EFFECTS OF GLUCOCORTICOIDS AND RETINOIC ACID ON ACTIVATED RAT MICROGLIAL CELLS IN PRIMARY CULTURE

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**International Journals:**

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**Conference Abstracts:**


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ABBREVIATIONS

ABC, avidin-biotin conjugate
AD, Alzheimer’s disease
AEA, endocannabinoid anandamide
AP-1, activating protein-1
APC, antigen-presentation cell
APP, amyloid precursor protein
Aβ, β-Amyloid
BBB, blood-brain barrier
BrdU, Bromodeoxyuridine
BSA, bovine serum albumin
B-SA, biotin-streptavidin
bZIP, basic region-leucine zipper
CCR2, chemokine (C-C motif) receptor 2
CJD, Creutzfeldt-Jakob disease
Ct, threshold cycle
DAPI, 4’, 6- diamidino-2-phenylindole dihydrochloride
Dex, Dexamethasone
DMEM, Dulbecco’s Modified Eagle Medium
DMSO, Dimethyl Sulfoxide
JNKs, c-jun N-terminal kinases/stress-activated protein kinases
EAE, experimental autoimmune encephalomyelitis
ECL, enhanced chemiluminescence
ERK1/2, extracellular-signal-regulated kinases
GAPDH, glyceraldehydes-3-phosphate dehydrogenase
GCs, Glucocorticoids
GM-CSF, Granulocyte-Macrophage colony stimulating factor
GR. Glucocorticoid receptor
GREs, glucocorticoid response elements
HIV associated dementia (HAD)
HRP, horseradish peroxidase
IFN-\( \gamma \), interferon - \( \gamma \)
IGF, insulin-like growth factors
IHC, Immunohistochemistry
IL-1\( \beta \), interleukin-1\( \beta \)
iNOS, inducible nitric oxide synthase
JNK, C-Jun N-terminal kinase
LPS, lipopolysaccharide
LBP, LPS binding protein
MAC-1, macrophage antigen complex-1
MAPK, mitogen-activated protein kinase
MCP-1, Monocyte chemoattractant protein-1
M-CSF, Macrophage colony stimulating factor
MHC, major histocompatibility complex
MKP-1, MAPK Phosphatase-1
MK2, MAPK-activated protein kinase 2
MS, multiple sclerosis
NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase
NO, Nitric Oxide
NFTs, neurofibrillary tangles
NF-\( \kappa B \), nuclear factor-\( \kappa B \)
PAP, peroxidase antiperoxidase
PARs, protease-activated receptors
PBS, phosphate-buffered saline
RT-PCR, reverse transcription-polymerase chain reaction
PI, Propidium iodide
PD, Parkinson’s disease
An inflammatory process in the central nervous system (CNS) is believed to play an important role in the pathway leading to neuronal cell death in a number of neurodegenerative diseases. The inflammatory response is mediated by the activated microglia, the resident immune cells of the CNS. In response to a variety of stimuli, microglia undergo rapid proliferation, secrete a number of proinflammatory cytokines, migrate to the injury sites, and remove the damaged cells by phagocytosis. Although microglia play a beneficial role in large by removing potentially toxic cellular debris, it remains controversial whether microglial cells have beneficial or detrimental functions in various neuropathological conditions. The chronic activation
of microglia may cause neuronal damage through the release of potentially cytotoxic molecules such as proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and reactive oxygen intermediates. Therefore, suppression of microglia-mediated inflammation has been considered as an important strategy in neurodegenerative disease therapy. Several anti-inflammatory drugs have been shown to repress the microglial activation and to exert neuroprotective effects in the CNS after different types of injuries. However, these drugs do not specifically target microglial cells and therefore, exhibit potential systemic side effects. In this study, we attempted to understand the potential mechanisms and signaling pathways by which two drugs, glucocorticoids (GCs) and all-trans-retinoid acid (RA), suppress the activation of microglial cells in CNS diseases.

**Dexamethasone inhibits chemotactic activity of microglia:** Microglial cells release monocyte chemotactic protein-1 (MCP-1) which is believed to amplify the inflammation process by recruiting macrophages and microglia to the inflammatory sites in CNS diseases. GCs are widely used anti-inflammatory and immunosuppressive drugs in several neurological diseases. Recently, it has been shown that GCs could inhibit LPS-induced MCP-1 production in the hippocampus and cortex (Szczepanik and Ringheim 2003; Little *et al.* 2006). However, the molecular mechanisms by which GCs regulate MCP-1 expression in activated microglial cells have not been elucidated. It has been reported that GCs counter-regulate mitogen-activated protein kinase (MAPK) signaling pathways, in particular p38 and Jun N-terminal kinase (JNK) pathways by inducing expression
MAPK phosphatase-1 (MKP-1) (Clark 2003). Moreover, activation of MAP Kinases in microglial cells, leads to phosphorylation of transcription factors such as c-Jun/AP-1 resulting in induction of expression of some target genes including TNF-α, and MCP-1 (Babcock et al., 2003; Waetzig et al., 2005). In view of these observations, it was hypothesized that GCs inhibit MCP-1 production via MKP-1-mediated inactivation of MAP Kinases, resulting in decreased microglial migration towards the sites of inflammation in the CNS. Hence, effects of dexamethasone (Dex), a synthetic GC on MAP Kinase pathways and expression of MCP-1 in activated microglia as well as migration of microglia have been investigated using the real time RT-PCR, immunocytochemistry, Western blot, ELISA and in vitro chemotaxis assay. The results indicate that Dex suppressed the mRNA and protein expression of MCP-1 in activated microglia resulting in inhibition of microglial migration. This has been further confirmed by the chemotaxis assay which showed that Dex or MCP-1 neutralization with its antibody inhibits the microglial recruitment towards the conditioned medium of LPS-treated microglial culture.

This study also revealed that the downregulation of the MCP-1 mRNA expression by Dex in activated microglial cells was mediated via MAPK pathways. It has been demonstrated that Dex inhibited the phosphorylation of JNK and p38 MAP kinases as well as c-jun, the JNK substrate in microglia treated with LPS. The involvement of JNK and p38 MAPK pathways in induction of MCP-1 production in activated microglial cells was confirmed as there was an attenuation of MCP-1 protein release when microglial cells were treated with inhibitors of JNK and p38. In addition,
Dex induced the expression of MAP kinase phosphatase-1 (MKP-1), the negative regulator of JNK and p38 MAP kinases in microglial cells exposed to LPS. Blockade of MKP-1 expression by triptolide enhanced the phosphorylation of JNK and p38 MAPK pathways and the mRNA expression of MCP-1 in activated microglial cells treated with Dex.

In brief, Dex inhibits the MCP-1 production and subsequent migration of microglial cells to the inflammatory site by regulating MKP-1 expression and the p38 and JNK MAPK pathways. This study reveals that the MKP-1 and MCP-1 as novel mediators of biological effects of Dex may help developing better therapeutic strategies for the treatment of patients with neuroinflammatory diseases.

Retinoic Acid inhibits neuotoxic effect and proliferation of microglia:
Retinoic acid (RA), the biologically active form of vitamin A, exhibits anti-proliferatory and anti-inflammatory activities in various cell types (Mathew and Sharma 2000). It has also been demonstrated that RA is synthesized in the adult vertebrate brain (Dev et al. 1993; Zetterstrom et al. 1999). In view of these observations, it is hypothesized that RA may modulate the inflammatory response and proliferation index of microglia. Hence, we have investigated the effects of RA on release of proinflammatory cytokines and proliferation in activated microglia using immunocytochemistry, Western blot, and ELISA. It has been shown that RA could inhibit microglial activation by suppressing their secretions of TNF-α as well as NO in primary microglia cultures exposed to LPS. This inhibition of TNF-α and NO syntheses by RA in the activated microglia appeared to be mediated via inhibition of
NF-κB translocation which could be caused by upregulation of RAR and TGF-β1 gene expression. It has also been shown that RA could inhibit syntheses of TNF-α and NO in activated microglia by MKP-1-mediated inhibition of JNK MAP kinase pathway.

Moreover, this study demonstrates that RA inhibits GM-CSF induced microglial proliferation by altering the expression of cell cycle associated proteins such as cyclin D1, E2F transcription factor 1 (E2F-1), Retinoblastoma (Rb) and p27. Based on the results, it is suggested that RA could be considered a potential therapeutic agent that may inhibit the expansion and activation of microglia in the neurodegenerative diseases. However, careful evaluation is needed before RA is considered for the treatment of neurodegenerative diseases as it modulates a wide variety of biological processes including proliferation, differentiation and apoptosis in various cell types.
Chapter 1

Introduction
Introduction

The central nervous system (CNS) consists of neurons and glial cells including astrocytes, oligodendrocytes and microglia. Microglia were first recognized in the brain by Nissl in 1899 (Nissl 1899) and constitute about 5-12% of the total glial population (Ling and Leblond 1973). They play an important role as resident immune cells in the healthy and diseased CNS. It is generally agreed that microglia are related to monocytes and belong to the mononuclear phagocyte lineage (Vilhardt 2005). Microglia display considerable phenotype heterogeneity during their life cycle such as ameboid, ramified and reactive microglia (Kaur and Ling 1991). Ameboid microglia found in the developing brain are phenotypically similar to reactive microglia found in the pathological conditions, both of which have a large spherical body and short processes. During postnatal stages of development, the ameboid microglia transform into resting ramified microglia which are distributed ubiquitously throughout the nervous system including the optic nerve and retina (Nissl 1899; Kaur et al. 2006b). When ramified microglia are activated in pathological conditions, they transform into ameboid morphology. Concurrently, they acquire functions such as induction of inflammation, phagocytosis and antigen presentation to circulating T cells in order to mobilize the defence system in the CNS (Aloisi 2001, Vilhardt 2005). Besides serving as resident immune cells in the brain, microglia also interact dynamically with neurons and other glial cells, thus fulfilling important neurotrophic roles (Vilhardt, 2005).
1.1 Origin of microglia

Despite intense study, the precise tissue origin and cell lineage of microglia are still at the centre of debate. Unlike astrocytes, oligodendrocytes and neurons, which are believed to be derived from neuroectoderm, microglia have been considered to have originated from (i) neuroectoderm, (ii) peripheral mesodermal/mesenchymal tissues, or (iii) from circulating blood monocytes (Chan et al. 2007). The view that is widely accepted by many is the latter - that microglia are derived from circulating mesodermal hematopoietic cells which in mammals originate from the yolk sac (Chan et al. 2007). It has been demonstrated that circulating monocytes or precursor cells of the monocyte-macrophage lineage invade the developing brain during embryonic, fetal or postnatal stages (Kaur et al. 2001) and transform into microglial cells which express several proteins, specific for cells of the monocyte/macrophage lineage (Kaur et al. 1984; Kaur and Ling 1991; Ling and Wong 1993). These findings, together with the phagocytic activity of microglia suggest that microglia are derived from circulating monocytes and belong to the mononuclear phagocytic system.

1.2 Functions of microglia

In the developing brain, ameboid microglia phagocytose, the cells undergoing apoptosis, and are also actively involved in the determination of cell fate (elimination/survival) (Vilhardt 2005). In the adult brain, ramified resting microglia serve as supportive glial cells and their phagocytic functions are
downregulated. However, upon stimulation, the ramified microglia undergo a series of morphological and functional changes in order to respond specifically and appropriately towards the insult by induction of inflammation, tissue repair, neurotropic support, or activation of lymphocytes. The upregulation of multiple immunological functions is referred to as activation and is paralleled by both morphological transformation and discrete temporal changes in gene expression (Vilhardt 2005).

1.2.1 Phagocytosis

As macrophages of the CNS, the phagocytic capacity of microglia is well demonstrated. Microglia are capable in displaying a phagocytotic response to β-amyloid (Aβ) (Wilkinson et al. 2006), degenerated myelin (Makranz et al. 2006), fibrillar prion peptide (Ciesielski-Treska et al. 2004), and apoptotic and nonapoptotic damaged cells (Petersen and Dailey 2004). Microglial interaction with these macromolecules and damaged cells is mediated through an ensemble of cell surface receptors, and thus prevents their harmful effects.

1.2.2 Release of cytokines and chemokines

Cytokines and chemokines secreted by microglia constitute microglial communication and effector system. Microglial cytokines and chemokines regulate innate defense mechanisms, help the initiation of immune responses, participate in the recruitment of leukocytes into the CNS, and support tissue repair
and recovery (Hanisch 2002).

Tumor necrosis factor-α (TNF-α) is one of the major proinflammatory cytokines with pleiotropic functions produced by microglia as well as blood-derived macrophages during CNS inflammation. In microglia culture, synthesis and release of TNF-α is induced by pathogens or pathogen components (such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ)). TNF-α has been implicated in the development of CNS inflammation mainly through their ability to induce expression of chemokines and adhesion molecules in cerebrovascular endothelial cells and astrocytes, which help leukocyte extravasation into the CNS (Lee and Benveniste 1999).

Chemokines are chemotactic cytokines which act through G-protein-coupled receptors. Chemokines could participate in directing microglia recruitment to injured CNS sites and sustain their activation, whereas chemokines produced by microglia are likely to contribute to leukocyte recruitment and amplification of CNS inflammation. Chemokines promote migration of microglia to a particular site in the brain during development and disease processes. In conjunction with integrins and endothelial cell-adhesion molecule, chemokines are believed to control the circulation of macrophages, leukocytes and other immune cells (Ambrosini and Aloisi 2004). A variety of inflammatory chemokines and chemokine receptors have been described in the brain during disease processes. Glial cells stimulated by LPS and inflammatory cytokines, TNF-α and IL-1
(interleukin-1), *in vitro* have been shown to produce several chemokines such as macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), MCP-1, IL-8 and RANTES (regulated upon activation, normal T cell expressed and secreted) (Lokensgard *et al.* 1997; Peterson *et al.* 1997; Ehrlich *et al.* 1998; Lipovsky *et al.* 1998; McManus *et al.* 1998). Microglia in culture also express chemokine receptors, CCR3, CCR5 (He *et al.* 1997), CXCR4 (chemokine (C-X-C motif) receptor 4)(Lavi *et al.* 1997) and CX3CR1 (chemokine (C-X3-C) receptor 1) (Nishiyori *et al.* 1998).

Among chemokines, monocyte chemotactant protein-1 (MCP-1, also known as CCL2), a member of β-chemokine subfamily, mediates the migration of microglia, monocytes and lymphocytes to the inflammation sites in the CNS (Cross and Woodroffe 1999; Taub *et al.* 1995; Gunn *et al.* 1997; Babcock *et al.* 2003) and is produced mainly by astrocytes and microglia (Hayashi *et al.* 1995). The MCP-1 acts on its targets by binding to its receptor, chemokine (C-C motif) receptor 2 (CCR2) which is a seven-transmembrane domain G-protein coupled receptor. The expression of MCP-1 and CCR2 has been shown to be induced following diverse CNS insults, including ischemia (Che *et al.* 2001; Minami and Satoh 2003; Vilhardt 2005), Alzheimer’s Disease (AD) (Fenoglio *et al.* 2004), HIV type-1-associated dementia (HAD)(Conant *et al.* 1998), multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (EAE) (Sorensen *et al.* 1999; Simpson *et al.* 1998; Glabinski *et al.* 2003). In an EAE animal model, MCP-1 induced the recruitment and activation of endogenous
microglia and blood-derived macrophages to demyelinated areas, promoting myelin phagocytosis (Ambrosini and Aloisi 2004). Moreover, the functional antagonism of MCP-1 attenuates leukocyte infiltration and decreases the severity of CNS injury (Calvo et al. 1996; Muessel et al. 2002). In a murine stroke model, MCP-1 deficiency has been shown to have a protective role in acute infarct growth (Hughes et al. 2002).

1.2.3. Release of proteases

Activated microglia produce a number of proteases, which contribute to various events in the CNS through proteolysis (Nakanishi 2003). These proteases include cathepsin, endosomal/lysosomal proteases, tissue-type plasminogen activator, and matrix metalloproteases, etc. They have been shown to play important roles in the MHC class II-mediated antigen presentation, processing of pro-inflammatory cytokines and microglia activation (Deussing et al. 1998; Chauvet et al. 2001; Kakimura et al. 2002). Some members of proteases are also involved in neuronal death and clearance of phagocytosed Aβ peptides (Flavin et al. 2000; Hamazaki 1996).

1.2.4 Generation of reactive oxygen species (ROS) and nitrogen intermediates

Microglia can produce ROS when activated by various stimuli. NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), which catalyses the production of superoxide from the oxygen, is implicated as the primary source
of microglial-derived extracellular ROS (Babior 2000). In addition to the production of extracellular ROS, NADPH oxidase is also thought to be a crucial component of microglial signalling (Babior 2000; Block et al. 2007). NADPH oxidase-generated intracellular ROS can act as second messenger to amplify the pro-inflammatory function through effects on kinase cascades and transcription factor activation (Block et al. 2007). However, high levels of intracellular ROS might result in microglial death (predominantly by apoptosis) (Sim et al. 2005). The overactivation of NADPH oxidase and the dysregulation of intracellular ROS in microglia are associated with neurodegenerative diseases such as Alzheimer’s Disease (AD) (Shimohama et al. 2000).

NO, which is produced by the action of nitric oxide synthase (NOS), possesses a diverse array of physiologic functions, such as muscle relaxation, immune modulation, and neuronal activity (Grisham et al. 1999). As a free radical, NO is one of the major contributors to the formation of reactive nitrogen species. On the other hand, NO directly reacts with proteins, especially heme-containing enzymes, such as guanylate cyclase, cytochrome P450, cyclooxygenase, and hemoglobin, to modulate their functions. In addition, NO can react with membrane lipids and induce lipid peroxidation. Indirectly, the combination of NO and superoxide can form highly reactive intermediates, such as peroxynitrite, that can induce DNA strand breaks, lipid peroxidation, and protein nitration (Beckman 1996). The reactive microglia in neurodegenerative diseases produce an increased amount of NO, which either directly or through its highly toxic derivative,
peroxynitrite augments neurotoxicity in the CNS (Possel et al. 2000). Inducible NOS, which is a calcium independent enzyme, catalyzes the production of NO. Several proinflammatory cytokines have been shown to induce iNOS expression (Knerlich et al. 1999).

1.2.5 Migration
A rapid increase in number of microglial cells at injury sites is partly resulted from recruitment from other CNS regions by chemotaxis. The migration of microglia is regulated by chemokines, which are small, basic proteins produced during injury and infection (Ambrosini and Aloisi 2004). Under resting conditions in vitro, microglia have perinuclear distribution of actin and tubulin and lack membrane localization of these proteins and process formation (Eugenin et al. 2005). When cells are treated with a chemotactic stimulus, rearrangement of actin and tubulin occurs to facilitate the process of attachment, protrusion and traction that allows microglia to migrate (Eugenin et al. 2005). Chemokine receptors can also be redistributed to the leading edge when microglia acquire a migratory phenotype (Eugenin et al. 2005). Chemokines orchestrate chemotaxis as a protective response to injury, however, the recruited cells can also have harmful consequences by promoting toxicity (Ambrosini and Aloisi 2004).

