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Anlys Olivera Date

Inhibition of the NF-κB signaling pathway by the curcumin analogs, 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31) and 3,5-Bis(2-pyridinylmethylidene)-1-methyl-4-piperidone (UBS109): in-vitro and in-vivo studies.

By

Anlys Olivera

Doctor of Philosophy

Division of Biological and Biomedical Sciences

Neuroscience

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Abstract

Inhibition of the NF-κB signaling pathway by the curcumin analogs, 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31) and 3,5-Bis(2-pyridinylmethylidene)-1-methyl-4-piperidone (UBS109): in-vitro and in-vivo studies.

By Anlys Olivera

Mounting data suggest that pro-inflammatory cytokines may play a role in depression, especially in individuals who are non-responsive to conventional antidepressant strategies. Nuclear factor κ B (NF-κB), a lynchpin in the inflammatory response, plays a key role in transmitting cytokine signals to the brain and the release of central pro-inflammatory cytokines, which in turn have been shown to induce depressive-like behaviors in laboratory animals and humans. Data indicate that curcumin, a natural ingredient of the curry spice turmeric, acts as a NF-κB inhibitor and exhibits anti-inflammatory and anti-cancer properties. Curcumin analogues with enhanced activity on the NF-κB pathway have been developed including EF31 and UBS109 whose potency for NF-κB inhibition has yet to be determined.

The anti-inflammatory effects of these compounds on the NF-κB pathway were explored in-vitro in mouse RAW264.7 using lipopolysaccharide (LPS), a potent inflammatory stimulus. Compared to curcumin, EF31 and UBS109 exhibited significantly more potent inhibition of LPS-induced NF-κB pathway activity, including IκB kinase β activity, NF-κB DNA-binding activity, and downstream pro-inflammatory cytokine mRNA and protein. Curcumin analogues also demonstrated potent toxicity in NF-κB-dependent cancer cell lines while having minimal and reversible toxicity in RAW264.7 macrophages.

To examine the effects of curcumin analogs in-vivo, a model of LPS-induced immune and behavioral alterations was developed using C57BL/6 mice. Treatment with LPS induced a bi-phasic behavioral response characterized by sickness behaviors at 6 hours followed by depressive-like behaviors at 24 hours. Moreover, NF-κB DNA-binding activity peaked early in the spleen and brain, while expression of pro-inflammatory cytokines in the brain corresponded with the expression of sickness and depressive-like behaviors at 6 and 24. Although curcumin analogs showed potent NF-κB inhibition in-vitro, neither reliably reduced LPS-induced NF-κB DNA-binding activity or cytokine mRNA expression in the periphery or brain.

Taken together, these data suggest that EF31 and UBS109 are potent inhibitors of NF-κB in-vitro, although modifications to the treatment paradigm in-vivo may be necessary to achieve inhibition of NF-κB, reduce inflammation, and consequently block LPS-induced behavioral alterations. Thus, both EF31 and UBS109 represent promising curcumin analogues for further therapeutic development.

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Chapter 1

Background: depression, inflammation, and the role of NF-κB

I. Depression symptoms, treatment, and current challenges

One of the earliest reports of depression dates back to ancient Egypt. The Ebers Papyrus, written more than three thousand years ago, contains a chapter called the Book of Hearts in which mental disorders, including depression, and suggested treatments are described (Okasha, 2005). Other references to depression can be found in the Old Testament and more notably in the writings of the Greek physician Hippocrates (Kovacs and Beck, 1978). Hippocrates referred to depressive symptoms as melancholia (Greek for black bile) around 400 B.C., for which he had a number of theories and treatment suggestions (Gilbert, 1992; Nestler et al., 2002). As the fields of psychology and psychiatry evolved, many theories were proposed for the causes and treatments of depressive disorders. But it was not until the late 20th century that depression was diagnosable based on a symptomatic criteria detailed in the Diagnostic and Statistical Manual (DSM).

Depression is surprisingly common, affecting 121 million people worldwide (twice as commonly reported in women as men), while still being underreported (Kessler et al., 2003). The National Center for Health Statistics conducted a survey between 2005-2006 which estimated that 5.4% (1 out of 20) of Americans ages 12 and older were currently experiencing depression, but only 29% of them reported contacting a mental health professional. Although depression is in the category of “mood” disorders, it is mentally, physically, and socially disabling, commonly described by patients as a mental paralysis, or a psychic pain.

Depression adversely affects not only a person's general health (altered sleep and appetite), but also social relations with family and friends (anhedonia, reduced sex drive, low mood, and low self-esteem), and work or school life (problems with memory and concentration, irritability, and lack of energy/ fatigue), accumulating a financial burden of approximately $83 billion annually in the U.S alone (Lopez and Murray, 1998; Crown et al., 2002). Originally, it was thought that depression was life threatening primarily due to the risk of suicide (in the U.S approximately 15% of patients with major depression die by suicide), but more recently, depression has been associated with cardiovascular disease, autoimmune disorders, cancer, and neurodegeneration which are a major cause of morbidity and mortality worldwide (Musselman et al., 1998; Raison and Miller, 2003; Lett et al., 2004; Raison et al., 2006; Anisman et al., 2008; Gold and Irwin, 2009). Not surprisingly, the World Health Organization (WHO) has ranked depression as the 4th leading contributor to the global burden of disease (expected to become the 2nd leading contributor by 2020) and the leading cause of disability worldwide (Lopez and Murray, 1998).

Depression is a chronic illness with an elusive underlying circuitry and few biological markers. One of the dominant theories of depression in the field is the monoamine hypothesis, which stipulates that depression results from a dysregulation in the neurotransmission of the monoamine systems including serotonin, norepinephrine, and dopamine (Hirschfeld, 2000). The monoamine hypothesis is supported by the mechanisms of current antidepressant drugs including monoamine oxidase inhibitors (MOAI), tricyclic antidepressants (TA),

and selective reuptake inhibitors (SRI). All of these drugs aim to increase the availability of monoamines at the synapse (Carlsson and Lindqvist, 1978; Millan, 2006). However, increasing the amount of monoamines available at the synapse is not sufficient to reverse behavioral symptoms in all patients. Moreover, antidepressant drugs are notorious for taking weeks to months before mood can be stabilized in patients, even though monoamine levels are increased shortly after treatment begins (Oswald et al., 1972; Parker et al., 2000). It has been hypothesized that behavioral symptoms are indicative of a de-stabilization of mood regulatory circuits (Duman et al., 1997; Nestler et al., 2002). Antidepressant treatment enables a slow adaptation that returns the activity of the mood regulatory circuits to basal levels, thereby alleviating the symptoms. New treatments are being developed; however, these treatments are designed to improve the actions of older antidepressants. There are a number of problems with current antidepressant treatments; only about 40% of patients exhibit complete remission after the first treatment, while approximately 10% become resistant to treatment over time (Nestler et al., 2002; Krishnan and Nestler, 2008). In addition, up to 80% of patients receiving antidepressant treatment only show partial responses, often requiring drug cocktails to treat the spectrum of symptoms, while up to 30% are non-respondent or treatment resistant (Nestler et al., 2002). Antidepressants are also plagued by side effects, slow onset of actions, and drug-drug interactions (Nestler et al., 2002). Apart from classical antidepressants, there are other forms of treatment, including psychotherapy, light therapy, and deep brain stimulation (DBS). DBS in particular has been used

successfully to alleviate depressive symptoms in treatment resistant patients (Ward et al., 2010). However, this is an invasive procedure that involves surgical implantation of probes that send electrical impulses to targeted brain regions which carries risks, complications, and side effects (Hariz, 2002; Appleby et al., 2007; Rabins et al., 2009). Despite the success of DBS in depressed patients, there remain a number of patients that are also non respondent to DBS methods (Mayberg et al., 2005). Depression has been described as a heterogeneous syndrome, whereby no one patient has the same experience. The heterogeneity of the symptoms and the inconsistency in responses to antidepressant treatments in subsets of depressed patients is indicative of a heterogeneous pathology, underscoring the need to identify novel neurobiological targets and for designing treatments that target a wider array of systems.

The causes of depression have been postulated to be as diverse as the symptoms. The role of inflammation in the development and maintenance of major depression has received much attention in the current literature (Dantzer, 2006; Raison et al., 2006; Dantzer and Kelley, 2007; Dantzer et al., 2008; Miller et al., 2009). Inflammation has been shown to promote depressive symptoms in humans and laboratory rodents via actions on multiple pathways and brain regions relevant to the neurobiology of depression (Miller et al., 2009). This chapter describes the link between inflammation and depression. More specifically, the role of the inflammatory transcription factor nuclear factor κ B (NF-κB) on neuronal function and behavior is highlighted. Finally, studies showing effects of anti-inflammatory agents on depressive symptoms are

presented here.

II. Inflammation and depressive symptoms

Recent data suggest that activation of peripheral immune responses may contribute to depression (Miller et al., 2009). During Hippocrates' time it was believed that secretion of black bile, a humour made in the spleen, could affect the brain and cause melancholia (Gilbert, 1992; Nestler et al., 2002). The humoral theory was an early attempt to explain mood disorders that has relevance in modern psychiatry. The spleen is a lymphoid organ that filters blood and mounts an aggressive immune response when a pathogen is detected. Pro-inflammatory cytokines and other inflammatory immune mediators produced and secreted by the spleen during infection coordinate the inflammatory response to pathogens and can induce neurobiological and behavioral alterations in laboratory animals and humans.

1. Immune-based behavioral alterations in laboratory animals

A number of animal models have been developed to measure immune-based behavioral alterations (Dantzer and Kelley, 2007; Dantzer et al., 2008). Although depression cannot be examined objectively in laboratory animals, there are behavioral symptoms which closely resemble those in depression and that can be measured. Behavioral alterations following an immune challenge are

termed sickness and depressive-like behaviors (described in Chapter 3). These include suppression of exploration and social activity, reduced grooming or body care activities, reduced motivated behaviors (including sex), and symptoms of anhedonia which have been reported in animal models involving an immune challenge such as autoimmune disorders [experimental autoimmune encephalomyelitis (EAE)], viral or bacterial infection, and administration of pro-inflammatory immune mediators such as pro-inflammatory cytokines (Dantzer and Kelley, 2007; Dantzer et al., 2008). A potent immune stimulus that has been shown to induce sickness and depressive–like behaviors is lipopolysaccharide (LPS) (Yirmiya, 1996; Dantzer et al., 2008). LPS is a component of gram-negative bacteria and is recognized by innate immune cells that then mount an inflammatory response (described in Chapter 3). Transduction of the inflammatory signal to the brain affects several key processes implicated in the pathology of depression such as monoaminergic activity, neuroendocrine function, and neuronal plasticity (see section VI). Of note, sickness and depressive-like behaviors can be blocked or attenuated with pretreatment of cytokine synthesis blockers (e.g. curcumin), cytokine receptor antagonists [e.g. Interleukin-1 receptor antagonist (IL-1RA)], antidepressants, and genetic manipulations [Tumor necrosis factor (TNF)-α knock-out] (Kelley et al., 2003; Godbout et al., 2005; Dantzer and Kelley, 2007; Dantzer et al., 2008; Gupta et al., 2009; Sachdeva et al., 2009).

2. Association of inflammation and depression in medically ill and healthy

patients

Pro-inflammatory cytokines have also been implicated as a contributing factor to depressive symptoms in medically ill and healthy patients (Figure 1-1). Depressive symptoms are commonly reported in patients suffering from disorders associated with inflammation including autoimmune disorders, (multiple sclerosis, Type 1 diabetes, lupus, asthma, allergies, and rheumatoid arthritis), infection [human immunodeficiency virus (HIV),hepatitis C, herpes, and influenza], cardiovascular disease (stroke and myocardial infarction), trauma, epilepsy, cancer, and neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) (Evans et al., 2005). Indeed, the prevalence of depression in the medically ill has been estimated to range between15% and 61% (Creed and Dickens, 2007). However, since depressive-symptoms closely resemble the symptoms of medical illness, depression is often underreported in medical settings. Depression in the medically ill has been shown to contribute to disability, reduced quality of life, increased morbidity and mortality, and medical related debt (Evans et al., 2005; Creed and Dickens, 2007; Iosifescu, 2007). For example, HIV-positive patients diagnosed with depression often show a more rapid disease progression (Rabkin, 2008). Moreover, treatment of depression in the medically ill comes with its own challenges. Overall, studies suggest that the medically ill may be more prone to treatment resistance, have poor or slower responses to antidepressants, and show increased rates of relapse (Iosifescu, 2007).

The findings in the medically ill patients are consistent with healthy patients showing increased inflammatory markers and are also treatment resistant to conventional antidepressants. Approximately 30% of healthy depressed patients that have no history of disease also exhibit elevated levels of pro-inflammatory cytokines including IL-1, IL-6, and TNF (IL-6 being the most reliable and robust finding) and their soluble receptors in peripheral blood and cerebral-spinal fluid (CSF), as well as peripheral blood elevations in acute phase proteins including C-reactive proteins (CRP), chemokines, and other inflammatory mediators including prostaglandins (Table 1-1) (Miller et al., 2009). Interestingly, in several studies, the concentrations of inflammatory markers correlated with the intensity of the depressive symptoms (Gold and Irwin, 2009; Miller et al., 2009; Dantzer and Kelley, 2010). A number of mechanisms for the inflammation present in healthy depressed patients have been proposed. These include cytokine production in adipose tissue and immune responses to bacterial products introduced by a leaky gut (Capuron et al., 2008; Maes, 2008; Shelton and Miller, 2011). Moreover, stress, which often precedes depression, promotes the production and release of pro-inflammatory cytokines (Bierhaus et al., 2006; Miller et al., 2009) (see section VII).

3. Immune-based depressive symptoms in healthy volunteers

Administration of an inflammatory stimulus including immune cytokines [e.g. Interferon (IFN)-α], vaccination with an inactive virus, or LPS to healthy

adults results in the development of depressive symptoms, including anhedonia, fatigue, impaired sleep, anorexia, and decreased memory and concentration (Yirmiya et al., 2000; Glaser et al., 2003; Raison et al., 2006; Miller et al., 2009). Of note, depressive symptoms are reported very quickly after administration of cytokines, and disappear soon after termination of cytokine treatment. For example, administration of a low dose of LPS to healthy volunteers significantly elevated depressive mood and anxiety, and impaired memory in the absence of flu-like symptoms(Yirmiya et al., 2000). LPS-induced elevations in cytokine blood levels positively correlated with the levels of depressive mood, anxiety, and memory impairments. Likewise, anxiety and depressed mood was found to be significantly increased in healthy volunteers that were administered Salmonella abortus equi endotoxin as a model of experimental inflammation (Reichenberg et al., 2001; Wright et al., 2005). Higher body temperature and marked reductions in food intake were also reported, while blood pressure, heart rate, and reported physical symptoms were unaffected. These findings suggest that inflammatory cytokines can impact mood circuits involved in the pathology of depression.

III. Innate immune cells, pathways, and cytokines

Innate immune cells are involved in the surveillance, detection, inactivation, and clearance of exogenous pathogens and tumour cells (Janeway et al., 1996). Immune cells are derived from progenitor cells created in the bone marrow. The myeloid progenitors give rise to monocytes/ macrophages,

microglia (brain resident macrophages), dendritic cells (DCs), mast cells, and granulocytes (neutrophils, basophils, and eisonophils). The lymphoid progenitors give rise to B (plasma and memory B cells) and T (killer/cytotoxic and helper T cells) cells, natural killer (NK) cells, and NKTcells. Upon detection of a pathogen, a layered immune defense ensues consisting of innate and adaptive immunity. The innate immune system, is the first to respond to pathogen infection with key characteristics: 1) fast, reacting within hours after infection, 2) non-specific, recognizing molecular patterns on bacteria or viruses, known as pathogen-associated molecular patterns (PAMPs), 3) without immunological memory, thus incapable of providing long lasting protective immunity against future infection, and 4) exposure to a pathogen results in an immediate maximal response.

Innate immunity is mediated by phagocytes (macrophages and neutrophils), DCs, and NK cells that are present in the blood and other bodily fluids (including lymph), and tissues. Activation of pattern recognition receptors (PRRs) or Toll-like receptors (TLRs) by a pathogen induces a cascade of cytosolic signals that terminate in the transcription and expression of inflammatory genes and ultimately the production of inflammatory mediators. Transduction pathways activated by pathogens include NF-κB, mitogen activated protein kinases (MAPKs), and Janus family tyrosine kinases/ signal transducer and activator of transcription (JAK/STAT) proteins. The inflammatory mediators such as cytokines IL-1, 6, 8, 12, and TNF-α, IFNs (IFN-α, β, κ, ω), and chemokines [monocyte chemotactic protein (MCP)-1, 2, macrophage inflammatory protein (MIP), and IL-8] activate surrounding cells and recruit other

immune cells to the site of infection by inducing vasodilation, expression of adhesion molecules, and extravasation of cells into site of injury or infection. In conjunction with other plasma proteins such as the complement system, cytokines and interferons inactivate the pathogen directly or induce death of the infected cell by activation of apoptotic pathways, lysis, or opsonization, which are then cleared by phagocytes In addition, the inflammatory cytokine IL-6 induces the release of CRP from the liver, which in turn, binds phosphocholine on the surface of damaged cells and other microbes to assist the functions of the complement system and facilitate phagocytosis by macrophages (opsonization). Depending on the degree and or extent of infection and/or tissue damage and destruction, inflammation can induce the proliferation of immune cells in the spleen and other lymph nodes and the release of cytokines and other inflammatory mediators throughout the body (Janeway Jr and Medzhitov, 2002).

IV. Brain-immune interactions

Pro-inflammatory cytokines have been implicated as a contributing factor to depressive-like behaviors in laboratory animals as well as clinical studies in healthy volunteers and the medically ill (Raison et al., 2006; Miller et al., 2009; Dantzer and Kelley, 2010). Mechanisms underlying cytokine-induced behavioral changes remain largely unknown, but a picture is beginning to emerge showing that a number of systems altered in major depression are accessible to and may be modulated by cytokines. The pattern of cytokine receptors in the brain is

associated with their physiological and behavioral effects. For example, IL-1β signaling in the hypothalamus mediates the anorexic effects of LPS, while IL-6 signaling in the hypothalamus mediates the fever response to LPS (Chai et al., 1996; Layé et al., 2000). Similarly, cytokine signaling in the basal ganglia has been implicated in alterations in motor activity (Capuron and Miller, 2004; Majer et al., 2008). Others have proposed that these behaviors are not simply a side effect of the immune response to a challenge, but rather, an orchestrated response intended to return the organism to homeostasis (Raison et al., 2006; Dantzer and Kelley, 2007). A wounded or infected animal would benefit from conserving energy through inactivity while tissue is being repaired or fighting an infection. Nevertheless, while behavioral alterations induced by acute inflammation can benefit the organism, persisting inflammation can promote sickness or depressive-like behaviors.

1. Cytokine receptor expression in the brain

Inflammatory signaling receptors are expressed throughout the brain (Figure 1-2). With the development of scientific methods such as immunohistochemistry, in-situ hybridization, cloning, and autoradiography, complex cytokine circuits within the brain have been identified. IL-1 receptors are expressed in neuronal and non-neuronal cells including vascular endothelial cells, ependymal cells lining the ventricular system, choroid plexus, meninges, oligodendrocytes, astrocytes, and microglia, as well as cells in the dentate gyrus,

mammillary body, arcuate nucleus, thalamus, amygdala, raphe nuclei, cerebellum, trigeminal nerve, hypoglossal nerve, striatum, pituitary, cerebral cortex, area postrema, hypothalamus, olfactory bulb, pons, and the spinal cord (Ban et al., 1991; Gabellec et al., 1995; Rothwell and Luheshi, 2000; Parnet et al., 2002). TNF-α receptors are expressed in the hypothalamus, striatum, hippocampus, thalamus, cortex, pons, and cerebellum (Kinouchi et al., 1991). IL-6 receptors are expressed in the hippocampus, hypothalamus, frontal cortex, striatum, pons, and the cerebellum (Yan et al., 1992; Gadient and Otten, 1994). Some areas of the brain constitutively express cytokine protein and receptor mRNA, suggesting that they may be involved in non-pathological brain functions, which is consistent with their roles in long-term potentiation, neuronal development, plasticity, and endocrinology (Kennedy and Jones, 1991; Jonakait, 1996; Jankowsky and Patterson, 1999; McCoy and Tansey, 2008).

2. Brain-immune signaling pathways

Following the detection of a pathogen or immune stimuli, the inflammatory signal can be transmitted to the brain in more than one way (Figure 1-3) (Miller et al., 2009). First, peripheral release of cytokines by macrophages in the gut, liver and spleen, increases the presence of these proteins in the blood. Cytokine proteins are then able to cross the blood brain barrier via active transport molecules or leaky regions in the blood brain barrier (e.g. circumventricular organs), entering the brain to activate microglia, neuronal and glial cells, as well

as DCs and macrophage cells in the choroid plexus and the meninges (Banks et al., 1989; Blatteis and Sehic, 1997; Banks, 2005). Part of amplifying an inflammatory response in the brain rests on the production of chemokines such as MCP-1, which in turn promotes the infiltration of peripheral macrophages, DCs, and lymphocytes (T and B) that could play a role in further propagation of the immune signal. Second, cytokine (IL-1β and TNF-α) and LPS-induced activation of afferent nerve fibers such as the vagus nerve during abdominal or visceral infection or the trigeminal nerve during oro-lingual infections provide a neural route of communication (Dantzer et al., 2008; Miller et al., 2009). The vagus nerve signals the nucleus of the solitary tract and the trigeminal nerve signals the spinal trigeminal nucleus, which relay information to other brain regions such as the hypothalamus. Third, cytokine or LPS in the blood can activate their corresponding receptors (cytokine receptors, TLRs) expressed on endothelial cells and perivascular macrophages resulting in the production and release of inflammatory mediators into brain parenchyma. Central inflammation then activates neuronal processes and neuroendocrine systems that contribute to the organism's response to infection, stress, or insult.

Not only can the immune system signal to the brain to modulate neuronal activity and behavior, but the central nervous system also has direct and indirect pathways by which to signal to the immune system and modulate inflammatory responses. One pathway is the direct neuronal innervation of primary and secondary lymphoid organs. Noradrenergic and peptidergic (tyrosine hydoxylase and neuropeptide-y positive nerve fibers) innervations have been found in close

proximity to immune cells in the thymus, bone marrow, spleen, and lymph nodes (Ader et al., 1995). The second pathway is via hormonal signaling involving the hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic-adrenal-medullary (SAM) axis (Bateman et al., 1989; Padgett and Glaser, 2003). Hormonal signaling via cortisol, norepinephrine (NE), and epinephrine (E) can have anti-inflammatory or inflammatory effects. Receptors for stress hormones are expressed by almost all immune cells (Smith et al., 1977; Werb et al., 1978; McKay and Cidlowski, 1999). For example, receptors for catecholamines (E and NE) and glucocorticoids are expressed in T and B lymphocytes, monocytes, and macrophages. Corticotropin releasing hormone (CRH) receptors are also found in T lymphocytes, monocytes, and macrophages. In the context of inflammation, the brain circuitry that mediates cytokine-induced alterations in neuroendocrine function, plasticity, and monoamine metabolism, remains largely unknown. However, blockade of these pathways via antagonists, inhibitors, genetic knock-outs, or nerve transaction can block transmission of peripheral inflammatory signals (Wrona, 2006).

V. The role of NF-κB in inflammation

NF-κB is a family of transcription factors (described in Chapter 2) found in virtually all cell types including cells of the immune system such as microglia, macrophages, T and B lymphocytes and granulocytes (Baeuerle and Baltimore, 1996). Like other transcription factors, NF-κB regulates the expression of an

array of genes, some of which are involved in inflammatory processes (both in the adaptive and innate immune response), cell proliferation and development, apoptosis, and survival. NF-κB was first identified by Sen and Baltimore more than two decades ago (1986) as an enhancer-binding protein that controls the expression of the Igκ-light chain in B-cells. NF-κB is considered to be a “fast-acting” or primary transcription factor, as it is always present in the cell in an inactive state and does not require de novo protein synthesis to be activated. This allows NF-κB to respond rapidly in the event of tissue infection, injury, or trauma. Bacterial products such as LPS, viral proteins, proinflammatory cytokines (e.g. IL-1β and TNF-α), neurotransmitters (glutamate), and reactive oxygen and nitrogen species all induce activation of the NF-κB pathway resulting in the production of cellular signaling molecules including cytokines, chemokines, adhesion molecules, acute phase proteins, inducible enzymes, major histocompatability proteins, and cyclooxygenase-2.

Considering the key role that NF-κB plays in inflammation, it is not surprising that it is also involved in transducing inflammatory signals from the periphery to the brain. NF-κB is found in various cell types of the CNS including neurons, astrocytes, oligodendrocytes, and microglia and mounting evidence indicates that blocking NF-κB signaling prevents the transmission of inflammatory signals from the periphery to brain and inflammation-induced behavioral alterations. Indeed, blocking NF-κB using α-tocopherol (vitamin E), also an antioxidant, attenuated LPS-induced production of pro-inflammatory cytokines in the brain and improved the recovery from sickness behavior induced by an

intraperitoneal (i.p.) injection of LPS (Godbout et al., 2005). Furthermore, blocking NF-κB using NEMO binding domain (NBD) peptide, blocks c-fos expression in areas activated by peripheral administration of IL-β and IL-1β induced sickness behaviors (Nadjar et al., 2005).

VI. Inflammation and biological alterations in depression: a role for NF-κB

Pro-inflammatory cytokines can modulate pathways known to be involved in the pathophysiology of depression, including neurotransmitter function, neuroendocrine function, and synaptic plasticity (Figure 1-4). In addition to variations in serotonin (5-HT), a monoamine neurotransmitter widely used in the limbic system, the 5-HT transporter and several 5-HT receptors including 5-HT1a and b, and 5-HT2a and c, catecholamines [dopamine (DA) and NE], acetylcholine (Ach), glutamate, gamma-aminobutyric acid (GABA), other peptides (including CRH, vasopressin, and substance P), and growth factors [brain-derived neurotrophic factor (BDNF)] have also been found to have altered synthesis, release, or metabolism in patients diagnosed with major depression (Nestler et al., 2002; Krishnan and Nestler, 2008; Miller et al., 2009; Nemeroff and Owens, 2009). Moreover, imaging studies show altered cerebral blood flow (indicative of alterations in neuronal activity) in the prefrontal cortex, cingulate cortex, hippocampus, amygdala, striatum, and the thalamus, and reductions in glial number, size, and neurons in some of these regions (especially the hippocampus) (Nestler et al., 2002; Krishnan and Nestler, 2008; Miller et al.,

2009). Mounting evidence show that inflammation can promote many of these alterations.

1. Neurotransmitter signaling

There are a number of ways by which cytokines can alter neurotransmitter signaling: 1) NF-κB-dependent expression of proinflammatory cytokines, including TNF-α and IFN-γ, can alter serotonergic signaling in brain regions implicated in major depression, such as the hypothalamus, hippocampus, amygdala and prefrontal cortex via activation of indoleamine 2,3 dioxygenase (IDO) (Miller et al., 2009; Dantzer and Kelley, 2010). IDO is an enzyme that metabolizes tryptophan (TRP), the primary precursor of serotonin, to kynurenine (KYN) and quinolinic acid (QUIN). Thus, an increase in IDO activity can lead to low availability of TRP in the brain resulting in decreased 5-HT synthesis, which may contribute to the serotonergic deficiency that is associated with depressive disorders. Moreover, NF-κB has been shown to regulate the expression of IDO. Indeed, suppression of NF-κB activity by antioxidant treatment resulted in a 40% reduction in the expression of IDO in macrophages (Alberati-Giani et al., 1997). Of note, blockade of IDO activation has been found to attenuate LPS-induced depression-like behaviors in mice (Dantzer et al., 2008). 2) Administration of KYN to mice induces depressive-like behaviors (Godbout et al., 2007; Miller et al., 2009). KYN is converted to kynurenic acid (KA), a glutamate receptor antagonist, and inhibitor of the α7-nicotinic acetylcholine receptor (α7nAChR). Both receptors

contribute to the release of glutamate, which in turn, regulates the release of DA in the striatum. Interestingly, treatment with galantamine, an α7nAChR agonist, increased DA levels thereby reversing the effects of KA (Wu et al., 2007) . 3) QUIN promotes neuronal death by generating oxidative stress and activation of the N-Methyl-D-aspartic acid (NMDA) receptor which results in glutamate release and possible excitotoxicity (Whetsell Jr and Schwarcz, 1989; Miller et al., 2009). Excitotoxicity can induce NF-κB pathway activity and secretion of cytokines, thereby contributing to the mechanisms described above. Of note, DA neurons are especially vulnerable to oxidative stress, NMDA mediated excitotoxicity and TNF-α induced apoptosis (Zhang et al., 1999; Casper et al., 2000; McCoy et al., 2006). 4) Another way by which inflammatory cytokines such as IFN-α can modulate 5-HT and DA release is via tetrahydrobiopterin (BH4) (Kitagami et al., 2003; Miller et al., 2009). BH4 is a cofactor to tryptophan hydroxylase (TRPH), nitric oxide synthase (NOS), tyrosine hydroxylase (TH), and phenylalanine hydroxylase (PHA). NF-κB-dependent expression of pro-inflammatory cytokines leads to the activation of NOS to produce nitric oxide (NO) as part of the immune response (Xie et al., 1994). Moreover, NF-κB also regulates expression of NOS (Barnes and Karin, 1997). An increase in NO production reduces the availability of BH4 to act as a cofactor to TRPH and TH, thereby reducing the synthesis of 5-HT and DA (Southan and Szabo, 1996; Miller et al., 2009). Accordingly, inhibiting NO prevents IFN-α's negative effects on BH4 and DA levels in the brain (Kitagami et al., 2003; Miller et al., 2009). 5) Finally, activation of MAPK signaling pathways, including p38 and extracellular signal-regulated kinases (ERK) 1/ 2,

has been shown to upregulate the activity and/or expression of DA, 5-HT, and NE transporters, which may further reduce the amount of dopamine, serotonin, and norepinephrine in the synapse (Moron et al., 2003; Zhu et al., 2006; Miller et al., 2009). A reduction in DA levels in the basal ganglia may account for the psychomotor slowing and the fatigue symptoms experienced by patients treated with INF-α or typhoid vaccination and patients diagnosed with depression. Similarly, cytokine-mediated reductions in 5-HT synthesis and metabolism have been correlated with depressive moods in animal models and clinical studies (Sanchez et al., 2007; Miller et al., 2009).

2. Neuroendocrine function

In depression, hypocorticolism, hypercorticolism, and glucocorticoid resistance are commonly reported (Lowy et al., 1984; Arborelius et al., 1999; Ehlert et al., 2001; Pariante and Miller, 2001; Carroll et al., 2007; Pace et al., 2007). Pro-inflammatory cytokines can also influence stress signaling. Cytokine receptors are expressed in the hypothalamus, pituitary, and adrenal gland, and cytokine-induced activation of these receptors stimulates the production and release of CRH, adrenocorticotropic hormone (ACTH), and cortisol (CORT), thereby modulating HPA activity (Turnbull and Rivier, 1995; Silverman et al., 2003). Alterations in HPA axis have been consistently described in healthy subjects as well as medically ill patients treated with cytokines (Raison et al., 2008; Miller et al., 2009).

Another mechanism by which cytokines may contribute to HPA axis dysregulation is through effects on the glucocorticoid receptor (GR). Pro-inflammatory cytokines can signal through a number of pathways including NF-κB, MAPK, and JAK/STAT. Proteins of the NF-κB pathway have been shown to interact with the GR in the cytosol, inhibiting its translocation to the nucleus where it acts as a transcription factor to modulate HPA axis function (McKay and Cidlowski, 1999). In addition, NF-κB can physically interact with the GR in the nucleus to prevent GR-DNA-binding, thus preventing transcription of relevant genes. Conversely, GR can also repress NF-κB activity in the nucleus. GR and NF-κB have a dual mechanism of repression; directly through physical interaction and indirectly by expression of a dominant negative regulators such as IκB, the inhibitory protein for NF-κB, and GR-β, the endogenous dominant negative inhibitor of GR-α (McKay and Cidlowski, 1999). For example, cytokine signaling through NF-κB can decrease the expression of GR-α, the active form of the receptor, and increase the expression of GR-β, a less active form of the receptor (McKay and Cidlowski, 1999). Glucocorticoids acting on the GR can have potent anti-inflammatory effects, thus by interfering with GR functions cytokines signaling through NF-κB may induce glucocorticoid resistance and contribute to the disinhbition of the inflammatory response (Barnes, 1998; De Bosscher et al., 2000).

3. Growth factors and neuronal plasticity

Consistent with their role in cell survival and apoptosis, cytokines and NF-κB have been shown to play a key role in the effects of stress on the expression of relevant growth factors as well as neurogenesis in brain regions such as the hippocampus (Dantzer et al., 2008; Koo and Duman, 2008; Miller et al., 2009; Koo et al., 2010). Chronic stress was found to increase signaling of IL-1β and NF-κB in mouse and rat hippocampus and hypothalamus (Nguyen et al., 1998; Koo and Duman, 2008). Moreover, NF-κB was found to mediate stress induced impairments in cell proliferation and neurogenesis (Koo et al., 2010). Indeed, inhibition of NF-κB was found to block stress-induced reductions in cell proliferation and neurogenesis in the hippocampus, as well as stress-induced depressive-like behaviors (Koo et al., 2010). Expression of IL-1β and TNF-α in the hippocampus is associated with reduced expression of the neurotrophic factor, BDNF, and its receptor tyrosine kinase B (Barrientos et al., 2003; Imamura et al., 2005; Guan and Fang, 2006; Koo and Duman, 2008). Stress-induced reductions in trophic factors have been shown to cause reversible atrophy of hippocampal dendrites and induce apoptosis in cortical and hippocampal areas (Watanabe et al., 1992; Koo and Duman, 2008; Koo et al., 2010). These alterations in trophic factors and neuroplasticity may account for the inflammation-induced impaired memory and cognition seen in laboratory rodents (Song et al., 2006). Likewise, healthy volunteers treated with a low dose of LPS exhibit acute impairments in memory (Yirmiya, 2000; Krabbe et al., 2005).

