or p38 inhibitor (SB202190) for an hour followed by another one hour of incubation with 30 μM andrographolide (with presence of inhibitors). Nuclear **(A)** and cytoplasmic fraction **(B)** were subjected to immunoblotting for Nrf2. Lamin B1 was used as loading control for nuclear protein while β-actin was used as loading control for cytoplasmic protein. Sample size n = 3. Data shown are mean ± S.E.M. Multiple pair-wise comparisons were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$); ns = not significant ($p > 0.05$)

5.1.9 ERK and p38 inhibitors attenuated andrographolide-induced mRNA expression of HO-1 and Nqo1

After demonstrating that inhibition of ERK and p38 could lead to reduction of Nrf2 level, expression of Nrf2 target genes, HO-1 and Nqo1 were examined. Primary astrocytes were either pretreated with or without MAPK inhibitors for 1 hour followed by andrographolide treatment for 4 hours (with presence of inhibitors). Increasing concentration of andrographolide leds to dose dependent increase of HO-1 and Nqo1. In corroboration with Nrf2 levels, inhibitors of ERK and p38 also attenuated HO-1 and Nqo1mRNA level induced by andrographolide (Figure 5.10). These results supported the role of ERK and p38 in mediating Nrf2 accumulation and thus transcription of anti-oxidative genes.

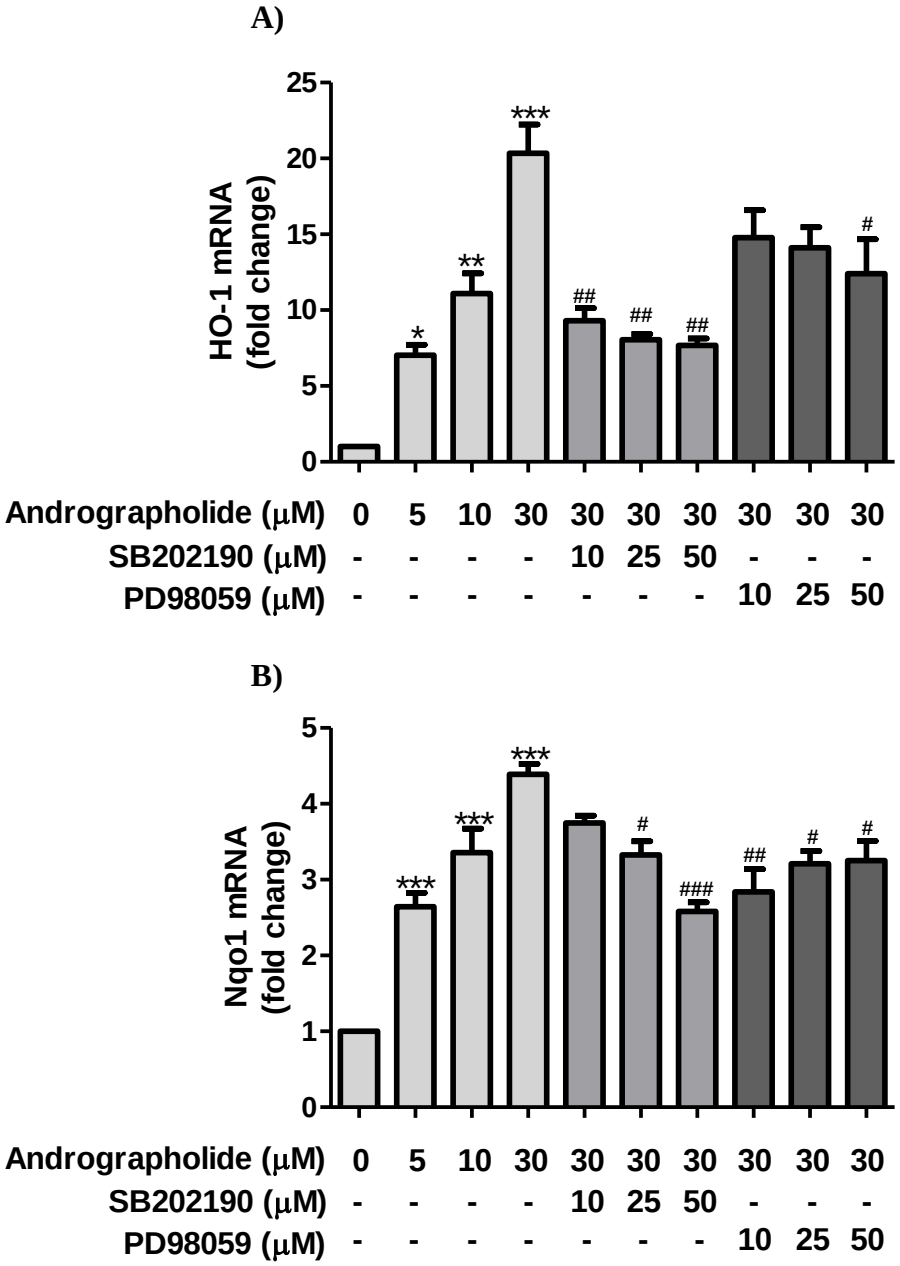


Figure 5.12 ERK and p38 inhibitors attenuated andrographolide-induced HO-1 and Nqo1 mRN expression. Primary astrocytes were pretreated with or without MAPK inhibitors for an hour followed by 4 hour of incubation with andrographolide (with presence of inhibitors). Transcript of HO-1 **(A)** and Nqo1 **(B)** were quantified with real-time PCR. Data shown are mean ± S.E.M. Andrographolide dose effects were compared with control with one way ANOVA followed by Dunnett's *post-hoc* tests. (*$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$). Sample size n = 3. Multiple pair-wise comparisons with andrographolide 30 μM were performed using one way ANOVA followed by Bonferroni's *post-hoc* tests. ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$)

5.2 Discussion

Neuroinflammatory processes induce oxidative stress which has detrimental effects on the vulnerable brain due to its high content of polyunsaturated fatty acids that are highly susceptible to lipid peroxidation (Mariani *et al.*, 2005). Lipid peroxidation decreases membrane fluidity and elasticity, leading to cell death. These modifications are particularly deleterious to cells with limited regenerative capacity like neurons (Richter, 1987). Hence, interventions that targets both neuroinflammation and oxidative stress are deemed to bring many medical benefits. As Nrf2 plays an essential role in cellular redox homeostasis and also some indications of andrographolide-mediated Nrf2 activation (demonstrated in the previous chapter), I further the investigation of andrographolide on its potential antioxidative effects by examining Nrf2 pathway. One of the cytoprotective enzymes regulated by Nrf2 is HO-1 which mediates heme catabolism (Jazwa *et al.*, 2010). Biliverdin generated from heme catabolism is quickly converted to bilirubin which had been shown to protect against lipid peroxidation as effective as α-tocopherol (Stocker *et al.*, 1987a; Stocker *et al.*, 1987b). Therefore, HO-1 was being used in this study as an indication of antioxidant effects and also a read out of Nrf2 transcriptional activity.