1.2.6 Upregulation of antigen-presentation cell (APC) capabilities
Antigen presentation is the critical event involved in the generation of T-cell responses against infectious agents or against self-components (Aloisi 2001). It requires interaction between the T-cell receptor and processed antigen peptides
bound to major histocompatibility complex (MHC) molecules on the surface of APC (Aloisi 2001). Microglial cells behave as poor APCs in their resting condition since the ability to present antigens to T cells is inhibited in the normal CNS (Bailey et al. 2006). However, microglia upregulate MHC-II expression in virtually all inflammatory and neurodegenerative conditions (Kreutzberg 1996). Microglial cells in these pathological conditions are able to take up, process, and present protein antigen to naive, memory, and differentiated T cells, leading to either T cells proliferation, cytokine secretion or both (Becher et al. 2000).

1.2.7 Proliferation

One of the main characteristic features of microglial cell population is their remarkable capacity to expand in response to injury or neurological disease processes (Ladeby et al. 2005). This expansion is considered predominantly to be due to proliferation of activated microglia and to a lesser extent migration of microglia from adjacent brain areas as discussed in detail before (Ladeby et al. 2005). Indeed, proliferating microglia have been implicated in the onset and/or progression of a number of CNS pathology such as trauma (Urrea et al. 2007), ischemia (Denes et al. 2007), Parkinson’s disease (Henze et al. 2005) and demyelination (Remington et al. 2007). These cells which respond to injury may participate in brain repair and functional recovery by phagocytosis of debris; however, they may also contribute to glial scar formation and excess release of cytotoxic factors (Byrnes and Faden 2007).
The most likely candidates that induce and sustain microglial proliferation are the colony stimulating factors (CSF), including macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF), both of which promote microglial proliferation in vitro as well as in vivo (Sawada et al. 1990; Lodge and Sriram 1996; Kloss et al. 1997; Sasaki et al. 2000), and are produced either by astrocytes or microglia themselves in an autocrine fashion (Nakajima et al. 2006). Other factors which have been shown to promote microglial proliferation and survival are NT-3 (Elkabes et al. 1996), IL-4 and IL-5 (Elkabes et al. 1996).

The mechanisms of the microglia proliferation appear to involve the regulation of cell cycle. The cell cycle is the series of events that can be divided into 4 phases: G1 phase (Gap1 phase), S phase (DNA replication phase), G2 phase (Gap2 phase), and M phase (mitotic phase). Progression through the cell cycle is controlled by the interaction of several factors including cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). Cyclins are key molecules in cell cycle control. Cyclin D (including D1, D2, and D3) forms a complex by binding with CDK4 and CDK6, and regulates the transition from G1 phase to S phase, which is a rate-limiting step in the cell cycle (Bruce et al. 2007). The best known effects of Cyclin D/CDK4,6 complex activity are mediated by a gene regulatory protein, E2F, which transactivates many genes that encode proteins required for S phase entry (Bruce et al. 2007). E2F function is in turn controlled by an interaction with the retinoblastoma protein (Rb), an
inhibitor of cell-cycle progression (Simin et al. 2004). During G1 phase, Rb binds with E2F and blocks the transcription of S-phase genes. When cells are stimulated to divide by extracellular signals, cyclin D/CDK4, 6 complexes induce phosphorylation of Rb, and release of E2F that activates transcription of S phase genes (Bruce et al. 2007). E2F-1 is one of the six members of the E2F family. Although E2F-1 activity is considered to be regulated mainly through its temporal association with Rb, recent research has shown a direct induction of E2F-1 by growth factors such as IGF-1 (Shen et al. 2004). P27 belongs to the family of CDKIs, is associated with a variety of cyclin-CDK complexes and inhibits their activity, leading to cell cycle arrest at G1/S phase (Simeone and Tari 2004).

Although microglia in mature CNS are considered terminally differentiated, they can re-enter the cell cycle after stimulation (Suh et al. 2005). Expression of cell cycle proteins such as cyclin D1 in microglia has been shown in the hippocampus of ischemic rat brain (Wiessner et al. 1996). Furthermore, Granulocyte-Macrophage colony stimulating factor (GM-CSF) administration has been shown to increase the expression of cyclin D1 and decrease the expression of p27 in a microglial cell line (GMI-M6-3) (Koguchi et al. 2003). Further understanding of mechanisms of microglial proliferation may improve therapeutic strategy that limits the microglial expansion and subsequent neurotoxicity in CNS diseases.
1.3 Microglial activation

1.3.1 Activation of microglia by various stimuli

Functions of microglial cells in the CNS appear to be complex as they exhibit both neuroprotective and neurotoxic effects. In the past decades, a large number of papers have focused on the understanding of mechanisms of microglial activation in response to neuropathological conditions \textit{in vivo} and \textit{in vitro}. For \textit{in vitro} analysis, microglial cells are activated by various inflammatory stimuli such as LPS, Aβ, thrombin and some proinflammatory cytokines including IFN-γ. The inflammatory response of activated microglia appears to be consistent although the nature of the stimuli varies.

1.3.1.1 Lipopolysaccharide

LPS is a cell wall component of Gram-negative bacteria and the most frequently used activator for microglia activation and inflammatory signaling. In experimental endotoxemia, LPS has been shown to enter the brain parenchyma by diffusion through regions with defective blood-brain barrier (BBB) function. After binding of LPS to LPS binding protein (LBP), its receptor CD14 presents the LPS-LBP complexes to Toll-like receptor 4 (TLR4) (Chen \textit{et al.} 2002). Interaction of TLR4 with the LPS-LBP-CD14 complexes triggers a signaling cascade involving activation of a set of transcription factors such as NF-κB and AP-1 (Rivest 2003). This ultimately leads to the expression of a wide array of inflammatory mediators including proinflammatory cytokines, chemokines and
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ROS which orchestrate inflammation and activation of adaptive immunity to eliminate invading microorganisms (Lacroix et al. 1998).

1.3.1.2. β-Amyloid

Aβ have been widely used to activate microglial cells in vitro. Extracellular Aβ deposit (or senile plaques) is one of the two characteristic lesions in the brains of individuals with AD. They induce the neurodegeneration both directly by interacting with components of the cell surface to trigger apoptogenic signaling and indirectly by activating microglia to produce excess amounts of inflammatory cytokines (Chiarini et al. 2006). Microglial cells activated with the treatment of Aβ1-42 or Aβ25-35 in vitro exhibit upregulation of mRNA and protein expression of proinflammatory cytokines, proteases and chemokines which include IL-1β, IL-8, IL-10, IL-12, TNF-α, MIP-1α, MIP-1β and MCP-1 (Nagai et al. 2001). These results indicate that reactive microglia play a significant role in the initiation and propagation of immune responses as the inflammatory mediators during the process of inflammation in neurodegenerative diseases.

1.3.1.3. Interferon -

Interferon (IFN)- also appears to be a key cytokine in the activation of microglial cells (Chao et al. 1993; Xu and Ling 1994). In murine models, microglial cells exhibit significantly increased myelin phagocytosis, proteolytic enzyme secretion, and oxidative stress in response to IFN- (Smith et al. 1998).
IFN-\(\gamma\) and microglial cells are thought to play important roles in initiation and development of multiple sclerosis. Recently, the molecular mechanisms of human microglial responses to IFN-\(\gamma\) have been studied by microarray. This microarray analysis which revealed a change in expression of number of genes, including transcriptionally induced chemokines, IFN-\(\gamma\) signaling factors, MHC genes and proinflammatory T-lymphocyte-related chemokine genes as well as genes involved in antigen presentation, provides a foundation for the molecular mechanisms of microglial activation by IFN-\(\gamma\) (Rock \textit{et al.} 2005). This study showed no increase in expression of NOS genes in response to IFN-\(\gamma\) which is in contrast to results found in the murine model. This difference suggests a possible species specific response of microglia to IFN-\(\gamma\).

1.3.1.4. Thrombin

In addition to its role in coagulation cascade, the serine protease thrombin has been shown to activate microglia through proteolytic activation of protease-activated receptors (PARs). Thrombin-induced microglial activation induces cytokine release, proliferation and intracellular calcium signaling (Moller \textit{et al.} 2006). Further, it has been shown that thrombin activates microglia by inducing NADPH oxidase and oxidative stress. The thrombin-induced microglial activation has also been shown to result in the production of toxic and inflammatory mediators leading to degeneration of dopaminergic neurons and hippocampal neurons (Beal 2002; Koutsilieri \textit{et al.} 2002; Gao \textit{et al.} 2003; Wu \textit{et al.} 2004).
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al. 2003; Block et al. 2004; Qin et al. 2004; Choi et al. 2005; Moller et al. 2006;).

1.3.1.5. Granulocyte-macrophage colony stimulating factor and macrophage colony stimulating factor

GM-CSF is a hematopoietic growth factor that promotes the survival and proliferation of microglia in culture. GM-CSF has numerous effects on microglia, ranging from induction of proliferation to changes in morphology and immune properties (Suzumura et al. 1990; Esen and Kielian 2007). It has been reported that GM-CSF induces the expression of genes related with chemotaxis, antigen processing, and innate immunity, suggesting that GM-CSF helps the transition of microglia into a more professional antigen presenting cell phenotype (Esen and Kielian 2007). Activated astrocytes appear to be the main source of GM-CSF in the CNS (Koguchi et al. 2003).

Macrophage colony stimulating factor (M-CSF), a hematopoietic growth factor, also activates microglial cells by enhancing the microglial proliferation and production of inflammatory cytokines and NO (Hao et al. 2002). Increased M-CSF receptor expression by activated microglia has been reported after ischemic and mechanical brain injury in mice (Raivich et al. 1998; Wang et al. 1999). A transient overexpression of M-CSF receptor on murine microglial cell lines resulted in microglial proliferation and increased expression of iNOS, proinflammatory cytokines, IL-1α, MIP-1α, IL-6 and M-CSF (Mitrasinovic et al. 2001). M-CSF acts as a mitogen on microglia in vitro (Lodge and Sriram 1996;
Sawada et al. 1990; Kloss et al. 1997) and mutation in the M-CSF gene inhibits microglial proliferation in vivo (Wegiel et al. 1998; Sasaki et al. 2000), indicating that M-CSF expression in microglia may be one of the molecular signals that initiate proliferation of microglia at the injury site.

1.3.2. Signaling pathways mediating microglial activation

1.3.2.1. Mitogen-activated protein kinase pathways

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that are highly evolutionarily conserved in eukaryotic species. MAPK pathways have important roles in various cellular processes, such as stress response, immune defence and proliferation (Liu et al. 2007). Four major MAPK pathways have been reported in mammals: 1) p38 MAPK; 2) extracellular-signal-regulated kinases (ERK1/2, also known as p44/42 MAPK); 3) c-jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs); 4) ERK5 (big MAPK1). The four MAPK signaling pathways are activated by distinct and sometimes overlapping sets of stimuli (Hommes et al. 2003). In general, ERKs are activated by growth factors, whereas the p38 and JNK are activated by stress stimuli (Ma et al. 2004).

Microglial response to extracellular stimuli is mediated by MAPK pathways. Several reports have demonstrated that MAPK pathways play a significant role in activation of microglial cells which in turn leads to release of neurotoxic
molecules and neuroinflammation (Lee et al. 2000; Li et al. 2001; Waetzig et al. 2005a). In vivo evidence also implicates that MAPKs play an important role in microglial activation in acute brain injury such as stroke and in chronic neurodegenerative diseases such as AD. Recently, MAPK-activated protein kinase 2 (MAPKAP kinase 2 or MK2), which is one of several kinases directly regulated by p38 MAPK, has been shown to play a role in neuroinflammatory and neurodegenerative pathology by inducing release of pro-inflammatory mediators in microglial cells (Culbert et al. 2006). The activation of MK2 expression was increased in microglial cells stimulated by LPS and IFN-γ, implicating a role for MK2 in eliciting a proinflammatory response of microglial cells.

Activation of these MAPK pathways leads to induction of a wide array of downstream targets, including protein kinases and nuclear transcription factors, which lead to transcription of MAPK-regulated genes. AP-1, a member of the basic region-leucine zipper (bZIP) family of DNA binding proteins, is one of the downstream targets of MAPK pathways. AP-1 is comprised of a Jun family member (c-Jun, v-Jun, Jun-B, or Jun-D) homodimerized with another Jun protein or heterodimerized with a Fos protein (c-Fos, Fos-). LPS has been shown to stimulate the dimerization via c-Jun phosphorylation by JNK (Smoak and Cidlowski 2004).

The MAPK pathways can be regulated by various mechanisms. One of the most efficient ways of deactivation is dephosphorylation of the kinases by phosphatases. A number of protein phosphatases has been discovered, including
MAPK phosphatase-1 (MKP-1). MKP-1 is a prototype of dual specificity phosphatase induced by cellular stresses, serum and growth factors and dephosphorylates MAP Kinases such as JNK, p38, and also ERK1/2 in some circumstances (Clark 2003).

1.3.2.2. Nuclear factor-κB pathway

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor whose involvement in microglia activation is well established in both *in vitro* and *in vivo* experimental systems (Quan *et al.* 2000; Nakamichi *et al.* 2007). NF-κB consists of homodimers or heterodimers assembled from subunits including p65 (Rel A), c-Rel, Rel B, p52/p100, and p50/p105. The p65/p50 heterodimer is the prototypical and most thoroughly studied NF-κB dimer (Baeuerle and Henkel 1994). NF-κB is latently present in the cytoplasm, under control of the associated protein I-κB. The first step in the activation of NF-κB is an I-κB kinase complex (IKK)-dependent phosphorylation of I-κB, followed by ubiquitinylation and degradation. This finally leads to release of the NF-κB protein which is translocated to the nucleus to exert its effects on gene regulation (Brasier 2006).

1.3.3. Microglia activation in brain disorders

It has been widely demonstrated that microglial cells are activated in neurodegenerative diseases such as AD, PD and Creutzfeldt-Jakob disease (CJD),
MS, HIV-associated dementia (HAD) and stroke. In these diseases, only resident microglial cells respond to inflammation in the absence of neutrophil infiltration and mononuclear cell perivascular cuffing. These microglial cells rapidly proliferate, become hypertrophic and express a plethora of marker molecules such as the macrophage antigen complex-1 (MAC-1), the cytotoxic molecules including ROS, NO and a variety of proinflammatory cytokines such as TNF-α, IL-1β. Although several reports indicate that the chronic inflammation could influence the pathogenesis of degenerative diseases, the potential detrimental or protective roles of activated microglial cells in these diseases remain to be elucidated.

1.3.3.1 Alzheimer’s disease

AD is the most common neurodegenerative disorder of the elderly, and it is characterized clinically by progressive memory loss, as well as other cognitive impairments. The neuropathological hallmarks of AD include abundant deposits of Aβ fibrils as senile plaques (SPs), massive accumulations of abnormal tau filaments (one type of mirotubule associated proteins) and intraneuronal neurofibrillary tangles (NFTs), and extensive neuronal degeneration associated with profuse reactive microglia. Apart from the SPs and NFTs, AD brains also exhibit a number of nondiagnostic pathological abnormalities, including a profound loss of synapses, massive neuronal degeneration, extensive gliosis, microglial proliferation/activation, and evidence of an unusual inflammatory
process (Clark and Trojanowski 2000).

Microglia activation is associated with the neuropathology of AD. It has been reported that Aβ-42 is far more toxic to neurons in vitro when they are cocultured with microglia (Qin et al. 2002). Moreover, cytokines produced by activated microglia can both stimulate neuronal production of amyloid precursor protein (APP), and promote its conversion to Aβ (Ge and Lahiri 2002). This suggests a vicious cycle in which neural production of Aβ leads to microglia activation, which in turn promotes neuronal cell death while further stimulating Aβ production (Blasko et al. 2004).

1.3.3.2. Parkinson’s disease

PD is the second most common aging-related neurodegenerative disease after AD. PD is clinically characterized by various symptoms including tremor, slowness of movement, stiffness and postural instability (Fahn and Przedborski 2000). These symptoms are primarily attributable to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibres in the striatum (Hornykiewicz and Kish 1987; Pakkenberg et al. 1991). The disease is suggested to be caused by some exogenous or endogenous substances that are neurotoxic to catecholamine neurons, which lead to mitochondrial dysfunction and subsequent oxidative stress resulting in the apoptosis of dopaminergic neurons. In addition to the neurodegeneration in SNpc, there is a robust microglial reaction which
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accompanies an inflammatory process in PD and experimental models of PD. The activated microglial cells which produce protective or toxic substances, such as cytokines including TNF-α and IL-6 proteins, neurotrophins, and ROS may be neuroprotective in the initial stage, and later become neurotoxic due to toxic change resulting in the death of CA neurons (Mogi et al. 1994b; Mogi et al. 1994a; Imamura et al. 2003; Nagatsu and Sawada 2006)

1.3.3.3. Multiple sclerosis

MS is one of the most common CNS diseases characterized by inflammatory reaction, demyelination and axonal loss in the brain, spinal cord and optic nerve (Sanders and De 2007). It is an autoimmune disorder and the acquired immune system plays a central role in the pathogenesis of MS although target antigens and effector mechanisms are still poorly defined (Hemmer et al. 2004). Microglia participate in nearly all phases of the disease process, including recognition of stranger/danger signals, presenting antigens to cells of the adaptive immune system and inducing an inflammatory response (Jack et al. 2005). Modulation of the dynamic properties of microglia so as to limit potential damaging innate response remains a therapeutic challenge (Jack et al. 2005).

1.3.3.4. HIV associated dementia

In recent years, considerable attention has been drawn to the factors involved in the development of HIV associated dementia (HAD) which is
characterized by cognitive, behavioral, motor deficits ranging from mild disease to profound dementia. The primary factor in the development of HAD is the infection of mononuclear phagocytes and microglial cells with HIV-1. The HIV-1 infected microglial cells and macrophages are activated and secrete both endogenous neurotoxins such as TNF-α, IL-1β, IL-8, eicosanoids and NO as well as neurotoxic viral proteins such as Tat, gp120 and gp41 (Brenneman et al. 1988; Nottet et al. 1995; New et al. 1997). These microglial cells can also induce the recruitment of additional microglia and macrophages to the site of infection by inducing endothelial cells to release chemokines such as MCP-1, MIP-1α and MIP-1β (Lipton and Gendelman 1995; Persidsky et al. 1999; Nelson et al. 2002). Overall, microglial cells are the principal target of HIV-1 in the brain and the activated microglial cells contribute significantly to the HIV-1 associated neuropathogenic processes (Nelson et al. 2002).

The prevalence of neurodegenerative diseases is increased with aging. Ageing seems to influence microglial activation that could contribute to an enhanced inflammatory response in the brain, leading to neurodegenerative diseases. Aged brains in rodents and human show increased numbers of activated phagocytic microglia and increase in IL-1β, OX42 and OX6 expressing microglial cells (Rozovsky et al. 1998; Sheng et al. 1998; Conde and Streit 2006). The age-related activation of microglia appears to be a reflection of a lifelong accumulation of minor insults, leading to an increased risk of neurodegenerative diseases. In addition, several recent reports demonstrate that microglial cells are
subjected to age-related structural deterioration and cellular senescence, which may be caused by various factors including genetic and epigenetic alterations and cellular accumulation of Aβ protein (Korotzer et al. 1993; Flanary and Streit 2004; Streit et al. 2004). This raises the possibility that microglial cells may become increasingly dysfunctional with ageing, thereby their normal neuroprotective functions are decreased, resulting in the increased risk of neurodegenerative diseases in aged population (Streit 2006).