In summary, pro-inflammatory cytokines may contribute to the development and maintenance of depressive symptoms by reducing availability

of neurotransmitters, promoting HPA axis activity and glucocorticoid resistance, and reducing neurogenesis and plasticity.

VII. Linking stress, inflammation, and depression: a role for NF-κB

An estimated 80% of patients report experiencing psychological or physical stress, anxiety, or major life events prior to the development of depressive disorders (Hammen, 2005). Stress has been shown to have deleterious effects on monoaminergic pathways and brain regions involved in mood regulation. Moreover, stress can promote immune-based and neurogenic inflammation, which has also been shown to contribute to depressive symptoms. A mechanism by which stress can have these effects is via activation of inflammatory mediators such as NF-κB. Within the CNS, stress has been shown to induce the release of large quantities of aspartate and glutamate, both of which have the potential to activate NF-κB (Palkovits et al., 1986; Moghaddam, 1993; Stein Behrens et al., 1994). Furthermore, glucocorticoids have also been reported to interfere with calcium mobilization inside the cells by inducing influx of Ca++ into the cytosol and inhibiting efflux via Ca++/ATPase and Ca++/Na+ exchangers, which may lead to excitotoxicity and thus activate the NF-κB pathway (Stein Behrens et al., 1992; Sapolsky, 1996; Lee et al., 2002). In addition, stress is associated with the production and accumulation of reactive oxygen species (attributed to glucocorticoid signaling), which also activate NF-κB (McIntosh and Sapolsky, 1996; Sapolsky, 1996).

Consistent with reports of stress-induced immune activation, acute psychosocial stress in the Trier Social Stress Test (TSST) has also been shown to induce NF-κB nuclear translocation in peripheral blood mononuclear cells (PBMC) within 10 minutes (Pace et al., 2006). Notably, NF-κB activation in PBMCs was associated with increases in catecholamines and cortisol levels. Following the TSST, NF-κB activity was up-regulated returning to basal levels within 60 minutes. However, some participants failed to down-regulate NF-κB activity within the same time period, suggesting an altered response to stress. A prolonged inflammatory state following exposure to a stressful event could place the individual at risk for stress-related diseases, including psychiatric disorders such as major depression. Indeed, NF-κB DNA-binding activity has been reported to be significantly higher in depressed males following a TSST which was also correlated with an increase in IL-6 (Pace et al., 2006).

VIII. Anti-inflammatory compounds and depression

Co-administration of anti-inflammatory agents and antidepressant drugs has been shown to effectively treat depressive symptoms. Cyclooxygenase-2 (COX-2) inhibitors are non-steroidal anti-inflammatory drugs (NSAIDs) that block the enzyme COX-2 responsible for the production of prostaglandin E2 (PGE2). PGE2 is an immune modulator that can stimulate the production of IL-6 and is involved in LPS-induced sickness behaviors (Pecchi et al., 2009). A subgroup of depressed patients show marked elevations of PGE2 in saliva and CSF, as well

as increased secretion of PGE2 by lymphocytes in-vitro, which is indicative of an inflammatory response (Lieb et al., 1983; Song et al., 1998; Leonard, 2001). When celecoxib, a COX-2 inhibitor, was administered in conjunction with reboxetine, a noradrenaline re-uptake inhibitor, to patients suffering from an acute depressive episode their scores in the Hamilton depression scale were significantly improved compared to a control group that only received reboxetine or placebo (Müller et al., 2006; Akhondzadeh et al., 2009). Moreover, rolipram, a selective phosphodiesterase IV inhibitor that increases the activity of tyrosine hydroxylase as well as the availability of norepinephrine in the rodent brain showed promising antidepressant effects (Kehr et al., 1985; Wachtel and Schneider, 1986; Sommer et al., 1995). Rolipram is also a suppressor of TNF-α production, and when co-administered with an antidepressant it was found to increase BDNF mRNA in the rat hippocampus in a shorter time frame than antidepressant treatment alone (Itoh et al., 2004). In clinical studies, rolipram successfully improved depressive symptoms even in a group that had previously been refractory to antidepressant therapy (Maubach et al., 1999). Moreover, a clinical study comparing the effects of rolipram to desipramine, a tricyclic antidepressant, showed that there were no differences between the two drugs in terms of clinical efficacy in depressed patients (Guiot-Goffioul et al.; Scott et al., 1991).

The natural compound turmeric which is derived from the root of the plant *Curcuma longa*, has been used for centuries in traditional Chinese medicine to treat inflammatory and depression-related diseases (Cronin, 2003; Anand et al.,

2007). Curcumin is the active ingredient in turmeric and it has also been used effectively in animal studies as an anti-inflammatory and antidepressant drug. Curcumin inhibits the activity of several targets in the inflammatory pathways, including NF-κB (Anand et al., 2008). Oral administration of curcumin in mice reduced immobility duration in the tail suspension and the forced swim tests (Xu et al., 2005b; Xu et al., 2005a). These effects were not dependent on changes in locomotor activity. Furthermore, oral administration of curcumin increased serotonin and noradrenaline levels in the frontal cortex and hippocampus, as well as increased dopamine levels in the frontal cortex and the striatum (Xu et al., 2005b; Xu et al., 2005a; Bhutani et al., 2009). Chronic i.p. administration of curcumin was also found to inhibit monoamine oxidase activity in the brain, suggesting that the antidepressant effects of curcumin may be dependent on central monoaminergic neurotransmitters (Xu et al., 2005b; Xu et al., 2006; Bhutani et al., 2009). In a separate study in rats, chronic oral administration of curcumin was found to reverse the effects of chronic unpredictable stress on depressive-like behaviors, HPA axis activity, and BDNF expression (Xu et al., 2006). Curcumin treatment increased hippocampal neurogenesis and significantly reduced stress-induced decreases in 5-HT1A mRNA (Xu et al., 2006; Xu et al., 2007). Interestingly, co-administration of curcumin and piperine (i.p.), a bioavailability enhancer, significantly potentiated the effects on behaviors and monoamines. Similarly, chronic oral administration of curcumin in an olfactory bulbectomy model of depression in rats, was found to reverse behavioral alterations in the open field test, and the passive avoidance test (Xu et

al., 2005a). These effects were correlated with increases in central monoaminergic neurotransmitters. Given the role of NF-κB and pro-inflammatory cytokines in immune-based behavioral alterations, it is reasonable to speculate that curcumin's widespread effects on monoamine metabolism and behavior may be through its effects on NF-κB and inflammation. Of note, there are currently no studies in the literature that have examined curcumin in the context of the behavioral effects of inflammatory stimuli including LPS. However, curcumin's use for clinical treatment is limited in part due to its reduced bioavailability, poor absorption and rapid metabolism (Anand et al., 2007). Structural modifications have been made to curcumin with the aim of improving bioavailability and absorption, and increase potency of NF-κB pathway inhibition. It is possible that by targeting inflammatory processes in conjunction with the monoamine systems, novel curcumin-derived compounds could provide a safe and effective alternative treatment for depression, especially for treatment resistant depression.

In conclusion, depression is a chronic disabling disease that involves a number of systems including stress and immune systems. Targeting a wider array of systems could improve current available antidepressant treatments and perhaps offer relief for the treatment resistant population.

Peripheral leukocytes	Adhesion proteins
Monocytes Neutrophils	sICAM-1 E-selectin
Acute-phase proteins	**Antibodies**
CRP α_1-acid glycoprotein, α_1- antichymotrypsin Haptoglobin Serum amyloid A	IgM
Cytokines	**Cell surface markers**
TNF-α IL-6 IL-1β IL-12 IL-2 * IFN *	CD25 CD3 CD4 CD8 HLA-DR
Cytokine receptors	**Transcription factors**
sIL-6R sIL-2R	NF-κB *
Chemokines	**Prostaglandins**
MCP-1	PGE2

Table 1-1. Inflammation markers reported in depression. * Increased activity following stimulation.

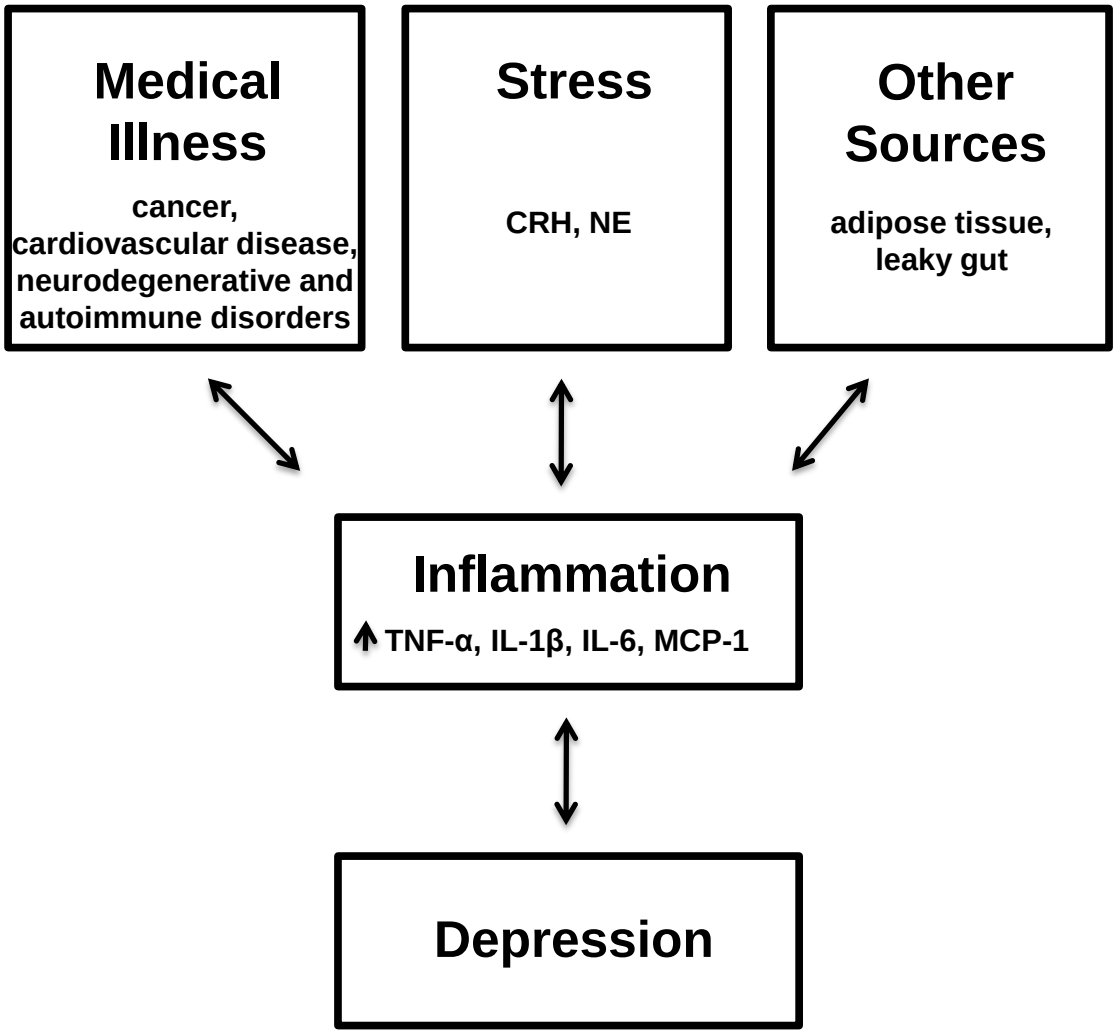


Figure 1-1. Inflammation and depression. Inflammatory signaling induced by medical illness, stress, adipose tissue, or a leaky gut may contribute to the development of depression. Moreover, depression may contribute to the inflammation in medical illnesses.

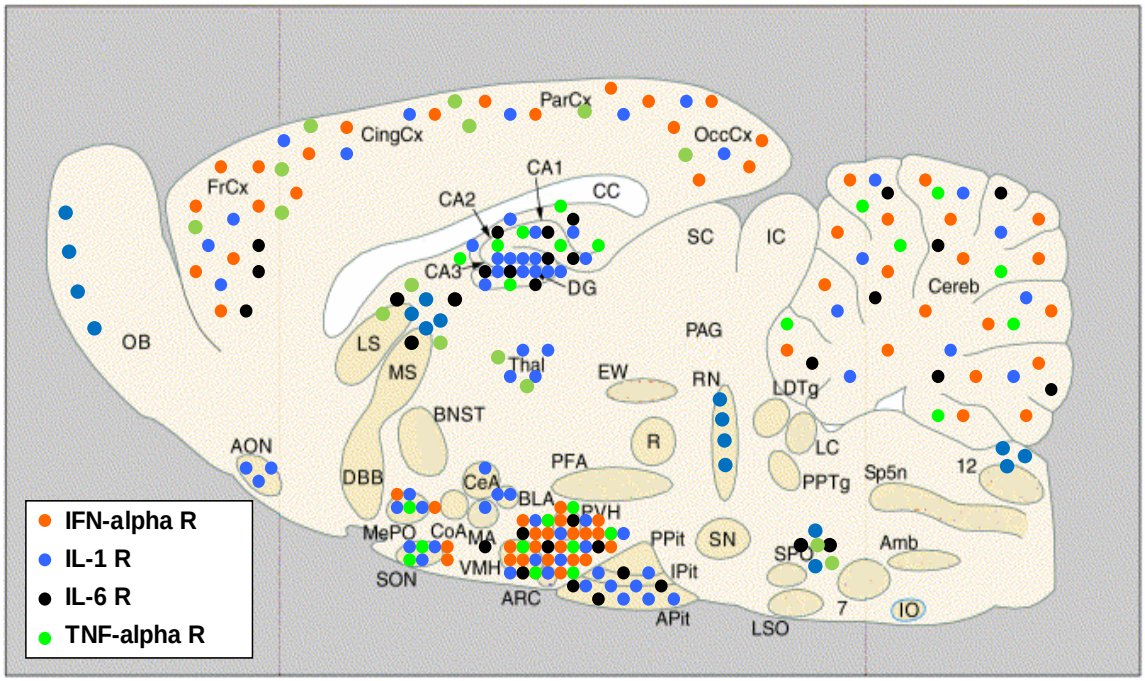


Figure 1-2. Cytokine receptor expression in the brain. Cytokine receptors are expressed in the hippocampus, prefrontal cortex, hypothalamus, striatum, and other brain regions that have been implicated in the pathophysiology of depression.

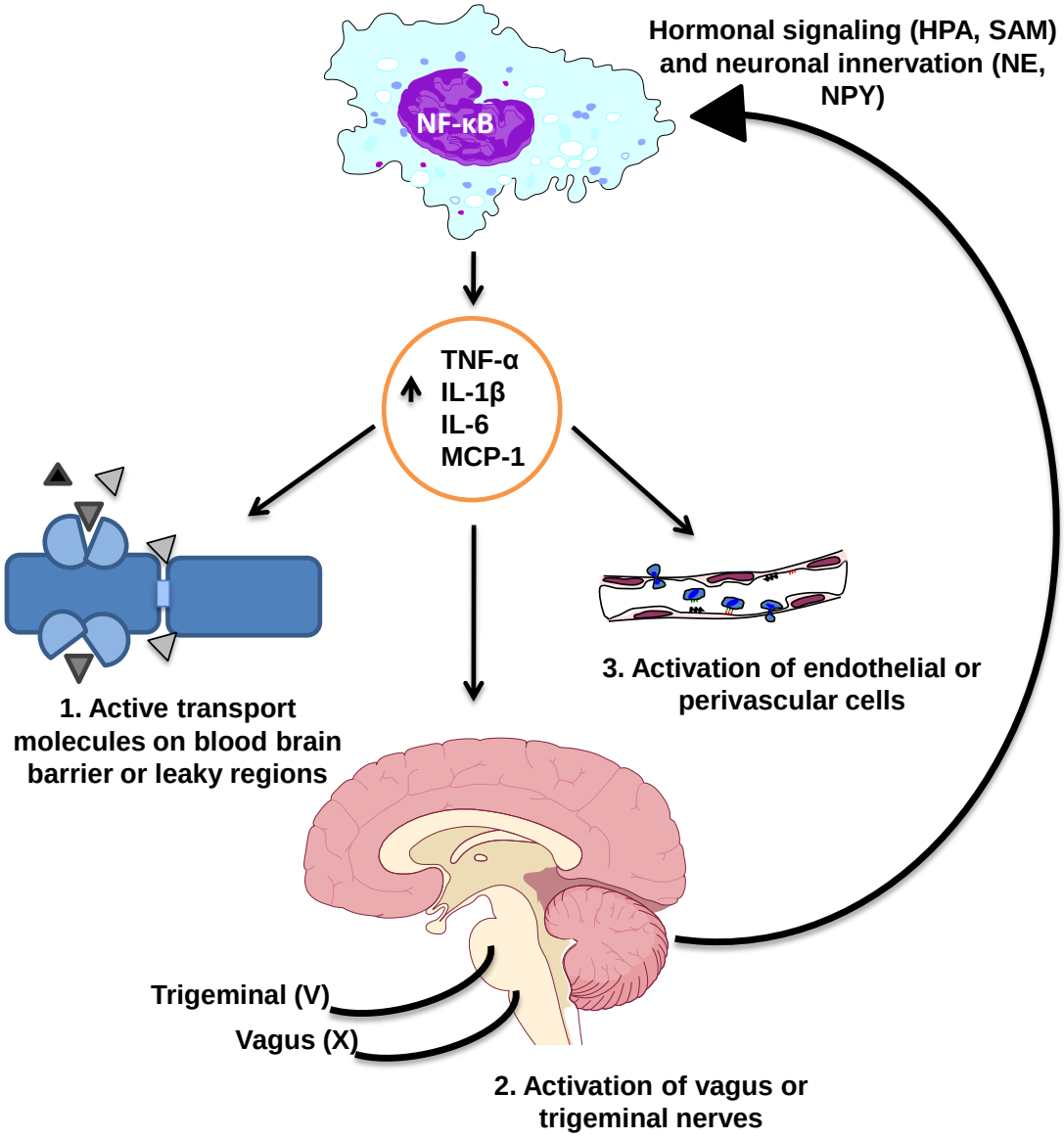


Figure 1-3. Brain-immune signaling pathways. Following the detection of a pathogen or immune stimuli, the inflammatory signal can be transmitted to the brain in more than one way. 1. Cytokines have access to the brain via active transport of cytokines across the blood brain barrier or through leaky regions. 2. Cytokines can activate afferent nerve fibers such as the vagus and trigeminal nerves. 3. Cytokines can signal through cytokine receptors on endothelial or perivascular cells in the brain. Cytokine signaling results in the propagation and amplification of inflammation in the brain and behavioral alterations.

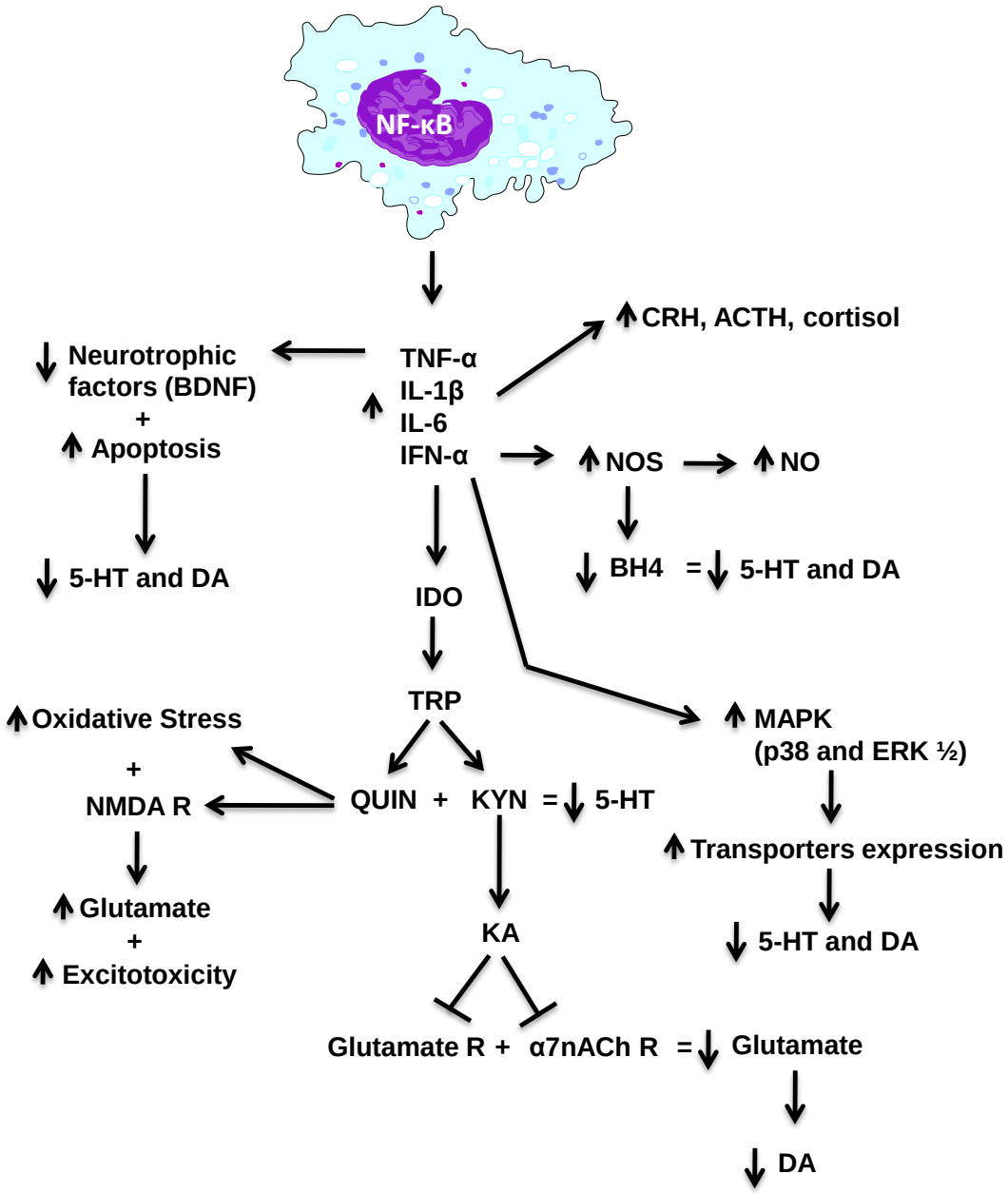


Figure 1-4. Inflammation and biological alterations in depression. NF-κB-induced cytokines modulate pathways known to be involved in the pathophysiology of depression, including neurotransmitter function, neuroendocrine function, and synaptic plasticity.

Chapter 2

Inhibition of the NF-κB signaling pathway by the curcumin analog, 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31): anti-inflammatory and anti-cancer properties

I. Introduction

Mounting data suggest that inflammation may serve as a common mechanism of multiple diseases including cardiovascular disease, diabetes, cancer, neurodegenerative diseases, and certain neuropsychiatric disorders (Tracy, 2003; Wellen and Hotamisligil, 2005; Aggarwal et al., 2006; Libby, 2006; Miller et al., 2009; Amor et al., 2010; Couzin-Frankel, 2010). The nuclear factor κ B (NF-κB) pathway has been identified as a key mediator of inflammation and therefore serves as an important target for drug development and discovery (Barnes and Karin, 1997; Giuliani et al., 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Aggarwal, 2004).

NF-κB is a family of transcription factors with five subunits (p52/p100/NF-κB2, p50/p105/ NF-κB1, RelB, c-Rel, and RelA/p65) that form hetero- and homodimers which remain inactive in the cytoplasm when associated with IκB proteins (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996) (Figure 2-1). The p50 and p65 heterodimers are the most prevalent activated form of NF-κB. Activation of IκB kinases [IκKα, IκKβ, and NF-κB essential modulator (NEMO)/ IκKγ] results in the phosphorylation of the inhibitory IκB (IκBα, IκBβ, and IκBε) proteins bound to NF-κB. NF-κB is consequently released and translocates to the nucleus where it can interact with other transcription factors and transcriptional co-factors to regulate expression of an array of genes, many of which are involved in inflammatory signaling (e.g. cytokines, chemokines, adhesion molecules, and acute phase proteins) as well as proliferation and

apoptosis (Baeuerle and Baltimore, 1996). The NF-κB pathway can be activated by multiple inflammatory stimuli including cytokines and pathogen-derived molecules such as lipopolysaccharide (LPS). LPS is a cell wall component of gram-negative bacteria, and with the aid of accessory proteins [LPS-binding protein (LBP) and cluster of differentiation 14 (CD14)], it is recognized by the toll-like receptor 4 (TLR-4) and myeloid differentiation factor 2(MD-2) complex on the surface of mononuclear myeloid cells (Chow et al., 1999). Activation and dimerization of the TLR4-MD2 monomer complex induces a cascade of signaling molecules that include myeloid differentiation factor 88 (MyD88), interleukin (IL)-1 receptor associated protein kinase (IRAK), tumor necrosis factor (TNF) receptor activated factor 6 (TRAF6), and that ultimately lead to activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways (Lu et al., 2008). LPS is a common inflammatory stimulus in clinical and laboratory studies, and its effects on NF-κB and inflammatory mediators have been well characterized both in-vivo and in-vitro (Ulevitch and Tobias, 1999; Aderem and Ulevitch, 2000).

The search for drugs that block NF-κB have identified several promising natural compounds, including curcumin. Curcumin is a component of the curry spice, turmeric, and is derived from the root of the plant *Curcuma longa*, a member of the ginger family. Curcumin has been prized for centuries throughout Asia not only for culinary applications, but also as a treatment for a variety of ailments ranging from acute infection to chronic disease (e.g. inflammatory bowel syndrome, diabetes, and asthma) (Shishodia et al., 2005; Jagetia and Aggarwal, 2007; Aggarwal and Harikumar, 2009). Curcumin has also been shown to exhibit

anti-inflammatory, anti-bacterial/ fungal/ viral, anti-cancer, and anti-oxidant activities in laboratory animals (Goel et al., 2008; Aggarwal and Sung, 2009). Although curcumin targets many transcription factors (ATF3, AP-1, STAT-3), protein kinases (PKA, PKC), enzymes, growth factors, inflammatory mediators, and anti-apoptotic proteins (Aggarwal and Harikumar, 2009), there has been considerable interest in curcumin's ability to inhibit NF-κB pathway activity (Figure 2-2). However, due to its low bioavailability, poor absorption and rapid metabolism (Anand et al., 2007; Anand et al., 2008), clinical trials have involved doses of curcumin as high as 12 g/day orally. Although this dosage regimen may be unacceptable to some patients, no dose-limiting toxicities have been reported (Cheng et al., 2001; Hatcher et al., 2008). Nevertheless, curcumin at high doses has shown potential efficacy in patients with advanced stage pancreatic cancer (Dhillon et al., 2008). These data suggest that curcumin has therapeutic potential that may be limited by dosing considerations.

Structural curcumin analogs have been created to optimize the therapeutic effects of curcumin by increasing potency, slowing metabolism, and increasing absorption. Two promising monoketone curcumin analogs include 3,5-Bis(2-fluorobenzylidene)-4-piperidone (EF24), and the more recently developed 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31), which replaces the carbon-flourine bond at the 1-position of the terminal benzene rings with nitrogen to give the corresponding pyridine rings (Figure 2-3). The NF-κB inhibition activity of EF24 has been previously reported (Kasinski et al., 2008). EF24 was found to block IκB kinase, phosphorylation of IκB, and the translocation of NF-κB to the

nucleus. EF31 has shown a better physical profile with increased stability and solubility (Sun and Snyder, unpublished data), however its effect on NF-κB activity has yet to be determined. In the current study, the capacity of EF31 to inhibit NF-κB and its downstream inflammatory mediators was examined in mouse RAW264.7 macrophages as well as NF-κB-dependent cancer cell lines. EF31 was found to be a more potent inhibitor of NF-κB than either EF24 or curcumin.

II. Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Cellgro (Manassas, VA). Weymouth's medium and Leibovitz's L-15 medium were purchased from ATCC (Manassas, VA). Curcumin was purchased from Sigma (St. Louis, MO) (C1386-5G), and the structurally related compounds EF24 and EF31 were prepared at Emory University as described previously (Adams et al., 2004). Dimethyl sulfoxide (DMSO) was used to dissolve all compounds, and all dilutions were made from a 10mM stock. LPS (Escherichia coli O55:B5) was obtained from Sigma and was suspended in saline to a final concentration of 1 μg/mL. Antibodies against NF-κB and the secondary goat anti-rabbit antibody conjugated to Fluor 488 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers for mouse TNF-α, IL-1β, IL-6, and GAPDH were obtained from Qiagen (Valencia, CA).

Cell culture

Mouse RAW 264.7 macrophage cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (HyClone Labs, Logan, UT) at 37 °C with 5% CO_2. A2780 cells, a human ovarian carcinoma cell line (Sigma), were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum at 37 °C with 5% CO_2. MDA-MB-231 cells, a human breast cancer cell line (ATCC), were cultured in Leibovitz's L-15 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum at 37 °C with 100% air. EMT6 cells, a mouse mammary carcinoma cell line (ATCC), were cultured in Weymouths's MB 752/1 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 15% fetal bovine serum at 37 °C with 5% CO_2. MIA PaCa-2 cells, a pancreatic carcinoma cell line (ATCC), were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, and 2.5% horse serum at 37 °C with 5% CO_2.

Assessment of transcription factor DNA-binding in RAW cells

RAW 264.7 mouse macrophage cells were plated at 3.4 X 10 cells/well in 60 mm X 15 mm dishes, incubated overnight, and were then incubated in triplicate with either EF24, curcumin, or vehicle (DMSO 1%) for one hour prior to LPS (1 µg/mL) or saline treatment. Nuclear proteins were collected 15 minutes

after LPS treatment using the extraction kit protocol from Active Motif (Carlsbad, CA). The 15 minute collection time-point after LPS treatment was derived from a time course study (see Figure 2-4A). Nuclear protein samples were analyzed in triplicate using the NF-κB DNA-binding ELISA kit (Active Motif, Carlsbad, CA) or the MAPK family DNA-binding ELISA kit (Active Motif).

Assessment of NF-κB nuclear translocation in RAW cells

RAW 264.7 mouse macrophage cells were plated at 20,000 cells/400 µl/well in 8-well glass chamber slides (Nunc, USA) and incubated overnight. Cells were then treated with the test compounds or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) for 15 minutes. Cells were then processed for confocal microscopy as previously described (Kasinski et al., 2008). Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed using 2% paraformaldehyde for 30 minutes at room temperature. Triton-X 100 (0.1%) was used to permeabilize the cells for 20 minutes. Cells were then washed (3X) with PBS and blocked using bovine serum albumin (1%) for one hour followed by an overnight incubation at 4°C with rabbit anti-p65 NF-κB antibody (1:500). Cells were washed with PBS and incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:1000) along with Hoechst 33342 (1 µM) for one hour at room temperature. After washing the cells with PBS, the chambers were removed and the slides were imaged using an LSM510 confocal microscope set for fluorescein isothiocyanate (argon/2 laser- excitation at 488 nm, emission at 510 nm) and 4,6-diamidino-2-phenylindole (diode laser- excitation at 405 nm, emission at 420

nm). Images were quantified and analyzed using Metamorph for Olympus (Olympus America, Center Valley, PA). The nuclear region was defined using the Hoechst 33342 staining and was then used to calculate the average of the nuclear NF-κB fluorescence intensity. Samples were run in triplicate and 20 representative cells were chosen at random in each treatment well (total of 60 cells per treatment group) to assess NF-κB nuclear translocation.

Isolation of total RNA and cytokine RT-PCR

RAW 264.7 mouse macrophage cells were plated at 1.8 X10 cells/well in 6-well plates and incubated overnight. Cells were then treated with the test compounds or vehicle (DMSO 1%) in triplicate for one hour prior to stimulation with LPS (1 µg/mL) or saline. Three hours after LPS treatment, cells were collected and total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) followed by reverse transcription PCR using SuperScript First-strand Synthesis System (Invitrogen) with random primers, according to manufacturer's protocols. This was followed by amplification of cDNA by RT-PCR (Applied Biosystems, 7500 Fast, Carlsbad, CA). For data analysis the deltaCt method was used. Fold-changes were calculated as difference in gene expression between LPS and vehicle controls.

Cytokine protein ELISA

RAW 264.7 mouse macrophage cells were plated at 1.8 X10 cells/well in 6-well plates and incubated overnight. Cells were then treated with the test

compounds or vehicle (DMSO 1%) in triplicate for one hour prior to stimulation with LPS (1 µg/mL) or saline. Six hours after LPS stimulation, cell supernatants were collected and centrifuged. Cytokine protein concentrations were assayed using mouse TNF-α, IL-1β and IL-6 ELISA from R&D (Minneapolis, MN) according to manufacturer's recommendations.

Assessment of IκKβ inhibition

Kinase recombinant protein of the catalytic domain, purchased from Invitrogen (Carlsbad, CA.), was incubated with the compounds or vehicle (DMSO 1%) in tripiclate for 30 minutes before inhibition of IκKβ activity was assayed using the Z'-Lyte kinase assay kit from Invitrogen. In a 10 µL kinase reaction, the IκKβ transfers the gamma-phosphate of ATP to a single serine/ threonine residue in the synthetic peptide substrate (2 µM). The peptide is labeled with two fluorophores (coumarin and fluorescein), one at each end, to make up a fluorescence resonance energy transfer (FRET) pair. In the development reaction, 5 µL of a site-specific protease recognizes and cleaves non-phosphorylated peptides. Cleavage disrupts FRET between the coumarin and the fluorescein on the peptide. Five µl of stop reagent is added to halt the development reaction before the plate is read. Plates were read using an Envision 2102 plate reader from Perkin Elmer. During detection, a ratio-metric read-out of the donor emission over the acceptor emission quantitates reaction progress. Percent phosphorylation was calculated using controls. ATP concentrations were equal to K_m values for the kinase.