In order to ensure the purity of nuclear and cytoplasmic preparation, cell lysate prepared from subcellular fractionation was immunoblotted for TATA-box binding protein (TBP), lamin B1, epidermal growth factor receptor (EGFR), GAPDH, and actin. Cytoplasmic preparation was clearly devoid of nuclear protein such as TBP and lamin B1 whereas negligible cytoplasmic protein like EGFR and GAPDH were detected in nuclear preparation (Appendix

6). Actin was detected in both nuclear and cytoplasmic fraction as reported by previous studies. Actin could form complex with DNA (Miller *et al.*, 1991) or associate with nuclear matrix (Nakayasu *et al.*, 1983). Actin has been proposed to have structural or regulatory role within chromatin-remodeling complexes and facilitates nuclear export of mRNA transcripts (Bettinger *et al.*, 2004).

Rat and human Nrf2 contains 597 and 605 amino acids respectively. The predicted molecular weight of Nrf2 is ~66 kDa. *In vitro* transcription and translation of full-length Nrf2 cDNA generates distinct band at ~96 kDa which is larger than the predicted 66 kDa (Moi *et al.*, 1994). Moi *et al.* proposed that such discrepancy could be due to high acidic residues content in Nrf2, resulting in anomalous migration in SDS/PAGE. Moreover, Kang *et al.* showed that Nrf2 is an actin-binding protein. The 100 kDa protein detected in immunoblots and immunoprecipitation assay contained both Nrf2 and actin (Kang *et al.*, 2002). Using antibodies from Santa Cruz and laboratory generated antibodies, Nrf2 overexpression significantly increased protein band at 100 kDa in neuronal and astrocytic cultures (Li *et al.*, 2005). Though the nature of anomalous migration pattern of Nrf2 is still undefined, growing evidence supports the biologically relevant size of Nrf2 to be ~95-110 kDa under denatured condition. Therefore, I reported Nrf2 as the evident band detected ~100 kDa in my study.

LPS activates Nrf2 as an adaptive mechanism to oxidative stress

LPS stimulation triggers production of ROS (Hsu *et al.*, 2002). In response to oxidative stress, astrocytes upregulate cytoprotective enzymes like HO-1 through activation of Nrf2. This was indicated by elevation of both transcript and protein level of Nrf2 and its target gene, HO-1. This shows that

cells have adaptive mechanism to endure oxidative stress. Potential antioxidant properties of andrographolide was revealed when andrographolide markedly enhanced LPS-induced HO-1 transcript level.

Andrographolide is a potent Nrf2 activator

I then followed up the study by looking at andrographolide effects on HO-1 and Nqo1, another gene targets of Nrf2. Nqo1 is a quinone reductase that catabolizes two-electron reduction of quinone to hydroquinones, diverting away from one-electron reduction that generates semiquinones and ROS (Vasiliou *et al.,* 2006). Moreover, Nqo1 has been shown to protect neuroblastoma cells against oxidative stress induced by glutamate (Murphy *et al.,* 1991). Andrographolide time-course experiments showed induction of both HO-1 and Nqo1 suggestive of antioxidant activity. Andrographolide triggered rapid HO-1 transcription. Noticeable elevation of HO-1 transcripts were observed as early as 1 hour andrographolide incubation and reached statistical significance by 2 hours (Figure 5.3). Increase in Nqo1 transcript was also evident by 4 hours of andrographolide treatment. Andrographolide strongly induced transcription of HO-1 and Nqo1, increasing their transcripts level up to 130 fold and 30 fold respectively. Dose response experiment of andrographolide demonstrated a substantial Nrf2 protein increase with concentration as low as 1 μM (Figure 5.5). Screening of 54 natural dietary compounds as potential Nrf2 activator had identified andrographolide as the compound with highest efficacy and has EC_{50} value lower than tert-Butylhydroquinone (tBHQ); an effective antioxidant frequently used to study Nrf2/ARE activation (Wu *et al.*, 2014b). This implies that andrographolide is a potent activator of Nrf2.

Andrographolide-mediated early induction of Nrf2 is independent of Nrf2 transcription

Time course experiment of andrographolide demonstrated that Nrf2 protein level increased remarkably at a very early time-course (30 minutes) and peaked at 4 hours. Intriguingly, elevation of Nrf2 protein level was not accompanied with mRNA increment. Nrf2 mRNA did not increase even up to 8 hours of andrographolide incubation (Figure 5.5). Subcellular fractionation also showed elevation of Nrf2 in both nuclear and cytoplasmic compartments upon andrographolide incubation. In parallel with HO-1 and Nqo1 rapid transcription in andrographolide time-course experiment, nuclear accumulation of Nrf2 occurs as early as 30 minutes. The instantaneous rise of nuclear Nrf2 promotes rapid transcription of HO-1 and Nqo1. Results indicate that andrographolide's regulation on Nrf2 is biphasic and early activation of Nrf2 is independent of Nrf2 mRNA transcription.

Andrographolide enhances Nrf2 protein stability by reducing ubiquitination

The early accumulation of Nrf2 protein in the absence of transcript level may infer that andrographolide reduced turnover rate for Nrf2. In order to examine this possibility, primary astrocytes were treated with cycloheximide (CHX) which is a 80S ribosome inhibitor that blocks protein translation and therefore prevent *de novo* protein synthesis (Egorova *et al.*, 2015). Nrf2 is known to have high turnover rate (Nguyen *et al.*, 2003; Stewart *et al.*, 2003). Nearly 50% protein reduction was observed within 10 minutes of CHX time-course treatment and only a trace amount of Nrf2 was detected after 40 minutes.

Nonetheless, when primary astrocytes were pretreated with andrographolide, Nrf2 protein level sustained and noticeable decrease was only detected after 40 minutes of CHX exposure (Figure 5.7). These findings indicate that andrographolide-mediated Nrf2 protein accumulation does not depend on *de novo* Nrf2 synthesis but rather due to enhanced Nrf2 protein stability.

Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1. Nrf2 interacts with Keap1 via high affinity ETGE and a low affinity DLG binding motifs in the Neh2 domain (Bryan *et al.*, 2013; Hun Lee *et al.*, 2013). Keap1 acts as a substrate adapter for Cullin-3 (Cul3) which forms an E3 ubiquitin ligase complex with ring-box 1 protein (Rbx1) (Furukawa *et al.*, 2005). The Keap1-Cul3-Rbx1 complex promotes ubiquitination of Nrf2 and targets it for degradation by 26S proteasome, maintaining Nrf2 expression at low level. As ubiquitination plays a major role in regulating Nrf2 stability, I further examined endogenous level of ubiquitinated Nrf2 in andrographolide treated primary astrocytes. Immunoprecipitation revealed that absolute amount of ubiquitinated Nrf2 did not change with andrographolide treatment. However, it was well-appreciated that andrographolide dose-dependently increased Nrf2 total protein level. Hence, the ratio of ubiquitinated to non-ubiquitinated Nrf2 in andrographolide treated cells was markedly reduced. Therefore, it is reasonable to conclude that andrographolide enhanced protein stability by impeding Nrf2 ubiquitination and degradation. One advantage of my current studies is the measurement of endogenous level of ubiquitinated Nrf2 in primary astrocytes instead of ectopically expressed Nrf2 and ubiquitin commonly used by many studies to examine Nrf2 ubiquitination and protein-protein interactions due to general low abundance of Nrf2 in unstimulated cells (Ichikawa *et al.*,

2009; Li *et al.*, 2012a; Sun *et al.*, 2007). Such results obtained from forced protein expression approach may lead to ectopically expressed, non-physiological functions of proteins under study.