1.4. The role of microglia during neurogenesis and synaptogenesis in the brain

Neurogenesis is a life-long process which occurs in the developing and adult brain. In the adult brain, the hippocampus is the unique structure which produces new neurons throughout adult life since it contains a population of neural stem cells. Inflammation and activation of microglia by irradiation in the brain has been shown to impair the hippocampal neurogenesis (Ekdahl et al. 2003). In addition, neurogenesis was found to be inhibited when neural stem cells were exposed to activated microglia in vitro (Kempermann and Neumann 2003). In view of these findings, it was suggested that activated microglial cells could play a significant role in inflammation-induced impairment of neurogenesis in the hippocampus. Proinflammatory mediators released by microglia appear to be important contributing factors to the block in neurogenesis since treatment with non steroidal anti-inflammatory drugs restores hippocampal neurogenesis (Monje
et al. 2003). However the exact mechanisms by which microglial activation impairs the neurogenesis in the adult brain are not clear and need to be explored.

Apoptosis which is a prominent feature during early neural tube development, occurs in the cells of developing brain vesicles on days 10 and 11 of gestation in order to halt premature neurogenesis (Akazawa, 2005). Several studies show that microglia invade neural tissue shortly before or concomitantly to the period of naturally occurring neuronal death and induce apoptosis in developing neurons via the release of diffusible factors such as nerve growth factors and superoxide ions (Frade and Barde 1998; Marin-Teva et al. 2004). These observations indicate potential role of microglia on cell fate specification during neurodevelopment. However, it should be further clarified in vivo as most of these results are based on in vitro studies.

Microglia have also been shown to be involved in developmental synaptogenesis. They invade the developing neural tube in embryos, increase in number significantly during first week of postnatal stage through P18 (Dalmau et al. 1998; Dalmau et al. 2003), the period when intense synaptogenesis occurs. In the developing brain, microglia express thrombospondins, a family of extracellular matrix proteins which induce synaptogenesis (Christopherson et al. 2005). Moreover, microglial activation followed by neuronal death has been shown to be associated with synaptogenesis in experimental axotomy as they secrete thrombospondins (Chamak et al. 1995; Moller et al. 1996; Moran and Graeber 2004). Microglia express a broad spectrum of cytokines, neurotrophins
and other molecules such as glutamate and NO which regulate synaptic functions. These results support the notion that microglia could regulate synaptogenesis and neuronal apoptosis, indicating a strong functional bond between neurons and microglia. Overall, it is fairly well demonstrated that microglia regulate several aspects of neuronal functions in normal and pathological conditions and are activated by neurodegeneration. However, the interaction between microglia and non-neuronal cell types including oligodendrocytes and astrocytes has not yet been investigated systematically. Systematic investigation on functional interaction between microglia and neuronal and non-neuronal cell types including oligodendrocytes and astrocytes in vivo would greatly help in the understanding how the brain functions in normal and pathological conditions.

1.5. Inhibition of microglial activation may improve therapeutic strategy for neurodegenerative diseases

Neurodegenerative diseases share certain common features such as microglial activation associated with excitotoxic neuronal death and dysfunction. There is a vicious cycle in which the neuronal death leads to microglial activation, which in turn promotes death and dysfunction of neurons. Hence, measures which inhibit microglial activation may have a favorable effect on the induction and progression of neurodegenerative diseases, independent of the particular trigger or target involved in a given disorder (McCarty 2006). Consistent with this possibility, some inhibitors of microglia activation may have potential to be used in the
therapy of neurodegenerative disorders.

Although several drugs alleviate symptoms of neurodegenerative diseases, chronic use of these drugs is often associated with debilitating side effects, and none seems to dampen the progression of these diseases. So far, the development of effective neuroprotective therapies is impeded by our limited knowledge of the pathogenesis of neurodegenerative diseases. Several studies have demonstrated that the inhibition of microglial activation may help development of better therapeutic strategies for neurodegenerative diseases.

1.5.1. Glucocorticoids

Glucocorticoids (GCs) are the most powerful endogenous immunosuppressors for the innate immune response and the subsequent inflammatory reaction (McKay and Cidlowski 1999). GCs exert their actions by binding to an intracellular GC receptor, a ligand-activated transcriptional regulator that belongs to the nuclear receptor superfamily (Schoneveld et al. 2004). Blockade of endogenous GCs exacerbates the LPS-induced inflammatory response leading to neurotoxicity in the rat brain (Nadeau and Rivest 2003). Dexamethasone (Dex), a potent anti-inflammatory and immunosuppressive glucocorticoid, is widely used in the treatment of inflammation and a number of autoimmune disorders (Wilckens and De 1997). The immuno-suppressive properties of GCs have been ascribed to their ability to suppress the synthesis of a number of cytokines including IL-12p40 (Ma et al. 2004). GCs have been shown
to mediate their biological effects on cytokine production in LPS stimulated monocytic cells primarily by down regulating MAPK activation as well as AP-1 and NF-κB activity (Jonat et al. 1990; Schule et al. 1990; Anuphan N et al. 1995; Scheinman RI et al. 1995; Blotta MH et al. 1997; Hirasawa N et al. 1998; Franchimont 2000; Steer et al. 2000; Yang-Yen et al. 1990). Several lines of evidence suggest that GCs induced sustained expression of MKP-1 in various cell lines such as Hela cells (Lasa et al. 2002), osteoblasts (Engelbrecht et al. 2003), macrophages (Roger et al. 2005) and epithelial cells (Imasato et al. 2002; Sakai et al. 2004). As MKP-1 is a negative regulator of MAPK pathway, action of GCs on MAPK pathway appears to be mediated via MKP-1 expression.

It is well known that activation of AP-1 and NF-κB nuclear translocation is transcriptional activation of most proinflammatory genes. GCs also appear to act on microglial proliferation as the number of microglial cells was found to be reduced significantly in the developing corpus callosum following Dex treatment (Kaur et al. 1994). However, GCs are known to produce various side effects; hence, it is suggested that studies on its downstream targets may yield a better clinical outcome.

1.5.2. Retinoic acid

Retinoic acid (RA, a vitamin A metabolite) plays essential roles in cell growth, differentiation and apoptosis. Besides that, several lines of evidence suggest that RA exerts an anti-inflammatory effect and attenuates the production
of inflammatory mediators such as TNF-α and iNOS in various cell types (Datta et al. 2001). It is well known that RA modulates the target cell activity by binding to one of its two receptors: retinoic acid receptors (RAR)-α, -β, -γ and retinoid X receptors (RXR)-α, -β, -γ, both of which are members of the steroid hormone receptor superfamily (Simeone and Tari 2004). RAR or RXR may modulate gene transcription by binding directly to promoters containing a RA receptor element (RARE) or via antagonistic cross-coupling of transcription factors such as NF-κB (Xu et al. 1997). Recent studies have demonstrated the synthesis of RA in the adult vertebrate brain (Dev et al. 1993; Zetterstrom et al. 1999). In the human brain, retinaldehyde dehydrogenase (RLDH), the enzyme that forms RA from retinaldehyde, has been shown to be present in the hippocampus, and the frontal and parietal cortex (Connor and Sidell 1997). Its function in control of growth and differentiation in the embryonic CNS as well as neuronal plasticity and neurogenesis in the adult CNS has been extensively investigated (McCaffery et al. 2006). However, its effects on microglial proliferation and their release of proinflammatory mediators have not been fully understood.

1.5.3. Minocycline

Minocycline, a semisynthetic tetracycline derivative, exhibits neuroprotective effects in neurodegenerative diseases including PD, Huntington’s disease, amyotrophic lateral sclerosis, focal and global ischemia and traumatic brain injury (TBI) (Chen et al. 2000; He et al. 2001; Sanchez Mejia et al.
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It has been shown to inhibit microglial activation in various pathological conditions by suppressing the ROS production and expression of proinflammatory cytokines (Amin et al. 1996; Yrjanheikki 1998; Yrjanheikki 1999; Tikka 2001; Tikka and Tikka 2001). In the mouse model of PD, minocycline mitigates the loss of dopaminergic cell bodies in the SNpc and of nerve terminals in the striatum, prevents microglial activation and reduces the synthesis of IL-1β. Minocycline has also been shown to inhibit the thrombin-induced microglial activation together with the upregulation of iNOS and NADPH-oxidase, two enzymes implicated in microglial derived production of NO and ROS, respectively (Cai et al. 2001; Du 2001; Wu et al. 2002; Choi et al. 2003; Choi et al. 2005). Recently, it has been further shown that minocycline inhibits retinal microglial activation in retinal neurodegenerative diseases by suppressing the production of inflammatory cytokines and NO (Wang et al. 2005).

1.5.4. Vitamin D

There is accumulating evidence suggesting an involvement of various vitamins in brain functions and anti-inflammatory processes in CNS injuries. Vitamin E, a powerful biological anti-oxidant, has been shown to play neuroprotective roles by preventing the free radical-induced neurotoxicity and suppressing microglial activation (Kamal-Eldin and Appelqvist 1996). The vitamin E-induced inhibition of microglial responses to LPS was correlated with
the suppression of the activation of p38 MAPK and NFκB, both of which regulate cytokine and iNOS expression (Lee et al. 1994). Vitamin D also exhibits immunomodulatory properties via its specific receptor, vitamin D3 receptor expressed in antigen-presenting cells, activated lymphocytes and microglia in the CNS (Lemire 2000; Lefebvre et al. 2003). It has direct anti-inflammatory effects on activated microglia by inhibiting the production of TNF-α, IL-6 and NO (Lefebvre et al. 2003).

1.5.5. Endocannabinoids

The cannabinoids, which are active components of Cannabis sativa (marijuana) have been shown to protect neurons from toxic insults such as excitotoxicity, traumatic injury and ischaemia both in vitro and in vivo (Mechoulam et al. 2002; van der et al. 2002). Recently it has been shown that the endocannabinoid system consisting of cannabinoid receptors such as CB1 and CB2, their endogenous ligands, enzymes for synthesis and degradation of endocannabinoids, is highly activated during CNS inflammation and the signaling of endocannabinoid suppresses microglial activation by inducing histone H3 phosphorylation, MKP-1 gene expression and subsequent ERK-1/2 dephosphorylation which in turn abolishes NO release resulting in neuroprotection (Hansen et al. 2001; Panikashvili et al. 2001; Marsicano et al. 2003; Eljaschewitsch et al. 2006b). These findings have led to a remarkable expansion of basic cannabinoid research which would enhance the therapeutic value of
cannabinoids in the treatment of neurodegenerative diseases.

### 1.5.6. TGF-β1

TGF-β1 has been considered as a potent anti-inflammatory cytokine and plays a pivotal role in maintaining balanced host responses in inflammatory and immunological conditions in the CNS (Letterio and Roberts 1998). Activation of microglia by brain ischemia, proinflammatory factors, LPS and Aβ leads to increased production of TGF-β1 that may counteract the proinflammatory reactions (Constam et al. 1992; Weissner et al. 1993; Chao et al. 1995; Dheen et al. 2005). Several studies have demonstrated the potential mechanism and signaling pathway for the repressive effect of TGF-β1 on overactivation of microglial cells. It has been reported that TGF-β1 suppresses microglial activation via inhibition of phosphatidylinositol 3-kinase (PI3K) activity and its downstream signaling molecules by inhibition of formyl peptide receptor like 2 (FPR2) expression through Smad-3 mediated pathway (Kim et al. 2004; Le et al. 2004). In experimental AD models, FPR2 mediates the proinflammatory activity of Aβ in mononuclear phagocytes which result in the release of neurotoxic effectors. These data indicate that the cross-talk between TGF-β1 and PI3K/Smad3/MAPK signaling pathways may be important therapeutic targets for treatment of neuroinflammatory diseases.
1.5.7. Chondroitin sulfate proteoglycan

Chondroitin sulfate proteoglycan, a matrix protein that occurs naturally in the CNS has been shown to participate in the activation of inflammatory response. Recently it has been shown that the disaccharidic degradation product of this proteoglycan modulates the inflammatory responses and markedly alleviates the clinical symptoms of immune-induced neuropathologies of the CNS such as EAE (Rolls et al. 2006). This effect was associated with a reduction in numbers of infiltrating T cells, marked microglial activation, decreased secretion of cytokines, TNF-α and decreased expression of NF-κB, suggesting that a compound degraded from an endogenous CNS-resident molecule may provide a potential therapeutic modality for inflammation-induced neurodegenerative diseases.

1.5.8. PPARγ agonists

The peroxisome proliferators-activated receptor γ (PPARγ) belongs to a large group of nuclear receptors and controls immune responses by acting as an agonist-activated transcription factor to regulate target gene expression (Bernardo and Minghetti 2006). PPARγ is expressed in primary microglia cultures and its agonists have been shown to downregulate microglia activation, by inhibiting TNF-α synthesis, MHC class II expression, and by inducing apoptosis which may contribute to the safe elimination of activated microglia (Bernardo et al. 2000; Bernardo et al. 2003). Although a majority of the data are derived from in vitro studies, an increasing number of studies in animal models further suggest that
PPARγ agonists can provide therapeutic benefits in MS, PD and AD (Bernardo and Minghetti 2006).

All the drugs and molecules described above attempt to suppress the neurotoxic effect of microglial cells in CNS diseases. It should be emphasized that the neuroinflammation in CNS diseases is amplified considerably by the rapid influx of microglial cells to injury sites in the CNS. The migration of microglia is regulated by various chemokines. Recently, it has been shown that expression of chemokines such as MCP-1 and IFN-γ inducible protein-10 (IP-10) by astrocytes plays a role in migration and activation of microglial cells and subsequent amplification of neurodegeneration in secondary progressive MS (Tanuma et al. 2006). Therefore, targeting chemokines may be one of the therapeutic options to inhibit neuroinflammation caused by proinflammatory cytokines released by activated microglial cells.

1.6. Aims of this study

An inflammatory process in the CNS is believed to play an important role in the pathway leading to neuronal cell death in a number of neurodegenerative diseases. The inflammatory response is mediated by the activated microglia, which normally respond to neuronal damage and remove the damaged cells by phagocytosis. Activation of microglia is a hallmark of brain pathology. The chronic activation of microglia may in turn cause neuronal damage through the release of potentially cytotoxic molecules such as proinflammatory cytokines.
including TNF-α and IL-1β, reactive oxygen intermediates, proteinases and complement proteins. Therefore, suppression of microglia-mediated inflammation has been considered as an important strategy in neurodegenerative disease therapy. Several anti-inflammatory drugs have been shown to repress the microglial activation and to exert neuroprotective effects in the CNS after different types of injuries. However, these drugs do not specifically target on microglial cells and therefore, exhibit potential systemic side effects. The purpose of this study was to understand the potential mechanisms and signaling pathways for the repressive effect of some drugs (i.e., glucocorticoids and retinoic acid) on activation of microglial cells in CNS diseases. The main objectives are:

1.6.1. To examine the effects of Glucocorticoids on the chemotaxic activity of activated microglia

GCs are widely used anti-inflammatory and immunomodulatory agents and inhibit expression of proinflammatory genes in various cell types (Wilckens and De 1997; De et al. 2003). Recently, it has been shown that GCs could inhibit LPS-induced MCP-1 production in the hippocampus and cortex (Szczepanik and Ringheim 2003; Little et al. 2006). However, the molecular mechanisms by which GCs regulate MCP-1 expression have not been elucidated. It has been further reported that GCs counter-regulate MAPK signaling pathways, in particular p38, and JNK pathways by inducing expression MKP-1 (Clark 2003). Activation of MAP kinases in microglial cells, leads to phosphorylation of transcription factors
such as c-Jun/AP-1 resulting in induction of expression of some target genes including TNF-α and MCP-1 (Babcock et al. 2003; Waetzig et al. 2005).

Based on these data, it is hypothesized that GCs inhibit MCP-1 production via MKP-1-mediated inactivation of MAP kinases, resulting in decreased microglial migration towards the sites of inflammation in CNS. To address this,

1. the effects of GCs on LPS-induced MCP-1 mRNA expression and protein release in the rat microglia of primary culture have been examined.

2. the mechanisms by which GCs inhibit MCP-1 production in activated microglia have been investigated.

3. we have also explored whether the inhibition of MCP-1 by GCs leads to a significant reduction in the chemotactic activity of activated microglia using chemotaxis assay.

1.6.2. To study the effects of all-trans-retinoic acid (RA) on microglial activation and proliferation

RA is an active metabolite of vitamin A. It acts through a family of nuclear transcription factors known as retinoid receptors (Marill et al. 2003). While the principal effects of RA on target cells are inhibition of proliferation and induction of differentiation (Maden 2002), RA has also been shown to exhibit immunomodulatory and anti-inflammatory activities in various cell types (Mathew and Sharma 2000). Further, it has also been demonstrated that RA is synthesized in the adult vertebrate brain (Dev et al. 1993; Zetterstrom et al. 1999).
In view of these results, it is hypothesized that RA modulates inflammatory response and proliferation index of microglia which are the characteristic features of activated microglia that respond to injury or neurological disease processes. To address this,

1. effects of RA on inflammatory response of activated microglia have been investigated.

2. the mechanisms by which RA inhibits GM-CSF induced microglia proliferation have been studied.

This research mainly focuses on the mechanisms of inhibitory effects of GCs and RA on activated microglia in primary culture. The findings would reveal GCs and RA as novel mediators of biological effects and may help to develop better therapeutic strategies for the treatment of CNS diseases.
Chapter 2

Materials and Methods
2.1. Animals and microglia primary culture

2.1.1. Animals

Wistar strain rats (postnatal 1-3-day) were used in this study. In the handling and care of animals, the International Guiding Principles for Animal Research, as adopted by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore, were followed. All rats were supplied by the Laboratory Animal Center, National University of Singapore, and were kept in the Department of Anatomy animal house before the experiments. Rat pups (n = 850) were used for primary microglia cultures for Dex related experiments, and rat pups (n = 350) were used for primary microglia cultures for RA related experiments.

<table>
<thead>
<tr>
<th></th>
<th>Dex related experiments</th>
<th>RA related experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocytochemistry</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Real time RT-PCR</td>
<td>36</td>
<td>No</td>
</tr>
<tr>
<td>ELISA</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>Western Blot</td>
<td>300</td>
<td>54</td>
</tr>
<tr>
<td>MTS assay</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Chemotaxis assay</td>
<td>24</td>
<td>No</td>
</tr>
</tbody>
</table>

For repeated experiments, another 593 rat pups were used.