Cell Viability/ proliferation assay

All cell lines were plated at 5000 cells/well in 96-well plates and incubated overnight. Cells were treated with test compounds or vehicle (DMSO 1%) in triplicate for either 1 or 48 hours before viability was assayed using the CellTiter 96 Aqueous non-radioactive cell proliferation assay (MTS) kit from Promega (Madison, WI).

Data Analysis

Overall treatment effects were determined in each experiment by either a one- or two-way analysis of variance (ANOVA) using GraphPad Prism 5. Significant interactions were followed by post-hoc Bonferroni test to determine differences between specific groups of interest. An α level of $p < 0.05$ was used in all statistical tests. A t-test was used to determine a significant difference between vehicle and LPS in Figure 2-5B.

III. Results

EF31 is a potent inhibitor of NF-κB DNA-binding

To establish the best time-point to measure NF-κB DNA-binding activity, mouse RAW264.7 macrophages were treated with LPS (1 or 10 µg/mL) or saline for 5, 15, 30, or 60 minutes after which nuclear proteins were collected and analyzed using a NF-κB DNA-binding ELISA. Treatment with LPS at both 1 and

10 µg elicited a peak in NF-κB DNA-binding by 15 minutes (Figure 2-4A). Statistical analysis revealed a significant time x treatment interaction ($F [6, 24] = 4.209$, $p = 0.005$). Post hoc analysis revealed no significant differences between 1 and 10 µg LPS. Within cells treated with 1 µg, only the 15 minute time-point was significantly different from the 5 minute time-point ($p < .05$).

To examine the activity of EF31 compared to EF24 and curcumin, mouse RAW264.7 macrophages were treated with curcumin, EF24, EF31 (1, 5, 10, 30, 50, or 100 µM), or vehicle for one hour, and NF-κB DNA-binding activity was assessed 15 minutes following treatment with LPS (1 µg/mL). Potent inhibition of NF-κB DNA-binding was observed at concentrations of 5–10 µM for EF31, 30–50 µM for EF24, and 50–100 µM for curcumin (Figure 2-4B). Analysis of these results with a two-way ANOVA revealed a significant treatment x concentration interaction ($F [10, 36] = 4.001$, $p=0.0010$). The dose response indicated that the IC_{50} value of EF31 ~5 µM, ~35 µM for EF24, and >50 µM for curcumin. Due to a wide concentration range of inhibition (50-100 µM), the IC_{50} for curcumin could not be determined. Of note, lower concentrations of EF31 were tested including 0.01 µM, and 0.10 µM with no effect on NF-κB DNA-binding (Figure 2-9A).

NF-κB nuclear translocation was assessed using fluorescence microscopy to determine the nuclear localization of p65 following LPS. The treatment protocol used in the experiment shown in Figure 2-4B was employed, i.e. nuclear localization of NF-κB was determined after treating with EF31, EF24 and curcumin (5, 10, 50 µM) or vehicle for one hour and then for 15 minutes with LPS (1 µg/mL). In the absence of LPS, p65 was observed almost exclusively in the

cytoplasm. However, the nuclear content of p65 increased dramatically following LPS as indicated by the overlapping of the p65-fluor 488 green fluorescence with the Hoesch blue staining (Figure 2-5A). A two-way ANOVA revealed a significant treatment x concentration interaction ($F [6, 24] = 3.914$, $p = 0.0072$). Nuclear p65 localization was significantly reduced by EF31 at concentrations between 5-50 μM ($p < 0.05$ vs. vehicle + LPS) (Figure 2-5B). Similar results were found with EF24, but higher concentrations of EF24 (10-50 μM) ($p < 0.05$ vs. vehicle + LPS) were required to inhibit LPS-induced translocation of NF-κB. Finally, curcumin tended to decrease in p65 nuclear localization at 50 μM ($p > 0.05$ vs. vehicle + LPS).

EF31 blocks LPS-induced cytokine mRNA expression in mouse RAW264.7 macrophages

To determine the best time-point at which to measure mRNA expression, mouse RAW264.7 macrophages were treated with LPS (1 or 10 μg/mL) or saline, and mRNA was extracted at different time-points (15 minutes, 1, 3, or 6 hours) as described in Methods and the expression of TNF-α, IL-1β, and IL-6 was measured using RT-PCR. Treatment with LPS at both concentrations resulted in a time dependent increase in mRNA expression for TNF-α, IL-1β, and IL-6 (Figure 2-6). The 3 hour time-point was chosen to examine the effects of the compound-dependent NF-κB inhibition on mRNA expression because it was the earliest time-point that showed a significant increase in mRNA expression compared to the saline treated group for the cytokines examined. Moreover, this

time-point has been previously published on mouse RAW264.7 macrophages treated with LPS to examine pro-inflammatory cytokine mRNA expression (Chen et al., 2002; Chen et al., 2006).

Mouse RAW264.7 macrophages were treated with curcumin, EF24, EF31 (5, 10, or 50 µM), or vehicle for one hour, and mRNA was extracted 3 hours following treatment with LPS (1 µg/mL) (Figure 2-7). The concentrations for the test compounds were chosen to cover the range of IC_{50} values revealed in the previous experiments. Expression of TNF-α, IL-1β, and IL-6 mRNA were inhibited in a dose–dependent manner with all compounds. Statistical analysis revealed a treatment x concentration interaction (TNF-α, $F [4, 18] = 7.712$, $p = 0.0008$; IL-1β, $F [4, 18] = 40.71$, $p < 0.0001$; IL-6, $F [4, 18] = 31.11$, $p < 0.0001$). Of note, no inhibition of LPS-induced TNF-α was found with curcumin.

EF31 is a potent inhibitor of cytokine protein release

To assess the effects of EF31 on LPS-induce cytokine protein release, mouse RAW264.7 macrophages were treated with curcumin, EF24, EF31 (5, 10, or 50 µM), or vehicle for one hour and then with LPS (1 µg/mL). Cytokine protein released in medium was measured at 16 hours post LPS treatment using a cytokine protein ELISA. The 16 hours time-point was selected as it had been previously published using mouse RAW264.7 macrophages treated with LPS (Means et al., 2000). EF31 inhibited cytokine protein release at all concentrations tested (5-10 µM), while both EF24 (10-50 µM) and curcumin (10-50 µM) required higher concentrations to achieve similar results. Statistical analysis revealed a

treatment x concentration interaction (TNF-α, $F [4, 18] = 398.3$, $p < 0.0001$; IL-1β, $F [4, 18] = 19.00$, $p < 0.0001$; IL-6, $F [4, 18] = 50.42$, $p < 0.0001$) (Figure 2-8). Of note, lower concentrations of EF31 (< 5 µM) did not block TNF-α protein release (Figure 2-9B). LPS + DMSO treated samples yielded protein levels of approximately 1,400 pg/mL for TNF-α,15 pg/mL for IL-1β, and 11,000 pg/mL for IL-6.

Mechanism for EF31-dependent inhibition of the NF-κB pathway

To examine whether EF31 directly inhibits IκKβ activity, recombinant IκKβ was pre-incubated with EF24, EF31 (0.0977, 0.391, 1.56, 6.25, 25, 100 µM), or vehicle for 30 minutes. The activity of IκKβ was measured using a Z'Lyte kinase assay kit. EF31 exhibited significantly greater inhibition of the recombinant kinase activity compared to EF24 ($p < 0.01$) for concentrations higher than 100 nM (Figure 2-10). The dose response indicated that the IC_{50} value of EF31 was ~1.92 µM and ~131 µM for EF24. Statistical analysis revealed a significant treatment x concentration interaction ($F [5, 24] = 198.0$, $p < 0.0001$).

EF31's inhibition of NF-κB DNA-binding activity is reversible

To examine whether EF31's inhibition of NF-κB DNA-binding activity is reversible, mouse RAW264.7 macrophages were treated with curcumin (50 µM), EF24 (50 µM), EF31 (10 µM), or vehicle for one hour. Cells were then washed and medium was replaced and treated with LPS (1 µg/mL). Nuclear proteins were collected at different time-points, and NF-κB DNA-binding activity was

measured using a NF-κB DNA-binding ELISA. NF-κB DNA-binding increased in a time-dependent manner (Figure 2-11). Statistical analysis revealed a significant effect of treatment ($F [3, 24]= 3.883$, $p = 0.0215$), but not time. Post-hoc analysis identified a significant difference between vehicle and EF31 at 15 minutes ($p < 0.05$), but not at 3 or 6 hours.

At concentrations that inhibit NF-κB, EF31 does not reduce cell viability/ proliferation in mouse RAW264.7 macrophages

To assess the effects of EF31 on cell viability, mouse RAW264.7 macrophages were treated with curcumin, EF24, EF31 (1, 5, 10, or 50 µM), or vehicle for one hour, and then with LPS or saline for 15 minutes. Cell viability was assayed using a cell proliferation (MTS) kit. Statistical analysis showed a concentration effect in the LPS treated groups ($F [3, 24]= 4.052$, $p = 0.0183$) and a significant treatment x concentration interaction in the saline treated groups ($F [6, 24]= 5.295$, $p= 0.0013$). A post-hoc analysis showed a significant difference between curcumin and both analogs, EF24 and EF31 at the 50 µM concentration ($p < .01$) in the saline treated groups only. However, EF31 showed no reductions in cell viability as compared to vehicle treatment for concentrations that were previously found to inhibit NF-κB DNA-binding (5-10 µM). Nevertheless, a higher concentration of 50 µM did reduce cell viability by approximately 50% (Figure 2-12). EF24 did not have an effect on cell viability for any of the concentrations tested, while curcumin showed significant reductions in cell viability at 50 µM (a concentration that inhibits NF-κB DNA-binding activity by about 50%). Of note,

these data also indicate that the EF31-mediated reduction in NF-κB DNA-binding activity in mouse RAW264.7 macrophages was not due to a loss of viable cells.

EF31 is cytotoxic in cancer cell lines

Human ovarian carcinoma cells (A2780), human breast cancer cells (MDA-MB-231), human pancreatic carcinoma cells (MIA PaCa-2), and mouse mammary carcinoma cells (EMT6), all of which depend heavily upon the NF-κB pathway for proliferation, were treated with curcumin, EF31 (1, 5, 10, or 50 µM), or vehicle for 48 hours. Cell viability was then assayed using a cell proliferation (MTS) kit. Statistical analysis showed a significant interaction between treatment x concentration in the MIA PaCa-2 cells ($F [3, 16]= 15.76$, $p < 0.0001$), A2780 cells ($F [3, 16]= 16.28$, $p < 0.0001$), and the EMT6 cells ($F [3, 16]= 55.74$, $p < 0.0001$), while the MDA-MB-231 cells showed a significant treatment effect ($F [1, 16]= 46.44$, $p < 0.0001$) and a significant concentration effect ($F [3, 16]= 6.118$, $p = 0.0057$). In all cancer cell lines, when compared to curcumin, EF31 was consistently more potent in reducing cell viability (Figure 2-13).

EF31 inhibits mitogen-activated protein kinase pathways

To explore whether the effects of EF31 on cytokine mRNA and protein extended to other kinase mediated pathways, activity in the MAPK pathway was assessed following treatment with EF31. Mouse RAW264.7 macrophages were treated with curcumin (50 µM), EF31 (10 µM), or vehicle for one hour prior to LPS (1 µg/mL) or saline treatment. Nuclear proteins were collected 15 minutes after

LPS treatment and MAPK transcription factor (ATF-2 and c-JUN) DNA-binding activity was measured using a DNA-binding ELISA. This time-point was chosen based on a MAPK transcription factor time course (Figure 2-14) showing that at 15 minutes the DNA-binding activity of both transcription factors (c-JUN and ATF-2) is significantly increased. EF31 significantly reduced the DNA-binding activity for both transcription factors in the LPS treated groups. A two-way ANOVA showed a significant treatment (compounds) x treatment (LPS or saline) interaction (ATF-2, $F [2, 12]= 285.5$, $p < 0.0001$; c-JUN, $F [2, 12]= 83.08$, $p < 0.0001$) (Figure 2-15). Similar results were found for curcumin; however, post-hoc analysis revealed that in the LPS treated groups, EF31-induced inhibition was significantly greater than curcumin for both transcription factors ($p < 0.001$).

IV. Discussion

The results presented here demonstrate that EF31 is a potent inhibitor of NF-κB DNA-binding and nuclear translocation. EF31 also attenuated induction of downstream pro-inflammatory cytokine mRNA and protein mediators, and directly inhibited IκKβ activity. Of note, these effects were observed at concentrations that showed no toxicity in mouse RAW264.7 macrophages, while maintaining potent toxicity in NF-κB-dependent cancer cell lines.

Regarding the mechanism of these effects, the data indicate that EF31, like EF24, directly interacts with IκKβ, thereby blocking the phosphorylation of IκB, a necessary step in the activation of NF-κB and its translocation to the

nucleus (Kasinski et al., 2008; Shu, 2008). IκB binds to NF-κB subunits where it is thought to interact with the NF-κB DNA-binding/nuclear localization region, preventing NF-κB translocation to the nucleus. Upon activation, the IκB kinases (IκKα, IκKβ, and NEMO) phosphorylate the inhibitory IκB proteins bound to NF-κB. IκB is then polyubiquitinated and degraded by the 26S proteasome, and consequently, the p65 nuclear localization sequence is unmasked and other amino acids are in turn phosphorylated allowing translocation to the nucleus and activation of gene transcription. The mechanism by which EF31 inhibits IκKβ remains to be elucidated. One possibility is that EF31 may partially occupy the ATP binding site of the kinase as has been suggested for EF24 and curcumin (Kasinski et al., 2008). By interacting with the ATP binding site on the IκKβ, EF31 would block the phosphorylation of the IκB protein and thereby block activation of NF-κB. Furthermore, curcumin has also been shown to indirectly inhibit IκKβ through interactions with other kinases such as Akt, protein kinase C (PKC), and MAPK, which have also been implicated in the activation of NF-κB (Dolcet et al., 2005). Whether EF31 is active against these kinases remains to be determined.

EF31 was also found to block downstream cytokine mRNA expression and cytokine protein release. Indeed, in mouse RAW 264.7 macrophages, cytokine mRNA expression was significantly reduced by EF31 as well as EF24 and curcumin in a dose dependent manner at concentrations between 5-50 μM. Treatment with these compounds also blocked cytokine protein release at 16 hours post LPS. However, both EF24 and curcumin required significantly higher concentrations to achieve results similar to EF31. EF31's increased potency may

be related to its structure. Both EF31 and EF24 are shorter than curcumin, which could confer an advantage by allowing these compounds to be more adaptable to the globular binding pocket of IκKβ (Kasinski et al., 2008). Moreover, the nitrogen-carbon bonds in the terminal rings of EF31 are less bulky than the fluorine-carbon bonds in EF24. The nitrogen allows for favorable steric effects in the kinase binding site, and because they are good proton acceptors, they also have an advantage in the formation of hydrogen bonds to the target proteins (Shi and Snyder, unpublished data). Nevertheless, further testing is required to determine the binding site of EF31 on IκKβ and the mechanism for increased potency.

Similar to EF24, EF31 exhibited more potent toxicity on NF-κB-dependent cancer cell lines than curcumin. The NF-κB pathway plays an important role in the survival and proliferation of the cancer cell lines tested (Bharti and Aggarwal, 2002; Sartor, 2002; Shibata et al., 2002; Adams et al., 2005; Aggarwal et al., 2006; Monks and Pardee, 2006; Selvendiran et al., 2007; Holcomb et al., 2008; Kasinski et al., 2008). Sustained cell viability has been shown to depend in part on NF-κB-regulated gene expression of survival proteins such as Bcl-xL and other anti-apoptotic signaling molecules. By blocking the activation of the NF-κB pathway and thus the expression of survival signals, apoptotic signaling pathways may be activated and thereby reduce cell viability and proliferation. Another mechanism that may be involved in EF31's effects on cancer cell viability is increased expression of the protein phosphatase and tensin homolog (PTEN). PTEN is a negative regulator of the kinase Akt, which is involved in the

survival of some cancer cell lines (Stambolic et al., 1998). Expression of PTEN results in cell cycle arrest and apoptosis, however, activation of the NF-κB subunit p65 has been shown to reduce expression of PTEN by interfering with transcription cofactors in the nucleus (Vasudevan et al., 2004). By blocking the disinhibition of the p65 subunit of NF-κB, EF31 in turn may increase the expression of PTEN. A similar mechanism of action has been reported for EF24 (Selvendiran et al., 2007). Moreover, there is evidence suggesting that EF24 and EF31 both use a redox-dependent mechanism to induce apoptosis in cancer cells (Adams et al., 2005; Sun et al., 2009). Finally, although low concentrations of EF31 (1-30 μM) had cytotoxic effects on the cancer cell lines tested, it did not show significant reductions in cell viability/proliferation in mouse RAW264.7 macrophages, possibly related to the specific roles of NF-κB in these cancer cell lines.

Another issue regarding the development of curcumin analogs for therapeutic use is whether or not the inhibition of NF-κB activity is reversible. EF31 is a Michael acceptor, with the potential to form carbon bonds that irreversibly bind to the ATP pocket of IκKβ and thereby could irreversibly block NF-κB activity. NF-κB is important for the survival of healthy cells as well as cancer cell lines, and if inhibition is irreversible, it could induce cellular apoptosis and destroy vulnerable cells/ tissues as well as compromise the immune response. Indeed, IκKα, IκKβ, and p65 knockout mice typically die as embryos or shortly after birth, while p50 KO mice are viable but show degenerative alterations including increased spontaneous cell death in the brain, abnormal

capillaries, and fewer myelinated axons of the optic nerve (Li et al., 2008). In the current studies, mouse RAW26.7 macrophages treated with EF31 and LPS regained NF-κB DNA-binding activity in a time-dependent manner, suggesting that the inhibition is reversible. Nevertheless, recovery of NF-κB activity measured using a DNA-binding ELISA could have also been due in part to cell proliferation, and not necessarily to uncoupling of the compound from the IκKβ. Although, NF-κB DNA-binding activity in the current studies serves as an indirect indicator of upstream IκB kinase activity, Sun et al. (2009) showed that binding of EF24 and EF31 to thiol (SH) is reversible. This suggests that binding of the compounds to the nucleophile SH of the cysteine in the binding pocket of the IκB kinase is also reversible, which is consistent with the recovery of downstream NF-κB DNA-binding in the current studies.

In addition to effects on NF-κB, at a 10 μM concentration, EF31 significantly reduced DNA-binding activity of the activator protein (AP)-1 transcription factors activating transcription factor-2 (ATF-2) and c-Jun, suggesting that EF31 may have broad effects on inflammatory signaling pathways including MAPK. These results are consistent with data showing that curcumin inhibits the activity of p38 and c-Jun N-terminal kinase (JNK); kinases involved in the downstream activation of MAPK transcription factors (Chen and Tan, 1998; Cho et al., 2005; Camacho-Barquero et al., 2007; Epstein et al., 2010). Of note, our results for MAPK transcription factor DNA- binding inhibition also showed a significant increase in the saline group treated with EF31. This may be related to interactions between the NF-κB and MAPK pathways. Nijboer

et al., reported a significant increase in AP-1 activity when NF-κB was inhibited in a model of hypoxic-ischemic brain damage (Nijboer et al., 2009). The mechanism by which NF-κB inhibition can up-regulate activity in the JNK/AP-1 pathway, may be via down-regulation of NF-κB-dependent expression of the proteins growth arrest and DNA-damage-inducible (Gadd45) β and x-linked inhibitor of apoptosis protein (XIAP). The proteins Gadd45β and XIAP have been shown to inhibit the activation of the JNK/AP-1 pathway by interacting with kinases MAPK kinase (MAP2K) 7 and transforming growth factor β-activated kinase (TAK) 1, respectively (Nijboer et al., 2009). Thus, the EF31-related increase in AP-1 transcription factor DNA-binding activity in our results may be due to potent inhibition of the NF-κB pathway and down-regulation of the inhibitory proteins Gadd45β and or XIAP. However, this remains speculative and requires further testing.

In summary, EF31 is one in a series of curcumin analogs that show potent inhibition of NF-κB DNA-binding activity and nuclear translocation in conjunction with potent toxicity in NF-κB-dependent cancer cell lines. Pending bioavailability and toxicity studies, EF31 may serve as a relevant endpoint or a further step in the development of curcumin analogues for therapeutic applications in inflammatory disorders and cancer.

V. Limitations and Future Directions

One major limitation in these studies is in the lower concentrations tested in-vitro. For the majority of the experiments presented here, concentrations

between 5 and10 µM were chosen to cover the range of IC_{50} values revealed in Figure 2-4B. In Figures 2-9A and 2-9B, EF31 concentrations between 0.01 and 1 µM did not produce significant inhibition of NF-κB DNA-binding activity or TNF-α protein production. However, Figure 2-8 showed complete inhibition of cytokine protein production in the groups treated with an EF31 concentration of 5 µM. These data suggest that somewhere between 1 and 5 µM, there is concentration threshold that produces 100% inhibition of cytokine proteins, and perhaps the response curve takes place in a very short span of concentrations. Data from curcumin could help explain these results. Curcumin is known to have several targets (Figure 2-2) with varying dose-response curves. It is possible that EF31 may also have varying dose-response curves for each of its targets. The production of cytokine proteins is affected by several of these targets. Thus, a dose higher than 1 µM may be required to inhibit multiple targets to produce complete inhibition of cytokine proteins. Moreover, Figure 2-4B shows that at 5 µM, EF31 blocks NF-κB DNA-binding by about 50%, although this translates into complete inhibition of cytokine protein production. It is also possible that a 50% inhibition of this pathway is sufficient to induce 100% inhibition of the downstream cytokine protein production. Indeed, it has been previously reported that even partial inhibition of NF-κB can have marked effects on inflammation (Kopp and Ghosh, 1994). An experiment using EF31 concentrations between 1 and 5 µM may help to generate a dose-response curve and identify the lowest possible concentration that induces 100% inhibition of cytokine protein production.

Another limitation based on the doses tested here is the possible clinical or therapeutic relevance. Doses of 5 µM or higher could present a problem when translated to clinical research. Curcumin doses of 12 grams and higher have been reported in clinical studies, however, the volume was not acceptable to the research volunteers. Nevertheless, EF31 has shown potent anti-cancer activity at concentrations lower than 1 µM (Shim, unpublished data). Overall, EF31 is a promising compound that could be improved upon as new analogs are created.

Lastly, there are a number of complementary experiments for future directions: 1) The current studies measured reversibility by indirectly measuring IκKβ activity. A more direct measure would be to use the same recombinant protein assay described in Methods to assess reversibility. This would involve pre-treatment of the recombinant proteins with EF31 and then testing its activity at different time-points. 2) Although EF31 exhibited dose-dependent cytotoxic effects in the cancer cell lines tested, it was significantly more potent in some cell lines than others. Understanding why the pancreatic carcinoma and the ovarian carcinoma cell lines responded better would be worthwhile. This could be examined by using DNA microarrays to identify differential gene expression, and using software such as The Transcription Element Listening System (TELis) database to identify transcription factors and signalling pathways that may be modulated directly or indirectly by EF31 treatment. 3) Finally, the interactions between the NF-κB pathway and the MAPK pathway could be explored further. Figure 2-15 showed an increase in MAPK transcription factor activity in the EF31 treated cells. The fact that EF31 may target more than one kinase or pathway

suggests that the effects seen here may not be dependent on NF-κB inhibition. Using a specific NF-κB inhibitor such as NEMO binding domain (NBD) peptide would help to determine if the increase in MAPK activity is directly related to NF-κB inhibition.

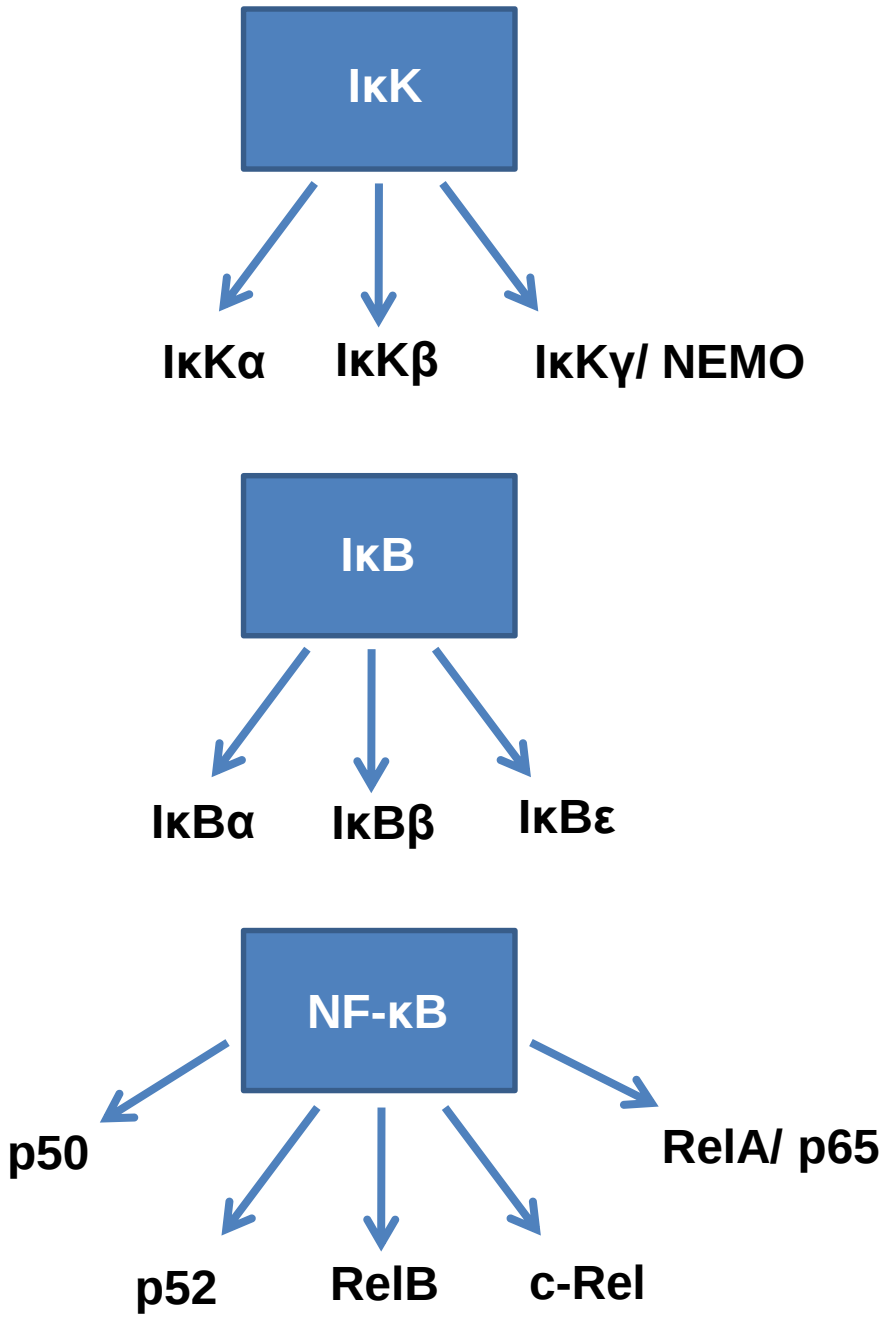


Figure 2-1. Members of the NF-κB pathway

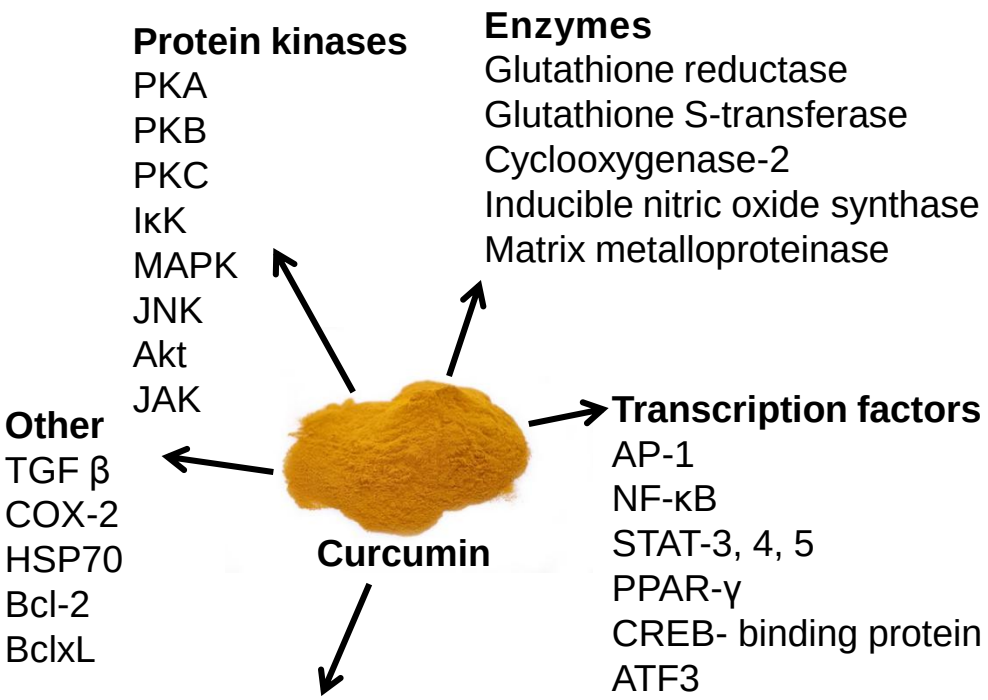
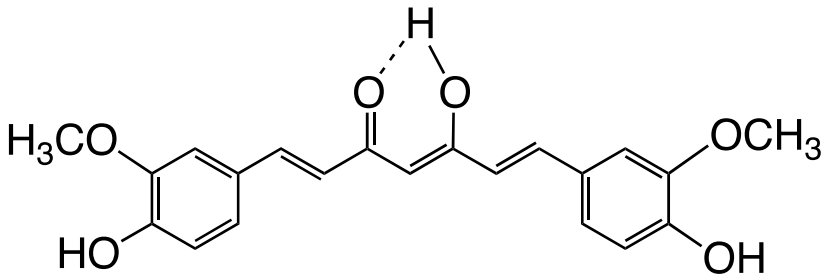
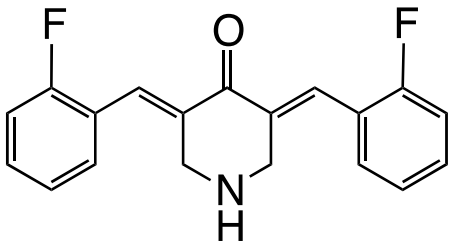


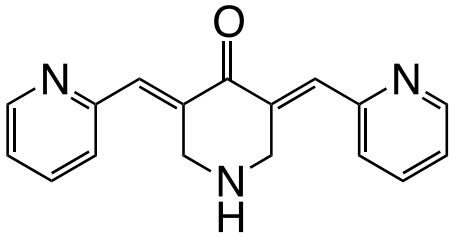
Figure 2-2. Molecular targets of curcumin. Curcumin targets a number of kinases, enzymes, proteins, and transcription factors that are involved in the inflammatory immune response.



Curcumin



EF24



EF31

Figure 2-3. Chemical structures of curcumin and the monoketone analogs. Modifications to curcumin include replacement of the carbon-flourine bond at the 1-position of the terminal benzene rings with nitrogen to give the corresponding pyridine rings on EF31.

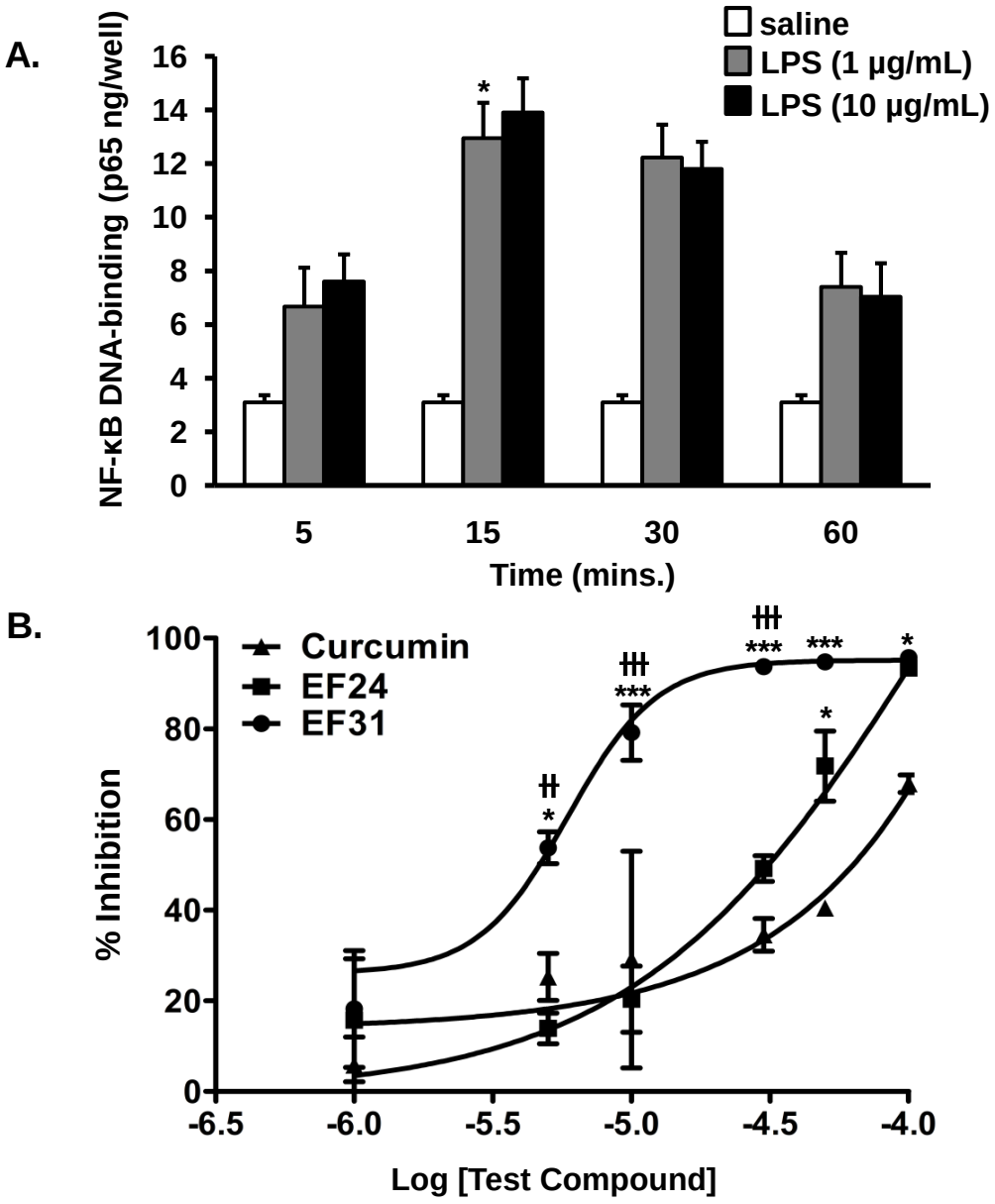


Figure 2-4. EF31 is a potent inhibitor of LPS-induced NF-κB DNA-binding activity. A) Mouse RAW264.7 macrophages were treated with LPS (1 or 10 µg/mL) or saline. Nuclear proteins were collected at different time-points (5 minutes- 60 minutes) and NF-κB DNA-binding activity was measured using a DNA-binding ELISA. All conditions were run in triplicate, and values shown are means (±SEM). * $p < 0.05$ vs. 5 minutes- LPS (1 µg/mL) treated group. B) Mouse RAW264.7 macrophages were pre-treated with curcumin, EF24, EF31 (1, 5, 10, 30, 50, or 100 µM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) for 15 minutes. Values shown are means (±SEM) expressed as percent inhibition from vehicle. * $p < 0.05$, *** $p < 0.001$ vs. curcumin at same concentration; ǂ ǂ $p < 0.01$, ǂ ǂ ǂ $p < 0.001$ vs. EF24 at same concentration.