Andrographolide neither enhance protein stability through regulation of Keap1 protein expression nor Nrf2 Ser40 phosphorylation

The cysteine-rich Keap1 is a sensitive sensor for oxidative stress. When exposed to oxidative stress or electrophiles, cysteine thiol groups in the intervening region (IVR) of Keap1 interact with ROS or electrophile and results in formation of disulfide bonds (Hun Lee *et al.*, 2013). This leads to Keap1 conformational changes which render its ability to sequester Nrf2 (Bryan *et al.*, 2013; Kansanen *et al.*, 2012). Keap1 was previously reported to be ubiquitinated in response to quinone-induced oxidative stress and resulted in subsequent degradation of Keap1 (Zhang *et al.*, 2005a). In my study, Keap1 expression did not change in response to andrographolide time-course treatment suggesting that andrographolide exert no effect on Keap1 protein level. My present study however, could not rule out that andrographolide may react and form adduct with Keap1. A few reactive cysteine residues (C257, C273, C288, C297, and C613) in Keap1 had been identified to react with Nrf2 inducers to form covalent adducts. This would induce Keap1 conformational changes and disrupt Keap1-Nrf2 association which eventually cause nuclear accumulation of Nrf2 (Bryan *et al.*, 2013; Dinkova-Kostova *et al.*, 2002; Kansanen *et al.*, 2012).

Nrf2 is an acidic protein. About 16% of its total amino acids are made up of serine, threonine, and tyrosine residues, making it a potential substrate for several signaling kinases (Rojo *et al.*, 2012). Protein kinase C (PKC) had been

shown to phosphorylate Nrf2 at Ser40 in the Neh2 domain (Bloom *et al.*, 2003; Huang *et al.*, 2002; Niture *et al.*, 2009). PKC phosphorylation was prevented when Ser40 was mutated to alanine residue. This mutation disrupts association between Keap1 and Nrf2, promoting nuclear accumulation of Nrf2 (Huang *et al.*, 2002; Niture *et al.*, 2009). In order to investigate if andrographolide promotes Nrf2/Keap1 complex dissociation and consequently leads to Nrf2 nuclear accumulation by inducing Ser40 phosphorylation, a time-course experiment was performed. Change in the absolute amount of phospho-Ser40 detected was negligible (Figure 5.9). On the other hand, total Nrf2 protein increased along the treatment time course. Thus, the proportion of phospho-Ser40 reduced as incubation time increases. My finding coincided with Li *et al.* who also reported minimal change in phos-Ser40 despite marked increase in total Nrf2 and reduction in Nrf2 ubiquitination (Li *et al.*, 2012a). I could therefore rule out the possibility of Nrf2 stabilization through Ser40 phosphorylation-mediated Nrf2 escape from Keap1.

ERK and p38 mediate Nrf2 activation potentially through GSK-3β inhibition

Emerging evidence showed that Nrf2 phosphorylation positively regulates Nrf2 stability. Using phosphatase inhibitors to induce Nrf2 hyperphosphorylation resulted in accumulation of Nrf2 and activation of ARE-mediated reporter gene (Nguyen *et al.*, 2003). Moreover, several xenobiotics like tert-Butylhydroquinone (tBHQ), Pyrrolidine dithiocarbamate (PDTC), and sulforaphane from cruciferous vegetables were shown to induce Nrf2 stability and transactivation activity through activation of ERK and p38 kinases (Keum *et al.*, 2006; Nguyen *et al.*, 2003; Zipper *et al.*, 2000). Consistent with these

studies, I discovered that andrographolide also activated ERK and p38 in a time- and dose- dependent manner (Figure 4.3 and Figure 4.4). Using ERK inhibitor (PD98059) and p38 inhibitor (SB202190), I demonstrated that Nrf2 accumulation in both cytoplasmic and nuclear compartments were attenuated (Figure 5.11). Transcript level of HO-1 and Nqo1 were also significantly reduced (Figure 5.12). These results indicate that ERK and p38 play a role in regulating Nrf2 activation. In actual fact, andrographolide and andrographolide derivatives have formerly shown to activate MAPKs and induce Nrf2 accumulation (Lee *et al.*, 2014; Lu *et al.*, 2014; Zhang *et al.*, 2013). Lu *et al.* reported that andrographolide induced Nrf2 by activating ERK but not p38. In contrast, Lee *et al.* showed that p38 mediates upregulation of Nrf2 and HO-1 but no significant ERK phosphorylation was detected in response to andrographolide treatment (Lee *et al.*, 2014). Thus, I have now demonstrated for the first time that andrographolide could activate both ERK and p38 MAPKs and inhibition of these two kinases would negatively regulate Nrf2, HO-1 and Nqo1. The discrepancy among the findings is largely unknown. One of the suspects why Lee *et al.* could not detect phosphorylation of ERK is probably due to insufficient andrographolide incubation time. The group incubated andrographolide for only 2 hours but my time-course experiment revealed that upregulation of ERK phosphorylation was only evident after 4 hours of incubation (Figure 4.3 C). The other reason for such discrepancy may be due to cells of different origins were used in the studies. Lu *et al.* and Lee *et al.* used human endothelial and hepatoma cell lines respectively while I cultured primary astrocytes for my studies.

Other than ERK and p38 MAPKs, JNK and Akt also potentiate Nrf2 activation (Li *et al.*, 2005; Wang *et al.*, 2008b; Yuan *et al.*, 2006). Induced Nrf2 accumulation was attenuated by LY294002 and wortmannin which inhibit phosphatidylinositol 3-kinase (PI3K), an upstream signaling molecule of Akt (Li *et al.*, 2005; Wang *et al.*, 2008b; Wu *et al.*, 2014a). Though MAPKs and Akt are known to activate Nrf2, the molecular mechanisms underlying their roles in Nrf2 activation remain unknown. Sun *et al.* identified a number of serine or threonine residues that MAPKs potentially target. Unexpectedly, none of the alanine substitution on these residues, neither single nor combined mutations caused an apparent reduction in Nrf2 accumulation. This indicates that MAPKs regulate Nrf2 activation through an indirect mechanism (Sun *et al.*, 2009). Nonetheless, some insight on how phosphorylation may activate Nrf2 was obtained when glycogen synthase kinase-3β (GSK-3β) was identified as a regulator of Nrf2. It was found that Akt activation oppose GSK-3β activity (Li *et al.*, 2014). While Nrf2 activation was upregulated by GSK-3β inhibitor, it was abated by PI3K inhibitors (Wu *et al.*, 2014a). It is well-recognized that Akt could inhibit GSK-3β by phosphorylating at its Ser9 residue (Van Weeren *et al.*, 1998). Interestingly, MAPKs like ERK also phosphorylate GSK-3β at Ser9 (Ding *et al.*, 2005). Meanwhile, p38 and potentially JNK inhibit GSK-3β by phosphorylating at Ser389 (Rojo *et al.*, 2012; Thornton *et al.*, 2008). Rada *et al.* found that GSK-3β can phosphorylate Nrf2 at Neh6 domain which is a docking site of β-TrCP adaptor protein that facilitate Nrf2 ubiquitination by recruiting Cullin1-Rbx1 E3 ligase complex. Hence, inhibition of GSK-3β by MAPKs and Akt would inhibit phosphorylation at Neh6 domain and prevented Nrf2 ubiquitination and degradation. In addition, report showed that mutation at