2.1.2. Materials

Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA, Cat. No. 11966025)

Insulin (Sigma-Aldrich, USA, Cat. No. I0516)

Non-essential amino acid (Invitrogen, USA, Cat. No. 11140050)
Materials and Methods

Antibiotic antimycotic solution (100×)(Sigma-Aldrich, USA, Cat.No. A5955)

Trypsin and EDTA (10×)(Invitrogen, USA, Cat. No. 15400-054)

Deoxyribonuclease I (Sigma-Aldrich, USA, Cat.No. D4527)

Fetal bovine serum (FBS, Invitrogen, USA, Cat. No. 10099)

70µm nylon mesh (BD Biosciences, USA, Cat. No. 352350)

75cm² tissue culture flasks (NUNC, Denmark)

*Lycopersicon esculentum* (tomato) lectin (Sigma-Aldrich, USA, Cat. No. L0401)

Propidium iodide (PI) (Sigma-Aldrich, USA, Cat. No. 70335)

2.1.3. Procedure

2.1.3.1. Removal of brain tissue

The rat pups were deeply anesthetized with pentabarbitone (50mg/kg body wt) and were decapitated. The heads were wiped with 70% ethanol and washed with cold PBS. The skin was incised along the midline in a caudal to rostral direction to expose the skull. The skull was penetrated with a tip of a pair of scissors medially just caudal to the cerebral hemispheres. This point was easy to penetrate as it lies at the juncture of two major sutures of the skull bone. The skull was cut-opened rostrally to the nose and then mediolaterally. Further from the original point of incision, the skull was cut mediolaterally and then in a rostral direction reaching the first set of cuts. In this way, two roughly rectangular flaps
of skull were removed. Underlying brain tissue was moistened with cold PBS. A pair of curved forceps was run around and under the hemispheres to scoop them out of the cortex. The brain tissue was placed onto a 100-mm-diameter dish with cold PBS and was rinsed three times. The cortices were collected into a 50-ml tube in PBS on ice and were rinsed three times with PBS. The unwanted brain region, such as olfactory bulb, basal ganglia, hippocampus, and thalamus, were removed firstly. The meninges and superficial vessels were removed subsequently. The remaining desired tissue was transferred to a new 60-mm-diameter dish, containing cold PBS. The tissue was finely dissected further to remove the remaining meninges and vasculature from the dish. Finally, the tissue was transferred to a new 60-mm-diameter dish containing 9ml cold DMEM medium.

2.1.3.2. Mechanical dissociation of brain tissue

A long pair of serrated forceps or hemostats was used to chop the tissue in the dish with gentle up-and-down motion. The tissue pieces were collected and mechanically dissociated by pipetting in and out with a 10ml plastic pipette in a 50-ml sterile conical tube on ice.

2.1.3.3. Enzymatic digestion

1ml of Trypsin-EDTA solution (0.5% Trypsine, 0.2% EDTA) and 100μl of DNase solution (50μg/ml DNase) were added into the tube, and mixed before the solution was transferred to a 75cm² tissue culture flask. The flask was placed in an
orbital shaker at 37°C and shaken at 200rpm/min for 15 min. Cells were passed through a 70μm cell strainer to remove remaining clumps of tissue and then transferred to a 50ml centrifuge tube again.

Finally the cell suspension was centrifuged at 1000rpm for 5 min, then seeded in culture dish consisting of DMEM supplemented with 10% fetal bovine serum at a density of 1.2×10^6 cells/ml and cultured at 37°C in humidified 5% CO₂/95% air. Medium was changed every 3-4 days and confluency was achieved after 10-12 days in vitro.

2.1.3.4. Microglia purification

Microglial cultures were prepared by a mild trypsinization method as described by Saura et al. (Saura et al. 2003). The mixed glial cultures were purified after 10 to 12 days. Briefly, the cultures were washed with PBS twice and incubated with a trypsin solution (1ml of 0.25% trypsin and 20μl of 0.5M EDTA diluted in 40ml of DMEM) for about 10min. This resulted in the detachment of an upper layer of cells which were removed subsequently. The microglial cells remained attached to the bottom of the flask and were cultured in DMEM supplemented with 10% fetal bovine serum.

For immunocytochemistry, cells were plated at 2.5×10^5 per well on a 24 multi-well culture dish and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 24h. For immunoblotting and RNA isolation, cells were plated at 1×10^6 cells per 75cm² flask. For enzyme-linked immunosorbent assay (ELISA),
cells were plated at $1 \times 10^4$ cells per well in 96-well plates. The following day, the cells were subjected to different treatments.

The purity of microglia was assessed immunohistochemically using *Lycopersicon esculentum* (tomato) lectin (1:100), a marker for microglia, and Propidium iodide (PI) (20μg/ml) a nuclear marker of all cells attached to the coverslips. The purity of microglial cultures was found to be around 96%.

### 2.2. Treatment of microglia culture

#### 2.2.1. Materials

- Lipopolysaccharide (LPS) (Sigma-Aldrich, USA, Cat. No. L2762)
- Dexamethasone (Dex) (Sigma-Aldrich, USA, Cat. No. D2915)
- SP600125, JNK inhibitor (Scientific, Inc. USA, Cat. No. S-2022)
- SB203580, p38 inhibitor (MERCK, Germany, Cat. No. 559400)
- Triptolide (Calbiochem, Germany, Cat. No. 645900)
- All-*trans*-retinoic acid (Sigma-Aldrich, USA, Cat. No. R2625)
- Granulocyte macrophage-colony stimulating factor (GM-CSF, BD Biosciences Pharmingen, USA, Cat No: 555111)
- Fetal bovine serum (Invitrogen, USA, Cat. No. 10099)
- Dimethyl sulfoxide (DMSO, Calbiochem, Germany, Cat. No. 317275)
- Ethanol (BDH Prolabo, Germany, Cat No: 15338-6F)
2.2.2. Procedure

In order to study the effects of LPS and Dex on activation of microglial cells, the cells were washed with PBS and incubated either with LPS (1μg/ml) or Dex or LPS plus Dex (10⁻⁶ M) in DMEM without serum. Effects of Dex on MAPK pathway were studied by adding Dex (10⁻⁶ M) 2 hours prior to LPS in microglia cultures. Further, microglia cultures were treated with either SP600125 (1μM) or SB203580 (1μM), inhibitors of JNK and p38 respectively, 30 min before LPS treatment to examine the effects of MAPK inhibitors on LPS-induced microglia activation. To investigate the role of MKP-1 on Dex-induced inhibition of microglial activation, microglial cells were pretreated with or without triptolide (0.5μM), 30min before the addition of Dex plus LPS. In all the experiments, control cells were treated with DMSO/ ethanol as the vehicle at final concentrations used in experimental cultures.

In order to study the effects of RA on GM-CSF-treated microglia, cells were grown in the DMEM containing 1% FBS for 12h, followed by incubation with serum-free medium for 24h, a standard means of synchronizing cells in the G0 phase of the cell cycle. At this time (designated time 0), the medium was replaced with DMEM containing 10% FBS and GM-CSF (10ng/ml) with or without RA (10μm). The vehicle, DMSO did not exceed 0.1%. Cells were then incubated at 37°C in a humidified atmosphere of air/5% CO₂ for the times indicated.
2.3. Immunofluorescence labeling

2.3.1. Principle

Immunohistochemistry (IHC), or immunocytochemistry, is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition. The basic principle of IHC is a sharp localization of target components in the cell and tissue, based on a satisfactory signal-to-noise ratio. A series of technical developments in IHC have created sensitive detection systems and the most commonly used is the enzyme-labeled system (for light microscope); the fluorescent dye-labeled system (for fluorescence microscope); and the electron-dense particles-labeled system (for electron microscope). To achieve greater sensitivity is always the critical issue for the IHC and a large number of detection techniques have been developed, including peroxidase antiperoxidase (PAP), avidin-biotin conjugate (ABC) and biotin-streptavidin (B-SA) methods.

The routine procedure for IHC includes fixation, blocking, the incubation with first antibody and detection of first antibody. Fixation is required for the IHC to stabilize the tissue or cells and protect them from the rigors of subsequent processing and staining techniques. Blocking of nonspecific background staining enhances the specificity of the primary antibody. Antibody molecules are labeled or flagged to permit their visualization. The detection system can be divided into direct conjugate-labeled antibody method and indirect conjugate-labeled antibody method. In the direct detection method, an antibody is labeled and then is directly applied to the tissue section or cells. In the indirect conjugate-labeled antibody
Materials and Methods

method, the primary antibody that has specificity against the antigen in question is added to the sample, followed by addition of the labeled secondary antibody, which has specificity against an antigenic epitope present on the primary antibody. Thus, the secondary antibody serves to label the primary antibody, which, in turn, is bound to the antigen in tissue (Petrusz et al. 1980; Wessel and McClay 1986).

2.3.2. Materials

MCP-1 (Santa Cruz, CA, USA, Cat. No sc-1785)
Rabbit phospho-c-Jun (ser73, Cell Signaling Technology, USA, Cat. No. 9164)
Fluorochrome-conjugated goat-anti-rabbit secondary antibody (Chemicon, USA, AP307F)
Fluorochrome-conjugated rabbit-anti-goat secondary antibody (Chemicon, USA, AP106F)
4’, 6- diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, USA, Cat. No. D1306)
Propidium iodide (PI) (Sigma-Aldrich, USA, Cat. No. 70335)
Fluorescent Mounting Medium (DakoCytomation, Denmark, Cat. No. S302380)
Phosphate Buffered Saline (PBS) (10X) liquid (Invitrogen, USA, Cat. No. 14200166)
0.1M phosphate-buffered saline (PBS)
Materials and Methods

0.1M PBS containing 0.1% Triton-X 100 (PBS-TX)

4% paraformaldehyde (PF):

Paraformaldehyde (Sigma)  4g

0.1M phosphate buffer (pH 7.4)  100ml

2.3.3. Procedure

Microglia attached to the coverslips were allowed to grow for 1 day before they are incubated with 1µg/ml of LPS in presence or absence of Dex (10⁻⁶ M) for either 30 min (for c-Jun and phospho-c-Jun detection) or 6h (For MCP-1). After the incubation, the cells were fixed in 4% PF for 20min, blocked in 1% bovine serum albumin (BSA) for 30min, and incubated overnight with specific primary antibodies that were raised in goat and directed against rat MCP-1 (1:100), or rabbit phospho-c-Jun (1:100). Subsequently cells were incubated with appropriate conjugated secondary antibodies (1:200). Finally, the cells were counterstained with nuclear markers either PI (5µg/ml) or DAPI (1µg/ml) and examined under a confocal microscope (Olympus Fluoview1000, Japan). For PI, λ<sub>excitation</sub> = 543 nm, λ<sub>emission</sub> = 560 nm; for DAPI, λ<sub>excitation</sub> =359nm, λ<sub>emission</sub> = 461; For FITC, λ<sub>excitation</sub> =488 nm, λ<sub>emission</sub> = 510nm; For Cy3, λ<sub>excitation</sub> = 568 nm, λ<sub>emission</sub> =630nm

Quantitative analysis of the number of phospho-c-Jun nuclear positive microglial cells was carried out by performing cell count in 8 randomly chosen microscopic fields containing about 40 cells in the control cultures and cultures treated with LPS in the presence or absence of Dex. The cells with blue nuclei were counted as phospho-c-Jun negative; the cells with pink nuclei were counted as phospho-c-Jun positive. Each experiment was done in triplicate.
2.4. RNA Isolation and Real time reverse transcription-polymerase chain reaction (RT-PCR)

2.4.1. Principle

The RNeasy technology (Qiagen, Germany) used in this study for RNA isolation combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Cell or tissue samples are first lysed in the presence of a highly denaturing guanidinium isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water. With this RNeasy procedure, all RNA molecules longer than 200 nucleotides are isolated while most RNAs shorter than 200 nucleotides such as 5.8S rRNA, 5S rRNA, and tRNAs are selectively excluded.

Polymerase chain reaction (PCR) is a technique used to amplify the number of copies of a specific region of DNA in order to produce sufficient amount of DNA to be adequately tested. There are three basic steps in a PCR, including denaturation, annealing, and DNA synthesis, which are repeated for 30 to 40 cycles. During these steps, the double-stranded sample DNA melts open to
become a single-stranded DNA which is then hybridized with a set of 5’ and 3’ end primers, short fragments of DNA that is base-paired to a specific region of single-stranded sample DNA. The 3’ end of the primer serves as the starting point for the new DNA synthesis in the presence of DNA polymerase and nucleotides. Since the reaction mixture contains primers complementary to both strands of sample DNA, the products of the DNA synthesis can themselves be copied (Mullis and Faloona 1987).

Reverse transcription combined with polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify the levels of mRNA expression (Murphy et al. 1990). The real time, fluorescence-based RT-PCR is a recent advance in quantitative RT-PCR technology which enables rapid and reproducible high-throughput RT-PCR quantification, with extremely high sensitivity. Hence, the real-time RT-PCR has become the method of choice for the quantification of gene expression (Bustin 2000; Ginzinger 2002). Real time RT-PCR incorporates a specific technology to detect the PCR product following each cycle of the reaction. This technology involves a DNA-binding fluorescent dye, such as SYBR green I, which binds in the minor groove of double-stranded DNA (Morrison et al. 1998). When the DNA is denatured, the unbound dye emits little fluorescence in the reaction system. During extension, increasing amount of dye binds to the newly formed double-stranded DNA, resulting in an increase in the fluorescence signal. Thus, the fluorescence measurement performed at the end of the extension step of every PCR cycle reflects the increasing amount of amplified DNA. After a few
cycles, the fluorescent signal is first recorded as statistically significant above background signal. This point is described as threshold cycle (Ct), which occurs during the exponential phase of amplification (Gibson et al. 1996). In addition, the specificity of the amplification and PCR product verification can be achieved by a melting curve of the PCR product (Ririe et al. 1997).

Real time RT-PCR data analysis can be classified as absolute quantification or relative quantification. The absolute quantification involves the construction of a standard curve based on known copy numbers to determine the input copy number of the transcript of interest by relating the PCR signal to the standard curve, whereas the relative quantification involves determining the change in gene expression level relative to another set of experimental samples such as untreated controls or samples at time zero in a time-course study (Livak and Schmittgen 2001). For most research applications, the relative quantification approach is more practical as it compares experimental samples against controls directly. Furthermore, a normalization of the target gene with an endogenous reference gene is required for the relative quantification. Usually, non-regulated genes or housekeeping genes like glyceraldehydes-3-phosphate dehydrogenase (GAPDH), albumin, actin, tubulin, cyclophilin, 18S rRNA or 28S rRNA are applicable as the reference genes (Marten et al. 1994; Thellin et al. 1999).

Using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), the data are presented as the fold change of target gene expression normalized to an
endogenous reference gene, relative to a calibrator. For the treated samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change of gene expression relative to the untreated control. For this method to be valid, the amplification efficiencies of the target and reference must be approximately equal.

2.4.2. Materials

RNeasy mini kit (Qiagen, Germany, Cat. No. 74106)

Molony murine leukemia virus reverse transcriptase (M-MLV) (Promega, USA, Cat. No. M1701)

Oligo(dT) primer (Promega, USA, Cat. No. C1101)

dNTP mix (Promega, USA, Cat. No. U1240)

RNase inhibitor (Promega, USA, Cat. No. N2111)

LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Applied Sciences, Germany, Cat. No. 03515885001)

TAE buffer (Invitrogen, USA, Cat. No. 15558034)

100bp DNA step ladder (Promega, USA, Cat. No. G6951)

LightCycler instrument (Roche Molecular Biochemicals)

GeneGenius (Syngene, UK)

Spectrophotometer (Eppendorf, Germany)

2.4.3 Procedure

2.4.3.1 Extraction of total RNA
Total RNA was extracted from the cells using RNeasy Mini Kit according to the manufacturer’s instructions. Approximately, $1 \times 10^6$ cells were used to extract the total RNA. Cells were lysed in 650$\mu$l of RLT buffer (containing a highly denatured guanidine isothiocyanate which inactivates RNase) and then scrapped from the flask with the scraper. The lysate was homogenized, then centrifuged for 30s at 14000g in a microfuge and the supernatant was mixed with 650$\mu$l of 70% ethanol to clear lysate. The sample was applied to an RNeasy mini spin column (silica-gel membrane, maximum binding capacity is 100$\mu$g of RNA longer than 200 bases) and spun for 30sec at 14000g and then flow-through was discarded. The RNA bound to the membrane was washed with buffer RW1 and RPE sequentially. High-quality RNA was then eluted in 20$\mu$l of RNase free water. The concentration and purity of the extracted RNA was evaluated spectrophotometrically at 260 and 280nm (Biophotometer, Eppendorf, Germany).

2.4.3.2 cDNA Synthesis

The cDNA synthesis was carried out in a total volume of 25$\mu$l containing the RNA template, Oligo(dT) Primer, M-MLV reverse transcriptase, transcription buffer, dNTP, RNase inhibitor and RNase-free water.

2$\mu$g of RNA was added with 1$\mu$l Oligo(dT)$_{15}$ primer into a microcentrifuge tube and then heated to 70° for 5min to melt secondary structure within the temple. The tube was immediately transferred to ice. The following components were added to the annealed primer/template in the order shown.
Materials and Methods

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MLV 5 × Reaction Buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>10 × dNTP Mix</td>
<td>1.25μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>0.675μl</td>
</tr>
<tr>
<td>M-MLV Reverse transcriptase</td>
<td>1μl</td>
</tr>
<tr>
<td>RNase free water to final volume</td>
<td>25μl</td>
</tr>
</tbody>
</table>

The reaction mix was mixed gently and incubated for 1h at 42°C. Finally the reaction was terminated by heating at 95°C for 5min. The cDNA synthesized was kept at 4°C.

2.4.3.3 Real time RT-PCR

Using the LightCycler instrument, the PCR was performed in a total volume of 10μl, containing 2μl genomic DNA, 10pmol of each primer, 1μl SYBR Green I, 4mM MgCl2 and water to the final volume. The sequences of the forward and reverse primers and the PCR conditions for each gene are given in Table 1.

Total RNA was extracted from these cultures using RNeasy mini kit according to the manufacturer's instructions and quantified spectrophotometrically. The reaction mixture containing 2μg of RNA, 2.5μM of oligo (dT) primer, and 200U of M-MLV in a total volume of 25μl was incubated for 1h at 42°C for reverse transcription, and the reaction stopped by heating for 10min at 70°C. Aliquot (0.5μl) of the each reverse transcription product was added to the reaction mixture (20μl) containing LightCycler-FastStart DNA Master SYBR Green I,
0.5µM of each primer corresponding to the rat MCP-1, CCR2, MKP-1 and β-actin (Table 2.), and 4mM MgCl₂ to amplify each of the genes in a LightCycler. The primer sequences are listed in Table 1. After pre-incubation at 95°C for 10min, the polymerase chain reaction (PCR) was performed as follows: 35 cycles of denaturation at 95°C for 15s, annealing at 60°C (for MCP-1 and CCR2) or 62°C (for MKP-1 and actin) for 5s, and elongation at 72°C for PCR product size per 25s. The expression level of each gene was quantified, expressed as C_t, the cycle number at which the LightCycler system detected the upstroke of the exponential phase of PCR product accumulation, and normalized by the level of expression of β-actin in each individual sample. The mRNA expression levels of various genes studied in the experimental groups and control groups were compared using the 2^[-ΔΔCt] method (Livak and Schmittgen 2001).

### Table 2. Primers for real timeRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| MCP-1| Forward: tctttctctccccactatgca  
                   Reverse: ggtgagacagcaagtggat | 103               |
| CCR2 | Forward: cgcagagttgacaagttgtg  
                   Reverse: gccatggatgaactgaggta | 233               |
| MKP-1| Forward: acttccctgttcctccct  
                   Reverse: acgtggaactcctcc | 302               |
| β-actin| Forward: tcacccacactgtgcctctacaag  
                   Reverse: ggatgccacaggatcctaccca | 327               |

### 2.4.3.4 Detection of PCR product

PCR products were run on agarose gel using electrophoresis. 2% agarose
Materials and Methods

gel was prepared by heating agarose in the TAE buffer. 5% of Ethidum bromide was added into the gel and the gel was cooled, poured onto a gel tray and allowed to solidify. 10μl of each real time PCR product was loaded in each lane of the gel and electrophoresed at 110V for 45min in TAE buffer with 10μl of 100bp DNA ladder. The PCR products in gel were visualized with an ultraviolet trans-illuminator and photographed. The 100bp DNA ladder was used to determine the size of the amplified DNA from 100 to 1000bp.