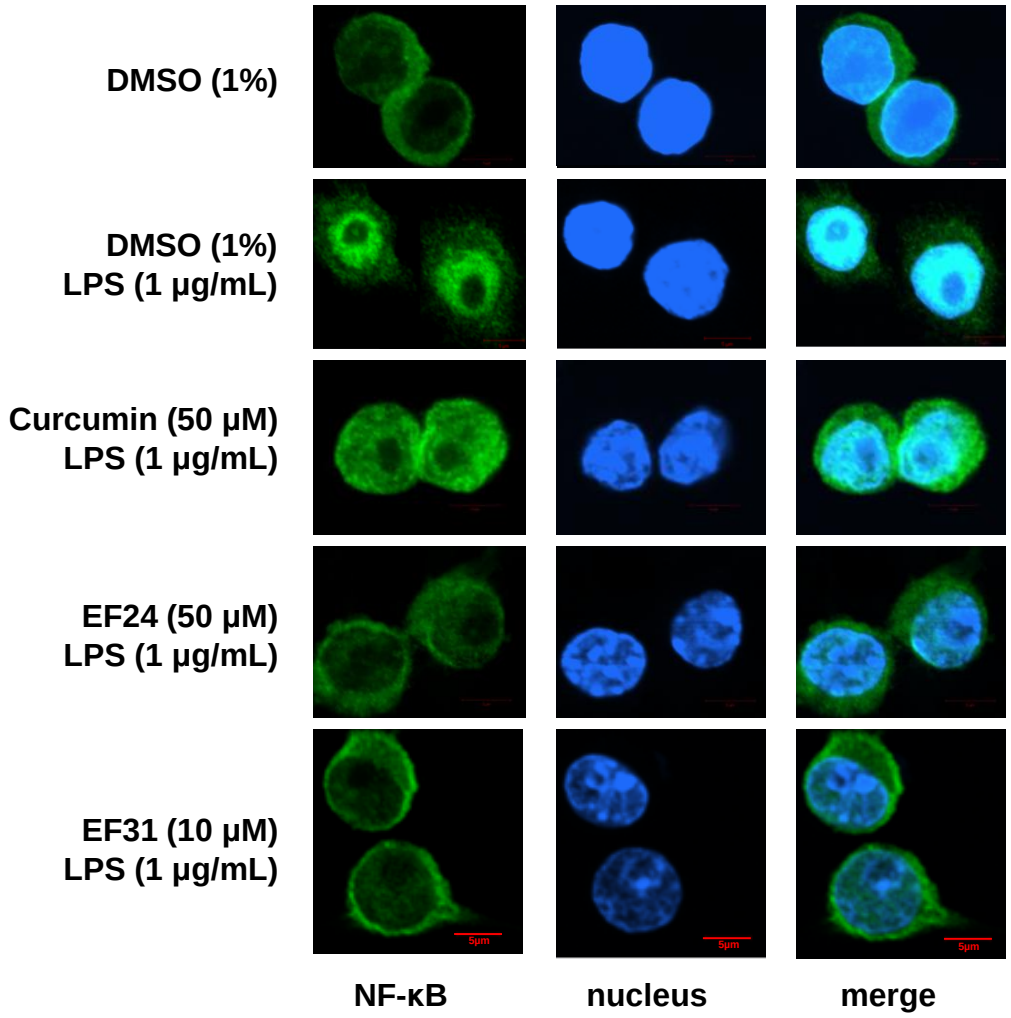


Figure 2-5A. EF31 impairs LPS-induced NF-κB nuclear translocation. Mouse RAW264.7 macrophages were pre-treated with curcumin, EF24, EF31 (5, 10, or 50 µM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) for 15 minutes. Cells were then fixed and processed as described in methods. Images were obtained using a LSM510 confocal microscope. Scale bar = 5µm.

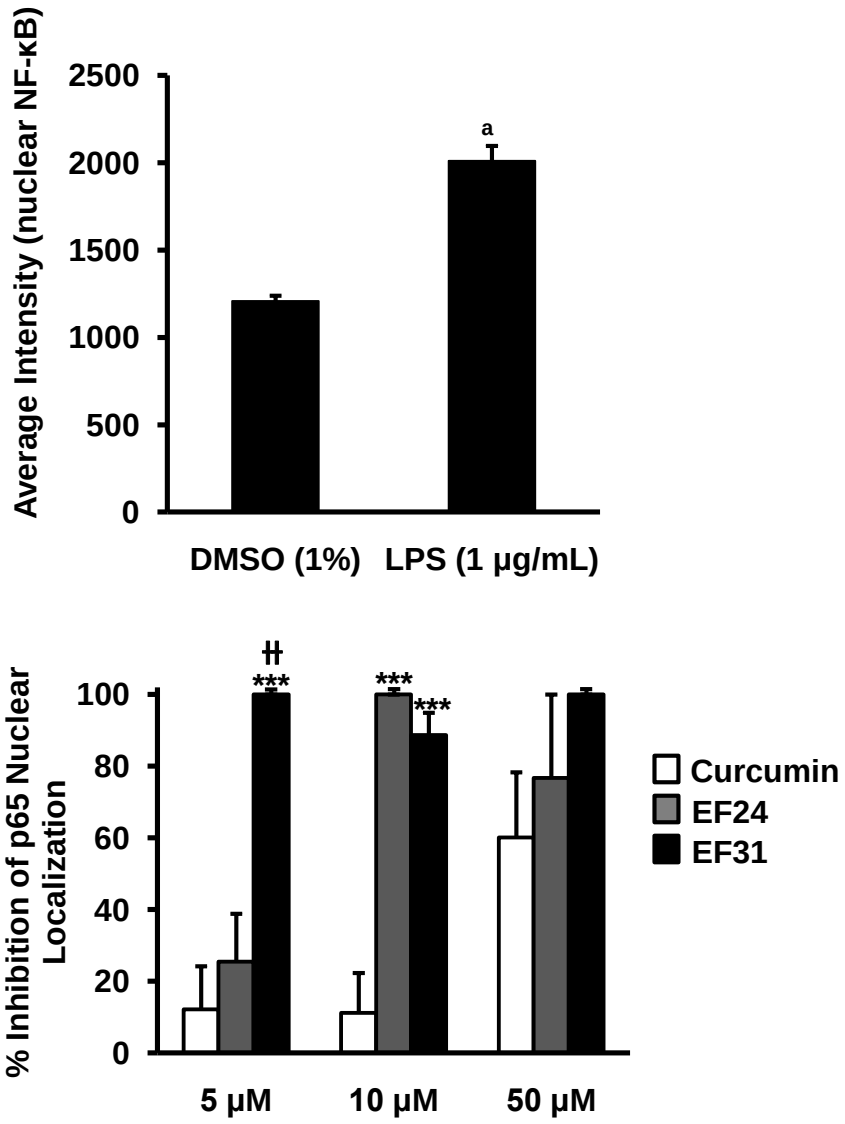


Figure 2-5B. EF31 impairs LPS-induced NF-κB nuclear translocation. The induction of NF-κB nuclear translocation by LPS was quantified by measuring nuclear p65 fluorescence intensity as described in Methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle. *** $p < 0.001$ vs. curcumin at same concentration; ǂ ǂ $p < 0.01$ vs. EF24 at same concentration; a $p < 0.001$ vs. vehicle.

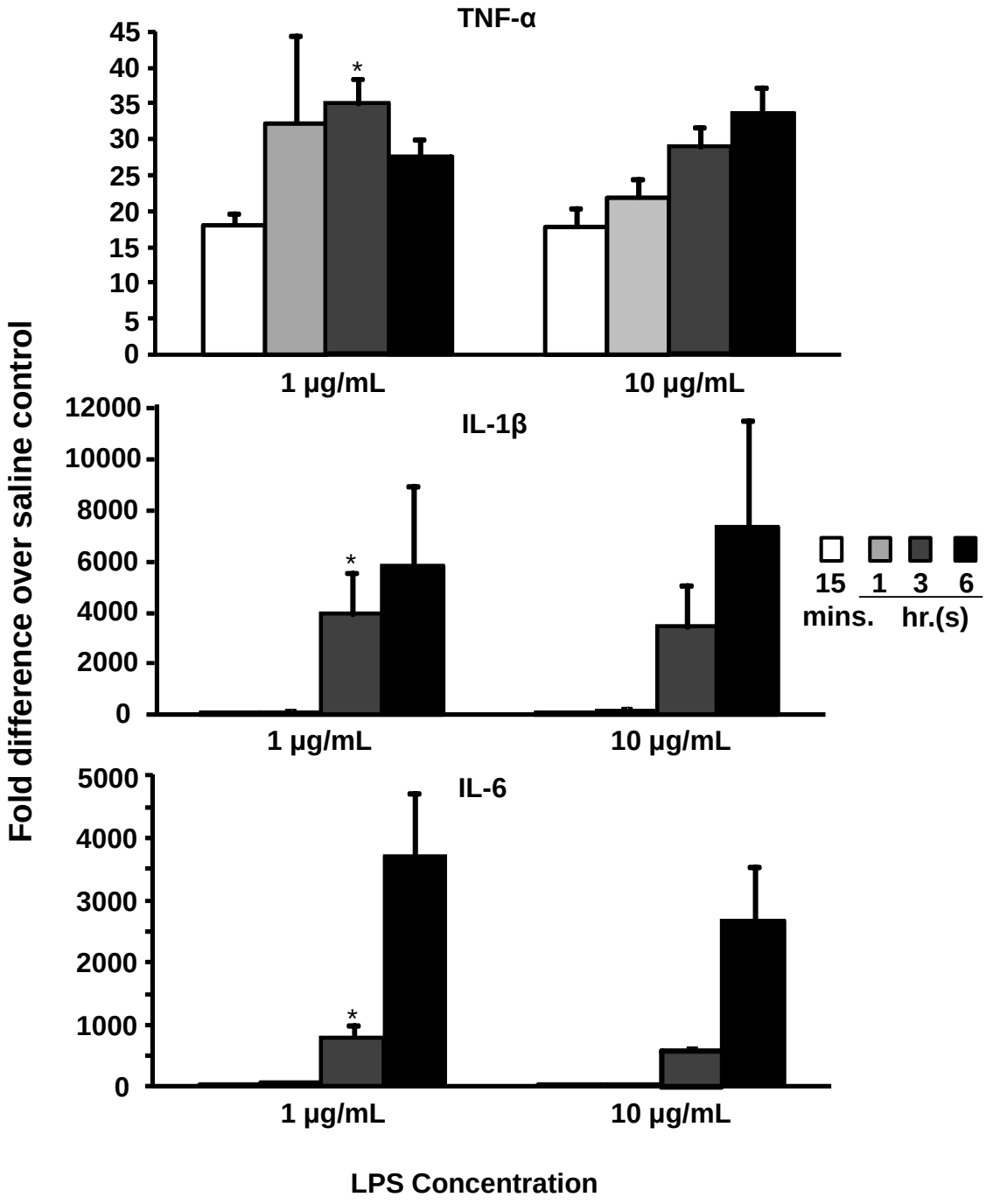


Figure 2-6. LPS-induced cytokine mRNA expression. Mouse RAW264.7 macrophages were treated with LPS (1 or 10 µg/mL) or saline for either 15 minutes, 1, 3, or 6 hours. Whole cell mRNA was then collected as described in Methods and measured using RT-PCR. All conditions were run in triplicate, and values shown are means (±SEM) expressed as fold difference over saline control. * $p < 0.05$ vs. saline.

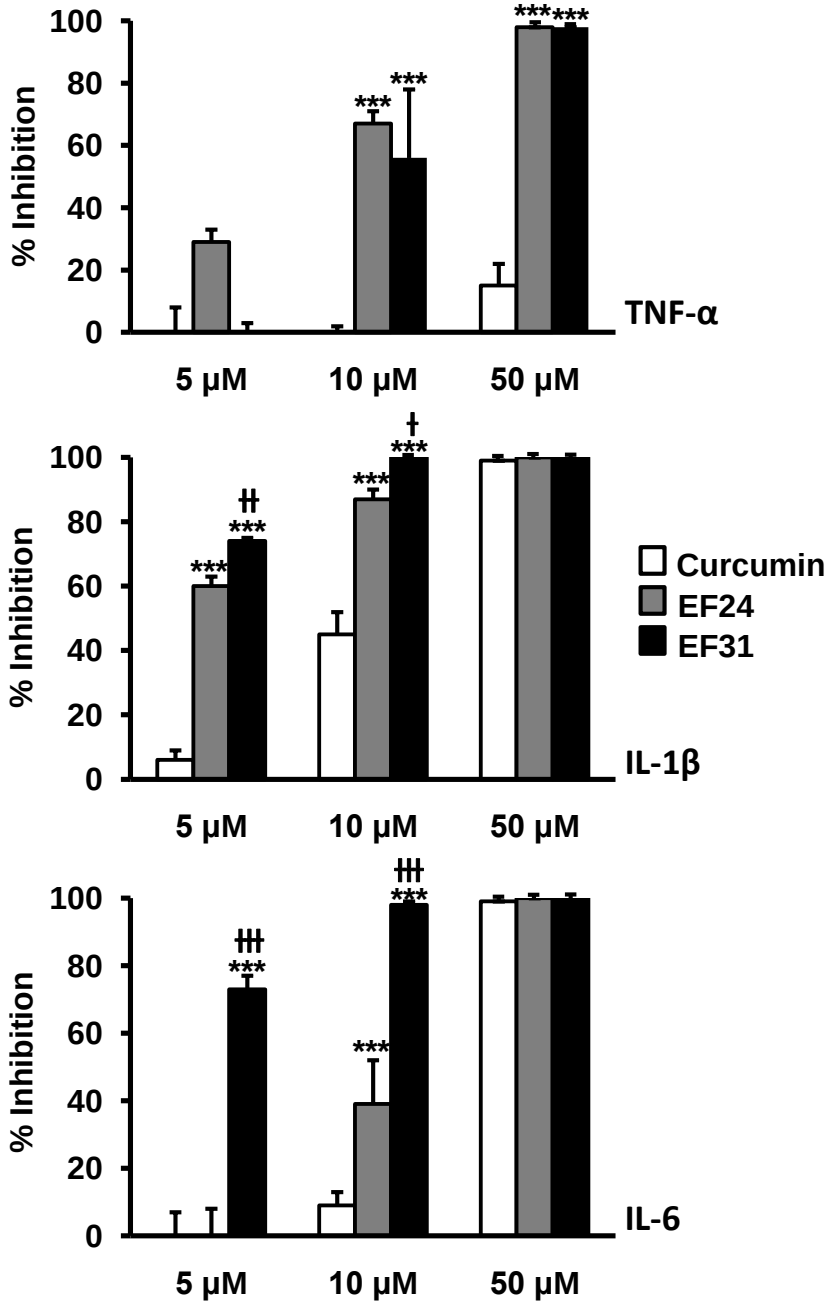


Figure 2-7. EF31 inhibits LPS-induced pro-inflammatory cytokine mRNA expression. Mouse RAW264.7 macrophages were pre-treated with curcumin, EF24, EF31 (5, 10, or 50 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) for 3 hours. Whole cell mRNA was then collected and measured using RT-PCR. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle. *** $p < 0.001$ vs. curcumin at same concentration; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. EF24 at same concentration.

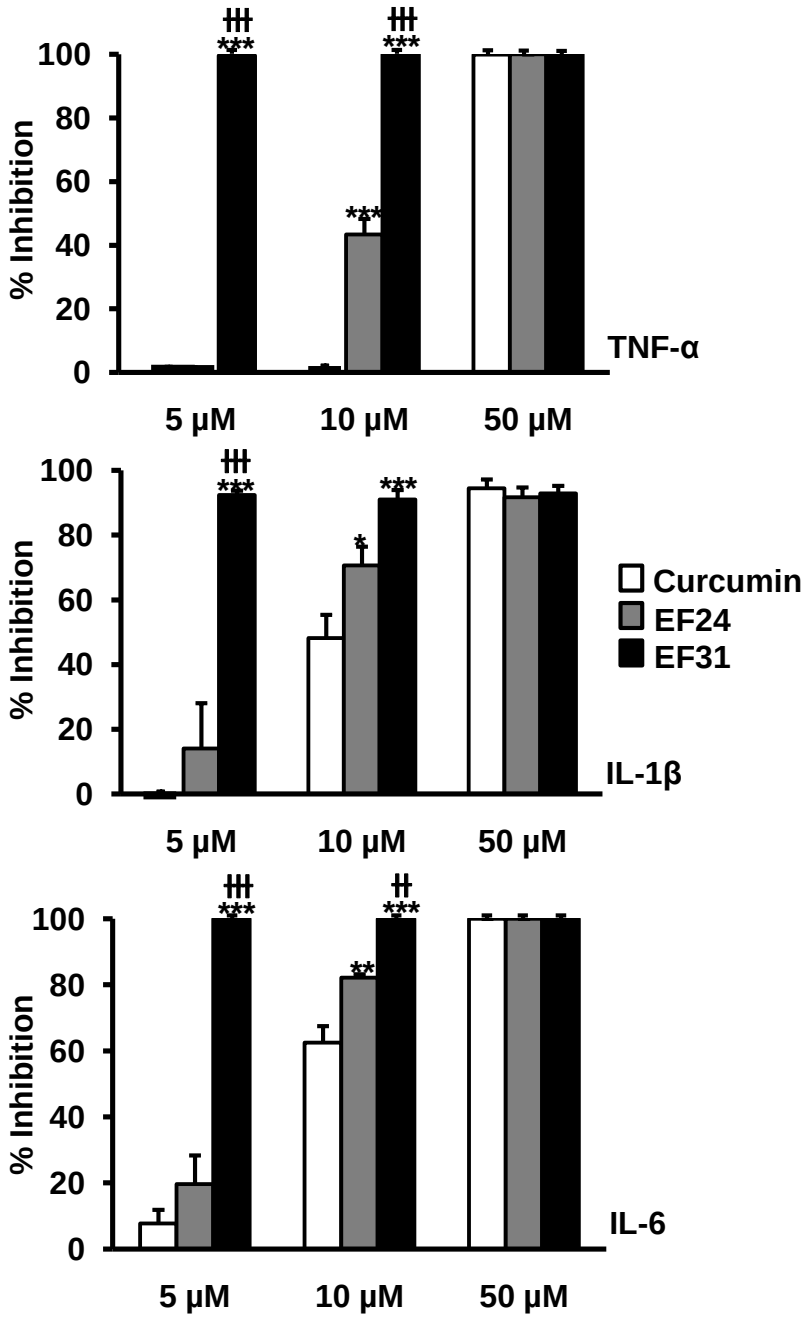


Figure 2-8. EF31 inhibits LPS-induced production of pro-inflammatory cytokine proteins. Mouse RAW264.7 macrophages were pre-treated with curcumin, EF24, EF31 (5, 10, or 50 µM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) for 16 hours. Medium was collected and the protein was then measured using cytokine ELISAs. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. curcumin at same concentration; ǂ ǂ $p < 0.01$, ǂ ǂ ǂ $p < 0.001$ vs. EF24 at same concentration.

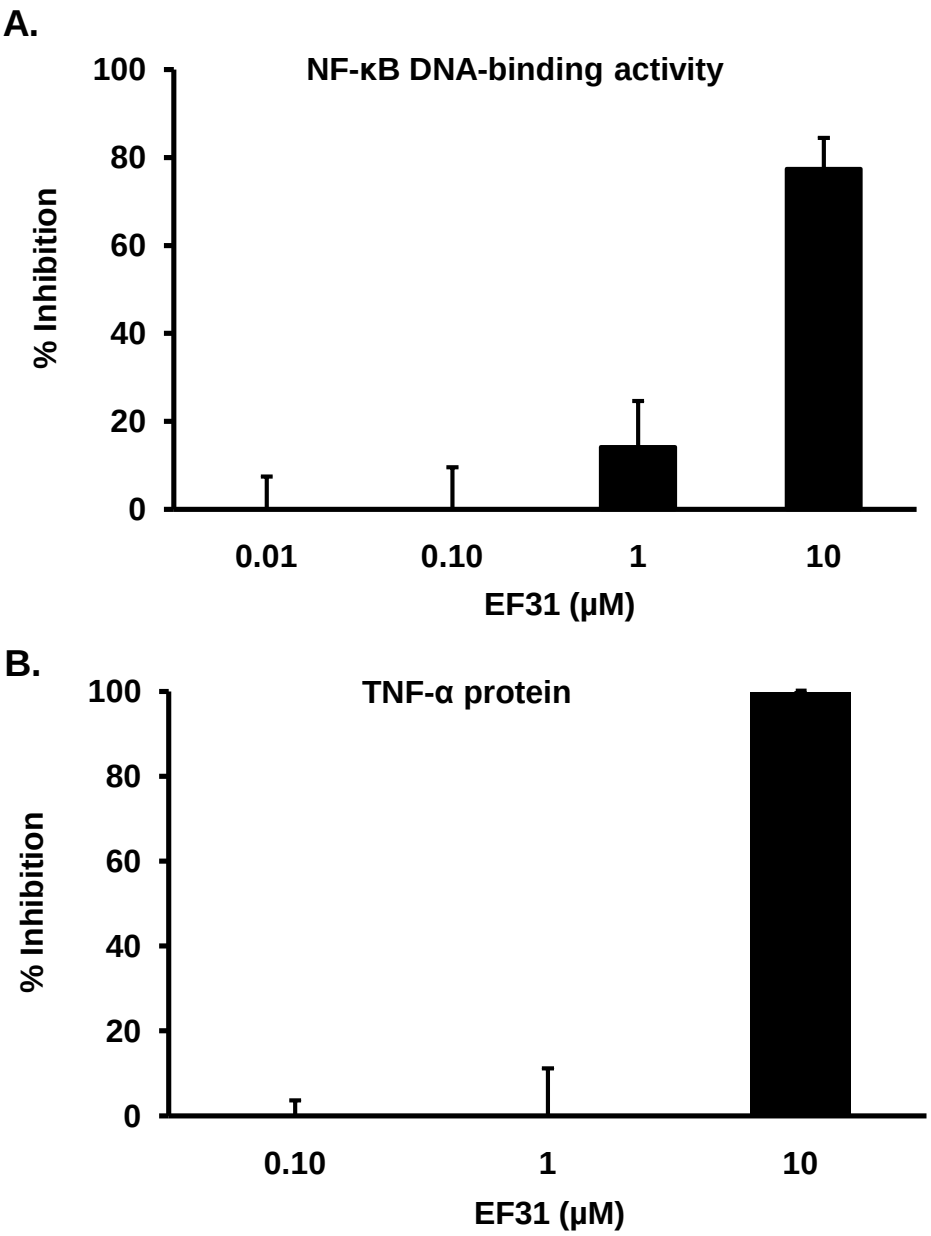


Figure 2-9. EF31 doses lower than 1 μM do not significantly block NF-κB DNA-binding or TNF-α protein release. A) Mouse RAW264.7 macrophages were pre-treated with EF31 (0.01, 0.10, 1, or 10 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) for 15 minutes. Nuclear proteins were collected and NF-κB DNA-binding was measured using a DNA-binding ELISA. B) Mouse RAW264.7 macrophages were pre-treated with EF31 (0.10, 1, or 10 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) for 16 hours. Medium was collected and the protein was then measured using cytokine ELISAs. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle.

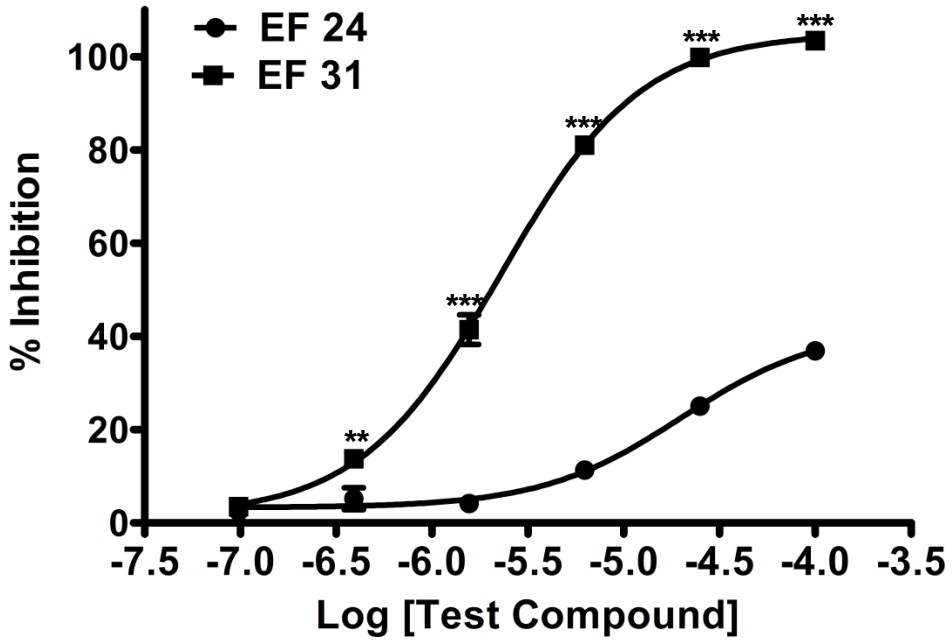


Figure 2-10. EF31 is a potent inhibitor of IκKβ activity. IκKβ recombinant protein was pre-incubated with EF24, EF31 (0.0977, 0.391, 1.56, 6.25, 25, 100 μM), or vehicle (DMSO 1%) for 30 minutes. Inhibition of IκKβ activity was measured using a Z'-Lyte kinase assay kit as described in Methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle. ** $p < 0.01$, *** $p < 0.001$ vs. EF24 at same concentration.

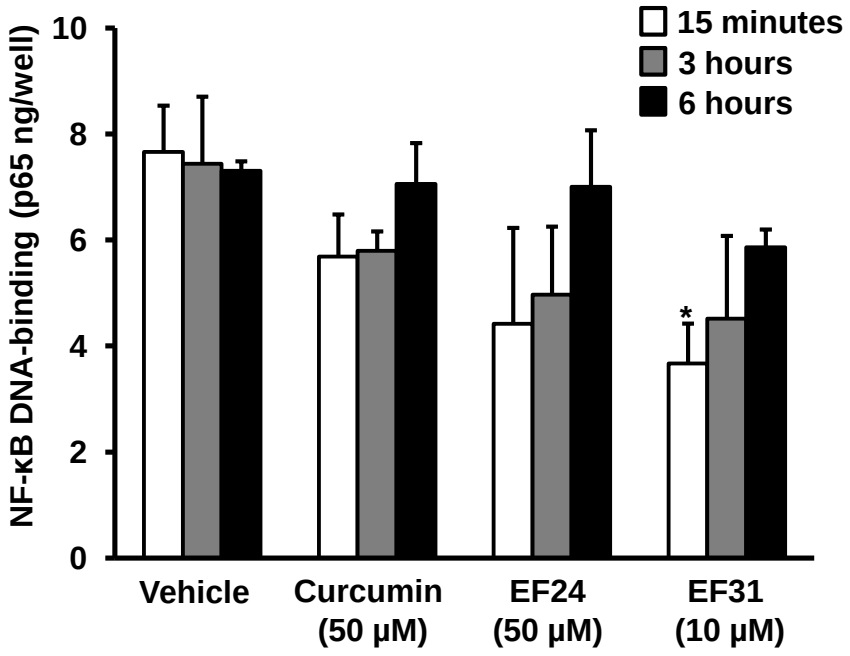


Figure 2-11. Inhibition of NF-κB DNA-binding by EF31 is reversible. Mouse RAW264.7 macrophages were pre-treated with curcumin (50 μM), EF24 (50 μM), EF31 (10 μM), or vehicle (DMSO 1%) for one hour. Cells were then washed and medium was replaced and treated with LPS (1 μg/mL) or saline. Nuclear proteins were collected at different time-points (15 minutes- 6 hours) as described in methods. NF-κB DNA-binding activity was measured using a DNA-binding ELISA. All conditions were run in triplicate, and values shown are means (±SEM). * $p < 0.05$ vs. vehicle + LPS at 15 minutes.

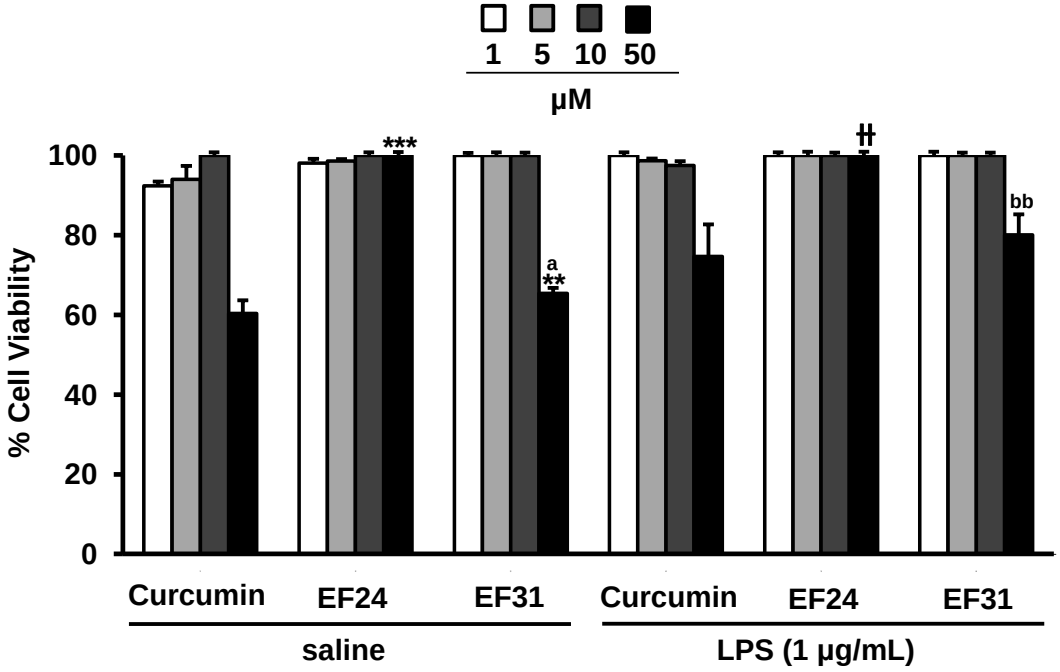


Figure 2-12. EF31 shows no reduction in cell viability at concentrations that inhibit NF-κB. Mouse RAW264.7 macrophages were pre-treated with curcumin, EF24, EF31 (1, 5, 10, or 50 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) or saline for 15 minutes. Cell viability/ proliferation was then measured as described in Methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent of vehicle. ** $p < 0.01$, *** $p < 0.001$ vs. curcumin at same concentration in the saline treated groups; a $p < 0.05$ vs. EF24 at same concentration in the saline treated groups; ǂ ǂ $p < 0.01$ vs. curcumin at same concentration in the LPS treated groups; bb $p < 0.01$ vs. EF24 at same concentration in the LPS treated groups.