Neh2 domain disrupted Neh2/Keap1 association while Neh6 domain mutation affected Nrf2 stability (Rada *et al.*, 2011). These results suggested alternative regulation of Nrf2 through Keap1-independent mechanism. Succeeding study by Li *et al.* demonstrated that proteasome inhibitor treatment elevated Nrf2 level in murine embryonic fibroblasts (MEF) expressing wild type Keap1, indicating Keap1-dependent Nrf2 regulation. Nevertheless, inhibiting protein degradation with proteasome inhibitor in Keap1 knockout MEF also enhanced Nrf2 level compared to untreated cells, suggesting a Keap1-independent mechanism in Nrf2 regulation (Li *et al.*, 2012a). Taken together, these findings shed light on how multiple signaling pathways may converge at GSK-3β level and regulate Nrf2 stability through Keap1-independent mechanism on top of the canonical Keap1-dependent mechanism.

CHAPTER 6: SUMMARY OF MAJOR FINDINGS AND CONCLUSIONS

Neuroinflammation is implicated in the pathogenesis of ischemic stroke, traumatic brain injury, multiple sclerosis, meningitis, and neurodegenerative diseases (Rivest, 2009; Wohleb *et al.*, 2013). Though inflammation is activation of host defense system in response to infection or insult to eliminate pathogens and toxic, excess and dysregualted inflammatory responses could be detrimental. Understanding of neuroinflammation mainly derives from investigation of microglia and there is a general lack of knowledge of astrocytes' role in mediating inflammatory responses. Astrocytes that was once believed to only serve supportive roles in central nervous system (CNS) have recently emerged as a crucial participant in brain development, function and disease. Like microglia, astrocyte express various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) receptors which allow them to respond to various stimuli. Activation of astrocyte initiates inflammatory signaling pathways, induces release of proinflammatory cytokines/chemokines and reactive oxygen species (ROS) that exacerbate various neurological diseases (Sofroniew *et al.*, 2010).

Currently available therapeutics for CNS disorders are scarce and many have their own limitations. Therefore, more effective and safer new therapeutic are critically needed. Anti-inflammatory agents should be evaluated as one of such approach due to the increasing evidence implicating neuroinflammatory processes in pathogenesis of various neurological disorder. Therefore, I examined andrographolide, artesunate, and plumbagin that have previously reported to exhibit anti-inflammatory effects. Andrographolide appears to be a potential candidate due to its low toxicity, lipid solubility and ability to cross

blood brain barrier (BBB) (Bera *et al.*, 2014b; Hancke *et al.*, 1995; Zheng, 1982).

Andrographolide has been reported to exhibit anticancer, anti-bacterial, anti-inflammatory, and antioxidative effects (Arifullah *et al.*, 2013; Lim *et al.*, 2012a; Sheeja *et al.*, 2006). Nevertheless, there is a dearth of studies on the effects of andrographolide in the CNS. Moreover, there are few studies of andrographolide in astrocytes. There is only one study thus far that investigated andrographolide's antineuroinflammatory effects on primary astrocytes. That study investigated andrographolide's effects on cytokines and oxidative stress, but did not access chemokines expression and astrocyte activation (Tzeng *et al.*, 2012). Hence in this thesis, I performed comprehensive experiments to address the above mentioned knowledge gap.

At the initial stage of my study, I used interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) to model conditions where cytokines are released to initiate and perpetuate neuroinflammation. As described in Chapter 3, I found that andrographolide exhibited higher efficacy in attenuating cytokines-mediated nuclear factor-κB (NF-kB) activation and CCL-5 secretion in astrocyte as compared to artesunate and plumbagin. Andrographolide attenuated NF-κB signaling through inhibition of IκBα Ser32 and p65 Ser536 phosphorylation which promotes inhibitory IκBα protein degradation and p65 transactivation activity respectively (Figure 6.1). Andrographolide also significantly decreased glial fibrillary acidic protein (GFAP) level which is a reactive astrogliosis marker.

I then further investigated andrographolide's ability to regulate neuroinflammation under bacterial infection by using lipopolysaccharide (LPS).

Andrographolide attenuated LPS-induced NF-κB and c-Jun N-terminal kinase (JNK) activation and various cytokines/chemokines including IL-1β and TNF-α; CC motif chemokines (CCL-2 and CCL-5); CXC motif chemokines (CXCL-1, CXCL-2, CXCL-5, and CXCL-10); CX3C motif chemokine (CX3CL-1). Treatment with tosyl phenylalanyl chloromethyl ketone (TPCK) (NF-κB inhibitor) and SP600126 (selective JNK inhibitor) prevented elevation of cytokines/chemokines. Thus, andrographolide was proposed to attenuate cytokines/chemokines expression through NF-κB and JNK pathways. Nonetheless, I found that LPS-induced IL-1β is likely a downstream response of JNK-dependent signaling whereas LPS-induced CX3CL-1 is mainly NF-κB dependent (Figure 6.1).

Interestingly, andrographolide treatment on primary astrocyte culture enhanced LPS-induced extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase phosphorylation (Figure 4.6, see also (Keum *et al.*, 2006; Nguyen *et al.*, 2003; Zipper *et al.*, 2000). Treatment with andrographolide alone activated ERK and p38 in a time- and dose- dependent manner (Figure 4.3 and Figure 4.4). It was found that andrographolide regulates ERK and p38 signaling in a cell-specific manner; activating ERK and p38 signaling in astrocytic culture and promotes apoptosis in glioblastoma while inhibiting ERK/p38-mediated inflammatory response and cancer development in other cell types. In non-cortical tissues, ERK and p38 activation may induce Nrf2 stability and transactivation activity, and thus are involved in modulating antioxidant protein expression (Keum et al., 2006; Nguyen et al., 2003; Zipper et al., 2000). Indeed, chemically inhibiting ERK and p38 attenuated nuclear factor (erythroid-derived 2)-like 2 (Nrf2) accumulation and Nrf2 transcriptional

activity as indicated by reduction of heme oxygenase -1 (HO-1) and NAD(P)H dehydrogenase, quinone 1 (Nqo1) mRNA level (Figure 5.12). This suggests that andrographolide-stimulated ERK/p38 signaling is cytoprotective.

Moreover, oral administration of andrographolide attenuated brain expression of various chemokines (CCL-2, CCL5, CXCL-1, CXCL-2, CXCL-9) in a mouse model of bacterial infection-related neuroinflammation (LPS treatment). This implies that peripherally administered andrographolide could exert anti-neuroinflammatory effects. GFAP expression was also decreased. My results suggested that andrographolide is a potential anti-neuroinflammatory therapeutic that can penetrate the BBB (Bera *et al.*, 2014a) and inhibit leukocytes recruitment to the CNS by modulating chemokines expression in astrocytes.