2.5. Enzyme-linked immunosorbent assays (ELISA)

2.5.1. Principle

Enzyme-linked immunosorbent assays (ELISA) are designed for detecting and quantitating substances such as proteins and hormones. In an ELISA, an antigen is immobilized to a solid surface in 96-well polystyrene plates. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate which produces a detectable product.

2.5.2. Materials

MCP-1 ELISA kit (Pierce, USA, Cat. No. ERMCP1)

TNF-α ELISA kit (BD Biosciences, USA, Cat. No. 550734)

ELISA plate reader (Molecular Devices, USA)
2.5.3. *Analysis of MCP-1 by ELISA*

Microglia cultures were treated with LPS, LPS plus Dex, LPS plus SP600125, or LPS plus SB203580 for 6h, and the culture medium from each experiment was collected. The concentration of MCP-1 released by microglial cells into the culture medium was measured using the rat MCP-1 ELISA kit, according to the manufacturer’s protocol. Briefly, samples including standards of known MCP-1 content were incubated in 96-well plates coated with MCP-1 antiserum for 2h. The samples were treated in subsequent steps with Enzyme Working Reagent for 30 min and TMB One-Step substrate reagent for 30min in the dark and the reaction plates were read within 30min in an ELISA plate reader at 450 nm.

2.5.4. *Analysis of TNF-α by ELISA*

The culture medium from the microglia culture (2.5 ×10^5 cells/well in a 24-well plate) was collected 6h after addition of LPS or RA or LPS plus RA in the culture and the concentration of TNF-α protein was measured using a solid-phase sandwich ELISA kit for rat TNF-α. Briefly, samples including standards of known TNF-α content were incubated in 96-well plates coated with TNF-α antiserum for 2h. Then samples were treated in subsequent steps with Enzyme Working Reagent (30min) and TMB One-Step substrate reagent (30min in the dark) and the reaction plates were read within 30min in an ELISA plate reader at 450nm.

2.6. Nitrite Assay
2.6.1 Principle

Nitric Oxide is rapidly oxidized to nitrite in culture medium and determination of nitrite concentration is used as a measure of NO production. This assay, which was first described by Griess in 1879, is based on a chemical reaction that uses sulfanilamide and N-1-naphthylethlenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.

2.6.2. Material

Griess reagent system (US Biological, USA; Cat. No. 2577-01)

2.6.3. Procedure

Culture medium from primary microglia cultures (2.5 × 10^5 cells/well in a 24-multi-well) was collected 6 h after addition of LPS or RA or LPS+RA in the culture. The quantity of nitric oxide in the culture medium was assessed using a colorimetric biogene kit with a Griess reagent system. Equal volumes of culture medium and the Griess reagent (0.1% N-(1-naphthyl)ethlenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H₃PO₄) were mixed and incubated for 10min at room temperature. The absorbance at 540 nm was determined with an E MAX precise microplate reader (Molecular Devices, USA).

2.7. Western Blot Assay

2.7.1. Principle
Western blotting has become one of the most common protein analysis techniques used in biomedical research. Western blotting provides a direct method for identifying, monitoring, and determining the relative amount of specific proteins in different samples. In general, a complex protein mixture (such as cell lysate or extract, or purified protein preparation) is fractionated on a gel by electrophoresis. After separation, proteins are transferred to a membrane, which can be nitrocellulose, polyvinylidene fluoride (PVDF) or nylon. Nitrocellulose and PVDF membranes are most commonly used for protein analysis. Primary antibodies are used to detect specific protein antigens on the membrane. Detection of antibody-antigen immune complexes on a membrane was accomplished using secondary antibodies covalently conjugated to horseradish peroxidase (HRP) enzyme. The chemiluminescence of HRP substrate is developed on standard X-ray film.

2.7.2. Materials

Protease inhibitor cocktail kit (Pierce, USA, Cat. No. 78410)

Mammalian protein extraction reagent (Pierce, USA, Cat. No. 78503)

Protein assay kit (Bio-Rad, USA, Cat. No. 5000002)

10% resolving gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>7.9ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>6.7ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>5.0ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2ml</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.2ml</td>
</tr>
</tbody>
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Materials and Methods

TEMED 0.008 ml

5% stacking gel:

H₂O 5.5 ml
30% acrylamide mix 1.3 ml
1.0 M Tris (pH 6.8) 1.0 ml
10% SDS 0.08 ml
10% ammonium persulfate 0.08 ml
TEMED 0.008 ml

1x SDS gel-loading buffer:

50 mM Tris.Cl (pH 6.8)
100 mM dithiothreitol
2% SDS
0.1% bromophenol blue
10% glycerol

Tris-glycine electrophoresis buffer:

25 mM Tris
250 mM glycine
0.1% SDS

Transfer buffer:

25 mM Tris
250 mM glycine
20% Methanol

1X TBS:

Tris base 2.42 g
NaCl 0.8 g
H₂O up to 1 liter; adjusted to pH 7.6 with 2N HCl

1X TBST:

1X TBS 1 liter
0.1% Tween 20
Materials and Methods

Rabbit anti- phospho-JNK, JNK (Cell Signaling Technology, USA, Cat. No. 9250)

Rabbit anti-phospho-p38, p38 (Cell Signaling Technology, USA, Cat. No. 9210)

Rabbit anti-phospho-ERK1/2, ERK1/2 (Cell Signaling Technology, USA, Cat. No. 9100)

MKP-1 (Santa Cruz, CA, USA, Cat. No. sc-1102)

rabbit anti-rat TNF-α antibody (Chemicon, USA, Cat. No. AB1837P)

rabbit anti-rat iNOS antibody (BD Transduction Laboratories; USA, Cat. No; 610332)

rabbit anti-Cyclin D1 (Santa Cruz Biotechnology, Inc, USA , Cat. No. sc-717)

Rabbit anti-P27 antibody (Cell Signaling Technology, USA; Cat. No. 2552)

Rabbit anti-α-tubulin antibody (Cell Signaling Technology, USA; Cat. No. 2144)

Rabbit anti-E2F-1 antibody (Santa Cruz Biotechnology, Inc, USA , Cat. No. sc-193)

Rabbit anti-phosphor-Rb (Ser 807/811) antibody(Cell Signaling Technology, USA; Cat. No. 9308)

Mouse anti-Rb antibody (BD Transduction Laboratories, USA, Cat. No. 300508)
Materials and Methods

Goat anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology, USA, Cat. No. 7074)

Mouse anti-β-actin monoclonal antibody (Sigma-Aldrich, USA, Cat. No. A1978)

Goat anti-mouse IgG conjugated with horseradish peroxidase (Pierce, USA, Cat. No. 31430)

Stripping buffer (Pierce, USA, Cat. No. 0021059)

2.7.3. Procedure

Protein extracts from microglial cultures subjected to various treatments for different periods were prepared using the protein extraction kit and protease inhibitor cocktail kit, and were quantified using a protein assay kit. Briefly, the cultures were washed with cold PBS twice and resuspended in 600μl of protein extraction buffer. Cells were then scraped from the culture flask. The supernatant was collected by centrifugation at 14,000 g at 4°C for 15 min and stored at -20°C for further use.

The concentration of protein extracts was determined using a protein assay kit. Four different concentrations (0.06, 0.12, 0.24, and 0.48mg/ml) of BSA were prepared as protein standards. 10μl of each standard or sample and 200μl of dye reagent were added together to each well of a 96-well plate, and mixed thoroughly. The mixtures were incubated at room temperature for 10 min, and the absorbance at 595nm was measured using a microplate reader (GENios, Tecan, Switzerland).
The concentration of protein extract of the samples was determined according to the standard curve of BSA.

Aliquots of protein extracts (20 µg each) were heated to 100°C for 3 min in 1x SDS gel-loading buffer to denature the proteins before the proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the proteins were transferred from the gel to the 0.2µm PVDF membranes using a semi-dry electrophoretic transfer cell (Bio-Rad, USA). Membranes were washed with TBST buffer and then blocked with 5% non-fat milk in TBST for 1h at room temperature. Subsequently, the membranes were incubated with appropriate primary antibodies overnight at 4°C in a shaker. The primary antibodies used are as follows: rabbit anti-phospho-JNK, JNK, phospho-p38, p38, phospho-ERK1/2, ERK1/2, MKP-1 and mouse anti-actin. The next day, blots were incubated with secondary IgG antibody conjugated with horseradish peroxidase for 1h at room temperature. Equal amount of protein loading was confirmed by reprobing with total kinases or actin antibodies. Immunopositive bands were visualized by enhanced chemiluminescence (ECL), and quantified by scanning densitometer and Quantity One software, version 4.4.1 (Bio-Rad, USA).

2.8. In vitro Chemotaxis Assay

2.8.1. Materials

48-well Boyden. microchamber (Neuro Probe, USA, Cat. No. AP48)
Materials and Methods

Fibronectin (BD bioscience, USA, Cat No: 354008)

2.8.2. Procedure

The in vitro migration of microglia in response to microglia conditioned medium was assessed using polyvinylcarbonate-free membranes with 10µm pore size in a modified 48-well microchemotaxis Boyden chamber (Neuroprobe, Cabin John, MD, USA) as described previously (Cross and Woodroofe 1999a). The membrane was coated with 20µg/ml of Fibronectin for 24h. Microglial cells were plated at 2.5×10^5 per well on a 24 multi-well culture dish for 1 day and incubated with LPS (1µg/ml) in the presence or absence of Dex (10^{-6} M) for 6h. The optimal dilution of media derived from the control, LPS and LPS+Dex treated microglial cultures was determined by measuring the chemotaxic effect of serial dilutions (1/2, 1/4 and 1/8). To examine the chemotaxis, 28µl of DMEM or conditioned medium collected from the above microglial cultures or rat recombinant MCP-1 protein (Chemicon, Cat. No. GF041) dissolved in DMEM with different concentrations was added to the lower chambers. In some experiments, rabbit anti-MCP-1 antiserum (10µg/ml) or another antiserum raised in rabbit (10µg/ml; anti-iNOS) as the control was added together with the LPS-treated microglia conditioned medium in the lower chambers in order to test the neutralization effects of anti-MCP-1. We also examined the chemotactic effect of microglial cells in LPS+Dex treated culture medium supplemented with rat recombinant MCP-1 protein (62.5ng/ml).
Materials and Methods

Microglial cells (1X10^6/ml) in 50μl of serum free migration medium, DMEM and 0.5% BSA were incubated in the upper chambers for 5h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. At the end of experiment, the upper surfaces of membranes were scraped free of cells and debris, fixed in 4% paraformaldehyde and stained using crystal violet. Cells that had migrated through pores and adhered to the lower surfaces of the membranes were counted under the light microscope and photographed.

2.9. Cell proliferation assay

2.9.1. Principle

MTS based colorimetric assay kit (Promega, USA) was used in the microglia proliferation assay. MTS is yellow tetrazolium salt, which can be converted by the activity of cellular mitochondrial dehydrogenases into orange formazyl products that are soluble in aqueous solutions. This conversion occurs only in metabolically active cells. The quantity of formazan products as measured at the 490nm absorbance is directly proportional to the number of living cells in culture.

To determine the number of viable cells in culture, cells are grown in a 96-well tissue culture plate and incubated with the yellow MTS solution for 4 hours. At the end of incubation, orange formazan solution is formed which can be quantified using a microplate reader (GENios, Tecan, Switzerland). An increase in number of living cells results in an increase in the overall activity of
mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed.

### 2.9.2. Materials

CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Cat. No. G3580, Promega, USA)

### 2.9.3. Procedure

To study the effects of RA on the proliferation of microglial cells in primary cultures, microglial cells were seeded on 96-well plates at a density of \(1 \times 10^4/\text{ml}\) in 100\(\mu\)l of control, or RA(10\(\mu\)m), GM-CSF (10ng/ml), or GM-CSF plus RA per well. After 1 day or 4 days of incubation, 20\(\mu\)l of MTS reagent was added into each well containing microglial cultures. To study the effect of JNK inhibitor SP600125 or p38 inhibitor SB202380 on microglial cell viability, microglial cells were seeded on 96-well plates at a density of \(1 \times 10^4/\text{ml}\) in 100\(\mu\)l of control, 1\(\mu\)M SP600125 or 1\(\mu\)M SB202380 per well. After 6h of incubation, 20\(\mu\)l of MTS reagent was added into each well containing microglial cultures. The cultures were incubated for 4h at 37\(^\circ\)C in a humidified atmosphere of 5% CO\(_2\) and 95% air before the absorbance at 490nm was measured using a microplate reader (GENios, Tecan, Switzerland). The absorbance is directly proportional to the number of microglia in each well at each time course. The relative increase of the number of microglia in each of the four groups was represented by the ratio of the absorbance to the control absorbance. The results are expressed as mean ± SD of
Materials and Methods

the ratios.

2.10. BrdU incorporation assay

2.10.1. Principle

Evaluation of cell cycle progression is essential for investigations in many fields of biological sciences. Measurement of \(^3\text{H}\) thymidine incorporation in cells that enter S phase during cell cycle has long been the traditional method for the detection of cell proliferation. Quantification of \(^3\text{H}\) thymidine incorporation is performed by scintillation counting or autoradiography. This technology is slow, labor intensive and has several limitations including the handling and disposal of radioisotopes and the necessity of expensive equipment.

A well-established alternative to \(^3\text{H}\) thymidine uptake has been demonstrated by numerous investigators (Gratzner 1982; Lanier et al. 1989; Wynford-Thomas and Williams 1986). Bromodeoxyuridine (BrdU), a thymidine analog, is incorporated into newly synthesized DNA strands of actively proliferating cells. Following partial denaturation of double stranded DNA, BrdU can be detected immunohistochemically using the anti-BrdU antibody. The proliferation index of the cells is analyzed by quantifying the percentage of BrdU positive cells.

2.10.2. Materials

5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich, USA, Cat. No. B5002)
Materials and Methods

Mouse anti-BrdU monoclonal antibody (Sigma-Aldrich, USA, Cat. No. B2531)

Cy3-conjugated goat anti-mouse IgG (Chemicon, USA, Cat. No. AP124C)

*Lycopersicon esculentum* (tomato) lectin (Sigma-Aldrich, USA, Cat. No. L0401)

Propidium iodide (PI) (Sigma-Aldrich, USA, Cat. No. 70335)

Fluorescent Mounting Medium (DakoCytomation, Denmark, Cat. No. S302380)

Phosphate Buffered Saline (PBS) (10X) liquid (Invitrogen, USA, Cat. No. 14200166)

0.1M phosphate-buffered saline (PBS)

0.1M PBS containing 0.1% Triton-X 100 (PBS-TX)

4% paraformaldehyde (PF)

4g paraformaldehyde

100 ml 0.1M PBS (PH=7.4)

2.10.3. Procedure

Microglial cells were transferred onto poly-L-lysine-coated coverslips in 24-well plates. Microglial cells were induced by GM-CSF for 1d before they were incubated with BrdU (10µmol/l) for 2h. The cells were washed twice with PBS and fixed with 4% PF for 20min at 4°C. Subsequently cells were treated with 2N HCl at 37°C for 30min, washed with PBS twice, blocked with 2% normal goat serum for 30min, and incubated with anti-BrdU monoclonal antibody (1:1000)
overnight at 4ºC. Further, the cells were washed with PBS, incubated with goat anti-mouse secondary antibody conjugated with Cy3 (1:200) for 1h at room temperature and counter-stained with lectin (1:100) for counting the total microglial cell number. The percentage of BrdU positive cells was determined by randomly scoring 5 fields for each experimental and control groups in each independent experiment (n=3). The proliferation index is expressed as mean ± SD of the percentage of BrdU positive cells.

2.11. Statistical Analysis

The data of experiments were presented as mean ± SD. The standard deviation (SD) was calculated using the formula given below:

$$SD = \sqrt{\frac{\sum x^2 - (\sum x)^2}{n-1}}$$

Where, \(x\) = individual observation, and \(n\) = number of observations

Statistical analyses for various experiments performed in this study were carried out by Student’s t-test or one-way ANOVA using the Microsoft Excel and GraphPad Prism 4 software. The data were considered as significant at \(p<0.05\).
Chapter 3
Results
3.1. Microglial cells in primary culture

Primary cultures of microglial cells with approximately 96% purity were prepared from brains of postnatal 1-2 days rats. The purity of these cells was confirmed by staining them with lectin, a known marker for rat microglia (Figs. 1A-C). The microglial cells exhibited a dendritic morphology with many highly ramified processes (Fig. 1A), and when activated with LPS in the serum-free medium, they became amoeboid in shape. The activated microglial cells appeared hypertrophic and contained abundant cytoplasmic inclusions (Fig.1B). A majority of the LPS-stimulated microglial cells pretreated with Dex showed ramified cytoplasmic processes of various degrees as observed in untreated culture (Fig. 1C).

3.2. Dex suppressed MCP-1 production in activated microglia via inhibition of MAP kinase pathway

3.2.1. Dex inhibited the MCP-1 mRNA expression in activated microglial cells

MCP-1 mRNA (91bp) was amplified from untreated microglial cultures by the real time RT-PCR (Fig. 2A, B). The effect of Dex on mRNA expression level of MCP-1 in LPS-treated microglia was analyzed (Fig. 2C). The mRNA expression level of MCP-1 was found to be significantly increased to about 22 fold in microglial cells treated with LPS. This increase was inhibited significantly, ranging from 94-98% by the addition of Dex in microglial cultures.
3.2.2. *Dex inhibited the MCP-1 expression and release in activated microglial cells*

Immunocytochemical analysis reveals that MCP-1 positive cells were hardly detectable in the untreated microglial cultures (Fig. 3A). However, LPS markedly induced the MCP-1 immunoreactivity in a majority of microglial cells in the primary culture (Fig. 3B). The intensity of MCP-1 immunoreactivity in the microglial cells treated with LPS appeared to be reduced by Dex (Fig. 3C). In line with these findings, MCP-1 release as revealed by ELISA was increased in microglia cultures treated with LPS and the Dex significantly inhibited the LPS-induced MCP-1 release from microglia cells (Fig. 4).

3.2.3. *Dex suppressed the LPS-induced JNK and p38 MAP kinases activation in microglial cells*

Since GCs have been shown to inhibit a number of MAP kinase family members and MAPK signaling transduction pathway, which plays a significant role in inducing the expression of inflammatory genes in many immune cell types (De et al. 2003), I examined whether Dex inhibits the LPS-induced activation of MAP kinases in primary microglial cultures. Western blot analysis showed that the LPS treatment induced rapid and time-dependent phosphorylation of JNK (Fig. 5A, B) and p38 (Fig. 6A, B) in microglial cells. The phosphorylated JNK and p38 levels increased at 15min, peaked at 30min and 1h and gradually declined to basal
levels at 4h. LPS-induced phosphorylation of JNK (Fig. 5A, B) and p38 (Fig. 6A, B) was significantly reduced at all time points studied when microglial cells were pretreated with Dex 2h prior to LPS treatment. In addition, LPS treatment induced phosphorylation of ERK1/2 in microglial cells (Fig. 7A, B). The phosphorylated ERK level was increased to peak within 30min of incubation. Unlike JNK and p38, however, ERK1/2 level was found to be decreased only modestly at 4h after achieving maximal activation following LPS treatment. In addition, Dex treatment did not alter the phosphorylation of ERK1/2 significantly in microglial cells treated with LPS.