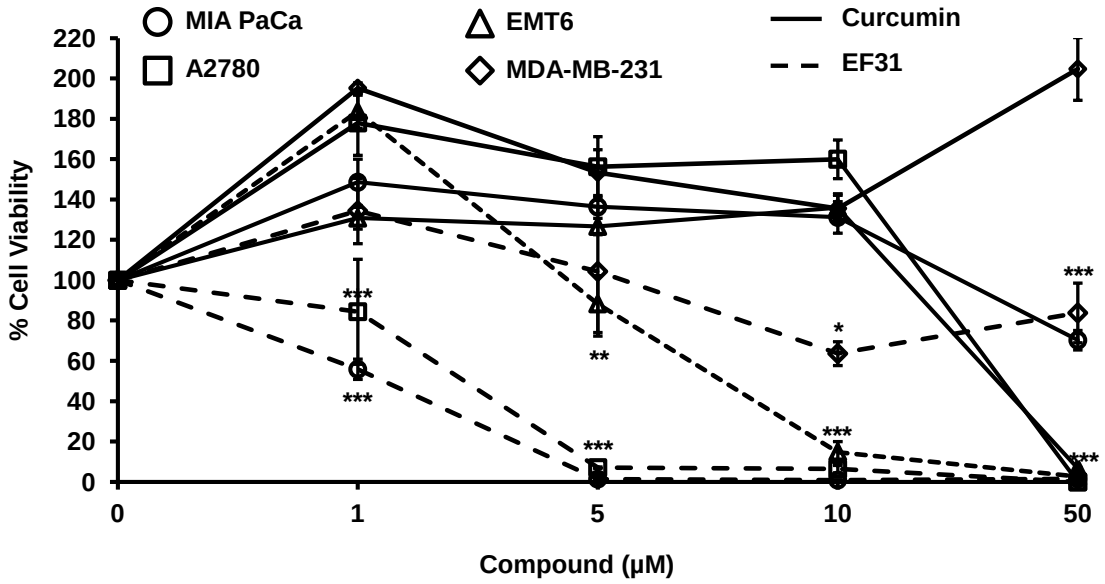


Figure 2-13. EF31 shows potent toxicity on NF-κB-dependent cancer cell lines. Cancer cell lines were treated with curcumin, EF31 (1, 5, 10, or 50 μM), or vehicle (DMSO 1%) for 48 hours. Cell viability/ proliferation was measured as described in methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent of vehicle. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. curcumin at same concentration.

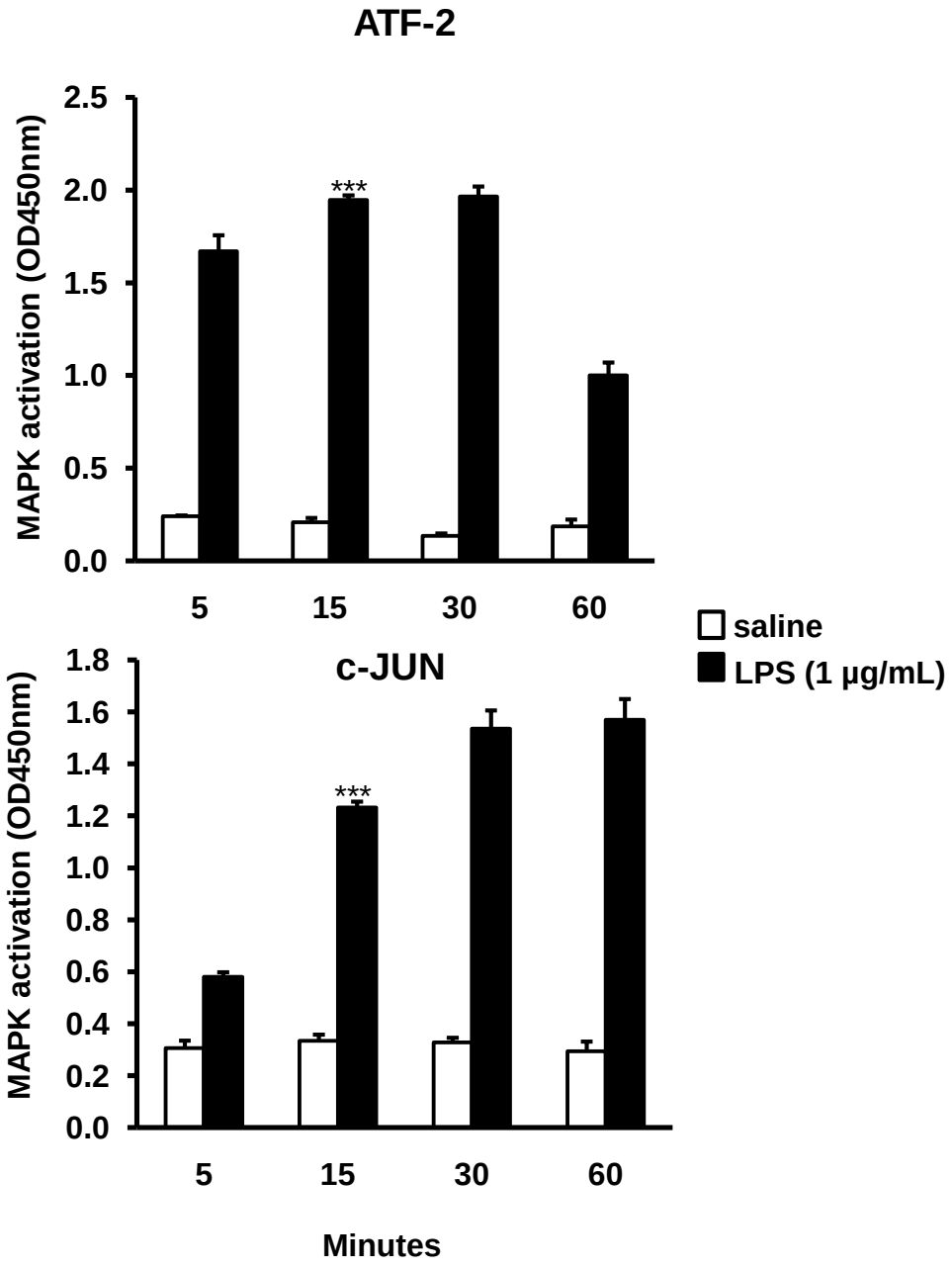


Figure 2-14. LPS-induced MAPK transcription factor activity. Mouse RAW264.7 macrophages were treated with LPS (1 µg/mL) or saline for either 5, 15, 30, or 60 minutes. Nuclear proteins were collected as described in Methods and transcription factor DNA-binding was measured using a DNA-binding ELISA. All conditions were run in triplicate, and values shown are means (±SEM). A two-way ANOVA showed a significant treatment (LPS or saline) x time interaction (ATF-2, $F [3, 16]= 46.08$, $p < 0.0001$; c-JUN, $F [3, 16]= 51.84$, $p < 0.0001$). *** $p < 0.001$ vs. saline.

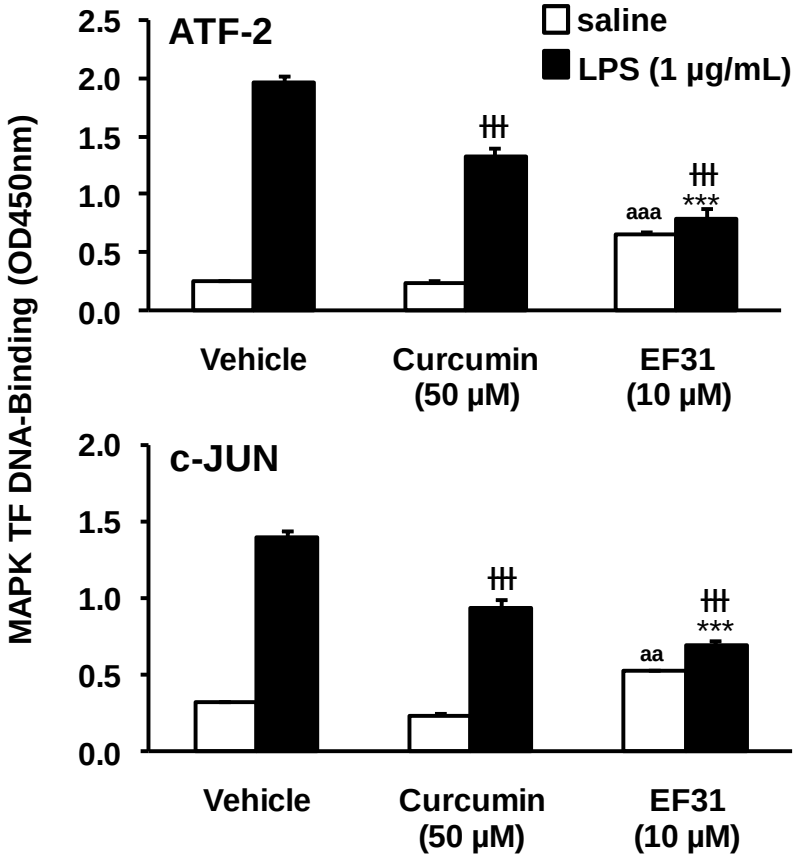


Figure 2-15. EF31 inhibits MAPK transcription factor DNA-binding activity. Mouse RAW264.7 macrophages were pre-treated with curcumin (50 µM), EF31 (10 µM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) or saline for 15 minutes. Nuclear proteins were collected as described in Methods and transcription factor DNA-binding was measured using a DNA-binding ELISA. All conditions were run in triplicate, and values shown are means (±SEM). *** $p < 0.001$ vs. curcumin + LPS; ǂ ǂ ǂ $p < 0.001$ vs. vehicle + LPS; aa $p < 0.01$, aaa $p < 0.001$ vs. vehicle + saline.

Chapter 3

Animal model of LPS-induced depressive-like behaviors: measures of immune response and behavioral alterations

I. Introduction

Depression is a common disorder with a lifetime prevalence of over 15% (Kessler et al., 2003). The World Health Organization has identified depression as one of the leading causes of disability worldwide (Lopez and Murray, 1998). The psychological symptoms of depression are not the only factors affecting the quality of life of the depressed patient. Major and minor depression can also have notable immunological consequences that may directly or indirectly result in immune-suppression as well as increased immune system activity, particularly increased innate immune (inflammatory) functioning (Kronfol, 2002; Raison et al., 2006; Irwin and Miller, 2007). This increase in inflammation may have clinical implications in terms of cardiovascular disease, diabetes and cancer, where inflammation is increasingly being recognized as a common mechanism of disease (Black and Garbutt, 2002; Coussens and Werb, 2002; Dandona et al., 2004; Wellen and Hotamisligil, 2005; Libby, 2006; Libby and Plutzky, 2007; Vaccarino et al., 2007). Inflammation may also contribute to the maintenance of depressive symptoms (Miller et al., 2009).

The body of evidence supporting a link between inflammation and depression in humans continues to grow. Building evidence suggests that a complex network links the immune system with the CNS in a bi-directional fashion. Innate immune pro-inflammatory cytokines have access to the brain and modulate pathways known to be involved in the pathophysiology of depression, including neurotransmitter function, neuroendocrine function, and synaptic

plasticity (Figure 3-1) (Miller et al., 2000; Raison et al., 2006; Irwin and Miller, 2007; Miller et al., 2009). For example, administration of pro-inflammatory cytokines [i.e. tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-2 and interferon (IFN)-α] to laboratory rodents and humans has been shown to alter mood and this has been correlated with changes in the metabolism of the monoamines, serotonin, norepinephrine, and dopamine in brain regions involved in mood regulation (Linthorst et al., 1995; Maes et al., 1997; Pauli et al., 1998; Song et al., 1999; Yirmiya et al., 1999; Reichenberg et al., 2002; Felger et al., 2007; Raison et al., 2009; Felger et al., 2011). Moreover, pro-inflammatory cytokines can modulate the hypothalamic-pituitary-adrenal (HPA) axis by inducing the release of corticotropin releasing hormone (CRH) and interfering with glucocorticoid receptor (GR)-mediated HPA negative feedback and anti-inflammatory effects (Besedovsky and Del Rey, 2000; Silverman et al., 2003; Silverman et al., 2005; Raison et al., 2008; Hu et al., 2009). Data also indicate that pro-inflammatory cytokines directly, and indirectly (via CRH), alter expression of growth factors including brain-derived neurotrophic factor (BDNF), thereby suppressing synaptic plasticity (i.e. neurogenesis and survival) (Raison et al., 2006; Koo and Duman, 2008; McNally et al., 2008; Spedding and Gressens, 2008; Miller et al., 2009).

Reliable models of inflammation are needed in order to further understand how inflammatory processes impact the central nervous system and behavior. Lipopolysaccharide (LPS) is an inflammatory agent that is widely used to examine cytokine-induced behavioral changes in humans and laboratory rodents

(Yirmiya et al., 2000; Guha and Mackman, 2001; Frenois et al., 2006; Dantzer and Kelley, 2007). LPS is a component of the cell wall of gram-negative bacteria and the structure contains an O polysaccharide/ O antigen, a core oligosaccharide, and Lipid A (Figure 3-2). The hydrophobic Lipid A attaches the LPS to the bacterial membrane, and it is also what activates immune cells (Raetz et al., 2009). When injected systemically, LPS induces an acute but potent innate immune/ inflammatory response. LPS activates TLR4 on the surface of mononuclear myeloid cells, which induces a cascade of signaling molecules that lead to the activation of the nuclear factor-κB (NF-κB) and mitogen activated protein kinase (MAPK) pathways (Figure 3-3) (Chow et al., 1999; Lu et al., 2008). NF-κB and MAPK pathways up-regulate expression of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, and IL-6), chemokines [i.e. monocyte chemotactic protein (MCP)-1], adhesion molecules, acute phase proteins (i.e. C-reactive protein), inducible factor enzymes [i.e. inducible nitric oxide synthase (iNOS)], major histocompatability (MHC) proteins, and cyclooxygenase-2 (COX-2) (Table 3-1) (Baeuerle and Baltimore, 1996).

In addition to inflammatory changes, a single administration of LPS is also known to induce profound changes in behavior (Figure 3-4) (Dantzer, 2006; Frenois et al., 2006; Dantzer and Kelley, 2007; Dantzer et al., 2008). Behavioral changes that take place after a single LPS injection and up to 24 hours post injection are termed sickness behaviors. Sickness behaviors include weakness, psychomotor retardation, cognitive impairments sleep alterations, reduced food intake and weight loss. Sickness behaviors in rodents are usually measured

between 2 and 8 hours after treatment with LPS. Depressive-like behaviors are apparent after sickness behaviors have abated and the stimulus such as LPS is no longer present. Characteristics of depressive-like behaviors also includes suppression of exploration and social activity, reduced grooming or body care activities, and symptoms of anhedonia. Depressive-like behaviors are usually measured between 21 and 72 hours in laboratory rodents.

LPS-induced peripheral immune activation has several paths (described in Chapter 1) by which to communicate and translate the inflammatory signaling to the brain (Maier et al., 1998; Singh and Jiang, 2004; Quan and Banks, 2007). NF-κB induced by LPS has been shown to be critically involved in the translation and amplification of the inflammatory signal in the brain. Indeed, inhibition of NF-κB signaling subsequent to treatment of mice with LPS has been shown to reduced the induction of the early immediate gene, c-Fos, in the brain and blocked the development of behavioral changes following peripheral administration of IL-1β (Nadjar et al., 2005).

The current study was undertaken in order to establish an animal model of LPS-induced activation of NF-κB and behavioral change. A 30 μg/mouse dose of LPS was chosen because it is comparable to doses that have been previously used to induce sickness and depressive-like behaviors while also activating NF-κB (Dunn and Swiergiel, 2005; Frenois et al., 2006; Godbout et al., 2007; O'Connor et al., 2008). LPS-induced NF-κB DNA-binding and other inflammatory immune markers and their relation to behavioral alterations were examined.

II. Methods

Mice

Adult male C57BL/6 mice (ages 6-8 weeks, 18-25g) were purchased from Charles River Laboratories (Wilmington, Mass.), and were individually housed in the Emory University Animal Care Facility for 14 days with a 12:12 hours reverse light:dark cycle (lights on at 2100 hr) prior to testing. Water and rodent chow were made available *ad libitum*. All mice were handled, tested, and sacrificed during the dark phase of the cycle using red lights. Mice showing reduced feeding, weight, or grooming, as well as any signs of infection or tissue damage were excluded from the studies prior to testing. Protocols were in accordance with institutional guidelines for animal care and use.

Treatment

Mice were randomly assigned to a treatment group (saline or LPS). LPS (Escherichia coli O55:B5) was obtained from Sigma (St. Louis, MO), suspended in saline (9% sodium chloride), and administered via intraperitoneal injection (i.p.) at a dose of 30 µg/mouse, in a total volume of 100 µL. Control animals received an i.p. injection of 100 µL sterile saline. For experiments testing the effects of NF-κB pathway inhibition, NEMO binding domain (NBD) inhibitory peptide coupled to the protein transduction sequence HIV-TAT was used. The coupling of the inhibitory protein with TAT facilitates cerebral uptake (Nijboer et al., 2008). TAT-NBD (YGRKKRRQRRR-TALDWSWLQTE) (administered i.p. at 20 mg/kg/100

µL), and control peptide TAT-NBD^{mut} (YGRKKRRQRRRTALDASALQTE) (administered i.p. at 20 mg/kg/100 µL) were purchased from the W.M. Keck facility (Yale University, New Haven, Ct.). All peptides were dissolved in DMSO (40 mg/mL) (Sigma) and diluted in PBS. Peptides were administered either one hour prior to or 12 hours post LPS or saline treatment.

Open-field test

Six or 24 hours after treatment with LPS or saline, mice were placed individually in the center of an open field apparatus (Plexiglas box- 40 X 40 X 30 cm3, with opaque plastic walls), and movements were recorded on video for a total of 5 minutes. Total distance moved in the entire arena during the 5 minutes was analyzed using Ethovision (Noldus, Leesburg, VA). Distance was tracked using the color subtraction method, detecting objects darker than the background. Each mouse was immediately returned to its home-cage after testing. All mice were tested at both time-points (6 and 24 hours).

Porsolt forced swim test

Twenty four hours after treatment with LPS or saline, mice were placed in a cylinder (diameter 16 cm, height 31 cm, water depth 20 cm) of tepid water (25±1 C) and were then observed and videotaped for 7 minutes. Following a habituation session (2 minutes), the amount of time swimming and the amount of time immobile was determined for 5 minutes using Observer (Noldus, Leesburg, VA). The swim test cylinder was cleaned between each test. A mouse was

judged to be immobile when it remained motionless, with only small movements to keep its head above water. Videotaped behavior was rated by an observer blind to LPS treatment.

Sucrose preference test

All mice were allowed access to both regular tap water and a 2% sucrose solution for five days before LPS administration to establish a baseline sucrose preference for each mouse. The sucrose solution was then available for an additional three days after LPS or saline treatment. A fresh batch of sucrose solution was made daily with tap water and sucrose powder (Sigma # 47289). The relative position of bottles was changed daily to avoid development of a place preference. The amount of tap water and sucrose solution consumed at each time-point (24, 48, and 72 hours) was recorded, and the grams of sucrose solution consumed per gram of body weight provided an indication of sucrose preference.

Social interaction test

Baseline social interaction was measured the day before treatment, and then again at 6, 24, or 48 hours after treatment with LPS or saline. The test consisted of placing a novel male juvenile mouse (C57BL/6, 2-3 wks old) in the home cage of a subject for a period of 10 minutes. A video camera was mounted over the subject's home cage for recorded behavioral analysis. A different juvenile was used for each testing session for a given subject. Novel juveniles

were only used in this assessment once per day. Separate groups of mice were used for each time-point (6 or 24 hours) to prevent habituation to the test. Social behavior was quantified as the amount of time the mouse interacts with the juvenile (e.g., anogenital sniffing, trailing, grooming, body contact, mounting, and attack) using Observer (Noldus). Results are expressed as the amount of time the mouse is engaged in social behaviors compared to baseline (pre-LPS) measures of interaction.

Assessment of NF-κB DNA-binding

Peripheral (spleen) and brain tissues (hippocampus, prefrontal cortex, and hypothalamus) were collected at different time-points (0.5- 24 hours) after treatment with LPS or saline. Of note, these brain regions have been selected on the basis that both pro-inflammatory cytokine production and receptors are present and their association with behavioral abnormalities in depressed patients (Ban et al., 1991; Laye et al., 1994; Besedovsky and del Rey, 1996; Capuron et al., 2002; Frenois et al., 2007; Miller et al., 2009). Nuclear proteins were obtained using an extraction kit from Active Motif (Carlsbad, CA) according to manufacturer's instructions. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL) per manufacturer's instructions. Nuclear protein samples were analyzed in triplicate using the NF-κB DNA-binding ELISA kit (Active Motif).

MCP-1 protein ELISA

Following treatment with LPS or saline, mice were rapidly decapitated under low stress conditions (within 1 minute of exposure to isoflurane anesthesia) at appropriate collection time-points (1-48 hours). Trunk blood was collected into chilled EDTA-coated microfuge tubes, and placed on ice until centrifugation to obtain plasma (10 minutes, 1100 RPM, 4°C). MCP-1 protein concentration in plasma was assayed using a mouse MCP-1/CCL2 ELISA from R&D (Minneapolis, MN) according to manufacturer's recommendations.

Isolation of total RNA and cytokine RT-PCR

Peripheral (spleen) and brain (hippocampus, hypothalamus, and prefrontal cortex) tissue sections were collected at different time-points (1-48 hours) after treatment with LPS or saline, and placed in RNAlater (Qiagen, Hilden, Germany). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by reverse transcription PCR using SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) with random primers, according to manufacturer's protocols. The cDNA was then amplified by RT-PCR (Applied Biosystems, 7500 Fast, Carlsbad, CA). Primers for mouse TNF-α, IL-1β, IL-6, and GAPDH were obtained from Qiagen. GAPdh was not affected by LPS and was used as a house keeping control gene for each plate. For data analysis the deltaCt method whereby fold-changes were calculated as difference in gene expression between LPS and vehicle controls.

Data Analysis

Overall treatment and time effects were determined in each experiment by either a one- or two-way analysis of variance (ANOVA) using GraphPad Prism 5. Significant interactions were followed by post-hoc Bonferroni test to determine differences between specific groups of interest. An α level of $p < 0.05$ was used in all statistical tests.

III. Results

LPS-induced alterations in the open field test in male C57BL/6 mice

To measure changes in motor activity as a result of LPS (Figure 3-5), mice (n= 6/ treatment group) were injected with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL), returned to the home cage, and after 6 hours they were placed in a rectangular enclosure for 6 minutes. The same test was administered again at 24 hours post LPS administration. Motor behavior was recorded for analysis using Noldus Ethovision. LPS decreased total distance moved and increased immobility at 6 hours. However, at 24 hours there were no significant effects of LPS on motor activity. Data analysis revealed a significant treatment x time interaction, and a post-hoc test showed a significant decrease in distance moved and increased immobility at 6 hours (LPS vs. saline, $p< 0.05$). In addition, there was a significant increase in distance moved and decreased immobility at 24 hours (LPS treated group at 24 hours vs. 6 hours, $p< 0.05$).

LPS-induced alterations in the Porsolt forced swim test in male C57BL/6

mice

To measure LPS-induced behavioral changes in the Porsolt forced swim test, a different cohort of male C57BL/6 mice (n= 8/ treatment group) were treated with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL) and tested at either 6 or 24 hours post LPS (Figure 3-6). The mice were placed in a glass cylinder filled with water (25 C), and behavior was recorded. Immobility was scored after the first 2 minutes of habituation. Mice treated with LPS showed a significant increase in immobility relative to saline-treated animals at 24 hours. Data analysis revealed a significant treatment x time interaction ($F [1, 28] = 5.2$, $p=0.030$), and a post-hoc test showed a significant increase in immobility in the LPS treated group at 24 hours ($p< 0.001$).

LPS-induced alterations in the sucrose preference test in male C57BL/6 mice

To measure changes in sucrose preference, male C57BL/6 mice (n= 6/ treatment group) were provided ad libitum access to both tap water and a 2% sucrose solution for five days prior to behavioral testing. Following the five days habituation period all mice showed a preference for the 2% sucrose solution as measured by drinking volume (Figure 3-7A). On the 6^{th} day, mice were injected with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL). Sucrose solution consumption relative to body weight was measured 24, 48, and 72 hours post LPS administration. Although there were no significant differences in tap water intake (Figure 3-7B), mice treated with LPS displayed a marked decrease in

sucrose solution consumption at 24 hours post treatment (Figure 3-7C). At 48 hours post injection, the LPS-treated group still showed a decrease in sucrose water consumption, and this effect lasted until 72 hours post LPS treatment, albeit there was a steady increase in sucrose solution consumption over time. Moreover, body weight also decreased during the first 24 hours but recovered steadily (Figure 3-7D). In the sucrose preference test, data analysis revealed a significant treatment x time interaction ($F [3, 44] = 12.8$, $p< 0.0001$), and a post-hoc test showed a significant decrease in sucrose solution consumption in the LPS treated group at 24 and 48 hours ($p< 0.001$).

LPS-induced alterations in the social interaction test in male C57BL/6 mice

To measure changes in social behaviors, baseline social interaction with a juvenile male C57BL/6 mouse was measured the day before treatment, and then again at 6, 24, or 48 hours after treatment with LPS or saline for a period of 10 minutes (n= 7/ treatment group). Social behavior was quantified using Observer (Noldus). Results are expressed as the amount of time the mouse engaged in social behaviors compared to baseline (pre-LPS) measures of interaction. Mice treated with LPS exhibited a significant decrease in social interactions at 6 hours post LPS (Figure 3-8). However, by 24 hours, social interactions recovered to nearly baseline levels. Data analysis revealed a significant treatment x time interaction ($F [3, 44] = 6.5$, $p= 0.0010$), and a post-hoc test showed a significant decrease in social interaction in the LPS treated group at 6 hours ($p< 0.001$).

LPS-induced NF-κB DNA-binding time course

To determine if NF-κB activity is present prior to LPS-induced behavioral alterations, male C57BL/6 mice (n= 6/ treatment group) were treated with either LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL), and sacrificed at 0.5, 1, 3, 6, or 24 hours post injection. Spleen, hippocampus, hypothalamus, and prefrontal cortex were dissected and used to generate nuclear extracts. The nuclear extracts were then analyzed using a NF-κB DNA-binding ELISA. Compared to saline-treated animals, NF-κB DNA-binding in the spleen and the brain of LPS treated mice was increased 30 minutes after injection and peaked at one hour post LPS administration (Figure 3-9), returning to baseline by 24 hours. Data analysis revealed a significant treatment x time interaction in the spleen, hippocampus, prefrontal cortex, and hypothalamus ($F [4, 43] = 2.655$, $p= 0.0457$, $F [4, 43] = 7.251$, $p= 0.0001$, $F [4, 42] = 6.806$, $p= 0.0003$, $F [4, 42] = 6.035$, $p= 0.0006$, respectively), and a post-hoc test showed a significant increase in NF-κB DNA-binding activity in the LPS treated group at one hour ($p< 0.001$) in all tissues collected. A second experiment measured NF-κB DNA-binding in the spleen and hippocampus at 12, 15, 18, 21, and 24 hours post LPS or saline injection (n=6/ treatment group) (Figure 3-10). Statistical analysis revealed no significant difference between treatment groups at any time-point.

LPS-induced cytokine mRNA expression time course

To determine if pro-inflammatory cytokine mRNA expression is active during sickness and depressive-like behaviors, male C57BL/6 mice (n= 6/

treatment group) were treated with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 1, 3, 6, 12, 24, or 48 hours before collection of spleen, hippocampus, hypothalamus, and prefrontal cortex. Total mRNA was then extracted from tissues and expression patterns of TNF-α, IL-1β, and IL-6 were examined with RT-PCR. Treatment with LPS significantly elevated expression of all cytokines in all the tissues collected relative to saline controls, peaking at one hour in the spleen and at 3 hours in the brain (Figures 3-11A-D). In the spleen, expression of all cytokines returned to saline levels by 12 hours post LPS. In contrast, in the brain, the expression of TNF-α and IL-1β showed a second peak at 24 hours post LPS. However, IL-6 mRNA did not show a second peak, returning to saline levels by 12 hours. Data analysis revealed a significant treatment x time interaction in the spleen, hippocampus, prefrontal cortex, and hypothalamus for TNF-α ($F [5, 51] = 33.88$, $p< 0.0001$, $F [5, 50] = 5.055$, $p= 0.0008$, $F [5, 50] = 9.097$, $p< 0.0001$, $F [5, 50] = 5.242$, $p= 0.0006$, respectively), and a post-hoc test showed a significant increase in mRNA expression in the LPS treated group at 1 and 3hours in the spleen ($p< 0.001$ and $p< 0.05$, respectively), and at 3, 6, and 24 hours in the brain ($p< 0.001$). For IL-1β mRNA expression, a significant treatment x time interaction was found in the spleen, hippocampus, prefrontal cortex, and hypothalamus ($F [5, 51] = 28.02$, $p< 0.0001$, $F [5, 50] = 2.704$, $p= 0.0307$, $F [5, 50] = 4.661$, $p= 0.0014$, $F [5, 50] = 7.591$, $p< 0.0001$, respectively), and a post-hoc test showed a significant increase in mRNA expression in the LPS treated group at one hour in the spleen ($p< 0.001$), at 1 and 3 hours in the hippocampus ($p< 0.001$), and at 3 and 6 hours in the

prefrontal cortex and hypothalamus ($p< 0.001$). For IL-6 mRNA expression, a significant treatment x time interaction was found in the spleen, hippocampus, prefrontal cortex, and hypothalamus ($F [5, 51] = 2.710$, $p= 0.0302$, $F [5, 50] = 10.39$, $p< 0.0001$, $F [5, 50] = 8.034$, $p< 0.0001$, $F [5, 50] = 8.577$, $p< 0.0001$, respectively), and a post-hoc test showed a significant increase in mRNA expression in the LPS treated group at 3 hours in the spleen and the brain ($p< 0.01$).

LPS-induced MCP-1 time course

To assess whether recruitment of monocytes and other immune cells to the brain may be related to the second peak of cytokine mRNA expression, male C57BL/6 mice (n= 6/ treatment group) were treated with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 1, 3, 6, 12, 24, or 48 hours before collection of trunk blood. MCP-1 protein concentration in plasma was assayed using a mouse MCP-1/CCL2 ELISA. MCP-1 protein peaked at 3 hours and returned to baseline by 24 hours (Figure 3-12). Data analysis revealed a significant treatment x time interaction ($F [5, 48] = 19.57$, $p< 0.0001$), and a post-hoc test showed a significant increase in plasma MCP-1 protein in the LPS treated group at 3 and 6 hours ($p< 0.001$).

Effects of NEMO inhibitor on TNF-α mRNA expression in the hippocampus

To examine the role of the NF-κB pathway in the second peak of cytokine mRNA expression, one group of male C57BL/6 mice (n= 5/ treatment group)were

treated with either TAT-NBD (i.p./20 mg/kg/100 µL/mouse), or control peptide (i.p./20 mg/kg/100 µL/mouse), one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). A second group of mice (n= 5/ treatment group) were treated with the peptides 12 hours post LPS or saline injection. Hippocampal tissue was collected at 24 hours post LPS injection and mRNA was extracted to measure expression of TNF-α using RT-PCR. Mice treated with TAT-NBD one hour prior to LPS showed a decrease in TNF-α mRNA expression, but not the mice treated 12 hours after LPS (Figure 3-13). Data analysis revealed a significant treatment effect (LPS vs. Saline) ($F [1, 17] = 31.52$, $p< 0.0001$), and a post-hoc test showed a significant difference between LPS and Saline in groups treated with the control peptide ($p< 0.05$) and TAT-NBD at 12 hours ($P< 0.01$).

IV. Discussion

Results presented here demonstrate that a single dose LPS treatment induces sickness and depressive-like behaviors at 6 and 24 hours after treatment, respectively. These behavioral changes were accompanied by systemic and central innate immune/ inflammatory alterations including increases in NF-κB DNA-binding activity peaking at one hour, pro-inflammatory cytokine mRNA expression (TNF-α, IL-1β, and IL-6) peaking at 3 hours and a second peak of TNF-α and IL-1β expression at 24 hours. Moreover, plasma MCP-1 protein peaked at 3 hours. A second peak of NF-κB DNA-binding was not

present between 12 and 24 hours but when TAT-NBD was administered prior to LPS, it significantly reduced TNF-α mRNA expression in the hippocampus at 24 hours. Taken together these results indicate that LPS-induced immune activity parallels the sickness behaviors and depressive-like behaviors in-vivo.

Motor activity was disrupted at 6 hours after treatment with LPS, which is typical of sickness behaviors and has been attributed to inflammation-induced pain, fever, and fatigue (Dantzer, 2001; Dantzer, 2004; Dantzer, 2006; Dantzer and Kelley, 2007). Moreover, mice exhibited reduced body weight which is consistent with previous reports showing that LPS suppresses ingestive behaviors (Yirmiya, 1996; Dantzer and Kelley, 2007). By 24 hours, however, motor activity recovered concurrent with a significant increase in immobility scores in the Porsolt forced swim test. These results suggests that motor activity does not account for the increase in immobility scores. The Porsolt forced swim test is an inescapable and stressful situation that has been used to test the effectiveness of antidepressant compounds. Behavioral changes in the Porsolt forced swim test have been interpreted as a measure of learned helplessness and reduced motivation (West, 1990; Contreras et al., 2001; Dantzer, 2006; Dantzer and Kelley, 2007), both of which are considered analogous to key features of major depression. Of note, immobility scores in the forced swim test can be reduced by treatment with conventional antidepressants (Porsolt et al., 1977; Cryan et al., 2005; Dantzer et al., 2008).

A second measure of depressive-like behaviors in the current studies was the sucrose preference test (Yirmiya, 1996; Dantzer et al., 2008). Consumption

of sucrose solution was reduced for up to 72 hours after treatment with LPS. However, tap water consumption was not affected, suggesting that the reduction in sucrose preference may reflect an impaired sensitivity to rewarding stimuli. These results are commonly interpreted as a sign of anhedonia in the literature, but an alternative interpretation that has also been proposed is conditioned taste aversion (CTA) (Tazi et al., 1988; Weingarten et al., 1993; Yirmiya, 1996; Dantzer, 2001). Indeed, presentation of the sucrose solution followed by an LPS injection has been shown to induce CTA (Tazi et al., 1988; Weingarten et al., 1993). Nevertheless, presentation of the sucrose solution hours after an LPS injection also shows similar decreases in sucrose preference, much like the results presented here (Yirmiya, 1996). In addition, pre-exposure to the sucrose solution has been shown to block formation of CTA (Revusky and Bedarf, 1967; Yirmiya, 1996). In the present study, mice had access to sucrose solution for 5 days prior to LPS administration. Finally, LPS did not completely abolish the intake of sucrose solution. Sucrose preference was significantly reduced after LPS treatment, but then showed a steady increase starting 24 hours after LPS. If it were the case that the mice developed an aversion to the sucrose solution, a recovery of preference would require extinction, which in turn, requires time. Since mice continued ingesting the sucrose solution, it suggests that an aversion may not account for the reduction. It should also be noted that although the effects of LPS on sucrose preference were evident for up to 72 hours, body weight recovered steadily, suggesting that there is an independent recovery of ingestive behaviors (i.e. food and water intake). Similar to the forced swim test,

pre-treatment with conventional antidepressants has been shown to attenuate or block LPS-induced behavioral alterations in the sucrose preference test (Willner et al., 1987; Muscat et al., 1992; Yirmiya, 1996).