Andrographolide has been previously reported to activate Nrf2 in the peripheral tissues (Chen *et al.*, 2014a; Guan *et al.*, 2013), however there is yet any study that examine andrographolide regulation on Nrf2 in CNS. Therefore, my study on astrocytes would allow a better understanding of the mechanism that underlies andrographolide's antioxidant effects in the CNS. Andrographolide's regulation on Nrf2 in astrocytes is summarized in Figure 6.2.

In conclusion, andrographolide's anti-neuroinflammatory and antioxidant mechanisms have been comprehensively investigated. My studies in cell lines, primary astrocytes as well as animal models have pointed to the efficacy of andrographolide to ameliorate astrogliosis as well as the accompanying upregulation of various cytokines and chemokines. Addditionally, andrographolide's antioxidant property is at least partly mediated by activation of Nrf2 signaling through Nrf2 protein stabilization and

ERK/p38 activation. Taken together, my study support that Andrographolide is a potential anti-neuroinflammatory therapeutic that ameliorates inflammatory response and upregulates antioxidative proteins. Thus, it may exhibit clinical benefits to CNS disorders where neuroinflammation is implicated for instance bacterial meningitis, stroke, and Alzhiemer's disease.

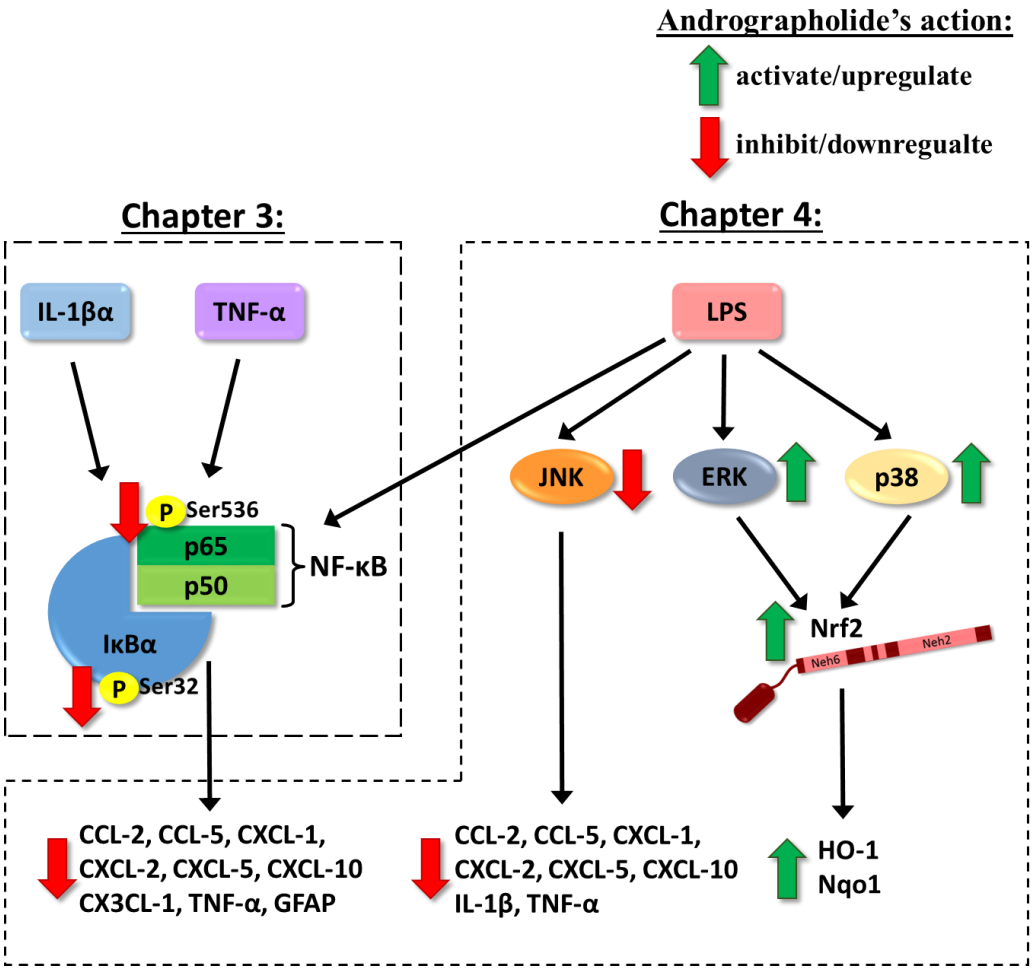


Figure 6.1 Summary of andrographolide's effects on NF-κB and MAPKs signaling pathways upon IL-1β, TNF-α, and LPS stimulation.

Chapter 5:

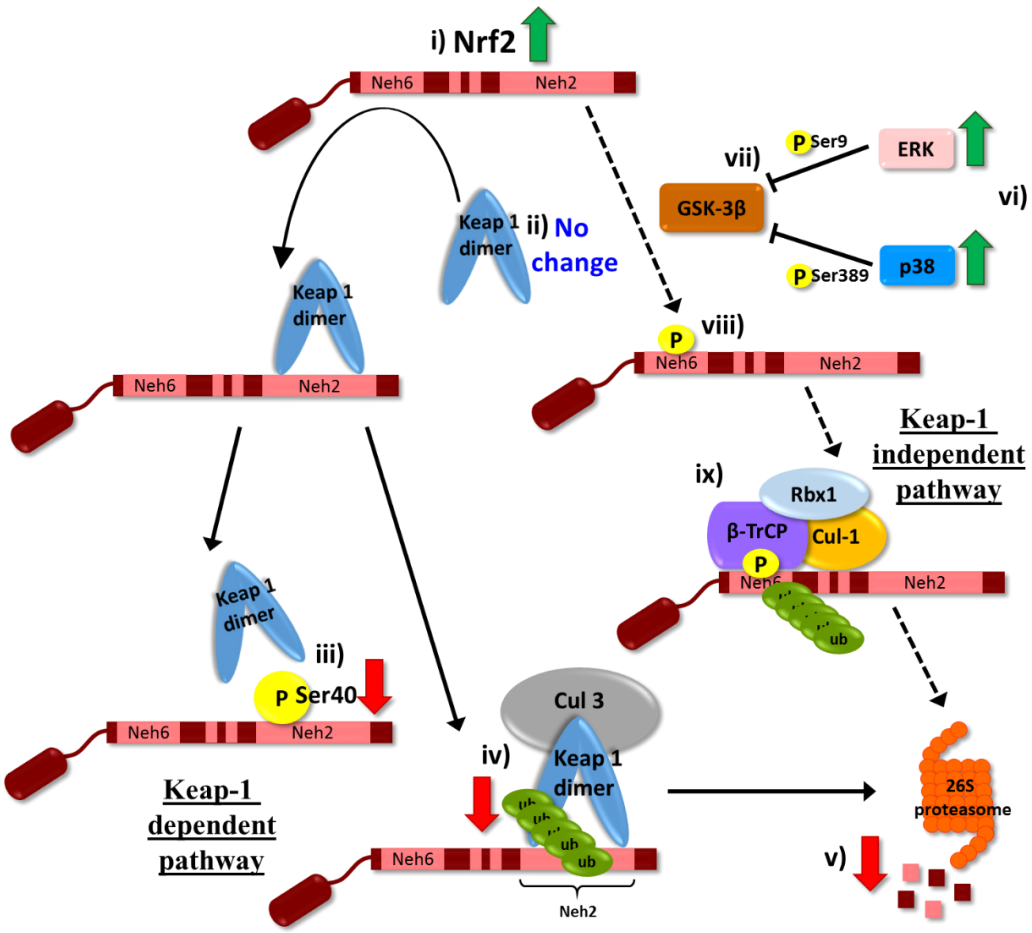


Figure 6.2 Andrographolide regulation on Nrf2 signaling.i) Andrographolide upregulated Nrf2 protein level independently of mRNA level. **ii)** Elevation of Nrf2 however was not due to andrographolide's inhibition on Keap1 expression level or **iii)** induction of Nrf2 Ser40 phosphorylation that promotes Keap1/Nrf2 dissociation. Nonetheless, **iv)** andrographolide attenuated Nrf2 ubiquitination which **v)** enhanced Nrf2 protein stability and decreased protein turnover rate. Interestingly, **vi)** andrographolide induced activation of ERK and p38 which at least partially contributed to Nrf2 activation. **vii)** ERK and p38 was previously reported to phosphorylate GSK-3β at Ser9 and Ser 389 respectively which inhibits its activity. On the other hand, **viii)** GSK-3β has been proposed to inhibit Nrf2 by phosphorylating it at Neh6 domain which is a docking site of β-TrCP adaptor protein. **ix)** β-TrCP could then facilitate Nrf2 ubiquitination and degradation by recruiting Cullin1-Rbx1 E3 ligase complex. Thus, I conclude that andrographolide reduces ubiquitination of Nrf2 therefore enhancing its protein stability and activates ERK and p38 which potentially regulate Nrf2 activity through a Keap1-independent pathway.

CHAPTER 7: FUTURE STUDY

Though ERK and p38 inhibition significantly attenuated andrographolide-induced HO-1 and Nqo1 mRNA expression, subsequent increase in inhibitor concentration had subtle effect on HO-1 and Nqo1 level (Figure 5.12). Highest inhibitor concentration (50 μM) used did not reduce HO-1 and Nqo1 level close to their basal expression. Hence, other mechanisms may be mediated by andrographolide to regulate expression of these cytoprotective genes. Andrographolide is a diterpenoid that can modify sulfhydryl groups of cysteine via adduct formation. It inhibits NF-κB DNA binding by forming a covalent adduct with Cys62 of p50 subunit (Xia *et al.*, 2004). As Keap1 and Neh2 domain are rich in cysteine, andrographolide is likely to impede Keap1/Neh2 interaction by forming adduct with either of them. Recently, p21 was discovered to compete with Keap1 to bind at Neh2 domain of Nrf2 which subsequently abrogated Nrf2 degradation (Chen *et al.*, 2009b). p21 is a cyclin-dependent kinase inhibitor that function as a tumor suppressor whereas andrographolide is well-known for its tumor suppression and one of its anticancer mechanism is through induction of p21 (Lim *et al.*, 2012a; Yan *et al.*, 2012). Hence, andrographolide may induce p21 and inhibit Keap1/Nrf2 association and prevented Nrf2 degradation. Unequivocal findings suggest numerous mechanisms could be involved in andrographolide mediated Nrf2 activation and more studies need to be done in order to validate these potential mechanisms.

Labdane diterpenoids are the major constituents of *A. paniculata*. Other than andrographolide, other labdane diterpenoids present include andrographiside, neoandrographolide, isoandrographolide, 14-deoxy-11,12-didehydroandrographiside and more (Lim *et al.*, 2012a). Though therapeutic

benefits of andrographolide has been slowly uncovered, the biological activities of most andrographolide derivatives are largely unknown. The anti-neuroinflammatory effects of these derivatives have yet been examined and therefore in the future study, these derivatives could be compared side by side to determine which derivative is the most potential anti-neuroinflammtory compound.

In my study, I showed that andrographolide elevated IL-6 expression. Instead of NF-κB and JNK, other signaling pathway seems to be involved in andrographolide-mediated upregulation of IL-6. Interestingly, Nrf2 has been reported to induce IL-6 expression via an antioxidant response element (ARE) located within IL-6 promoter. Nrf2 knockout mice failed to induce IL-6 expression and suffer from oxidative stress and neuronal death in response to oxidant treatment (Wruck *et al.*, 2011). Thus, proposing that andrographolide mediates IL-6 expression through Nrf2 activation. Nonetheless, the precise mechanism involved in andrographolide-mediated IL-6 production needs to be validated and the exact role of IL-6 elevation in neuroinflammation remains to be elucidated.

Anti-neuroinflammatory effects of andrographolide was investigated in mice induced with neuroinflammation arisen from peripheral infection and results showed a significant downregulation of several chemokines measured. Therefore, in the future study, effects of andrographolide can be examined in animal models with profound inflammation originated from the CNS for instance Alzheimer's disease transgenic mouse model. Moreover, behavioral studies like Morris water maze and passive avoidance test could be performed to investigate the effects of andrographolide in improving memory deficits.