3.2.4 *Dex inhibited LPS-induced c-Jun phosphorylation in microglial cells*

As JNK mediates N-terminal phosphorylation of c-Jun (De et al. 2003), effects of Dex on the c-Jun phosphorylation in LPS-treated microglia was examined. While the immunoreactivity of phospho-c-Jun was undetectable in untreated microglial cells, it was markedly induced in a majority of microglial cells in cultures treated with LPS (Figs. 8A, B, D). Dex decreased the number of phosphorylated c-Jun immunoreactive cells significantly in microglial cultures treated with LPS (Fig. 8C, D).

3.2.5 *Dex suppressed MCP-1 release by inhibiting the JNK and p38 MAPK pathway in activated microglia*

It was hypothesized that Dex suppresses MCP-1 production in activated
Results

microglial cells by altering the MAPK pathways, since phospho-c-Jun has been shown to induce the expression of MCP-1 in microglia (Waetzig et al. 2005). In order to address this, MCP-1 production was measured in LPS-induced microglial cells exposed to pharmacological agents, SP600125 and SB203580 which are selective inhibitors of JNK and p38 respectively. Incubation of microglial cells with SP600125 or SB203580, 30min prior to LPS treatment for 6h decreased the LPS-induced MCP-1 release by 24% and 40%, respectively (Fig. 9). Both the inhibitors of JNK and p38 mimic the inhibitory effect of Dex on MCP-1 production, suggesting that Dex suppresses the MCP-1 production by inhibiting the JNK and p38 MAPKs. Addition of SP600125 (1μM) or SB203580 (1μM) in microglial cultures did not affect the viability of microglia as revealed by the MTS assay (Fig. 10).

3.2.6. Dex inhibited MCP-1 production in activated microglia via MKP-1 dependent JNK and p38 MAPK pathways

3.2.6.1. Dex induced MKP-1 mRNA and protein expression:

GCs are known to inhibit the activation of JNK and p38 by up-regulating the expression of MKP-1 in several cell types (Lasa et al. 2002). I therefore investigated whether MKP-1 was involved in the inhibitory effect of GCs on the MAPK pathway in primary culture of microglia. The real time RT-PCR analysis showed that MKP-1 mRNA expression level was elevated by 27 fold or 19 fold in microglial cells exposed to LPS or Dex respectively, in comparison to that of
control (Figs 11A-C). Dex treatment further enhanced MKP-1 gene expression to 149 fold in the LPS-treated microglia (Fig. 11C). Western blot analysis showed that the MKP-1 protein level (42KDa) was very low in untreated microglial cells (Figs. 12A, B). LPS stimulated MKP-1 protein expression significantly in microglial cells within 15min of treatment. The expression level reached its maximum by 1-2h, and then decreased to basal level. Dex treatment further enhanced LPS-induced MKP-1 protein expression significantly in microglial cells from 30min onwards. However, the MKP-1 protein expression levels induced by Dex alone in microglial cells were constant at all time points studied (Fig. 13A, B).

3.2.6.2 Inhibition of MKP-1 expression by triptolide blocked the inhibitory effect of Dex on phosphorylation of JNK and p38

We next investigated whether inhibition of MKP-1 expression blocked the inhibitory effect of Dex on phosphorylation of JNK and p38 in activated microglial cells. Western blot analysis revealed that triptolide inhibited the Dex-mediated upregulation of MKP-1 protein expression in activated microglial cells (Fig. 14). Furthermore, the inhibitory effects of Dex on the phosphorylation of JNK and p38 in activated microglial cells were abrogated by the triptolide. The triptolide alone had no effect on MAPKs phosphorylation. Neither Dex nor triptolide inhibited the ERK1/2 phosphorylation in activated microglial cells.
3.2.6.3 *Inhibition of MKP-1 expression suppressed Dex-induced downregulation of MCP-1 mRNA expression in activated microglia*

Further, effect of triptolide on MCP-1 mRNA expression in Dex-treated activated microglial cells was examined. The quantitative analysis by real time RT-PCR showed that triptolide pretreatment increased MCP-1 mRNA expression to 2.9 fold in microglial cells treated with LPS plus Dex in comparison to that of cells treated only with LPS plus Dex (Fig. 15). These results suggest that Dex suppresses the MCP-1 production in activated microglial cells by enhancing the expression of MKP-1 and subsequent inhibition of the JNK and p38 phosphorylation.

3.2.7. *Dex inhibited the mRNA and protein expression of CCR2 in activated microglia*

The PCR product of CCR2 (233bp) was amplified in microglia cultures on Gel (Fig. 16A). The effect of Dex on mRNA expression level of CCR2 in LPS-treated microglia has been analyzed by the real time RT-PCR(Fig. 16B, C). The quantitative analysis revealed that the LPS increased CCR2 mRNA expression level to 11 fold in microglial cultures and the increase was reduced by about 32% with the addition of Dex in microglial cultures. CCR2 immunoreactivity was hardly detectable in the untreated microglial cells (Fig. 17A). It was, however, markedly increased in most of the cells in the microglial cultures challenged with LPS (Fig. 17B). Treatment of Dex significantly
Results

suppressed the intensity of the CCR2 immunoreactivity in microglial cells exposed to LPS (Fig. 17C).

3.2.8. *Dex inhibited MCP-1-mediated migration of microglia to medium from activated microglial cultures*

As it has been shown that Dex inhibits MCP-1 production in activated microglial cells in this study, effects of Dex and MCP-1 on the migration of microglia was investigated by an *in vitro* chemotaxis assay. The chemotaxis assay confirmed that MCP-1 induced the migration of microglia in a dose-dependent manner (Fig. 18). The maximum effect of MCP-1 on migration of microglia was at the concentration of 62.5ng/ml. We then investigated chemotactic effects of the conditioned-medium derived from the control (untreated) microglial cultures, microglia cultures treated with LPS with or without Dex (10^{-6} M) on the migration of microglial cells. The optimal dilution of these media determined by chemotaxis assay was found to be 1/4 dilutions (Fig. 19). The strongest chemoattraction was observed in LPS medium diluted 1/4. The optimal conditioned-medium from LPS-treated microglial cultures was able to attract more microglial cells than the medium from control microglial culture or DMEM. LPS-induced microglial migration was inhibited significantly by both Dex and 10μg/ml of anti-MCP-1 antiserum (Fig. 20A, B). When the LPS medium was supplemented with unrelated antiserum from the same species of anti-MCP-1 as the control, the migration of microglia was not inhibited. Moreover, the inhibition by Dex was partly reversed
when recombinant MCP-1 (62.5 ng/ml) was supplemented in the LPS plus Dex treated medium, confirming that inhibition of chemotactic activity by Dex is mediated \textit{via} downregulation of MCP-1.

3.3 RA inhibited inflammatory response of activated microglia by suppressing TNF-\(\alpha\) and iNOS expression

3.3.1 RA suppressed the expression of TNF-\(\alpha\) and iNOS in the microglia exposed to LPS

Immunohistochemically, both TNF-\(\alpha\) and iNOS positive cells were hardly detectable in the untreated microglial cells (Fig. 21A, D). LPS markedly induced the TNF-\(\alpha\) and iNOS immunoreactivity in most of the cells in the microglial cultures (Fig. 21B, E). These cells appeared to be reactive as they showed an increase in size and abundant cytoplasm with cytoplasmic inclusions. Significantly, RA (10\(\mu\)M) reduced the intensity of the TNF-\(\alpha\) and iNOS positive staining and the number of TNF-\(\alpha\) and iNOS positive cells in LPS-treated microglial cultures (Fig. 21C, F). Furthermore, these cells displayed phenotypic features as in the untreated cultures. The effect of RA on LPS-induced TNF-\(\alpha\) and iNOS protein expression has also been analysed by Western blot. LPS has been found to induce both TNF-\(\alpha\) and iNOS protein expression in the microglia of primary cultures, whereas RA suppressed significantly the LPS-induced TNF-\(\alpha\) and iNOS protein expression in microglia (Fig. 22A, B). The inhibitory effects of RA on TNF-\(\alpha\) and iNOS protein expression in LPS-stimulated microglia were further confirmed by ELISA
for TNF-α and nitrite assay, respectively. ELISA revealed that RA reduced the release of TNF-α from LPS-stimulated microglia by 44%, and nitrite assay indicated that RA reduced the production of NO in the microglia exposed to LPS by 58% (Fig. 22C).

3.3.2. RA inhibited JNK phosphorylation and induced MKP-1 expression in LPS-stimulated microglia

As RA suppressed the LPS-induced expression of proinflammatory gene, TNF-α and iNOS which are known to be regulated by JNK MAP kinase signaling pathway, we examined whether JNK MAPK signaling pathway was involved in RA action on inflammatory response of microglia by Westen blot analysis. LPS has been found to induce JNK phosphorylation within 30min in microglial cells (Fig. 23). Pretreatment of RA (10μm) for 2h significantly inhibited the phosphorylation of JNK in LPS-treated microglial cells. MKP-1 is a dual-specificity phosphatase which dephosphorylates and inactivates JNK. To determine whether the suppression of JNK MAP kinase by RA in activated microglial cells is mediated by MPK-1, the protein expression level of MKP-1 has been analysed in microglial cells exposed to RA and LPS. The MKP-1 expression was found to be induced in microglial cells exposed to LPS for 30min. In addition, pretreatment of these activated microglial cells with RA for 2h further increased the induction of MKP-1 expression (Fig. 23). RA-induced upregulation of MKP-1 was consistent with the inhibitory effect of RA on JNK MAPK, suggesting that
the inhibitory effect of RA on the phosphorylation of JNK may be mediated via upregulation of MKP-1 expression.

3.4. RA inhibited GM-CSF-induced microglial proliferation by regulating cell cycle-associated proteins

3.4.1 RA inhibited GM-CSF-induced proliferation of microglia

The proliferation rate of microglia was detected by BrdU incorporation assay. Since the role of GM-CSF in inducing the proliferation of microglia has been well established, I explored whether RA has any effect on GM-CSF-induced proliferation of microglia. GM-CSF treatment increased the number of BrdU positive microglial cells in the primary culture (Fig. 24A, B, D). However, the number of BrdU positive microglial cells was significantly decreased in culture exposed to RA together with GM-CSF (Fig. 24C, D). The identity of the proliferating cells was confirmed to be microglia by labeling the cells with lectin, the marker of microglial cells. This result implies that RA inhibits the proliferation of microglia. This was further confirmed by MTS assay (Fig. 25), which showed that RA inhibits the proliferation of microglia induced by GM-CSF. In addition, RA did not affect the viability of microglial cells in culture.

3.4.2. RA altered expression of cell cycle associated proteins in GM-CSF stimulated microglia

The mechanisms by which RA inhibit the GM-CSF-induced microglial
Results

proliferation has been analysed. I initially analyzed cyclin D1, which is the regulatory subunit of the cyclin D/CDK4/6 holoenzyme and one of the rate-determining factors for cells to progress through the G1/S checkpoint in the late G1 phase of cell cycle (Bruce et al. 2007). The protein expression of cyclin D1 in microglial cells was increased by GM-CSF (10ng/ml) treatment, and this increment was inhibited by RA (10μM) (Fig. 26). This result suggests that RA inhibits the proliferation of microglial cells at least in part by reducing the protein expression of cyclin D1.

The activity of cyclinD1 is regulated by a group of cyclin-dependent kinase inhibitory proteins, among which p27 has been shown to play a critical role in the negative control of the G1/S cell cycle transition (Koguchi et al. 2003). Therefore, the expression level of p27 protein was determined by Western blot analysis in GM-CSF stimulated microglia culture in the presence or absence of RA (Fig. 26). The protein expression of p27 was decreased in microglial cells exposed to GM-CSF and the decrease was restored by the addition of RA. Taken together, these results imply that RA inhibits the cell cycle progression of microglia by inducing the expression of p27, the inhibitor of CDK.

Since the cyclin D/CDK complex is a major negative regulator of Rb protein at the G1 phase of the cell cycle, I analyzed the level of total and phosphorylated Rb in GM-CSF or GM-CSF plus RA treated microglial cells. The protein level of total Rb was decreased in microglial cells by GM-CSF treatment and this decrease was reversed by RA. However, in contrast to total Rb, the
phosphorylated RB (phospho-Rb) was significantly increased by GM-CSF and the
increase was reduced by RA treatment (Fig. 26). These results confirm that RA
alters the functionality of Rb by increasing the expression of total Rb and by
reducing the phosphorylation of Rb.

It has been shown that E2F-1 which is bound to Rb, induces expression of
genes involved in cell cycle progression once it is released upon phosphorylation
of Rb (Bruce et al. 2007). Since the phosphorylation of Rb was inhibited by RA in
GM-CSF-induced microglial cells, I have analysed the effect of RA on the
expression of E2F-1 in GM-CSF induced microglia cells. It has been found that
E2F-1 expression was induced in microglial cells treated with GM-CSF. This
induction was found to be decreased by the addition of RA in microglial culture
(Fig. 26).
Chapter 4

Discussion
4.1 Dex suppresses the migration of activated microglia

4.1.1 Dex suppresses MCP-1 production and subsequent microglial migration

It is well established that activated microglial cells become hypertrophic, undergo rapid proliferation and migrate to the inflammatory sites where they produce excess amount of neurotoxic and proinflammatory mediators which cause deleterious damage to neurons in chronic neuropathological conditions (Vilhardt 2005). Several drugs have been used to limit the microglia activation by suppressing the release of neurotoxic mediators, and the proliferation as well as the migration of microglia. It has been widely demonstrated that GCs suppress the activation of microglial cells by inhibiting their secretion of proinflammatory mediators. The present study has shown that Dex, the synthetic GC, inhibits the migration of microglial cells by suppressing the release of MCP-1, a chemokine which regulates migration of activated microglial cells to the inflammatory sites in the CNS.

Activation of microglia and associated induction of chemokines have been well documented in various acute and chronic neuropathological conditions (Ambrosini and Aloisi 2004). Activated microglia release chemokines which facilitate the chemotaxis of leukocytes and microglial cells themselves and amplify the CNS inflammation (Hanisch 2002; Ambrosini and Aloisi 2004). Although microglial activation mainly contributes to neuroprotection, it has been widely accepted that inappropriately or persistently activated microglia could cause neuronal damage in chronic CNS diseases (Ambrosini and Aloisi 2004).
Hence, understanding the mechanisms that inhibit neurotoxic effect of microglial cells and that modulate the chemokine network may offer new specific windows for treatment of immune-mediated brain diseases.

Previous studies have shown that Dex inhibits the microglial activation by downregulating the neurotoxic and proinflammatory mediators such as NO, TNF-α and IL-6 (Chao et al. 1992; Golde et al. 2003; Lieb et al. 2003;). In the present study, Dex has been found to inhibit the LPS-stimulated mRNA expression and protein release of MCP-1 in activated microglial cells, as demonstrated in the brain endothelial cells (Harkness et al. 2003). MCP-1 binds with its receptor CCR2 to induce a variety of effects including transient mobilization of intracellular calcium and subsequent changes in actin polymerization which contributes to the microglial migration when activated (Cross and Woodroofe 1999). The rapid influx of microglial cells to the injury sites in CNS diseases contributes to phagocytosis and controlling the neuroinflammation. However, overcrowding of these cells around the injury site could exacerbate the neuroinflammation (Ambrosini and Aloisi 2004). The decreased expression of MCP-1 by Dex as demonstrated in this study could reduce the microglial migration so that augmentation of inflammation is inhibited. The chemotaxis assay also further confirmed that Dex or MCP-1 neutralization with its antibody inhibits the microglial recruitment towards the conditioned medium derived from LPS-treated microglial cultures, indicating that suppressive effect of Dex on migration of microglial cells is mediated by the downregulation
of MCP-1 expression.

The efficacy of GCs is well established in the treatment of bacterial meningitis, brain edema, and a number of autoimmune diseases such as multiple sclerosis (Gomes et al. 2005; Sloka and Stefanelli 2005). The present study demonstrates that GCs could block the development and progression of the neurological disorders by inhibiting the MCP-1 expression and subsequent microglial recruitment to the injury sites. This is further strengthened by recent reports which demonstrated that proteolytic activation of MCP-1 by protease tissue plasminogen activator (tPA) enhances the microglial migration and that GCs inhibit tPA expression and corresponding enzyme activities (Eberhardt et al. 2005; Sheehan et al. 2007), suggesting that the suppressive effect of Dex on microglial migration is mediated via inhibition of MCP-1. However, it needs to be emphasized that involvement of other chemotactic proteins in migration of microglia can not be excluded since there was only a partial inhibition of microglial migration after MCP-1 neutralization with its antibody.

4.1.2 Downregulation of MCP-1 expression in activated microglial cells by Dex is mediated via MKP-1-dependent inhibition of JNK and p38 MAPK pathways

I next investigated the molecular mechanisms by which Dex inhibits MCP-1 production in activated microglial cells. GCs have been consistently shown to inhibit the expression of pro-inflammatory genes by antagonizing the MAPK pathways, in particular the p38 and JNK pathways. To this end, the inhibition of
Discussion

JNK and p38 MAPK pathways by GCs has been found to be mediated via induction of MKP-1 (Chen et al. 2002). However, there is very little information about the molecular basis linking GCs to MCP-1. This study provided initial clue that MAPK pathway is involved in inhibition of MCP-1 release by GCs in the activated microglia. Members of the MAPK families mediate transcriptional and posttranscriptional changes in gene expression in response to proinflammatory stimuli such as LPS (Hommes et al. 2003). MAPK activity is regulated negatively by MKP-1, the expression of which was found to be induced by GCs in myeloid and non-myeloid cells (Kassel et al. 2001; Chen et al. 2002; Lasa et al. 2002; Engelbrecht et al. 2003). Several pro-inflammatory stimuli, such as LPS also induce MKP-1 expression which, however, activates a negative feedback loop down-regulating the production of pro-inflammatory cytokines such as TNF-α and IL-1 and IL-6 (Chen et al. 2002; Shepherd et al. 2004; Zhao et al. 2005). Recently it has been shown that endocannabinoid anandamide (AEA) which is released after brain injury and is believed to attenuate neuronal damage, induces MKP-1 expression and subsequent ERK-1/2 MAPK dephosphorylation in activated, but not in resting microglial cells which in turn abolishes NO release and finally results in neuroprotection (Eljaschewitsch et al. 2006). In the present study, Dex induced MKP-1 expression and inhibited phosphorylation of JNK and p38 MAPKs in activated microglial cells. The phosphorylation of c-Jun, the JNK substrate, was also suppressed in LPS-stimulated microglial cells exposed to Dex. It appears that the inhibition of phosphorylation of JNK and p38 MAPKs in
activated microglial cells exposed to Dex is due to induced expression of MKP-1 as it has been shown to be a negative regulator of MAP Kinases in various other cell types (Imasato et al. 2002; Sakai et al. 2004; Roger et al. 2005). This effect was further confirmed since blocking of MKP-1 induction by triptolide prevented the inactivation of JNK and p38 in activated microglial cells by Dex. Moreover, recent studies in MKP-1 knockout mice showed that Dex was unable to inhibit the LPS-induced JNK and p38 activation in bone marrow-derived macrophages (Abraham et al. 2006). It has also been reported that Dex accelerates deactivation of p38 and JNK following LPS stimulation in wild-type but not in the MKP-1-deficient peritoneal macrophages elicited by thioglycollate (Wang and Liu 2007). Taken together, these results strongly support the notion that Dex inhibits JNK and p38 phosphorylation in microglial cells by inducing MKP-1 expression, resulting in downregulation of proinflammatory genes. However, the MKP-1-mediated signaling pathway appears to be complex since the phosphorylation of ERK1/2 MAPK was unaffected in activated microglia by Dex, while GCs could inhibit the phosphorylation of ERK1/2 by inducing rapid up-regulation of MKP-1 expression in other cell types such as osteoblast cell lines (Engelbrecht et al. 2003). This indicates that GCs action is cell type-dependent. Moreover, the upregulation of MKP-1 expression was evident in activated microglial cells only after 30min of incubation with LPS plus Dex, although the downregulation of JNK and p38 MAP kinases occurred within 15min of incubation with LPS plus Dex. This prompts to speculate the possibility that
inhibition of p38 and JNK by Dex also involves phosphatases other than MKP-1, particularly in early phase (Cadalbert et al., 2005).