Our results do not completely agree with prior reports on the effects of LPS on performance in the social interaction test. Specifically, mice treated with LPS showed a significant decrease in social interactions at 6 hours, but not at 24 hours. Decreased social interaction at 6 hours is considered a sickness behavior and attributed to the same factors affecting motor behavior (Dantzer et al., 2008). However, previous studies have also shown that the effects of LPS on social interaction can last up to 24 hours, albeit with a higher dose (100 μg) than the one used here (30 μg) (Yirmiya, 1996). Nonetheless, a 10 μg dose of LPS also failed to elicit reduced social interactions at 24 hours in mice (Godbout et al., 2005). This suggests that a higher dose of LPS (~100 μg) may be required to induce changes in social interaction at 24 hours. Likewise, treatment with an anti-inflammatory agent such as α-tocopherol or a conventional antidepressant such as imipramine attenuates or blocks LPS-induced alterations in social behaviors (Yirmiya, 1996; Godbout et al., 2005; Henry et al., 2008).

The behavioral changes presented in the results have been linked to innate immune activation, including NF-κB pathway activity and induction of pro-inflammatory cytokine mRNA expression. Indeed, attenuated behavioral responses to LPS have been reported in IL-1β knockout mice (Bluthé et al., 2000). NF-κB has been identified as a key signaling molecule involved in the expression of pro-inflammatory cytokines and the amplification of inflammatory

responses in the periphery and the brain. Interestingly, as evidenced in Figure 3-9, NF-κB activity peaks early (one hour) in both the periphery and brain. This suggests that the inflammatory response in the periphery is rapidly communicated to the brain in a manner that maintains a similar time course of NF-κB activation in periphery and central nervous system. Moreover, consistent with the literature, cytokine mRNA expression paralleled the behavioral alterations. TNF-α, IL-1β, and IL-6 peaked in the spleen at one hour and in the brain at 3 hours (a time when sickness behaviors are displayed), suggesting that although NF-κB activity happens at a similar time course in the periphery and the brain, the brain requires a longer time to amplify that inflammatory response via expression of cytokine mRNA.

In addition to the first peak of cytokine mRNA expression, there was a second peak of TNF-α and IL-1β in the brain at 24 hours. By this time, cytokine mRNA expression in the periphery has long ceased, suggesting that it is no longer driving the central inflammatory response. The finding of a second LPS-induced peak of cytokine mRNA in the brain that parallels depressive-like behaviors and is independent of peripheral inflammation and sickness behaviors is novel. There are currently no reports of a second peak at 24 hours in this model of cytokine-induced depressive-like behaviors. Nonetheless, a bi-phasic response in cytokine mRNA expression has been reported in other models. For example, mice treated with a 50 μg (intraveneous) injection of LPS resulted in a peak of cytokine mRNA in peripheral tissues (including liver, spleen, and lungs) of mice between 1 and 3 hours and a second peak between 10 and 12 hours

(Han et al., 2002). Blockade of inflammatory signaling, including blocking NF-κB pathway activity, cytokine production, or cytokine receptor signaling has been shown to attenuate or inhibit the development of depressive-like behaviors in mice (Bluthé et al., 2000; Yirmiya, 2000; Godbout et al., 2005; Dantzer et al., 2008; Koo et al., 2010).

In terms of the mechanism behind the second peak of cytokine mRNA expression, there are a number of inflammatory pathways that could be involved, including the NF-κB, MAPK, and Janus family tyrosine kinases/ signal transducer and activator of transcription (JAK/STAT) proteins pathways. The first peak of mRNA is a direct response to the LPS challenge, but the second peak could be induced by the lingering inflammatory pathway activity in the brain. For example, inflammation that persists between 8 and 12 hours driven by expression of pro-inflammatory cytokines may be enough to induce a second peak of NF-κB activity. Nevertheless, NF-κB did not show increased activity between 12 and 24 hours, suggesting that it may not be associated with the second peak of cytokine mRNA expression. Interestingly, the same study that reported a second peak of mRNA between 10 and 12 hours after LPS also reported a preceding second peak of NF-κB activity between 8 and 12 hours (Han et al., 2002). Thus, it is possible that NF-κB activity is present between 6 and 12 hours, which we did not measure. If this is the case, it seems unlikely that the mRNA increase would be evident more than 12 hours later (at 24 hours). On the other hand, the NF-κB pathway has self-regulatory mechanisms intended to inhibit its own activity, which could explain the absence of a second peak of NF-κB activity. For

example, NF-κB, in addition to inducing the expression of inflammatory mediators, also induces the expression of the inhibitory protein IκB which blocks NF-κB nuclear translocation (Baeuerle and Baltimore, 1996). Furthermore, experiments in which TAT-NBD was used to block NF-κB activity, it only affected cytokine expression of TNF-α at 24 hours when administered before LPS, and thereby, before the first peak. It should also be noted, that if indeed a second peak of NF-κB activity takes place between 6 and 10 hours post LPS, then TAT-NBD treatment at 12 hours would be ineffective. However, these results suggests that the first peak may be necessary and / or sufficient to induce the second peak of mRNA expression, and that NF-κB may be involved in the first but not the second peak. Taken together, the current studies cannot rule out the possibility of NF-κB activity as a mechanism for the second peak mRNA, which requires further exploration.

It has been previously suggested that much of the inflammatory response in the brain is driven by immune cells that are recruited from the periphery (Hughes et al., 2002; Rankine et al., 2006; Thompson et al., 2008; D'Mello et al., 2009). One chemokine that has been identified in the recruitment of immune cells to the brain is MCP-1 (Thibeault et al., 2001; Charo and Ransohoff, 2006; Semple et al., 2009). MCP-1 can be produced by activated endothelial cells, astrocytes, and microglia, and attracts mainly monocytes/ macrophages and T-lymphocytes. Figure 3-12 shows that MCP-1 plasma levels peaked at 3 hours and returned to baseline levels by 24 hours post LPS. These results suggest that there may be an early recruitment of immune cells throughout the body and into

the brain, which may be sufficient to amplify the low level inflammatory signals between 12-24 hours, and induce a second peak of cytokine mRNA expression. However, whether recruitment of cells is necessary for the second peak remains to be determined.

In summary, LPS induced a behavioral profile that coincides with inflammatory markers. Determining the role and mechanism for the second peak of mRNA may elucidate novel biomarkers and targets for the treatment of depressive-like behaviors.

V. Limitations and Future Directions

The major limitation in this chapter is the NF-κB DNA-binding time course. Although we did not find a second peak of NF-κB DNA-binding activity in the brain that corresponds to the second peak of cytokine mRNA expression, LPS-induced bi-phasic NF-κB activation in peripheral tissues has been previously reported (Han et al., 2002). Given the role that the NF-κB pathway plays in the induction of the inflammatory immune response, it is reasonable to presume that it would be active prior to the second peak. It is possible that NF-κB activity peaks again sometime between 6 and 12 hours which was not included in the time course presented here. A second possibility is that NF-κB peaks for a second time after the second peak of mRNA expression. Cytokine mRNA expression suggests that cytokine protein is produced and released in the brain during this time period. Cytokines receptor pathways such as IL-1β R and TNF-α

R activate NF-κB. Whether NF-κB activity peaks a second time and its relations to the second peak of mRNA expression requires further testing. Moreover, NF-κB is not the only inflammatory pathway activated by LPS or by cytokine expression. The role of the MAPK and JAK/STAT pathways in the cytokine mRNA expression presented here also remains to be determined.

A future direction could involve investigating the role of MCP-1 in the second peak of mRNA expression. MCP-1 protein in the plasma peaked early at one hour and returned to baseline at 12 hours post LPS (Figure 3-12). Activation of microglia and endothelial cells results in the release of TNF-α which in turn promotes the production of MCP-1 (Hayashi et al., 1995). MCP-1 recruits immune cells to the brain which can then amplify the inflammatory response. One possibility that fits with the data presented here is that LPS-induced inflammatory cytokines in the brain (for which mRNA expression peaks at 3 hours) may be driving the MCP-1 expression. MCP-1 recruits immune cells including monocytes to the brain which then help to amplify the inflammatory activity resulting in the second peak of mRNA expression. This would be consistent with the mRNA expression time course in Figure 3-11 A-D showing that there is low level inflammation between 6 and 12 hours post LPS that is then amplified to produce a second peak at around 24 hours. One way to assess the role of MCP-1 in the second peak of mRNA is to use MCP-1 knockout mice. Recruited peripheral immune cells could then be identified by using flow cytometry and immunohistochemistry. It is possible that in MCP-1 knockout mice the first peak of mRNA expression in response to LPS detection is still present,

but the second peak could be blocked in the absence of MCP-1 signaling. This would be consistent with previous publications showing that MCP-1 knockout animals have an attenuated inflammatory response to LPS in the brain (Hughes et al., 2002; Rankine et al., 2006; Thompson et al., 2008).

Another future direction could involve looking at the role of platelet-activating factor (PAF) in the second peak of cytokine mRNA expression. Hans et al. (2008), found that PAF, which is released in response to the LPS injection, activates the early phase of NF-κB activation. This NF-κB activity leads to induction of pro-inflammatory cytokine (TNF-α and IL-1β) expression, which in turn promote the synthesis of PAF. A second peak in PAF is thought to be the mechanism for the second phase of NF-κB activation. Finding the second peak of PAF expression may help to predict the time-point for a second peak in NF-κB activity in the model presented here. Moreover, blocking PAF has been shown to block the second phase of LPS-induced NF-κB activity, and the second peak of cytokine mRNA expression in the periphery (Han et al., 2002), suggesting that the second peak is mediated by PAF and NF-κB activity. It is possible that pinpointing the time-point for the second peak in NF-κB activity in the brain could help to target the second peak of mRNA expression independently of the first peak.

Finally, the role of the second peak of mRNA in depressive-like behaviors remains to be tested. It is possible that it may have a unique relation to the persistent depressive-like behaviors at 24 hours. Future studies could address this question by treating with an anti-inflammatory agent after the LPS challenge

to block only the second peak and measure behavioral changes at 24 hours.

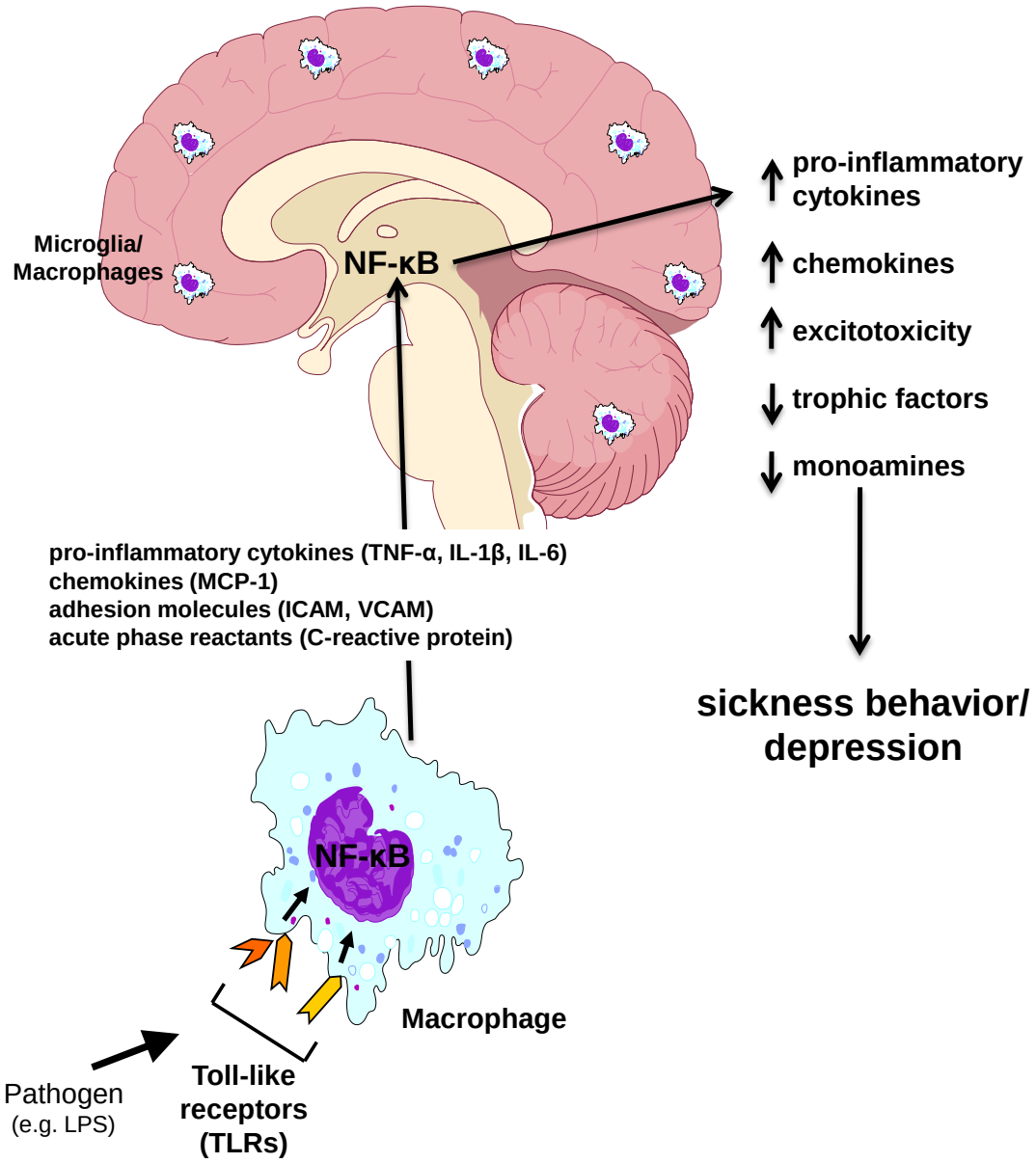


Figure 3-1. Brain-immune interactions. Activation of immune cells in the periphery or central nervous system results in the release of inflammatory mediators that have access to the brain. These inflammatory mediators induce the propagation of the inflammatory signal in the brain via activation of microglia and other cells. An increase in inflammatory mediators has been shown to increase excitotoxicity, decrease the expression of trophic factors, and decrease monoamine availability. These changes have been implicated in the expression of sickness and depressive-like behaviors in laboratory animals.

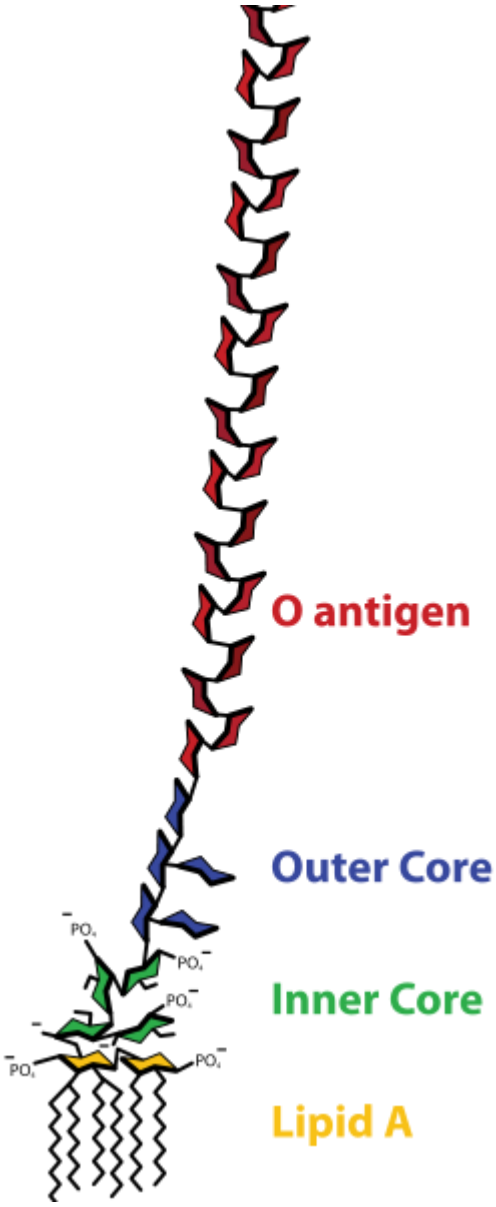


Figure 3-2. LPS structure. LPS is made up of an outer O antigen, an inner core and a hydrophobic Lipid A. Lipid A attaches to the membrane of LPS and is recognized by TLR-4 on the surface of innate immune cells.

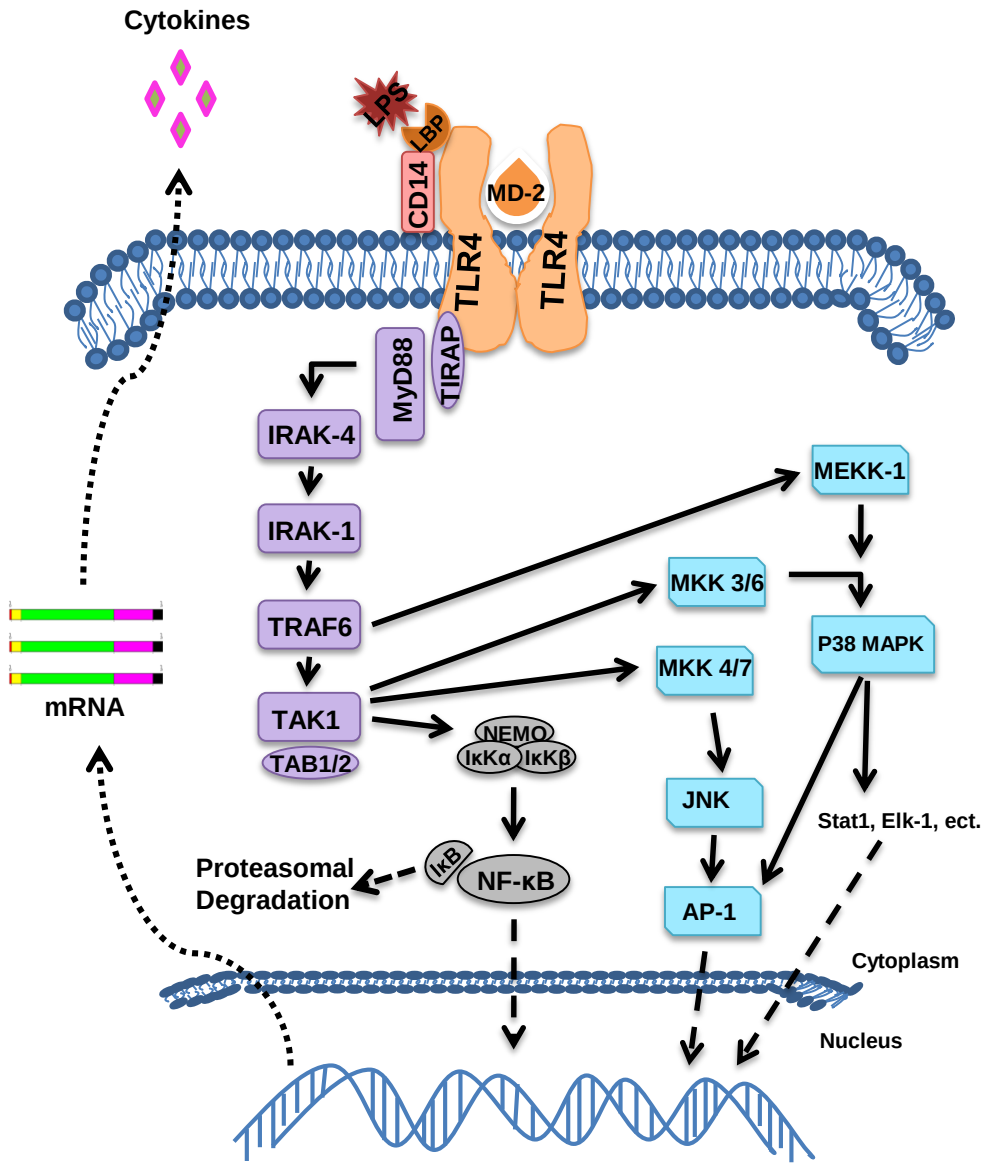


Figure 3-3. LPS-TLR-4 signaling pathway. LPS is a potent inflammatory stimulus that activates several immune inflammatory pathways including NF-κB and MAPK leading to transcription of pro-inflammatory mediators such as cytokines.

NF-κB pathway	LPS signaling
NF-κB p100, p105, c-rel, IκB α and β	MYD88, MD-2, TLR-2, TLR-4, and CD14
Cytokines	**Chemokines**
IL-1 α and β, TNF-α and β, IL-6, IL-12, IL-18, IFN-β1	MIP 1 α and β, MIP 2 α, CCL2/MCP-1, MCP-3, CCL3, CCL-4, CCL-8, CCL5, CCL7, CCL9, CXCL16, CX3CL1, CXCL9, CXCL11
Adhesion	**Pro-apoptotic**
ICAM-1, VCAM-1	Fas-LR, PTGES, TNFR1
Other	
MHC III, Heat shock protein, iNOS, COX-2, M-CSF, CD40, CD69, CD83, and STAT1, C-reactive protein	

Table 3-1. LPS-induced gene expression. Genes listed here are involved in the inflammatory immune response.

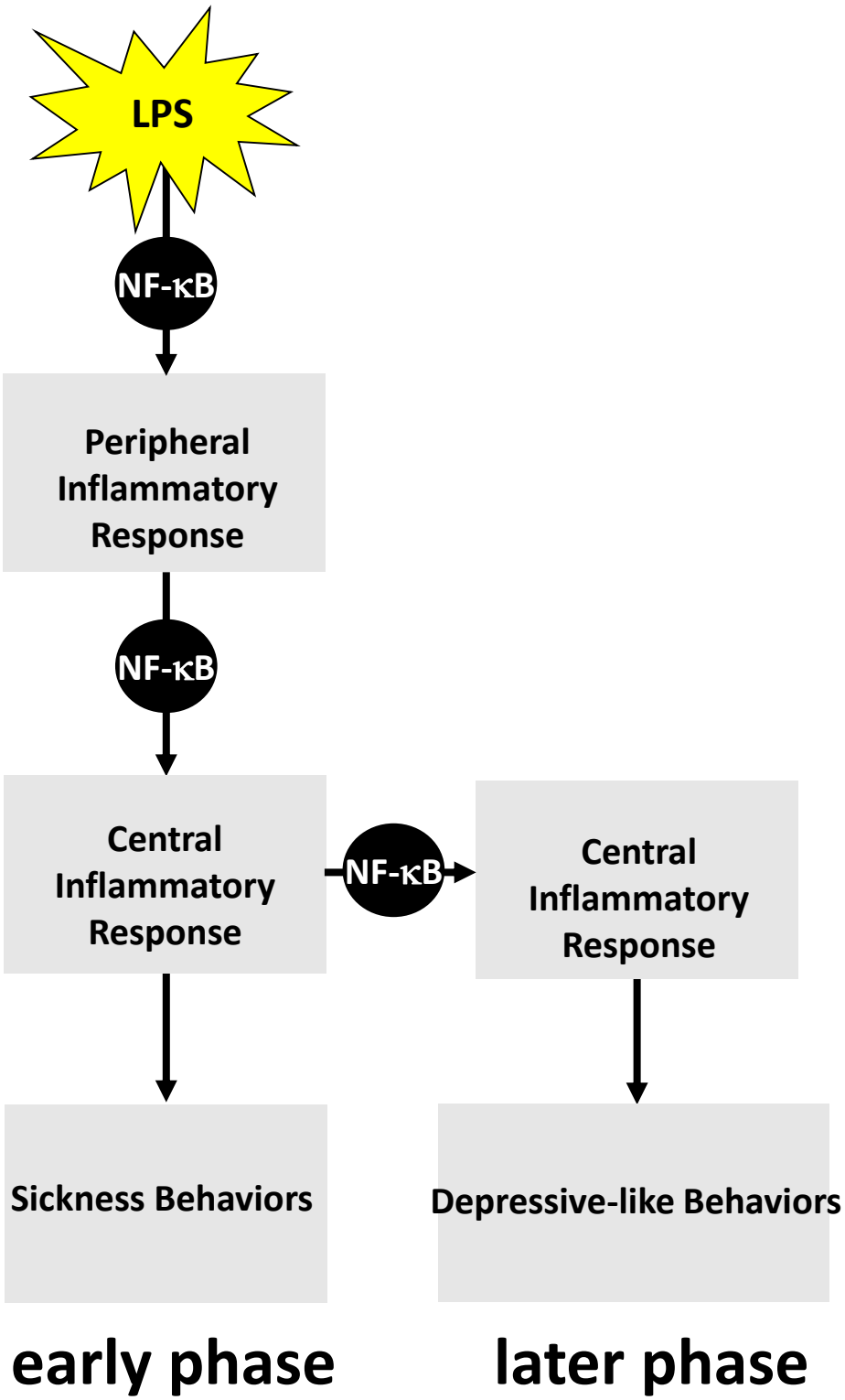


Figure 3-4. LPS-induced immune and behavioral effects. Treatment with LPS induces a bi-phasic immune and behavioral response.

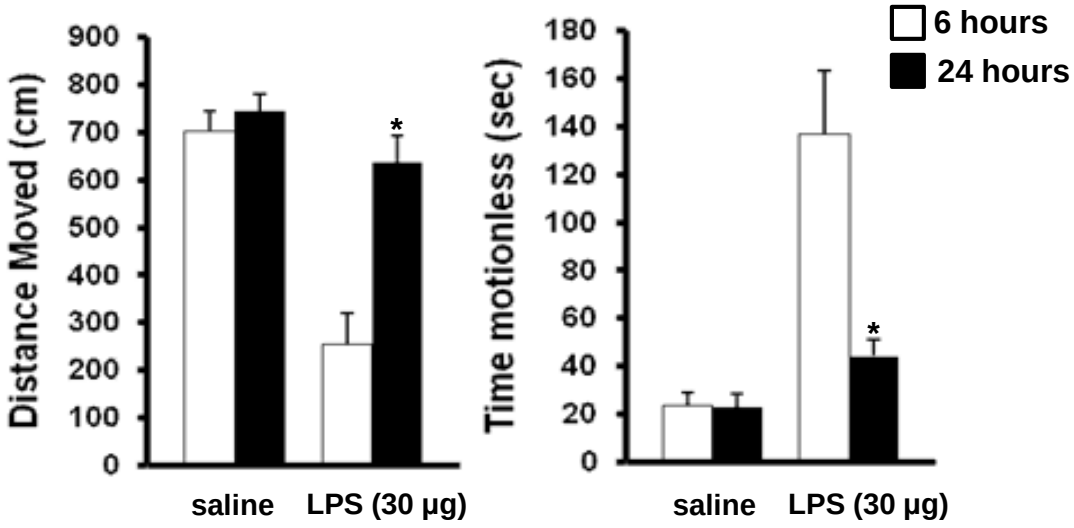


Figure 3-5. LPS-induced alterations in the open field test in male C57BL/6 mice. Mice (n= 6/ treatment group) were injected with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL) and placed individually in the center of an open field apparatus (Plexiglas box- 40 X 40 X 30 cm3, with opaque plastic walls) at 6 and 24 hours post LPS. Movements were recorded on video for a total of 5 minutes and total distance moved in the entire arena was analyzed using Ethovision. Values shown are means (±SEM). * $p < 0.05$ vs. LPS at 6 hours.

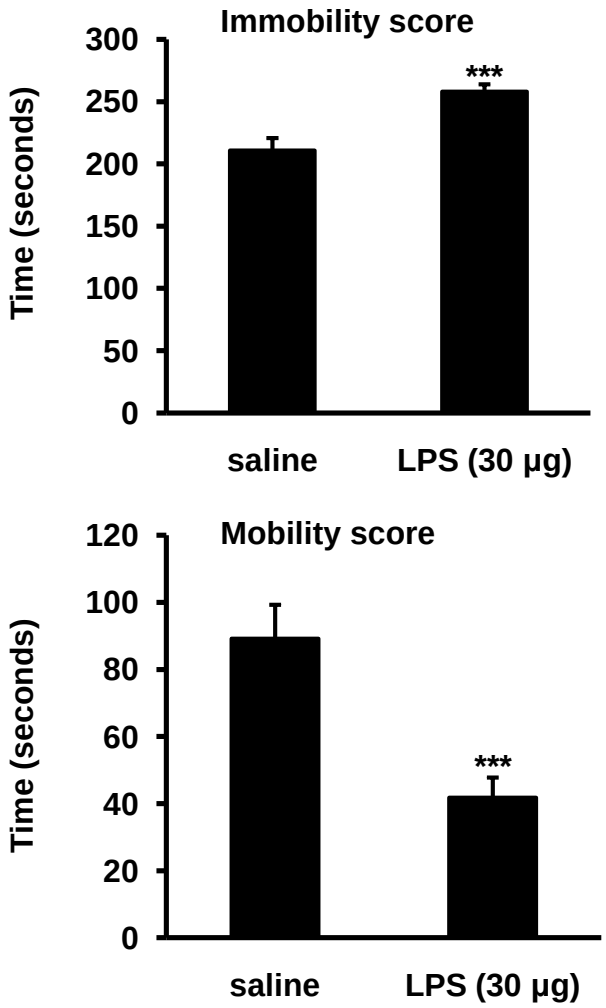


Figure 3-6. LPS-induced alterations in the Porsolt forced swim test in male C57BL/6 mice. Mice (n= 8/ treatment group) were injected with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL) and tested at either 6 or 24 hours post LPS. The mice were placed in a glass cylinder filled with water (25 C) and behavior was recorded. Immobility was scored after the first 2 minutes of habituation. Values shown are means (±SEM). *** $p < 0.001$ vs. saline.

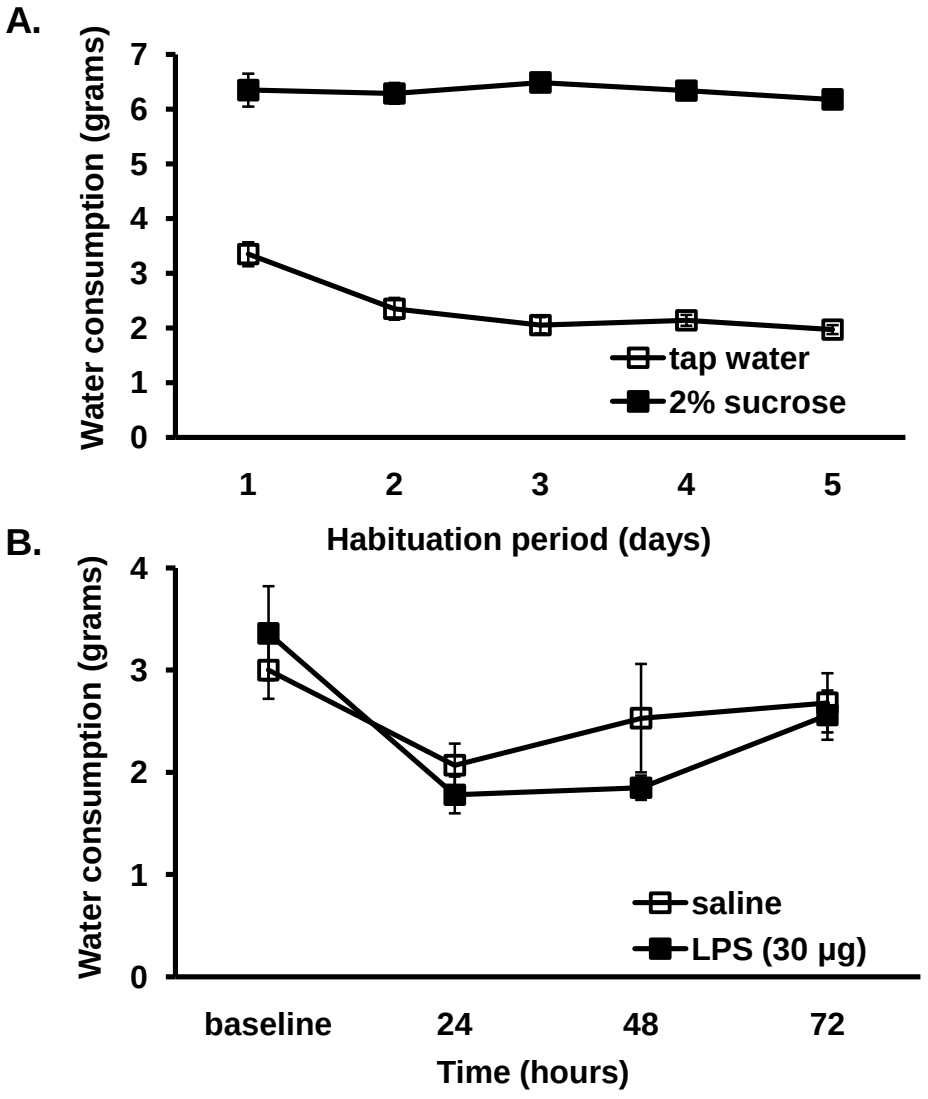


Figure 3-7. LPS-induced alterations in the sucrose preference test in male C57BL/6 mice. A) Mice (n= 8/ treatment group) were provided ad libitum access to both tap water and a 2% sucrose solution for five days prior to behavioral testing. Following the five days habituation period all mice showed a preference for the 2% sucrose solution as measured by drinking volume. B) On the 6th day, mice were injected with LPS (i.p./30 µg /100 µL) or saline (i.p./100 µL). There were no significant changes in tap water consumption between the saline and LPS treated groups. Values shown are means (±SEM).