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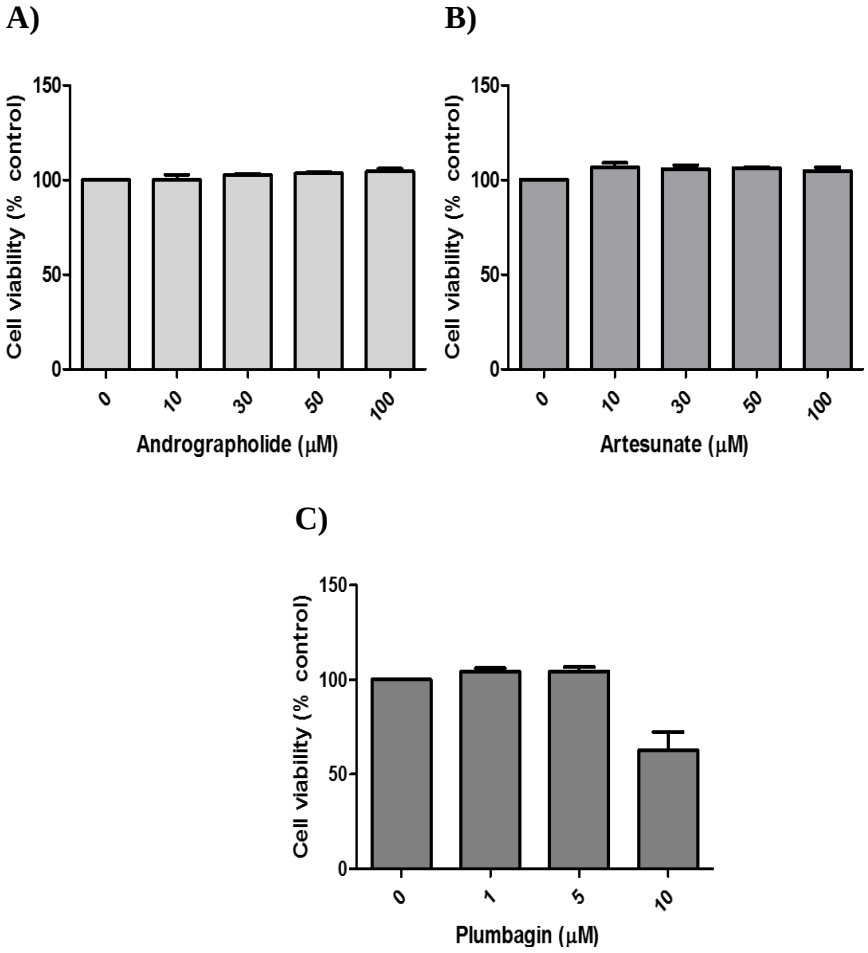
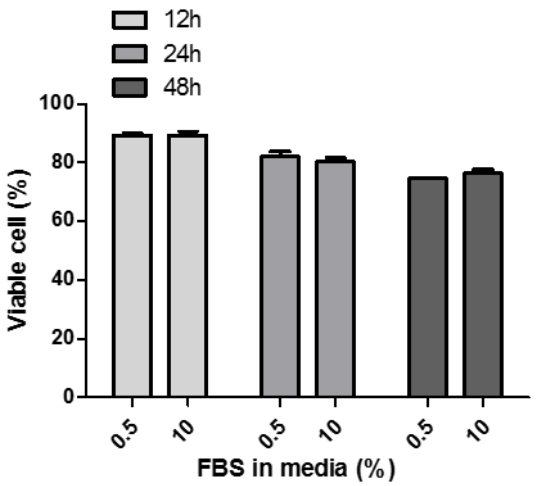
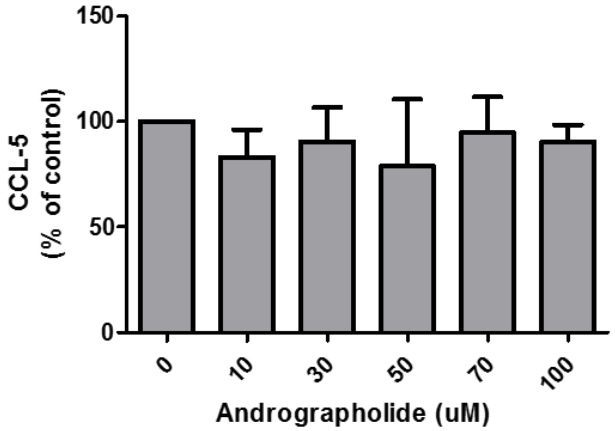
APPENDIX



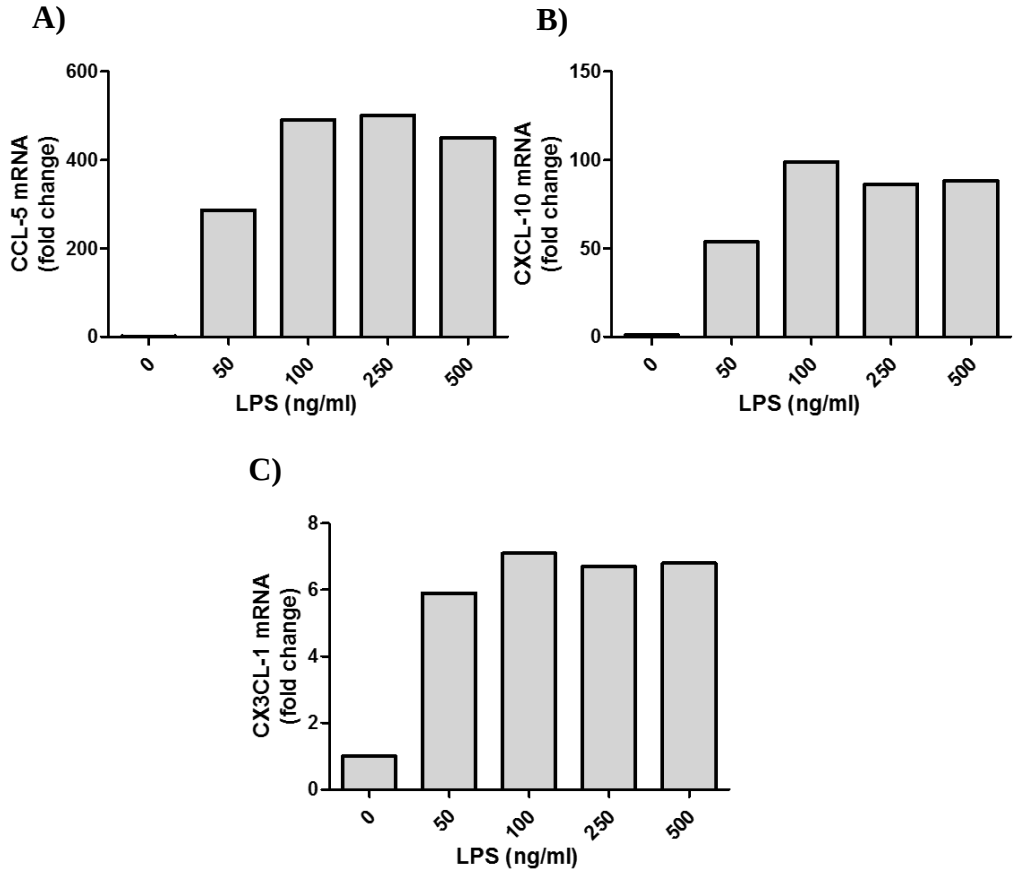
Appendix 1: Plumbagin reduced U373 cell viability. MTS assay of U373 cells incubated with various concentration of andrographolide **(A)**, artesunate **(B)**, or plumbagin **(C)** for 24 hours. Data shown are mean ± S.E.M of two separate experiments.



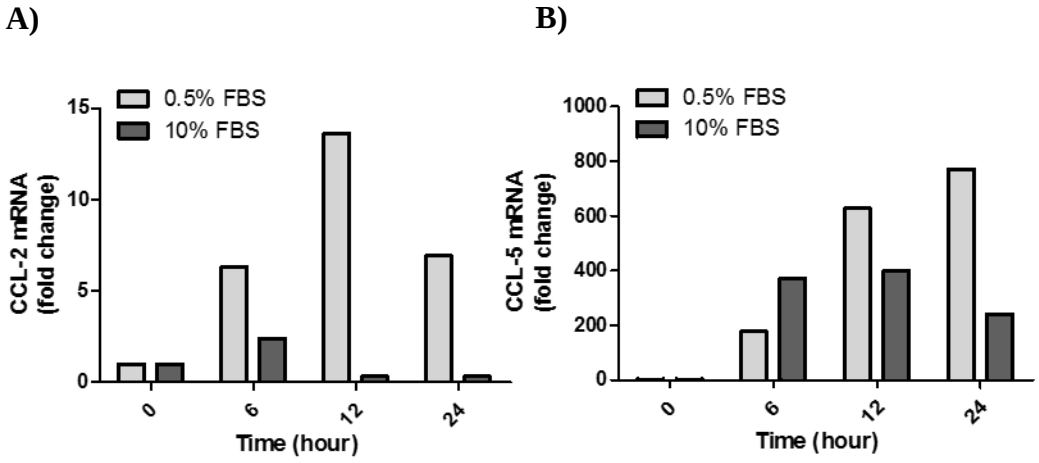
Appendix 2: Cell viability of primary astrocytes does not differ in 0.5% and 10% FBS supplemented media. Cell viability of primary astrocytes grown in 0.5% or 10% FBS supplemented media up to 48 hours was determined with Muse Count and Viability assay. Sample size n = 3. Data shown are mean ± S.E.M.