Activation of MKP-1 has also been shown to inhibit the production of pro-inflammatory cytokines such as TNF-α in several cell types (Chen et al. 2002). The present study revealed that induced MKP-1 expression in activated microglial cells treated with Dex contributed to downregulation of the MCP-1 mRNA expression. This effect appeared to be mediated via MAPK pathways since the inhibition of MKP-1 expression by triptolide enhanced the phosphorylation of JNK and p38 MAPK and the mRNA expression of MCP-1 in activated microglial cells treated with Dex. Further, the involvement of JNK and p38 MAPK pathways in induction of MCP-1 production in activated microglial cells was confirmed as there was an attenuation of MCP-1 protein release when microglial cells were treated with inhibitors of JNK and p38 signaling pathways. Moreover, MCP-1 promoter has been shown to contain functional consensus sequences of c-Jun, a primary transcription factor which is phosphorylated by JNK (De et al. 2003; Waetzig et al. 2005). Hence, the inhibition of c-Jun phosphorylation by Dex in activated microglia cells suggests that Dex inhibits MCP-1 via MAPK pathways. However, it should be noted that the inhibition of MKP-1 protein expression by triptolide treatment could not restore the MCP-1 mRNA expression completely in microglial cells exposed to LPS plus Dex. This could be due to the fact that the triptolide, an active component purified from the medicinal plant *Tripterygium wilfordii Hook F.*, is a pleiotropic causing widespread effects including the
Discussion

inhibitory effect on MCP-1 expression via inhibition of Stat3 phosphorylation and NF-κB activity (Liu et al. 2006). As mentioned earlier, MKP-1 mediated signaling pathway appears to be complex, since its expression is induced by various immune suppressive factors including GCs, TGF-β and endocannabinoid anandamide. Hence further studies are required to unravel the regulatory mechanisms of MKP-1 by GCs in microglial cells in the CNS.

4.2. RA suppresses activation and proliferation of microglia

4.2.1. RA inhibits the neurotoxic effect of activated microglia by suppressing the expression of TNF-α and iNOS

This study demonstrates that RA inhibits the neurotoxic effect of microglia by suppressing the expression levels of proinflammatory cytokine, TNF-α and iNOS induced by LPS. Microglial activation and associated induction of proinflammatory cytokines and NO have been well documented in various neurodegenerative diseases, such as AD and PD. It is believed that the activated microglia exert cytotoxic effects in the brain through two different processes, such as phagocytosis of cellular debris and the release of a large variety of potentially noxious substances (Banati et al. 1993). Both TNF-α and iNOS have been implicated as important mediators of the inflammatory response and they contribute to most of the neurotoxic activity exerted by the activated microglia (Combs et al. 2001). Although the microglial activation is elicited by neuronal damage, it has been widely accepted that their cytotoxic effects in turn contribute
to the pathogenesis of neurodegeneration (Kalaria 1999). Hence, understanding the mechanisms that inhibit microglial activation may provide insights into potential interventions for immune mediated neurodegenerative diseases. In general, the inhibition of microglial activation has been characterized by down regulation of proinflammatory cytokines and NO.

RA is an active metabolite of vitamin A. It acts through a family of nuclear transcription factors known as retinoid receptors (Marill et al. 2003). While the principal effects of RA on target cells are inhibition of proliferation and induction of differentiation (Maden 2002), it has also been shown that RA exhibits immunomodulatory and anti-inflammatory activities in various cell types (Mathew and Sharma 2000). For example, RA has been shown to induce TNF-α mRNA destabilization in an RXR dependent manner in the rat hepatic macrophages (Motomura et al. 2001). Further, RA also reduced NO production induced by IL-1 and IFN-γ in rat pancreatic beta cells (Kang et al. 2004). In the present study, RA has been found to inhibit the expression of TNF-α and iNOS in activated microglia.

I have also investigated the mechanisms by which RA inhibits the TNF-α and iNOS expression in activated microglia. Previous studies have suggested that RA plays a role in regulation of the activation of JNK MAP kinase signaling pathways in cardiomyocytes (Palm-Leis et al. 2004). In the present study, RA has been found to inhibit LPS-induced rapid phosphorylation of JNK MAP kinase in
microglial cells. JNK has been implicated as an essential mediator of pro-inflammatory functions such as TNF-α production in microglial cells (Waetzig et al. 2005). Inhibition of JNK suppresses the c-Jun phosphorylation and subsequent transcription of c-Jun/AP-1 target genes, including TNF-α and iNOS (Waetzig et al. 2005). In the present study, the inhibition of TNF-α and iNOS expression by RA in activated microglia appears to be mediated via inhibition of JNK MAP kinase phosphorylation.

MAP kinase activity is negatively regulated by MAPK phosphatases (MKPs) (Clark 2003). RA appears to inhibit MAP kinases activity in activated microglia by inducing MKP-1, a member of the MKP family, as it was found to induce the expression of MKP-1 in LPS-stimulated microglial cells. It has also been reported that RA inhibits the proliferation of various cell types including cardiomyocytes and hepatocytes in different experimental conditions by MKP-1 mediated MAP kinases inactivation (Palm-Leis et al. 2004). Taken together, it is suggested that RA acts on the activated microglia by inducing the expression of MKP-1 which negatively regulates the JNK phosphorylation, resulting in inhibition of neurotoxicity and possibly, proliferation of microglia,

It has also been shown that RA inhibits the expression of iNOS and TNF-α by down-regulating NF-κB transcriptional activity. A mechanistic link between iNOS and NF-κB has been demonstrated as the iNOS promoter contains several NF-κB binding sites (Xie et al. 1993). This link has been further confirmed as the
iNOS expression was shown to be inhibited by downregulation of NF-κB activity (Pahan et al. 1997). In addition, it has been shown that NF-κB forms a transcriptionally inactive complex with retinoid-activated RXR (Na et al. 1999). The RXR forms a heterodimeric complex of RARβ/RXR (Soprano and Soprano 2002) through which RAR-dependent signaling is transduced. Taken together, it was suggested that RA acts on the microglia by inducing the expression of RARβ which downregulates the NF-κB transcriptional activity, thereby iNOS expression is inhibited in activated microglia treated with RA. In addition, RA-induced inhibition of TNF-α expression in the microglia is likely to be NF-κB dependent as it has been reported that inhibition of active NF-κB nuclear translocation by the SN-50 peptide prevents the Aβ-stimulated increase of TNF-α promoter activity (Combs et al. 2001)

TGF-β1 acts as an immunosuppressor and inhibits the production of inflammatory cytokines by the stimulated microglia (Chao et al. 1995). RA has been shown to induce TGF-β1 expression in the various cell types (Defacque et al. 1999). I have also shown that upregulation of TGF-β1 expression observed in the activated microglia treated with RA could contribute to the inhibition of iNOS expression (Dheen et al. 2005), via downregulation of NF-κB transcriptional activity (Xie et al. 1994) as TGF-β1 has been reported to increase the expression of IκB, the inhibitor of NF-κB (Arsura et al. 1996).
4.2.2. RA inhibits GM-CSF-induced microglia proliferation by suppressing the cell cycle related protein.

Microglia proliferation plays an important role in various neurological diseases such as trauma (Urrea et al. 2007) and ischemia (Denes et al. 2007). Although the microglial proliferation is elicited by neuronal damage and contributes to neuroprotection in some circumstances (Denes et al. 2007), it has been widely accepted that the release of neurotoxic mediators by microglia in turn contributes to the pathogenesis of neurological diseases. Hence, understanding the mechanisms that inhibit microglial proliferation would provide a new therapeutic strategy for treatment of neuroinflammation associated disorders in the CNS.

Microglial proliferation is induced by certain mitogens including GM-CSF, M-CSF and some cytokines such as IL-3 (Flanary and Streit 2006). Vitamin E has also been shown to facilitate the proliferation of microglia in vitro (Flanary and Streit 2006). However, an in vivo analysis revealed that M-CSF and GM-CSF are the major mitogens of microglia (Koguchi et al. 2003). GM-CSF regulates several cell cycle associated proteins including p27, cylin D, and cyclin E (Koguchi et al. 2003), which control microglial proliferation.

The cell cycle progression occurs in 4 phases: G1 phase, S phase, G2 phase, and M phase which are controlled by the interaction of several factors including cyclins, CDKs and CDKIs. Cyclin D forms a complex by binding with CDK4 and CDK6, and regulates the transition from G1 phase to S phase (Bruce et al. 2007). Effects of cyclin D/CDK4,6 complex activity are mediated by a gene regulatory
protein, E2F, which binds with retinoblastoma protein (Rb), an inhibitor of cell-cycle progression (Simin et al. 2004) and transactivates many genes required for S phase entry (Bruce et al. 2007). When cells are stimulated to divide by extracellular signals, cyclin D/CDK4,6 complexes induce phosphorylation of Rb, and release of E2F that activates transcription of S phase genes (Bruce et al. 2007).

In the present study, I showed that GM-CSF stimulates the proliferation of microglia by inducing hyperphosphorylation of Rb and accumulation of E2F-1.

RA has been shown to modulate a wide variety of cellular processes; however the role for RA in the regulation of microglial proliferation has not been explored. In the present study, RA has been found to inhibit the GM-CSF-induced microglial proliferation by suppressing the expression of cyclin D1, E2F-1, and hyperphosphorylated Rb and increasing the expression of p27, the CDK inhibitor. It appears that RA inhibits the proliferation of microglia by inducing cell cycle arrest at G1 phase. This is in accordance with the effects of RA in other cell types such as breast cancer cells (Wilcken et al. 1996). In human bronchial epithelial cells, posttranslational mechanisms have been shown to contribute to the suppression of cyclin-CDK complexes by RA (Sueoka et al. 1999). Further, suppression of cyclin D1 by RA in microglia exposed to GM-CSF may be associated with inhibition of E2F-1 expression, since cyclin D1 is an upstream kinase involved in induction of E2F-1 transcription (Jia et al. 2006).

The suppression of cycin D1 by RA in microglial cells exposed to GM-CSF appears to be mediated via induction of p27 which belongs to a family of CDKIs.
and inhibits activity of cyclin/CDK complexes, resulting in cell cycle arrest at G1/S phase (Koguchi et al. 2003). Overall, RA induces p27 which suppresses the activity of cyclin D1, leading to inhibition of E2F-1 transcription required for the induction of expression of S phase genes, thereby resulting in cell cycle arrest at G1/S phase in microglial cells exposed to GM-CSF.

Rb, a negative regulator of cellular proliferation, is one of the target proteins of cyclin D-CDK complex. Inactivation of total Rb and activation of Rb phosphorylation are critical steps for cell cycle progression. The cyclin D-CDK complex activates phosphorylation of Rb, which induces physical dissociation of E2F transcription factor from the Rb and expression of genes required for the cell cycle at S phase (Gallo and Giordano 2005). Moreover, phosphorylation of Rb is also required for inactivation of Rb (Lundberg and Weinberg 1998). In the present study, GM-CSF appeared to induce Rb phosphorylation and inactivate total Rb in microglial cells. However, RA has been found to reverse these processes as observed in other cell types such as human monocytic THP-1 cells (Chen and Ross 2004), resulting in arrest of cell cycle progression at G1/S phase in microglial cells.

Other potential mechanisms may also be involved in the antiproliferative effects of RA on microglia as RA has been shown to inhibit microglial expression of insulin-like growth factors-1(IGF-1), a mitogen in various cell types (Dheen et al. 2005; Kaur et al. 2006). Further, a systematic investigation may be required to understand the antiproliferative effects of RA in microglial cells.
Overall, this study demonstrates the mechanisms by which RA inhibits proliferation and neurotoxic effect of microglia. RA suppresses the neurotoxic effect of microglia by inhibiting the mRNA and protein expression of proinflammatory cytokine, TNF-α and iNOS in the primary cultures of rat microglia. On the other hand, RA suppresses GM-CSF-induced microglia proliferation by altering the expression of cell cycle associated proteins such as cyclin D1, E2F-1, Rb and p27. The RA-induced inhibition of TNF-α and iNOS syntheses in the activated microglia appears to be mediated via MKP-1-induced inhibition of JNK MAPK pathway and downregulation of the NF-κB transcriptional activity. Based on the results, it is suggested that RA could be considered a potential therapeutic agent that may limit the neuroinflammatory response and rapid proliferation of microglia in the neurodegenerative diseases. However, further systematic evaluation such as Genearray analysis is required as RA modulates a wide variety of biological processes, including proliferation, differentiation, and apoptosis in various cell types.
Chapter 5

Conclusion
Microglia, the resident immune cells of the CNS, have emerged dramatically over the years as a key player engaged in mediating the inflammatory response to neuronal cell death in various neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, prion diseases and HIV-dementia. Activation of microglia is considered to be a hallmark of brain pathology. Although they normally respond to neuronal damage and remove the damaged cells by phagocytosis, it remains controversial whether the reactive microglial cells have beneficial or detrimental functions in various neuropathological conditions. Mounting evidence indicates that chronic activation of microglia causes neurotoxicity through the release of potentially cytotoxic molecules such as proinflammatory cytokines including TNF-α and IL-1β, reactive oxygen intermediates, proteinases, NO and complement proteins. Therefore, suppression of microglia-mediated inflammation has been considered as an important strategy in neurodegenerative disease therapy. Several drugs and molecules have been shown to repress the microglial activation and to exert neuroprotective effects in the various neuropathologic conditions. However, these drugs are not targeted specifically against microglial cells and hence may result in various forms of undesirable side effects. In view of this, it was suggested that a systematic molecular analysis of mode of action of these drugs on microglial cells is required to enhance the safe treatment strategy for neurodegenerative diseases. Moreover, activation of microglia becomes an increasingly common phenomenon in most of the neurodegenerative diseases which continue to be of global importance. The
present study was carried out to explore various forms of potential therapeutic options (using two drugs, Dex and RA) to inhibit the function of reactive microglia which amplify the inflammation-related neuronal injury in neurodegenerative diseases.

In the present study, Dex, a widely used anti-inflammatory and immunosuppressive drug in several neurological diseases and RA (a vitamin A metabolite) have been found to inhibit microglial activation. Particularly, Dex appears to have a dual role: 1. inhibition of inflammatory genes in microglia as reported in several studies and 2. inhibition of MCP-1 mediated migration of microglia to the inflammation sites in CNS. This study reveals that Dex inhibits the synthesis of MCP-1 which is believed to amplify the inflammation process by recruiting macrophages and microglia to the inflammatory sites in the CNS, by inducing MKP-1 expression which negatively regulates the p38 and JNK MAPK pathways (Fig 27). It is suggested that inhibition of MCP-1 by Dex suppresses the MCP-1 mediated-migration of microglia to the injury site, so that overpopulation of microglia that exacerbates the inflammation around the injury site in CNS is controlled. Suppression of the chemotaxic effect of microglia by Dex appears complementary to inhibition of inflammatory response of microglia.

One of the prominent features in the CNS pathology is the rapid microglial proliferation which has been implicated in the progression of inflammation and neuronal degeneration. As RA has been shown to act as a potent anti-proliferative and anti-inflammatory compound in various cell types including peripheral
macrophages, monocytes and hematopoietic progenitor cells, I have investigated the effects of RA on microglial activation and proliferation in vitro. The results obtained clearly indicate that RA suppresses the activation of microglial cells exposed to LPS and β-amyloid peptide in primary culture by inhibiting the expression and production of TNF-α and NO. This inhibition of TNF-α and NO synthesis by RA in the activated microglia appeared to be mediated via inhibition of NF-κB translocation which could be caused by upregulation of RAR and TGF-β1 gene expression. It has also been shown that RA could inhibit synthesis of TNF-α and NO in activated microglia by MKP-1-mediated inhibition of JNK MAP kinase pathway (Fig 27).

Moreover, this study demonstrates that RA inhibits GM-CSF induced microglial proliferation by altering the expression of cell cycle associated proteins such as cyclin D1, E2F-1, Rb and p27. Based on the results, it is suggested that RA could be considered a potential therapeutic agent that may inhibit the expansion and activation of microglia in the neurodegenerative diseases. However, careful evaluation is needed before RA is considered for the treatment of neurodegenerative diseases as it modulates a wide variety of biological processes including proliferation, differentiation and apoptosis in various cell types.
Fig 27. An illustration demonstrates that LPS and other stimuli activate microglial cells by inducing the release of proinflammatory mediators and chemokines via MAPKs i.e., p38 and JNK. Dex inhibits microglial activation and migration by suppressing the release of proinflammatory mediators and chemokines via induction of MKP-1 which negatively regulates MAPKs. RA binds with its receptor complex and inhibits the microglial activation by suppressing the release of TNF-α and NO via MKP-1-mediated inhibition of JNK MAP kinase pathway and inhibition of NF-κB translocation which could be caused by upregulation of RAR and TGF-β1 gene expression. Adapted and modified from Dheen et al., 2007.
Conclusion

Scope for the Future Study

Microglia, once considered not even to exist or in oblivion in the 50s-70s of the last century, have emerged dramatically over the years to be a key player engaged in a pivotal role that may lead to a fuller understanding of mechanism of various neurodegenerative diseases. Several studies on neurodegenerative diseases point to a hypothesis that multiple primary causes for these diseases may be ultimately linked to a final common signal transduction pathway involving microglial cells, leading to neuroprotection or neurodegeneration. There is ample evidence demonstrating the microglial activation and their secretory activity in response to various neurodegenerative diseases and remarkably, the response of microglial cells to various stimuli is relatively consistent. As far as can be ascertained, the majority of the studies on expression and secretory patterns of cytokines and chemokines in activated microglial cells has been based on *in vitro* analysis. There is an apparent lack of information on the signaling pathways that are elicited in microglial cells when activated *in vivo*. This direction of study is of clinical importance as it could open up new vistas of research to develop safe mode of therapeutic strategy for neurodegenerative diseases.