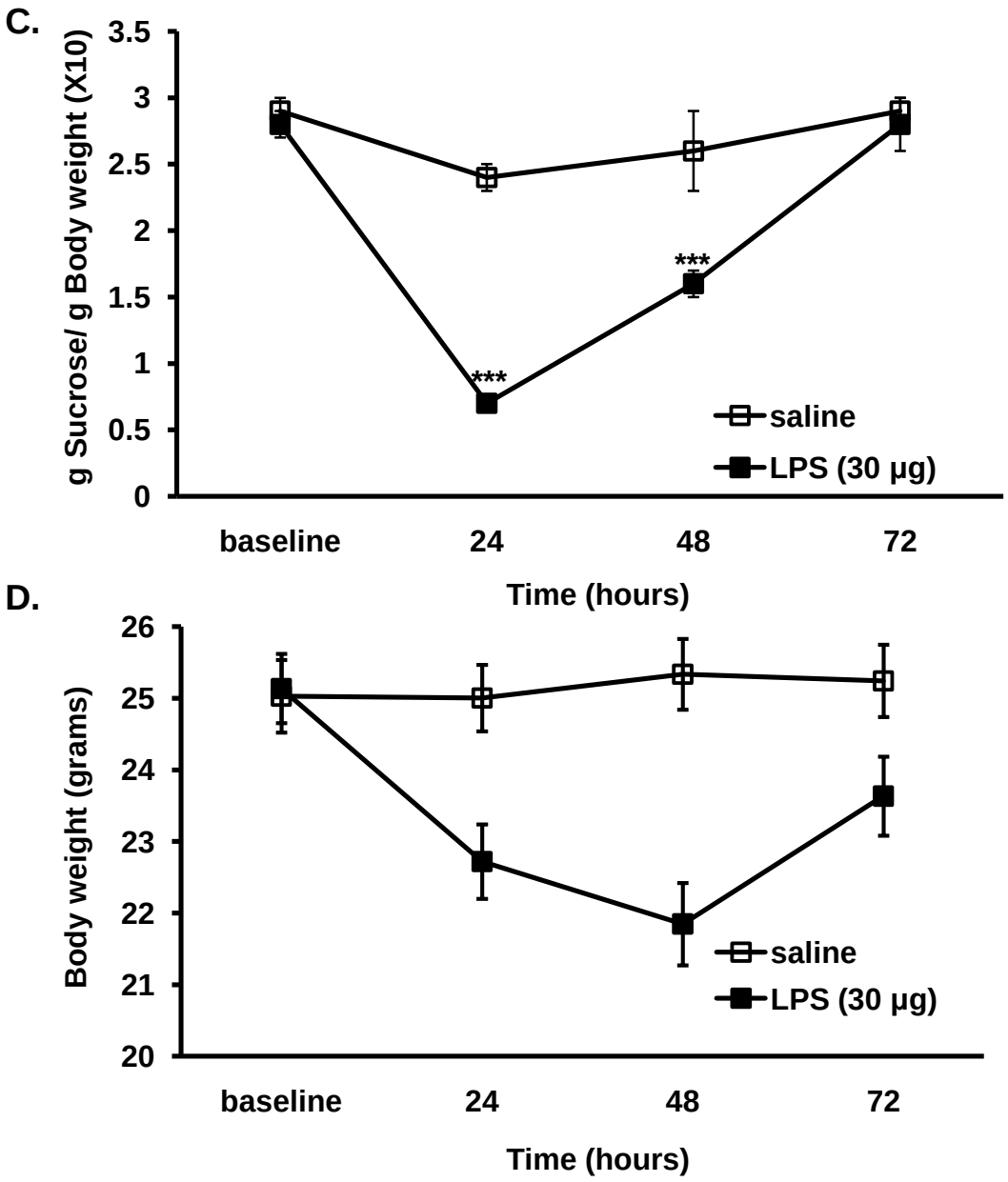


Figure 3-7. LPS-induced alterations in the sucrose preference test in male C57BL/6 mice. C) Sucrose solution consumption relative to body weight was measured 24, 48, and 72 hours post LPS administration. D) Changes in body weight after LPS administration. Values shown are means (±SEM). *** $p < 0.001$ vs. saline at same time-point.

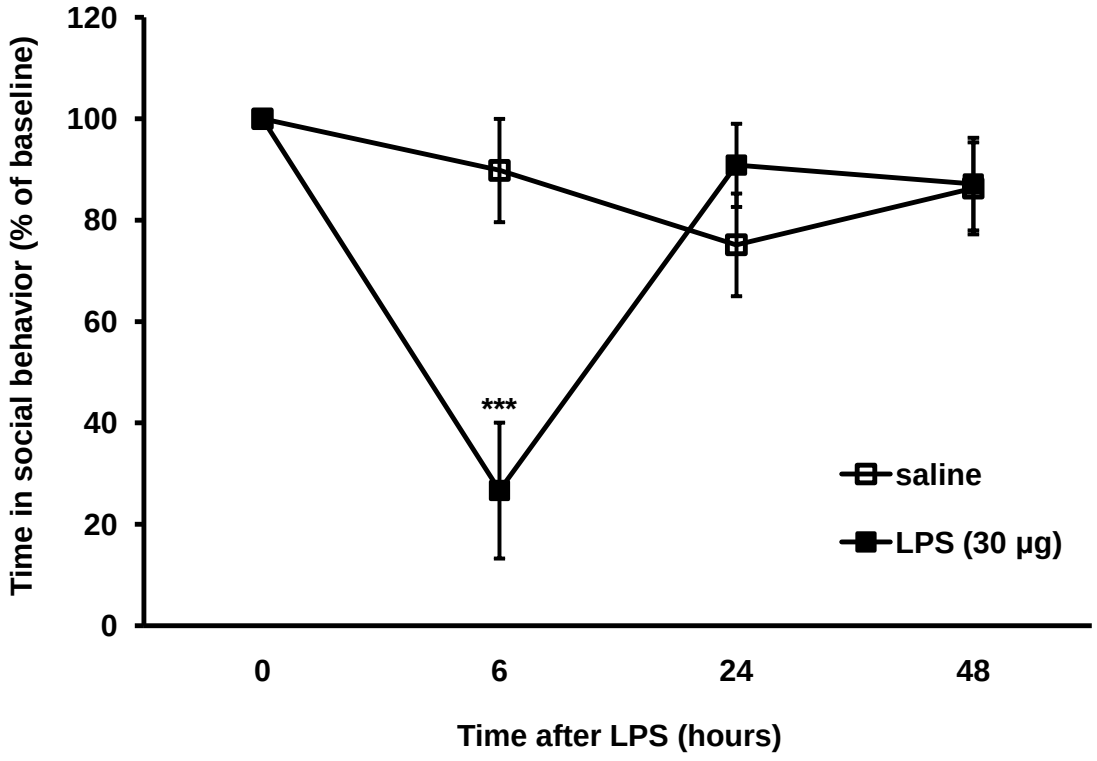


Figure 3-8. LPS-induced alterations in the social interaction test in male C57BL/6 mice. Baseline social interaction with a juvenile male C57BL/6 mouse was measured the day before treatment, and then again at 6, 24, or 48 hours after treatment with LPS or saline for a period of 10 minutes (n= 7/ treatment group). Social behavior was quantified using Observer. Results are expressed as the amount of time the mouse is engaged in social behaviors compared to baseline (pre-LPS) measures of interaction. Values shown are means (±SEM). *** $p < 0.001$ vs. saline at same time-point.

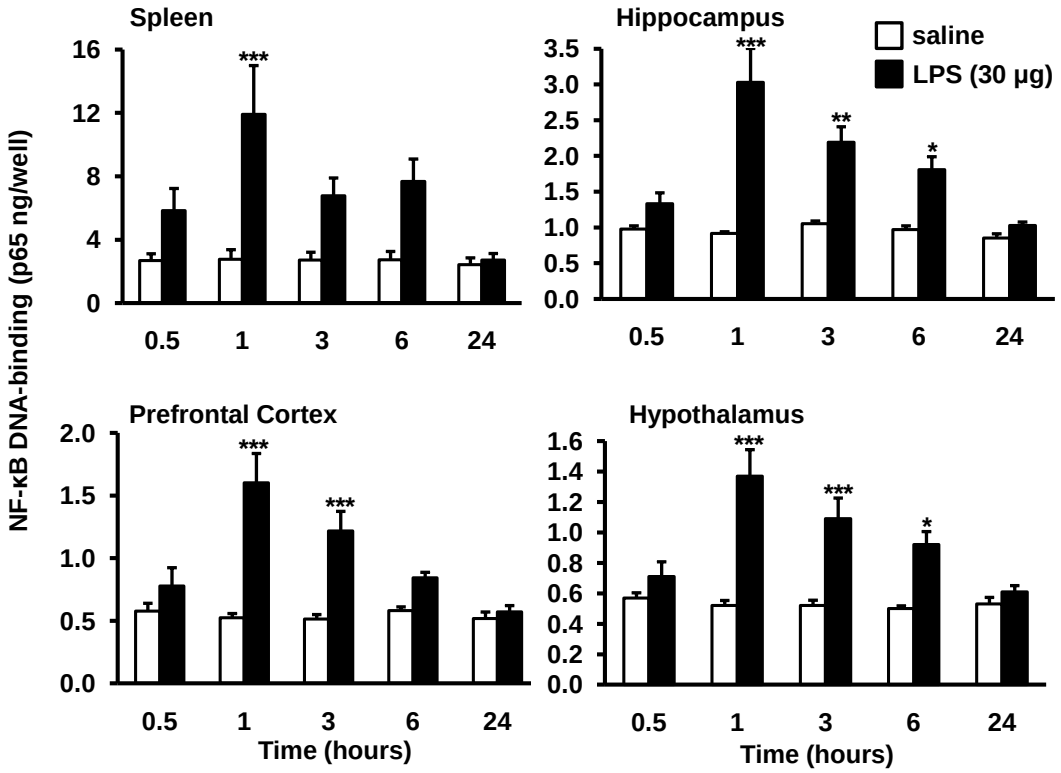


Figure 3-9. LPS-induced NF-κB DNA-binding in male C57BL/6 mice. Mice (n= 6/ treatment group) were treated with either LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL), and sacrificed at 0.5, 1, 3, 6, or 24 hours post injection. Spleen, hippocampus, hypothalamus, and prefrontal cortex were dissected and used to generate nuclear extracts. The nuclear extracts were then analyzed using a NF-κB DNA-binding ELISA. Values shown are means (±SEM). * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ vs. saline at same time-point.

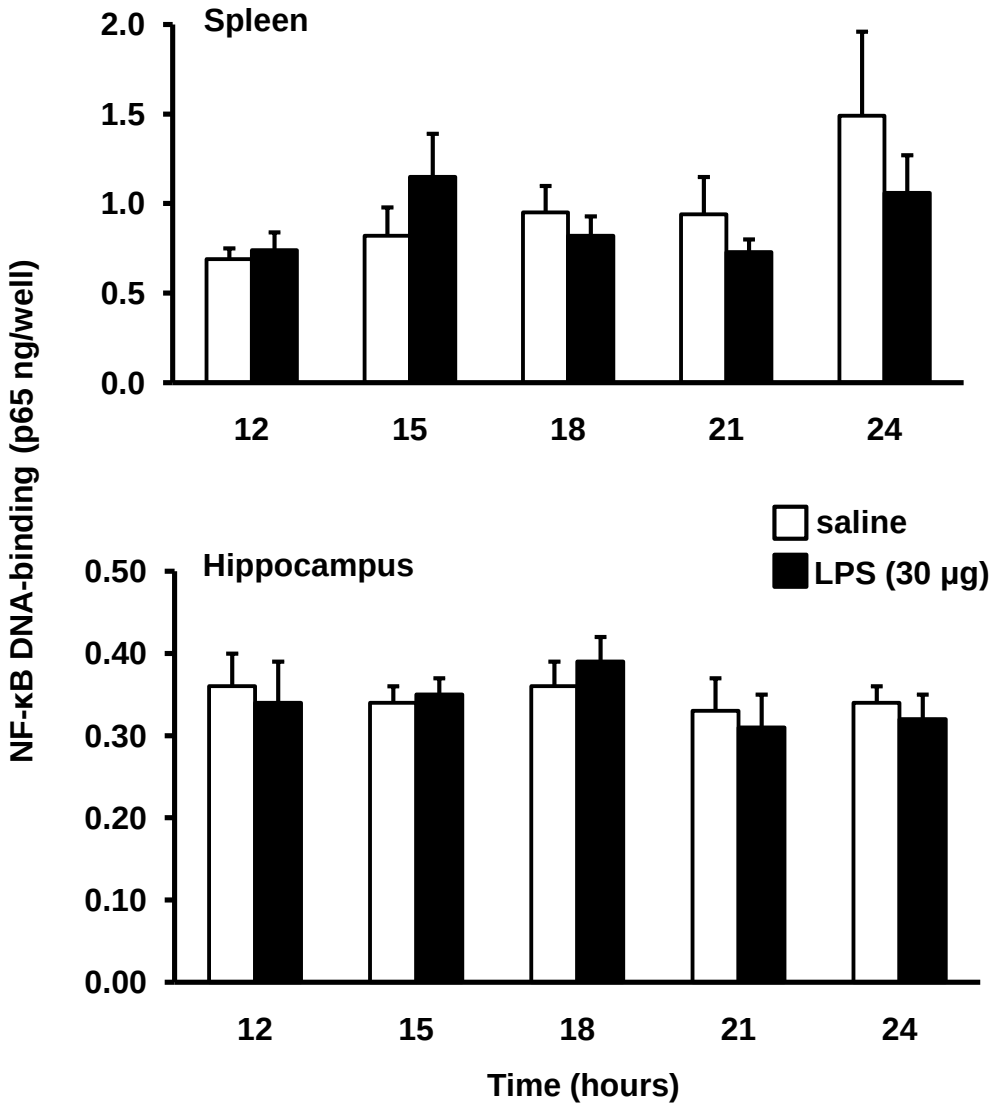


Figure 3-10. LPS-induced NF-κB DNA-binding in male C57BL/6 mice. Mice (n= 6/ treatment group) were treated with either LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL), and sacrificed at 12, 15, 18, 21, or 24 hours post injection. Spleen and hippocampus were dissected and used to generate nuclear extracts. The nuclear extracts were then analyzed using a NF-κB DNA-binding ELISA. Values shown are means (±SEM).

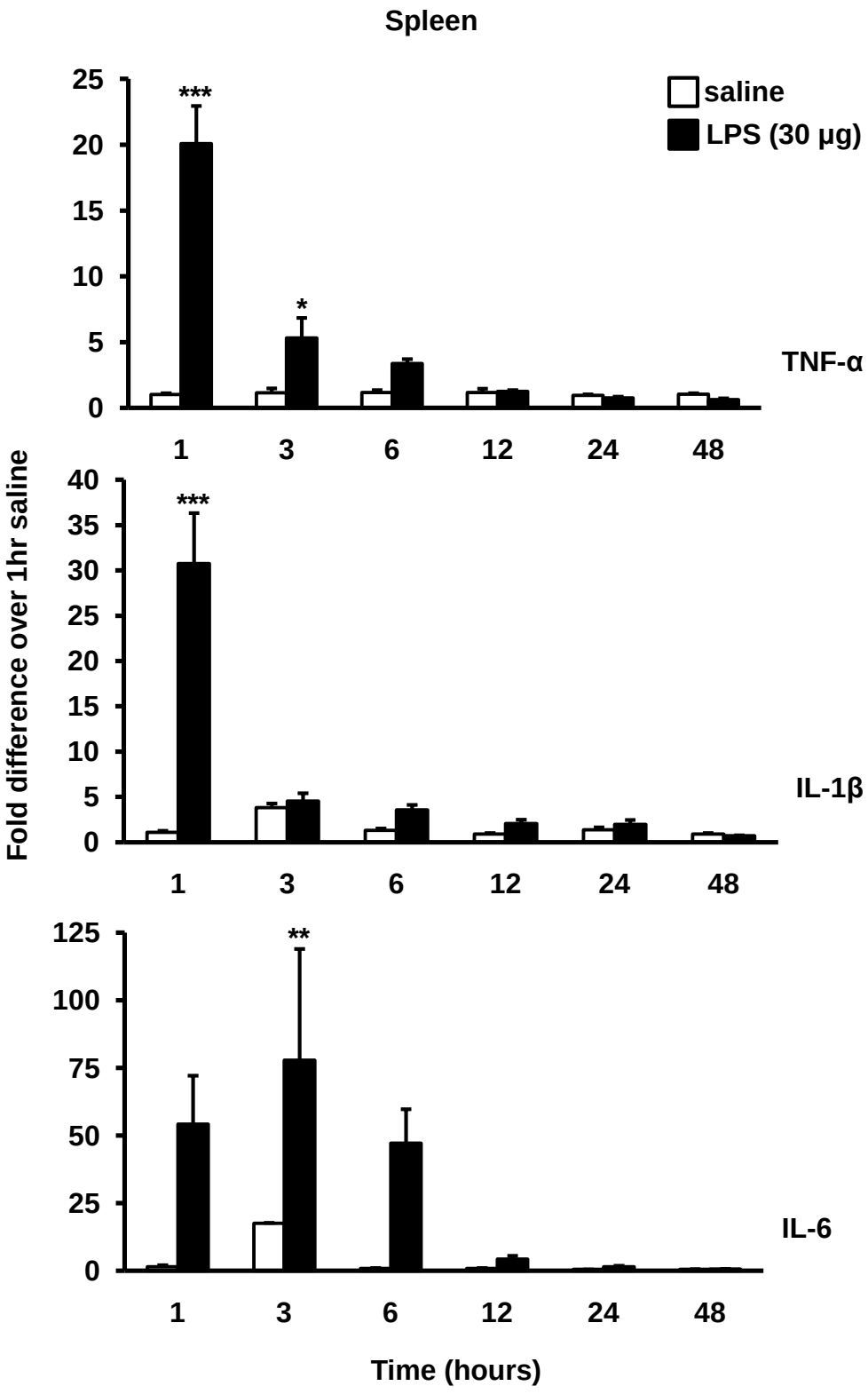


Figure 3-11A. LPS-induced cytokine mRNA expression in male C57BL/6 mice.

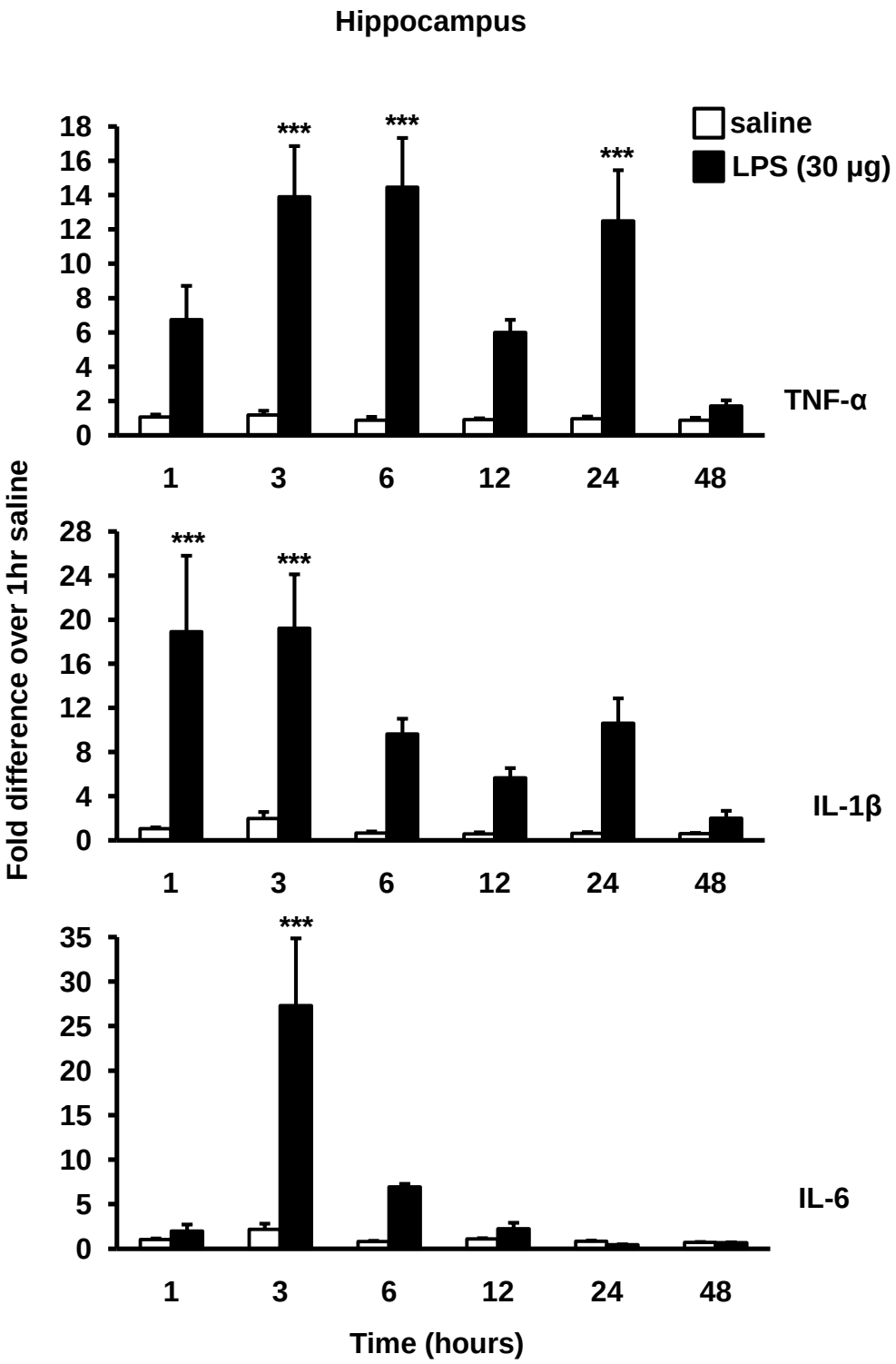


Figure 3-11B. LPS-induced cytokine mRNA expression in male C57BL/6 mice.

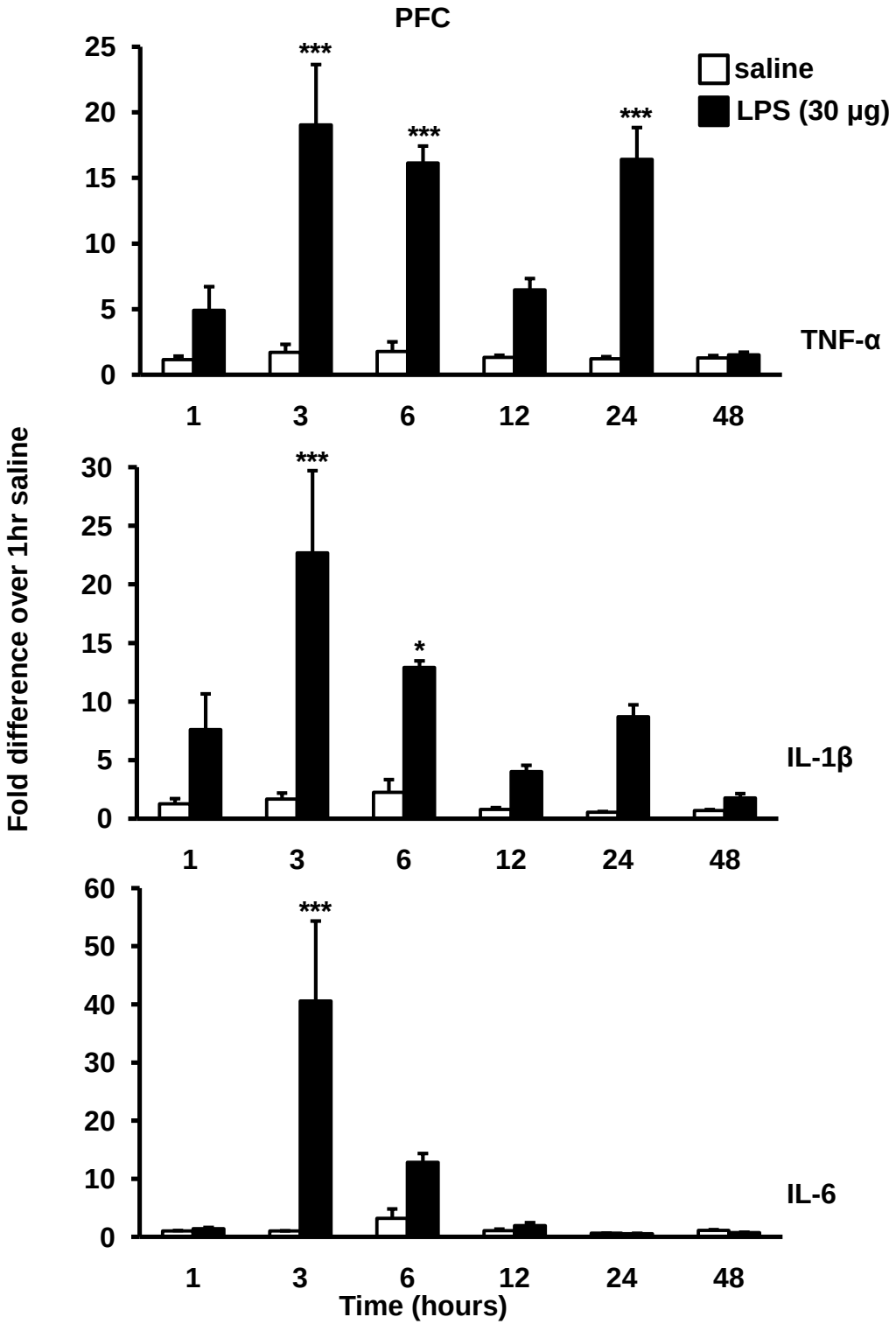


Figure 3-11C. LPS-induced cytokine mRNA expression in male C57BL/6 mice.

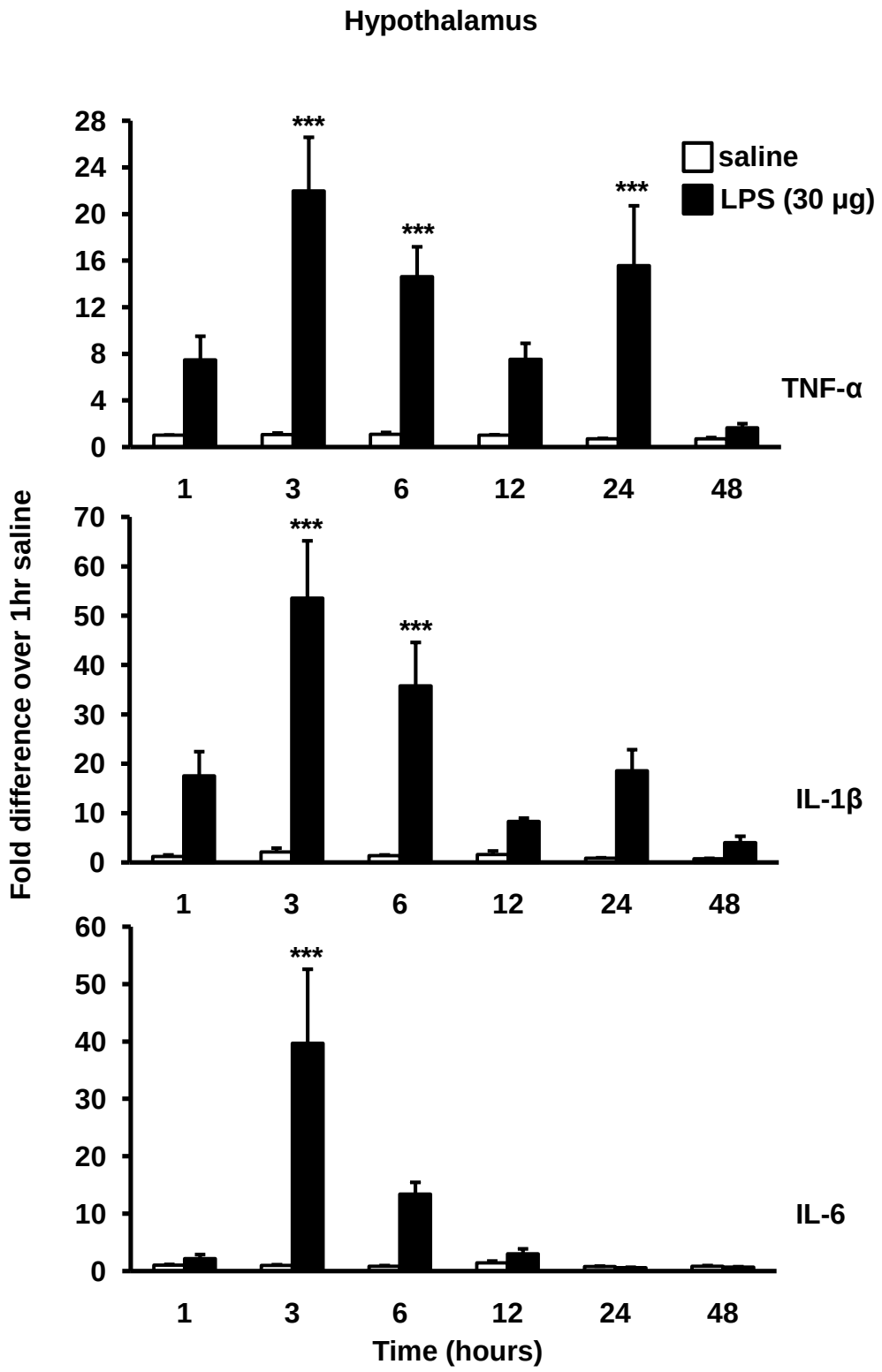
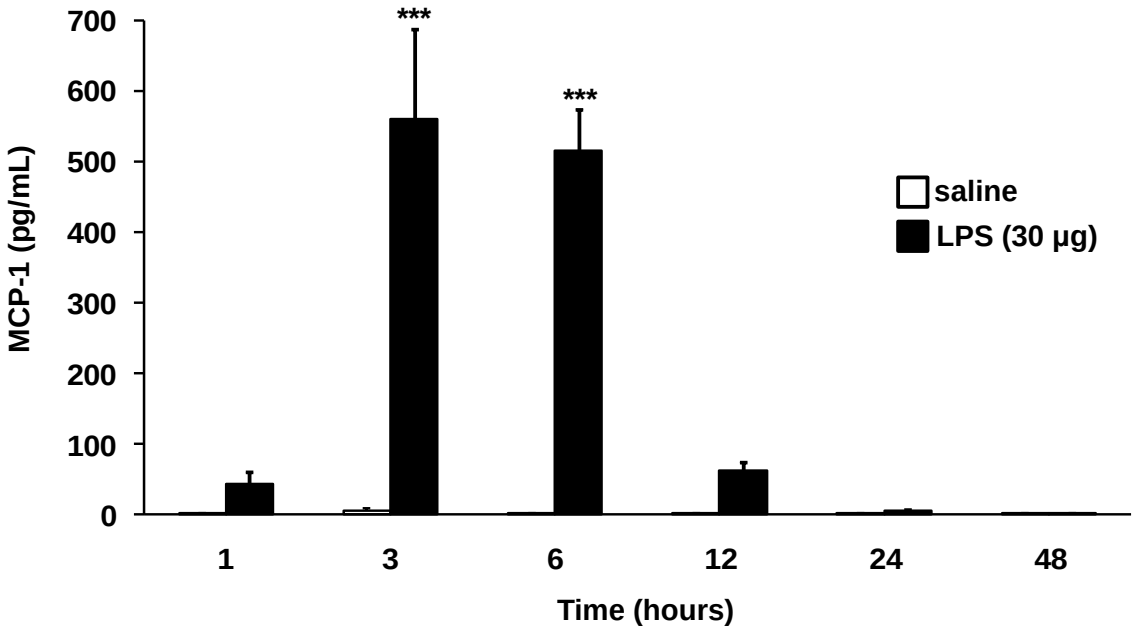
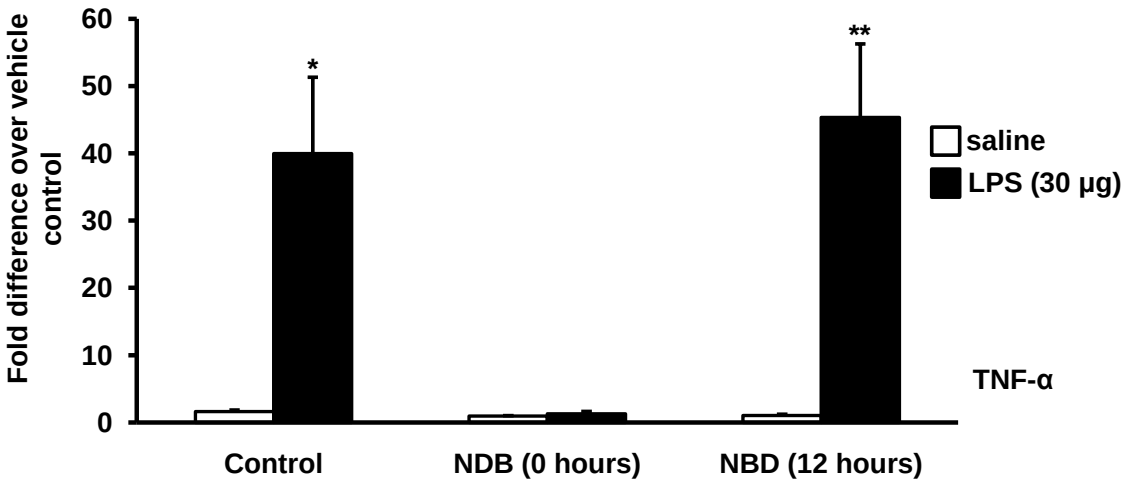


Figure 3-11D. LPS-induced cytokine mRNA expression in male C57BL/6 mice.