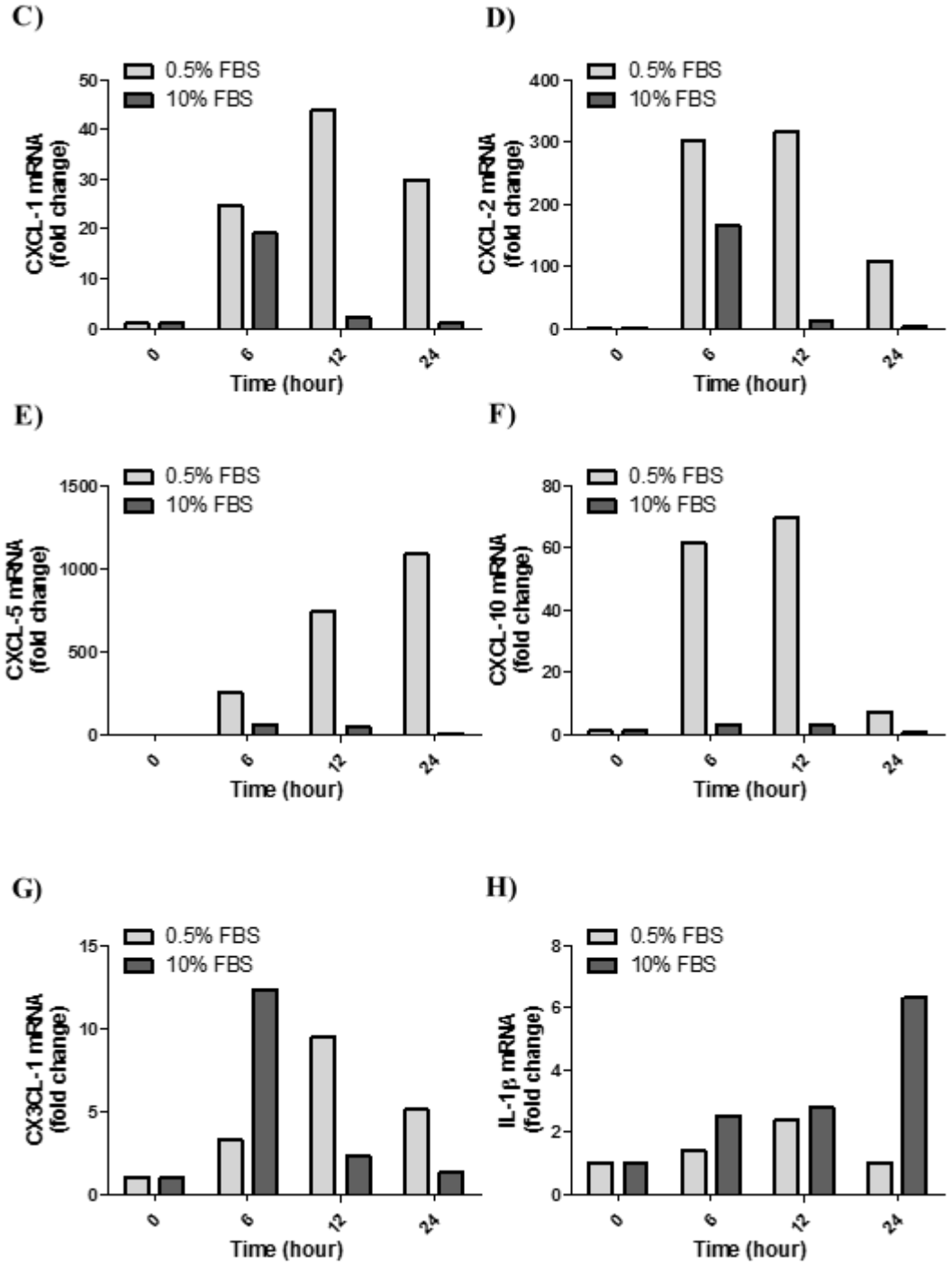


Appendix 3: Andrographolide alone does not induce CCL-5 secretion. Upon 24 hours of andrographolide incubation, 0.5% FBS supplemented media was collected from primary astrocytes and processed for Luminex assay. Data shown are mean ± S.E.M of two separate experiments.

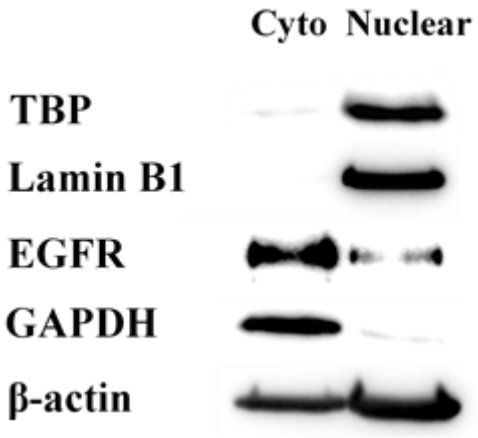


Appendix 4: Stimulation of primary astrocytes with various doses of LPS. Primary astrocytes were stimulated with increasing doses of LPS 12 hours in 0.5% FBS supplemented media. Transcript level of various cytokines were analyzed by real-time PCR. Results exhibit a LPS dose dependency in cytokines mRNA expression.





Appendix 5: mRNA expression profile of various cytokines in LPS-stimulated primary astrocytes. Primary astrocytes were stimulated with LPS 100 ng/ml for indicated timing in 0.5% FBS or 10% FBS supplemented media. Transcript level of various cytokines were analyzed by real-time PCR. In most cases, 0.5% FBS condition induces higher level of mRNA expression and it usually peaks at 12 hours LPS stimulation. Therefore, cell treatment was in 0.5% FBS media with LPS stimulation being kept at 12 hours for real-time quantification of cytokines mRNA level.



Appendix 6: Determination of subcellular fractionation efficiency via immunoblotting. Primary astrocytes cultured in 10% FBS supplemented media were harvested and subjected to subcellular fractionation. Both cytoplasmic (Cyto) and nuclear (Nuclear) fractions were immunoblotted for nuclear protein, TATA box binding protein (TBP) and lamin B1; cytoplasmic protein, epidermal growth factor receptor (EGFR), GAPDH, and β-actin. 10 μg protein was loaded for each sample.