Several drugs and molecules have been shown to suppress the microglial activation in chronic pathologic conditions. It needs to be emphasized that these drugs are not targeted specifically against the microglial cells and hence may result in various forms of undesirable side effects. A systematic molecular analysis of mode of action of these drugs on microglial cells would greatly enhance the
Conclusion

treatment strategy for neurodegenerative diseases. Moreover, activation of microglia becomes an increasingly common phenomenon in most of the neurodegenerative diseases which continue to be of global importance. Thus, future research needs to focus on identification of microglia specific proteins that would be of great therapeutic value and may serve as useful biomarkers for diagnosis and prognosis of neurodegenerative diseases.
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Figs. 1 A-C. Confocal images of microglia labeled with lectin (green) and nuclear marker, PI (red). In the control (A), microglial cells exhibit dendritic morphology with many highly ramified processes (arrows). After stimulation with LPS for 6 h, the cells transformed into amoeboid cells which appear hypertrophic and contain abundant cytoplasmic inclusions (arrows, B). Majority of the microglial cells treated with Dex and LPS exhibit ramified morphology (arrows, C). Scale bar = 50μM.
Fig. 1

Lectin + PI

Control

LPS treated

LPS + Dex treated
Figures

Figs. 2 A-C. Real time RT-PCR analysis demonstrating the inhibitory effects of Dex on MCP-1 mRNA expression in microglia treated with LPS. A. The PCR product of MCP-1 gene (91 bp) amplified in microglial cells is shown. B. The melting curve of the real time RT-PCR with a single peak shows the specificity of the product. C. After LPS treatment for 6 h, MCP-1 mRNA expression level is elevated to about 22 folds in comparison to that of control. Dex decreased the mRNA expression of MCP-1 significantly in microglial cells treated with LPS. Data represent mean±SD (n=4 independent experiments). Control vs LPS treated cultures; LPS vs LPS+Dex treated cultures. *p<0.01.
Fig. 2

A

MCP-1 (91bp)

B

C

MCP-1 mRNA Expression
(Fold Change)

Con  LPS  LPS+Dex

*
**Fig. 3:** Confocal images of microglia showing MCP-1 immunoreactivity (green) and PI (red), the nuclear marker. Note that MCP-1 positive cells are hardly detectable in the untreated microglial cultures (A), while majority of the microglial cells exhibit MCP-1 immunoreactivity in cultures treated with LPS (B, arrows). Dex decreased the staining intensity of MCP-1 immunoreactivity and the number of MCP-1 positive cells in the microglial cultures treated with LPS (C). Scale bar = 50μM.
Fig. 3

MCP-1 + PI

A. Control

B. LPS treated

C. LPS+Dex treated
Fig. 4 Analysis by ELISA shows that MCP-1 release is increased significantly in microglial cultures treated with LPS; the increase is inhibited by Dex. Data represent mean±SD (n=3 independent experiments). Significance of difference (Control vs LPS treated cultures; LPS vs LPS+Dex treated cultures) is indicated by *p<0.01.
Fig. 4

The graph shows the release of MCP-1 in pg/ml under different conditions: Con (control), LPS, and LPS+Dex. The LPS condition shows a significantly higher MCP-1 release compared to the other two conditions, indicated by an asterisk (*) on the graph. The error bars represent the standard error of the mean.
**Fig. 5** Western blot analysis shows that Dex inhibits the phosphorylation of JNK in microglia treated with LPS. **A:** LPS treatment induces rapid and time-dependent phosphorylation of JNK in microglial cells. The induction of phosphorylation of JNK is significantly reduced at 15min, 30min, 1h in microglial cells pre-treated with Dex, 2h prior to LPS stimulation. Lower panels of Western blot show no differences in total JNKs. Blots are representative of three independent time course studies. **B:** Data are presented as signal intensity of phospho-JNK relative to total JNK (mean ± SD). Significant difference between LPS and LPS plus Dex group is indicated by *p<0.05.
Fig. 5

A. Western blot analysis of phospho-JNK and Total JNK in response to LPS and LPS+Dex at different time points (15m, 30m, 1h, 4h).

B. Bar graph depicting relative signal intensity of phospho-JNK and Total JNK at different time points (Con, 15min, 30min, 1h, 4h) for LPS and LPS+Dex treatments. Asterisks indicate statistically significant differences.
Western blot analysis shows that Dex inhibits the phosphorylation of p38 in microglia treated with LPS. **A:** LPS treatment induces rapid and time-dependent phosphorylation of p38 in microglial cells. The induction of phosphorylation of p38 is significantly reduced at 15min, 30min, 1h in microglial cells pre-treated with Dex, 2h prior to LPS stimulation. Lower panels of Western blot show no differences in total p38. Blots are representative of three independent time course studies. **B:** Data are presented as signal intensity of phospho-p38 relative to total p38 (mean ± SD). Significant difference between LPS and LPS plus Dex group is indicated by *p<0.05.
Fig. 6

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**A**

Phospho-p38

Total p38

**B**

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<td>4</td>
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Fig. 7 Western blot analysis shows that Dex does not inhibit the phosphorylation of ERK1/2 in microglia treated with LPS. **A:** Phosphorylation of ERK1/2 is also induced in microglial cells treated with LPS, but it appears to be unaltered by Dex treatment. **B:** Data are presented as signal intensity of phospho-ERK1/2 relative to total ERK1//2 (mean ± SD). There is no significant difference between LPS and LPS plus Dex group.
Fig. 7

A

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<th>LPS+Dex</th>
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Phospho-ERK

Total-ERK

B

Relative Signal Intensity

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LPS
LPS+Dex
Fig. 8 A-C: Confocal images showing microglia immunostained with the N-terminal phosphorylated c-Jun (phospho-c-Jun, red) and lectin (green) and counterstained with DAPI (blue). Note the enhanced nuclear expression of phospho-c-Jun (pink, arrows) in microglia treated with LPS for 30 min (B). C. Dex treatment 2h prior to LPS decreases phospho-c-Jun expression significantly in microglial cells (arrows). D. Quantitative analysis shows the decrease in number of phosphorylated c-Jun immunoreactive cells in microglia cultures treated with LPS for 30min and Dex for 2h prior to LPS treatment. Data represent mean±SD (n=3 independent experiments). Significance of difference (control vs LPS and LPS vs LPS+Dex) is indicated by *p< 0.05. Scale bar = 50 μM.
Fig. 8

**Lectin + phospho-c-Jun + DAPI**

Control

LPS

LPS + Dex

**Phospho-c-Jun Immunoreactive microglia (%)**

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<th>Con</th>
<th>LPS</th>
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<tr>
<td>Control</td>
<td>5</td>
<td>60</td>
<td>30</td>
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* indicates statistically significant difference compared to control group.
Fig. 9 Analysis by ELISA shows that LPS-induced release of MCP-1 is partly suppressed by pre-incubation of microglial cells for 30 min with SP600125, the JNK inhibitor (24%), and SB202380, the p38 inhibitor (40%). Data represent mean±SD (n=4 independent experiments). Significance of difference (control vs LPS, LPS vs LPS+SP and LPS vs LPS+SB) is indicated by *p< 0.05.
Fig. 9

MCP-1 release (%) vs. treatment conditions:
- Con
- LPS
- LPS+SP
- LPS+SB

Significant differences are indicated by asterisks (*).
**Fig. 10** Analysis by MTS shows that neither SP600125 nor SB202380 alone has effects on microglial cell viability. Data represent mean±SD (n=4 independent experiments). There is no significant difference between control and JNK inhibitor SP600125 (1μm) treated cultures or between control and SB202380 (1μm) treated cultures.
Fig. 10

MTS assay (Ratio of Absorbance)

- Con
- SP600125
- SB202380
Fig. 11 Real time RT-PCR analysis demonstrates that Dex increases MKP-1 mRNA expression in microglia treated with LPS. A. The PCR product of MKP-1 gene (302 bp) amplified in microglial cells is shown. B. The melting curve of the real time RT-PCR with a single peak shows the specificity of the product. C. Real time RT-PCR analysis shows that pre-incubation of microglial cells with Dex, 2h prior to LPS treatment enhances MKP-1 mRNA expression significantly. MKP-1 mRNA expression level is elevated to 27 folds or 19 folds in microglial cells treated with LPS for 1h or with Dex alone for 2h respectively in comparison to that of control. Dex treatment further increases MKP-1 gene expression to 149 folds in the LPS-treated microglia. Data represent mean±SD (n = 4 independent experiments). Significant (control vs LPS, control vs Dex, LPS vs LPS+Dex) is indicated by *p<0.05.
Fig. 11

A

MKP-1 (302bp)

B

Fluorescence (F1/dT)

Temperature (°C)

C

MKP-1 mRNA Expression (Fold Change)

0 20 40 60 80 100 120 140 160 180

Con LPS Dex LPS+Dex

*
Fig. 12: Western blot analysis shows that Dex increases MKP-1 protein expression in LPS-treated microglia. A. LPS stimulated MKP-1 protein expression in microglial cells, reaching its maximal level by 1-2 h, and then decreased to basal level. Pre-incubation of microglial cells with Dex 2h prior to LPS treatment further enhances MKP-1 protein expression significantly at almost all the time points studied. B. Data represent the mean signal intensity of MKP-1 relative to control (mean ± SD, n= 3 independent experiments). Significant difference between LPS and LPS plus Dex group is indicated by *p<0.05.
Fig. 12

A

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>LPS</th>
<th>LPS+Dex</th>
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<tr>
<td>15m</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>30m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td></td>
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<td>2h</td>
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<td></td>
</tr>
<tr>
<td>4h</td>
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</tbody>
</table>

MKP-1

B

Relative Signal Intensity

- Con
- 15min
- 30min
- 1h
- 2h
- 4h

LPS
LPS+Dex
Fig. 13A: Western blot analysis shows that MKP-1 protein expression level induced by Dex alone in microglial cells is constant at all time points studied. B. Data represent the mean signal intensity of MKP-1 relative to control (mean±SD, n= 3 independent experiments).
Fig. 13

<table>
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<tr>
<th>Con</th>
<th>2.25h</th>
<th>2.5h</th>
<th>3h</th>
<th>4h</th>
<th>6h</th>
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</thead>
<tbody>
<tr>
<td>MKP-1</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**A**

**B**

Relative Signal Intensity

con  2.25h  2.5h  3h  4h  6h
Fig. 14: The phosphorylation of p38 and JNK is induced in activated microglial cells exposed to Dex by blocking of MKP-1 induction with triptolide. Triptolide alone has no effect on phosphorylation of MAPKs. Phosphorylation of JNK and p38 MAPKs is induced in microglial cells treated with LPS for 1h, and this induction is suppressed by Dex treated for 2h prior to LPS treatment. The inhibitory effect of Dex is reversed by pretreatment of microglial cells with triptolide. Neither Dex nor triptolide altered ERK1/2 phosphorylation. The blot is representative of three independent experiments.
Fig. 14

<table>
<thead>
<tr>
<th></th>
<th>MKP-1</th>
<th>phospho-JNK</th>
<th>phospho-p38</th>
<th>phospho-ERK1/2</th>
<th>actin</th>
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</thead>
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<tr>
<td>LPS</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dex</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triptolide</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 15: The quantitative analysis by real time RT-PCR shows that triptolide pretreatment increased MCP-1 mRNA expression to 2.9 folds in microglial cells treated with LPS and Dex for 6h. Data represent mean±SD (n= 4 independent experiments) LPS+Dex vs LPS+Dex+Triptolide: *p < 0.05.
Fig. 15

MCP-1 mRNA Expression (Fold Change)

Con  LPS  LPS+Dex  LPS+Dex+triptolide

*
**Fig. 16A-C** Dex inhibits CCR2 mRNA expression in activated microglia.  

A. The PCR product of CCR2 gene (233bp) amplified in microglial cells is shown.  

B. The melting curve of the real time RT-PCR with a single peak shows the specificity of the product.  

C. Real time RT-PCR analysis shows that the CCR2 mRNA expression level is increased to 11 folds in microglia treated with LPS, when compared to that of control.  

Dex decreased the mRNA expression level of CCR2 by 32% in the LPS-treated microglia. Data represent mean±SD (n=4 independent experiments). Control vs LPS treated cultures; LPS vs LPS+Dex treated cultures. *p<0.01.
Fig. 16

A

CCR2 mRNA Expression (Fold Change)

Con                 LPS           LPS+Dex

B

Temperature (°C)

Fluorescence -dF/dT

C

CCR2 mRNA Expression (Fold Change)

Con                 LPS           LPS+Dex

*
Fig. 17A-C Confocal images of microglial cells showing CCR2 immunoreactivity (green) and nuclear marker, PI (red). In the control (A), CCR2 positive cells are hardly detectable. The CCR2 immunoreactivity appears to be markedly increased in majority of the microglial cells treated with LPS for 6h (B, arrows). Dex decreased the staining intensity and the number of CCR2 positive cells in the LPS-treated microglia cultures (C). Scale bar = 50 μm
Fig. 17

CCR2 + PI

A

Control

B

LPS

C

LPS+Dex
Fig. 18 *In vitro* chemotaxis assay reveals that MCP-1 induces the migration of microglia in a dose-dependent manner. The maximum effect of MCP-1 on migration of microglia is found to be at the concentration of 62.5 ng/ml.
Fig. 18

Migration of Microglia (%)

MCP-1 concentration (ng/ml)

DMEM 31.25 62.5 125 250

* * *
The optimal dilution of media derived from the DMEM, control, LPS and LPS+Dex treated microglial cultures was determined by measuring the chemotaxic effect of serial dilutions (1, 1/2, 1/4 and 1/8). $1/4$ dilution was considered to be the optimal dilution for chemotaxis assay.
Fig. 19

Migration of Microglia

Dilutions of Media

DMEM
con
LPS
LPS+Dex
Fig. 20: Effects of Dex, recombinant MCP-1, anti-MCP-1 antiserum and unrelated antiserum on LPS-stimulated migration of microglia. A. Chemotaxis assay results show that a few violet-stained microglia (arrows) transmigrate through the membrane of insert in a transwell chamber containing DMEM medium. Optimised condition medium derived from LPS-treated microglial cultures attract more microglial cells than the control medium. The numbers of transmigrated microglia are markedly reduced in Dex+LPS treated medium. The inhibitory effect of Dex on chemotaxis is partly reversed when the medium is supplemented with 62.5ng/ml of recombinant MCP-1 protein. The medium of LPS treated culture with rabbit anti-MCP-1 antiserum attracts less microglia than that from LPS+unrelated antiserum (rabbit anti-iNOS). B. Quantitative analysis shows that LPS-induced migration is suppressed significantly by both Dex and anti-MCP-1 antiserum. Data represent the mean percentage of migrated cells per microscopic field relative to spontaneous migration towards the DMEM medium (0.5% BSA). Mean ±SD (n=4 independent experiments). *p < 0.05.
Fig. 20

**A**

Migration of Microglia (%)

**B**

Migration of Microglia (%)
**Fig. 21** Confocal images of microglia showing TNF-α and iNOS immunoreactivity (green) and nuclear marker, PI (red). In the control microglia cultures (A, D), TNF-α and iNOS positive cells are hardly detectable. The number of TNF-α and iNOS positive cells are increased in the cultures treated with LPS for 6 h (B, E). These cells appear to be increased in size and show abundant cytoplasm with numerous cytoplasmic inclusions (arrows). RA decreased the staining intensity and the number of both TNF-α and iNOS positive cells in the primary microglia cultures treated with LPS (C, F). These cells appear smaller and display reduction of cytoplasm. Scale bar = 50μM. *Adapted from Jun Yan’s thesis (2002).*
Fig. 21

<table>
<thead>
<tr>
<th>TNF-α + PI</th>
<th>iNOS+PI</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="A" alt="Control" /></td>
<td><img src="D" alt="Control" /></td>
</tr>
<tr>
<td><img src="B" alt="LPS" /></td>
<td><img src="E" alt="LPS" /></td>
</tr>
<tr>
<td><img src="C" alt="LPS+RA" /></td>
<td><img src="F" alt="LPS+RA" /></td>
</tr>
</tbody>
</table>
Fig. 22 Western blot shows that RA inhibited the LPS-induced expression of TNF-α and iNOS proteins in microglia (A,B). C. TNF-α and NO production by microglia either unstimulated or incubated with LPS, RA or LPS+RA for 6 h was determined by ELISA and nitrite assay, respectively. The results show that RA inhibited the release of TNF-α and NO by LPS-treated microglia. Results are expressed as percentage of change compared with the control. Data represent mean ± SD (n = 3). Significant differences between LPS-treated cultures and RA+LPS-treated cultures are indicated by *p < 0.01, **p < 0.001.
Fig. 22

A

TNF-α (17KDa)

iNOS (130KDa)

Con  LPS  LPS+RA

B

Protein Intensity (% of control)

Con  LPS  LPS+RA

C

TNF-α and NO release (% of control)

Con  RA  LPS  LPS+RA
Fig. 23 Western blot analysis shows that RA inhibits JNK phosphorylation and induces MKP-1 expression in LPS-treated microglia. LPS treatment for 30 min induced the expression of phosphorylated JNK and pre-incubation with RA 2h prior to LPS treatment inhibited the phosphorylation of JNK. The total JNK expression appears to be unaltered by LPS and RA. LPS treatment for 30 min also induced MKP-1 expression, which was further enhanced when microglial cells were pre-incubated with RA prior to LPS treatment. Each blot represents three independent experiments.
Fig. 23
Fig. 24. RA inhibits GM-CSF-induced microglial proliferation as revealed by BrdU incorporation.  

A. Confocal image showing microglia immunostained with BrdU (red) and counterstained with lectin (green).  

B. Note the increased number of BrdU positive cells (arrows) in microglia culture treated with GM-CSF for 24h.  

C. RA treatment together with GM-CSF decreased the number of BrdU positive cells significantly in microglial culture.  

D. Quantitative analysis shows the decrease in number of BrdU immunoreactive cells in microglia cultures treated with RA and GM-CSF for 24h. Data represent mean±SD (n=3 independent experiments). Significance of difference (control vs GM-CSF and GM-CSF vs GM-CSF+RA) is indicated by *p< 0.05. Scale bar = 50 μM
Fig. 24

Lectin+BrdU

A. Con

B. GM-CSF

C. GM-CSF+RA

D. % of BrdU positive cells

- Con
- GM-CSF
- GM-CSF+RA

* significant difference
Fig. 25  MTS analysis further confirms the inhibitory effect of RA on microglial proliferation. The number of microglial cells is increased in primary culture incubated with GM-CSF for 1 day, whereas the increase is arrested when the culture is exposed to RA together with GM-CSF. RA itself has no effect on the proliferation of microglia. Data represent mean ± SD (n=3 independent experiments). Significance of difference (control vs GM-CSF and GM-CSF vs GM-CSF+RA) is indicated by *p< 0.05.
Fig. 25

![Graph showing MTS assay results for Con, RA, GM-CSF, and GM-CSF+RA](image-url)
**Fig. 26** Western blot analysis of the effects of RA on expression of cell cycle related proteins in microglia treated with GM-CSF. RA suppressed the expression of cyclin D1 and E2F-1 induced by GM-CSF in microglia. In contrast, expression of P27, cyclin-dependent kinase inhibitory protein is decreased in microglia treated with GM-CSF and restored by RA. GM-CSF also increased the level of phosphorylated Rb and decreased the level of total Rb in microglia. RA suppressed the phosphorylation of Rb and restored the level of total Rb in microglia exposed to GM-CSF. Expression of tubulin confirms the equal amount of samples loaded. The blots represent one of three independent experiments.
Fig. 26