Figures 3-11A-D. LPS-induced cytokine mRNA expression in male C57BL/6 mice. Mice (n= 6/ treatment group) were treated with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 1, 3, 6, 12, 24, or 48 hours before collection of spleen, hippocampus, hypothalamus, and prefrontal cortex. Total mRNA was then extracted from tissues and expression patterns of TNF-α, IL-1β, and IL-6 were examined with RT-PCR. Results are expressed as fold difference over the saline treated group at one hour. Values shown are means (±SEM). * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ vs. saline at same time-point.



Figures 3-12. LPS-induced MCP-1 plasma levels in male C57BL/6 mice. Mice (n= 6/ treatment group) were treated with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 1, 3, 6, 12, 24, or 48 hours before collection of trunk blood. MCP-1 protein concentration in plasma was assayed using a mouse MCP-1/CCL2 ELISA. Values shown are means (±SEM). *** $p < 0.001$ vs. saline at same time-point.



Figures 3-13. LPS-induced TNF-α mRNA expression in the hippocampus of male C57BL/6 mice. Mice (n= 5/ treatment group) were treated with either TAT-NBD (i.p./20 mg/kg/100 µL/mouse), or control peptide (i.p./20 mg/kg/100 µL/mouse), either 1 prior to or 12 hours after treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Hippocampal tissue was collected at 24 hours post LPS injection and mRNA was extracted to measure expression of TNF-α. Results are expressed as fold difference over the saline treated group. Values shown are means (±SEM). * $p < 0.05$,** $p < 0.01$ vs. saline at same time-point.

Chapter 4

Effects of 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31) and 3,5-Bis(2-pyridinylmethylidene)-1-methyl-4-piperidone (UBS109) on LPS-induced NF-κB pathway activity in male C57BL/6 mice

I. Introduction

A high percentage of patients diagnosed with major depression do not experience complete resolution of symptoms or are resistant to conventional antidepressant treatments (Nestler et al., 2002). Indeed, approximately 30% of patients are refractory to all currently available antidepressants (Nestler et al., 2002). For some of these patients, efficacy is an issue, and for others, non-compliance due to intolerable side effects associated with conventional antidepressants results in only partial or incomplete responses to the medication. Patients showing partial or incomplete responses to treatment have a higher risk of relapse which significantly affects their quality of life (Judd et al., 2000). The need to identify novel treatment strategies for depressed patients who are resistant or only partially respond to conventional antidepressants has been highlighted in the current literature (Nestler et al., 2002; Raison et al., 2006; Wijeratne and Sachdev, 2008; Miller et al., 2009; Philip et al., 2010; Shelton et al., 2010). Recent data indicate that inflammation may play a significant role in the development and/or maintenance of depressive symptoms (Raison et al., 2006; Miller et al., 2009). Increased inflammatory markers in the blood or cerebral spinal fluid (CSF) have been associated with reduced probability of responding to conventional antidepressants. Likewise, patients who are resistant to treatment have a higher probability of expressing increased levels of inflammatory markers. Evidence for immune-brain interactions in major depression has identified novel targets in the innate immune system that may help to customize alternative therapeutic strategies for patients with treatment

resistance (Miller et al., 2009).

One such target is nuclear factor κ B (NF-κB). As discussed in previous chapters, NF-κB plays a major role in the induction of the inflammatory immune response. It is also a key player in transmitting inflammatory signals from the periphery to the brain where the signal is in turn amplified. Inflammatory cytokines in the brain have been associated with the development of depressive symptoms in humans and laboratory animals (Miller et al., 2009; Dantzer and Kelley, 2010). Indeed, inhibition of NF-κB signaling, cytokine expression or cytokine receptor activity has been shown to attenuate or block depressive symptoms (Godbout et al., 2005; Dantzer and Kelley, 2007; Koo et al., 2010). These data suggest that drugs that block the NF-κB pathway may be useful for inhibiting behavioral changes brought on by peripheral inflammatory responses as seen in treatment resistant depressed patients.

Curcumin, which has been used in traditional Chinese medicine to treat a number of ailments including depression, has been previously shown to have anti-inflammatory and antidepressant properties in laboratory animals. Curcumin directly inhibits the NF-κB pathway by inhibiting the activity of kinases including Iκ Kinase (IκK) β and Akt (Anand et al., 2008). In addition, curcumin also affects monoaminergic systems in brain regions implicated in major depression (Xu et al., 2005b; Xu et al., 2007). However, despite the anti-inflammatory and antidepressant effects of curcumin, the compound has limited therapeutic potential due to poor absorption, low blood concentrations, poor distribution to tissues (low bioavailability), and rapid metabolism (Anand et al., 2007). Curcumin

plasma levels have been shown to peak soon after oral administration (between 45-60 minutes), and is quickly metabolized by the liver (Holder et al., 1978; Wahlström and Blennow, 1978; Ravindranath and Chandrasekhara, 1982). Reports on the half-life of curcumin and whether the metabolites are active NF-κB inhibitors are inconsistent. Moreover, studies show that curcumin is poorly absorbed from the gastrointestinal tract, and has poor tissue distribution (Ravindranath and Chandrasekhara, 1980; Ravindranath and Chandrasekhara, 1981). Being that curcumin is highly lipophilic, it is also often found in fat, which could explain why the plasma concentrations and tissue distribution is low. It has been suggested that much of the effects of curcumin may take place in the gut, and not necessarily at the target tissues such as the brain (Epstein et al., 2010). Massive doses of curcumin are required to achieve clinically relevant blood concentrations and effects on pancreatic cancer (Cheng et al., 2001; Hatcher et al., 2008). Nevertheless, it is a natural anti-inflammatory that is considered safe without any reported major side effects, and provides a chemical structure that can be modified to develop potent anti-inflammatory analogues.

Modifications have been made to the chemical structure which aims to address some of the limitations of curcumin. 3,5-Bis(2-pyridinylmethylidene)-1-methyl-4-piperidone (UBS109) and 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31) are structurally related to curcumin with modifications made to the aromatic rings, the beta-diketone moiety, and the two flanking double bonds conjugated to the beta-diketone moiety (Figure 4-1). In terms of solubility, EF31 is insoluble in water. However, UBS109 exhibits better solubility in water (Sun

and Snyder, unpublished data). Moreover, their calculated LogP values are similar (EF31= 2.1 and UBS109= 2.3) (Moore and Snyder, unpublished data), and they both show increased stability (Sun and Snyder, unpublished data). In the current studies, the anti-inflammatory and anti-cancer activity of UBS109 in-vitro was determined using the same measures discussed in Chapter 2. UBS109 was found to be similar to EF31 in terms of potency of NF-κB pathway inhibition in-vitro. The activity of UBS109, EF31, and curcumin were then examined in-vivo using the mouse model of LPS-induced depressive-like behaviors characterized in Chapter 3. Given the potent anti-inflammatory activity of these compounds in-vitro, it was hypothesized that oral administration of UBS109 and EF31 would block LPS-induced NF-κB DNA-binding and downstream inflammatory markers, including cytokine mRNA expression (Figure 4-2). This would suggest that these compounds could potentially block LPS-induced depressive-like behaviors in mice and thus merit further development for therapeutic applications.

II. Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Cellgro (Manassas, VA). Weymouth's medium and Leibovitz's L-15 medium were purchased from ATCC (Manassas, VA). Curcumin was purchased from Sigma (St. Louis, MO) (C1386-5G), and the structurally related compounds EF24 and EF31 were prepared at Emory University as described

previously (Adams et al., 2004). For in-vitro studies, dimethyl sulfoxide (DMSO) was used to dissolve all compounds, and all dilutions were made from a 10mM stock. LPS (Escherichia coli O55:B5) for cell culture was obtained from Sigma and was suspended in saline (9% sodium chloride) to a final concentration of 1 µg/mL. LPS (Escherichia coli O127:B8) for mice was obtained from Sigma, suspended in saline (9% sodium chloride), and administered via intraperitoneal injection (i.p.) at a dose of 30 µg/mouse, in a total volume of 100 µL. Control animals received an i.p. injection of 100 µL sterile saline. Primers for mouse TNF-α, IL-1β, IL-6, and GAPDH were obtained from Qiagen (Hilden, Germany). GAPdh was not affected by LPS and was used as a house keeping control gene for each plate.

Cell culture

Mouse RAW 264.7 macrophage cells (ATCC) were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (HyClone Labs, Logan, UT) at 37 °C with 5% CO_2. A2780 cells, a human ovarian carcinoma cell line (Sigma), were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum at 37 °C with 5% CO_2. MDA-MB-231 cells, a human breast cancer cell line (ATCC), were cultured in Leibovitz's L-15 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum at 37 °C with 100% air. EMT6 cells, a mouse mammary carcinoma cell line (ATCC), were cultured in Weymouths's MB 752/1 supplemented with 100 U/mL penicillin, 100

µg/mL streptomycin, and 15% fetal bovine serum at 37 °C with 5% CO_2. MIA PaCa-2 cells, a pancreatic carcinoma cell line (ATCC), were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, and 2.5% horse serum at 37 °C with 5% CO_2.

Mice

Adult male C57BL/6 mice (ages 6-8 weeks, 18-25g) were purchased from Charles River Laboratories (Wilmington, Mass.), and were individually housed in the Emory University Animal Care Facility for 14 days with a 12:12 hours reverse light:dark cycle (lights on at 2100 hr) prior to treatment. Water and rodent chow were made available *ad libitum*. All mice were handled and sacrificed during the dark phase of the cycle using red lights. Mice showing reduced feeding, weight, or grooming, as well as any signs of infection or tissue damage were excluded from the studies prior to treatment. Protocols were in accordance with institutional guidelines for animal care and use.

Assessment of NF-κB DNA-binding

RAW 264.7 mouse macrophage cells were plated at 3.4 X 10 cells/well in 60 mm X 15 mm dishes, incubated overnight, and were then incubated in triplicate with test compounds, or vehicle (DMSO 1%) for one hour prior to LPS (1 µg/mL) or saline. Nuclear proteins were collected 15 minutes after LPS treatment using the extraction kit protocol from Active Motif (Carlsbad, CA). The 15 minute collection time-point after LPS treatment was derived from a time

course study (Figure 2-4A). Nuclear protein samples were analyzed in triplicate using the NF-κB DNA-binding ELISA kit (Active Motif, Carlsbad, CA).

Mice were pre-treated orally (255 µL) with test compound or vehicle (various vehicles were used, see results) and then were randomly assigned to a treatment group (saline or LPS). Mice were then treated with either LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Spleen and hippocampus were collected at either 6 or 24 hours after treatment with LPS or saline. Nuclear proteins were collected using the extraction kit protocol from Active Motif according to manufacturer's instructions. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL) per manufacturer's instructions. Nuclear protein samples were analyzed in triplicate using the NF-κB DNA-binding ELISA kit (Active Motif).

Isolation of total RNA and cytokine RT-PCR

RAW 264.7 mouse macrophage cells were plated at 1.8 X10 cells/well in 6-well plates and incubated overnight. Cells were then treated with the test compounds or vehicle (DMSO 1%) in triplicate for one hour prior to stimulation with LPS (1 µg/mL) or saline. Three hours after LPS treatment, cells were collected and total RNA was extracted using the RNeasy Mini kit (Qiagen) followed by reverse transcription PCR and amplification of cDNA by RT-PCR (Applied Biosystems, 7500 Fast, Carlsbad, CA).

Mice were pre-treated orally (255 µL) with test compounds, or vehicle (various vehicles were used, see results). Mice were then treated with either LPS

(i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL), and sacrificed at 6 or 24 hours post injection. Spleen and hippocampus tissue sections were collected and placed in RNAlater (Qiagen). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by reverse transcription PCR using SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) with random primers, according to manufacturer's protocols. The cDNA was then amplified by RT-PCR (Applied Biosystems, 7500 Fast). For data analysis the deltaCt method was used. Fold-changes were calculated as difference in gene expression between LPS and vehicle controls.

Cell viability/proliferation assay

All cell lines were plated at 5000 cells/well in 96-well plates and incubated overnight. Cells were treated with UBS109 or vehicle (DMSO 1%) in triplicate for either 1 or 48 hours before viability was assayed using the CellTiter 96 Aqueous non-radioactive cell proliferation assay (MTS) kit from Promega (Madison, WI).

Data Analysis

Overall treatment and time effects were determined in each experiment by either a one- or two-way analysis of variance (ANOVA) using GraphPad Prism 5. Significant interactions were followed by post-hoc Bonferroni test to determine differences between specific groups of interest. An α level of $p < 0.05$ was used in all statistical tests.

III. Results

Effects of UBS109 in-vitro

UBS109 is a potent inhibitor of NF-κB DNA-binding in mouse RAW264.7 macrophages.

To examine the activity of UBS109 compared to curcumin, mouse RAW264.7 macrophages were pre-treated with UBS109, curcumin (1, 5, 10, 30, 50, or 100 μM), or vehicle for one hour, and NF-κB DNA-binding activity was assessed 15 minutes following treatment with LPS (1 μg/mL) using a NF-κB DNA-binding ELISA. Potent inhibition of NF-κB DNA-binding was observed at concentrations of 5–10 μM for UBS109, and 50–100 μM for curcumin (Figure 4-3). Analysis of these results with a two-way ANOVA revealed a significant treatment x dose interaction ($F [5, 24] = 19.14$, $p< 0.0001$). The dose response indicated that the IC_{50} value of UBS109 was ~2 μM, and >50 μM for curcumin. Due to a wide concentration range of inhibition (50-100 μM), the IC_{50} for curcumin could not be determined. Post-hoc analysis revealed significant differences between curcumin and UBS109 at doses 5-50 μM ($p< 0.001$).

UBS109 blocks LPS-induced cytokine mRNA expression in mouse RAW264.7 macrophages.

To examine the effects of UBS109 on pro-inflammatory cytokine mRNA expression, mouse RAW264.7 macrophages were pre-treated with UBS109,

curcumin (10 or 50 µM), or vehicle for one hour, and mRNA was extracted 3 hours following treatment with LPS (1 µg/mL) and the expression of TNF-α, IL-1β, and IL-6 was measured using RT-PCR (Figure 4-4). The doses for the test compounds were chosen to cover the range of IC_{50} values revealed in the previous experiments. Expression of TNF-α, IL-1β, and IL-6 mRNA were inhibited in a dose–dependent manner with all compounds. Statistical analysis revealed a treatment x dose interaction (TNF-α, $F [2, 12] = 10.46$, $p = 0.0023$; IL-1β, $F [2, 12] = 58.64$, $p < 0.0001$; IL-6, $F [2, 12] = 208.9$, $p < 0.0001$). Of note, no inhibition of LPS-induced TNF-α was found with curcumin.

At doses that inhibit NF-κB, UBS109 does not reduce cell viability/ proliferation in mouse RAW264.7 macrophages.

To assess the effects of UBS109 on cell viability, mouse RAW264.7 macrophages were pre-treated with USB109 (1, 5, 10, or 50 µM), or vehicle for one hour, and then with LPS or saline for 15 minutes. Cell viability was assayed using a cell proliferation (MTS) kit. UBS109 did not significantly reduce cell viability/ proliferation in mouse RAW 264.7 macrophages in any of the doses tested (Figure 4-5). Statistical analysis did not show an effect of dose or treatment. Of note, these data also indicate that the UBS109-mediated reduction in NF-κB DNA-binding activity in mouse RAW264.7 macrophages was not due to a loss of viable cells.

UBS109 is cytotoxic in cancer cell lines.

To examine the cytotoxic activity on UBS109 on cancer cell lines tested in Chapter 2 (Figure 2-13), human ovarian carcinoma cells (A2780), human breast cancer cells (MDA-MB-231), human pancreatic carcinoma cells (MIA PaCa-2), and mouse mammary carcinoma cells (EMT6), all of which depend heavily upon the NF-κB pathway for proliferation, were treated with UBS109 (1, 5, 10, or 50 μM), or vehicle for 48 hours (Figure 4-6). Cell viability was then assayed using a cell proliferation (MTS) kit. Cell viability in cancer cell lines was reduced in a dose-dependent manner. Statistical analysis revealed a significant treatment effect for A2780, MDA-MB-231, MIA PaCa-2, and EMT6 cells ($F [3, 11] = 68.76$, $p < 0.0001$, $F [3, 11] = 11.45$, $p = 0.0029$, $F [3, 11] = 25.16$, $p = 0.0002$, $F [3, 11] = 176.7$, $p < 0.0001$, respectively). A post-hoc showed a significant difference in the MIA PaCa-2 cells (1-50 μM, $p = 0.0002$), A2780 cells (5-50 μM, $p < 0.0001$), EMT6 cells (5-50 μM, $p < 0.0001$), and the MDA-MB-231 cells (10-50 μM, $p = 0.0029$) compared to DMSO treatment.

Effects of UBS109 and EF31 in-vivo

Oral treatment with EF31 or UBS109 did not significantly reduce LPS-induced NF-κB DNA-binding activity in the spleen or brain.

To examine the effects of EF31 on LPS-induced NF-κB DNA-binding in-vivo, adult male C57BL/6 mice were pre-treated orally with either EF31 (25, 50, or 100mg/kg/255 μL), or vehicle [PEG300/DMA (85%/15%)] one hour prior to treatment with LPS (i.p./30 μg/ 100 μL/mouse) or saline (i.p./100 μL). Spleen,

hippocampus, prefrontal cortex, and hypothalamus were dissected one hour post LPS (n= 5 mice/treatment group) and used to generate nuclear extracts. The nuclear extracts were analyzed using a NF-κB DNA-binding ELISA. The one hour time-point was chosen based on the data presented in Chapter 3 (Figure 3-9), showing that NF-κB DNA-binding in the spleen and brain peak at one hour post LPS. Overall, EF31 did not reduce NF-κB DNA-binding in any of the tissues collected (Figure 4-7). Statistical analysis revealed a significant treatment (LPS or saline) effect in the spleen, hippocampus, prefrontal cortex, and hypothalamus ($F [1, 30] = 38.10$, $p < 0.0001$, $F [1, 29] = 57.37$, $p < 0.0001$, $F [1, 30] = 42.03$, $p < 0.0001$, $F [1, 30] = 32.09$, $p < 0.0001$, respectively). These data suggest that EF31 did not block LPS-induced inflammatory signaling in the periphery or the brain.

In a separate cohort, adult male C57BL/6 mice were pre-treated orally with either UBS109 (50 or 150mg/kg/255 µL), or vehicle [PEG300/DMA/Cyclodextrin (28%/5%/30%)] one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Spleen and hippocampus were dissected one hour post LPS (n= 5 mice/treatment group) and used to generate nuclear extracts. The nuclear extracts were analyzed using a NF-κB DNA-binding ELISA. Statistical analysis revealed a significant treatment (LPS or saline) effect in the spleen and hippocampus ($F [1, 28] = 13.16$, $p = 0.0011$, $F [1, 28] = 12.25$, $p = 0.0016$, respectively). Nevertheless, UBS109 did not reduce NF-κB DNA-binding in any of the tissues collected (Figure 4-8). These data suggest that UBS109 did not block LPS-induced inflammatory signaling in the periphery or the brain.

Oral treatment with EF31 and UBS109 did not reliably attenuate LPS-induced cytokine mRNA expression in the brain.

To examine the effects of EF31 on cytokine mRNA expression in-vivo, adult male C57BL/6 mice were pre-treated orally with EF31 (150mg/kg/255 µL) or vehicle [9 parts Methyl cellulose (.5%) + 1 part PEG200/255 µL], for two consecutive days, followed by treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 6 or 24 hours before sacrifice. Total mRNA was extracted from the hippocampus (n= 3/ treatment group) and the expression of TNF-α and IL-1β was measured using RT-PCR. In the hippocampus, both TNF-α and IL-1β expression were increased at 24 hours, a time at which depressive-like behaviors are apparent in the absence of decreased locomotor activity (Figure 4-9). These results were consistent with the two peaks of mRNA reported in Chapter 3 (Figure 3-11B). At 24 hours, EF31 reduced both TNF-α and IL-1β expression by ~50% in the hippocampus. These data indicate that oral administration of EF31 can reduce LPS-induced cytokine expression in the hippocampus in a time dependent fashion. Statistical analysis for TNF-α indicated a significant treatment x time interaction ($F [2,12] =11.27$, $p<0.01$). Statistical analysis for IL-1β also indicated a significant treatment x time interaction ($F [2,12] =29.52$, $p<0.01$). Post hoc analysis revealed that EF31 had no effect on LPS-induced TNF-α and IL-1β expression in the hippocampus at 6 hours (data not shown). But by 24 hours, LPS-induced expression of both cytokines in the hippocampus was attenuated by EF31 (trend for TNF-α). Nonetheless, the effects of EF31 on cytokine mRNA

expression could not be replicated on two separate occasions raising concerns regarding the reliability or reproducibility of these effects.

To examine the effects of UBS109 on cytokine mRNA expression in-vivo, adult male C57BL/6 mice were pre-treated orally with either UBS109 (50mg/kg/255 µL), curcumin (150mg/kg/255 µL), or vehicle [PEG300/DMA/Cyclodextrin (28%/5%/30%)] one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Total mRNA was extracted from the hippocampus (n= 6 mice/ treatment group) and the expression of TNF-α, IL-1β, and IL-6 was measured using RT-PCR. Statistical analysis revealed a significant time effect in the hippocampus for TNF-α, IL-1β, and IL-6 mRNA expression ($F [3, 68] = 20.86$, $p < 0.0001$, $F [3, 68] = 19.41$, $p < 0.0001$, $F [3, 68] = 59.18$, $p < 0.0001$, respectively). However, neither UBS109 nor curcumin reduced cytokine expression in the hippocampus (Figure 4-10). These data suggest that UBS109, like curcumin, did not block LPS-induced inflammatory signaling in the periphery or the brain.

IV. Discussion

The results presented here demonstrate that UBS109 is a potent inhibitor of NF-κB DNA-binding and downstream pro-inflammatory cytokine mRNA expression in-vitro. Of note, these effects were observed at doses that showed no toxicity in mouse RAW264.7 macrophages, while maintaining potent toxicity in NF-κB-dependent cancer cell lines. In-vivo experiments showed no effects of

EF31 or UBS109 on LPS-induced NF-κB DNA-binding activity and no reliable effects were found on LPS-induced cytokine mRNA expression in the periphery or the brain of male C57BL/6 mice. Taken together these results indicate that UBS109 is a potent inhibitor of the NF-κB pathway in-vitro. However, in-vivo effects of curcumin analogs were inconsistent and may require a different treatment paradigm and/or a different mouse model of increased innate immune/ inflammatory system activity.

The results presented in Chapter 2 using EF31 in-vitro were replicated here with UBS109. Given that these two compounds are very similar in structure and activity, it is reasonable to speculate that they work via the same mechanisms (i.e. direct inhibition of IκKB). Indeed, preliminary data using the same recombinant kinase inhibition assay described previously (Chapter 2), showed that UBS109 is a potent inhibitor of IκKB activity (Brown, unpublished data). However, whether UBS109 is active against other kinases such as Akt and MAPK remains to be determined. Like EF31, reversibility in NF-κB inhibition also requires further testing. Nonetheless, both compounds have been shown to have similar toxicity profiles in mice (Shoji, unpublished observations).

In terms of the in-vivo effects of UBS109 and EF31, the results were not consistent. Although EF31 was shown to reduce cytokine mRNA expression in the brain in one experiment, the results were not reproducible and EF31 did not block NF-κB DNA-binding. In addition, UBS109 did not affect cytokine mRNA or NF-κB DNA-binding activity in the spleen or the brain. Of note, curcumin was included in these experiments and it also failed to block cytokine mRNA

expression. There are a number of factors that could explain the results produced in-vivo. Some pharmacokinetics has been worked out for EF31 and UBS109, showing that they peak early in plasma (<one hour) but remain at lower concentrations for several hours after treatment (Figure 4-11). This suggested that the best time to administer the compounds is at least one hour prior to LPS treatment. Since no effects of the compounds on NF-κB DNA-binding activity was obvious, it is possible that the treatment paradigm used here may not be optimal. Perhaps multiple treatments or a different treatment schedule would produce different results. Of note, a reduction in cytokine mRNA expression was evident only after multiple administrations of EF31 (once a day for two consecutive days). This suggests that perhaps multiple doses of the compounds may achieve inhibition of the NF-κB pathway. Nevertheless, tissue distribution and absorption for these compounds remains to be determined. It is possible that like curcumin, these compounds are not reaching target organs when administered orally and thus fail to block LPS-induced inflammatory markers.

Given the link between inflammation and depressive-like behaviors, it was hypothesized that the antidepressant effects of these compounds would be dependent on the anti-inflammatory effects. However, curcumin has been shown to have antidepressant effects in the absence of LPS. Xu et al. (2005) reported a significant reduction in the duration of immobility in the tail suspension test and the Porsolt forced swim task in mice pre-treated with curcumin (p.o./5-10mg/kg). Moreover, the 10mg/kg dose of curcumin was found to increase serotonin and noradrenaline levels in the frontal cortex and hippocampus, as well as dopamine

levels in the frontal cortex and the striatum. Curcumin was also found to inhibit monoamine oxidase activity in the mouse brain. These data suggest that curcumin may be producing the antidepressant effects via manipulations of the monoaminergic systems in the brain. The monoaminergic systems play a crucial role in mood regulation and have been implicated in the pathophysiology of major depression. These data also suggest that oral administration of curcumin can still have effects in the central nervous system, even though curcumin has not been shown to cross the blood brain barrier in healthy laboratory animals. Nonetheless, in an animal model of advanced Alzheimer's disease, it was reported that orally administered curcumin was able to cross blood brain barrier to bind amyloid plaques in the brain, although the ability to cross blood brain barrier was reported as "weak" (Yang et al., 2005; Garcia Alloza et al., 2007). However, advanced neurodegenerative diseases and chronic inflammation are known to compromise the integrity of the blood brain barrier, making it more permeable to large molecules such as curcumin. Finally, antidepressant effects of curcumin were comparable to those produced by imipramine and fluoxetine, two commonly used antidepressants (Xu et al., 2005a). Subsequent studies have implicated 5-HT1a 1b, and 5-HT2C receptors in the antidepressant effects of curcumin in addition to upregulating the expression of brain-derived neurotrophic factor and promoting neurogenesis in the hippocampus (Xu et al., 2007). It is possible that curcumin analogs tested here may still show an antidepressant effect in the LPS model of cytokine induced depressive-like behaviors despite a lack of an anti-inflammatory effect by interacting with the monoaminergic systems

and neurotrophic factors in the brain. Nonetheless, this remains speculative.

Another possible explanation for the lack of anti-inflammatory effects in-vivo is the inflammatory stimulus. LPS is a potent acute inflammatory stimulus that is also commonly used to study sepsis. It is possible that the stimulus may be overpowering the anti-inflammatory effects of the compounds. Perhaps there is a threshold for inflammation at which these compounds can no longer subdue the inflammatory signaling. Again, this also remains speculative and requires further testing. Nevertheless, it is possible that using a different animal model of depressive-like behaviors such as a psychosocial stress model in which inflammation is still relevant, may yield better results with curcumin compounds. Indeed, curcumin has been shown to attenuate or block stress-induced behavioral alterations by targeting monoaminergic systems and trophic factors known to be affected by NF-κB (Xu et al., 2005a; Xu et al., 2006; Xu et al., 2007; Anand et al., 2008).

In summary, both EF31 and UBS109 are curcumin analogs that show potent inhibition of NF-κB DNA-binding activity in-vitro, in conjunction with potent toxicity in NF-κB-dependent cancer cell lines. Furthermore, the treatment paradigm tested here may require modifications to achieve in-vivo inflammatory effects.

V. Limitations and Future Directions

One major limitation in the current studies is the limited pharmacokinetics

(PK) information available that could be used to develop an optimal treatment regime. Several treatment protocols were employed for the administration of EF31 and UBS109. These included testing several different vehicles as well as dosing times. At least 3 vehicles were tested here, and preliminary PK studies (Figure 4-11) also tested a number of other commercial vehicle options. The PEG300/DMA/Cyclodextrin (28%/5%/30%) vehicle produced higher levels of UBS109 in plasma as detected by mass spectrometry. However, whether this vehicle is most optimal for absorption and tissue distribution remains to be determined. Nonetheless, data presented here does not show an advantage for this vehicle over the other vehicles tested for the inhibition of NF-κB DNA-binding activity and cytokine mRNA expression. Moreover, in the current studies, mice were treated one hour prior to LPS. This time-point was chosen based on preliminary PK findings (Figure 4-11). However, this also did not produce favorable results, suggesting that perhaps treating multiple times or closer to LPS treatment may be necessary. Again, without information about the absorption, metabolism, and tissue distribution of these compounds, it is very difficult to estimate the best time-point for treatment and develop an optimal treatment regime relevant to the animal model used in the current studies.

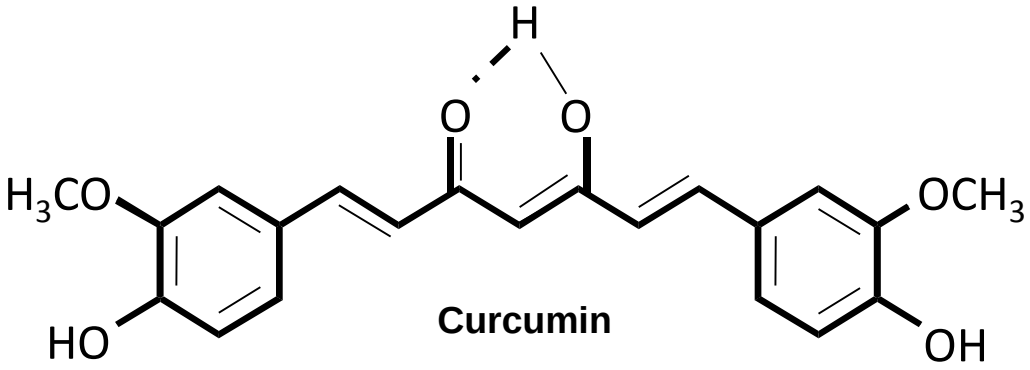
Lastly, there are a number of complementary experiments for future directions. The concerns raised in Chapter 2 for EF31 are applicable to UBS109. It remains to be determined how UBS109 binds to IκKβ, whether it is a reversible binding, and whether it binds to other kinases involved in the inflammatory response. These concerns could be addressed by using X-ray crystallography to

determine the binding site, and recombinant kinase proteins to test inhibition and reversibility in inflammatory pathways.

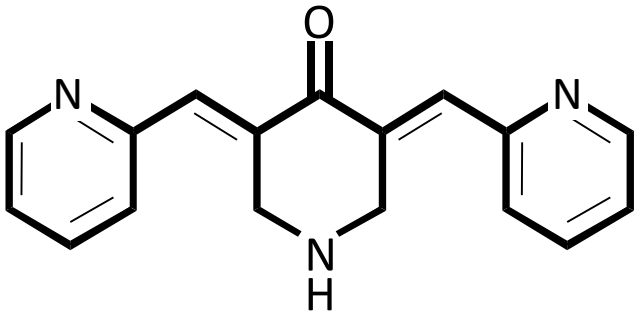
Like EF31, UBS109 exhibited dose-dependent cytotoxic effects in the cancer cell lines tested here, but it was significantly more potent in some cell lines than others. The breast cancer cell line (MDA-MB-231) shows greater resistance to both EF31 and UBS109. Theoretically, curcumin analogs should have access to the same kinases in the MDA-MB-231 cell line as it does in macrophages. Thus, it is reasonable to speculate that it may be inhibiting the NF-κB pathway in these cells. NF-κB is linked to the regulation of cancer cell proliferation, survival, and apoptosis. Inhibiting NF-κB was expected to produce a greater effect in the MDA-MB-231 cell line. Since it only reduced cell viability by approximately 25-30% at the higher doses tested, it implies that there may be something unique about this cell line that allows it to efficiently proliferate when the NF-κB pathway is inhibited. Cancer is a very complicated disease and thus, it is possible that either NF-κB does not play a significant role in this cell line as it was initially predicted, or there may be a redundant pathway. Since NF-κB has been shown to be involved in the proliferation of this cell line (Jiang et al., 2004), it seems more likely that there is a redundant pathway and that perhaps the analogs tested here do not target the pathways that are substituting for NF-κB. Again, it would be worthwhile to further investigate the role of NF-κB in this cell line and how it is affected by curcumin analogs.

Finally, the effects of EF31 and UBS109 on monoaminergic pathways remain to be determined. As described in the discussion section, curcumin's

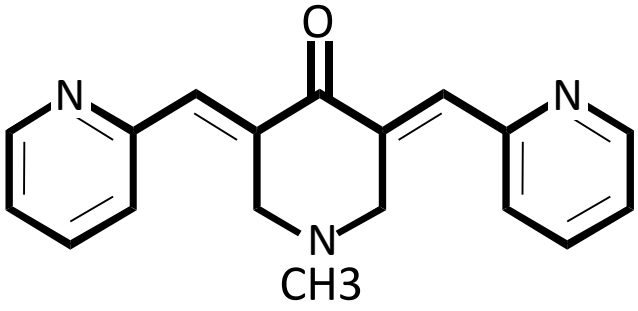
antidepressant effects have been linked to increases in serotonin and dopamine levels, as well as the expression of serotonin receptors and neurotrophic factors (Xu et al., 2005b; Xu et al., 2006). It is likely that curcumin analogs also produce these effects, and perhaps due to increased potency, they may exhibit more pronounced antidepressant effects. The antidepressant effects of EF31 and UBS109 in the absence of an inflammatory stimulus could be addressed by oral treatment in C57BL/6 mice and measuring behaviors in the Porsolt forced swim test, the open field test, and the sucrose preference test (described in Chapter 3).



Curcumin



EF31



UBS109

Figure 4-1. Chemical structures of curcumin and the monoketone analogs. EF31 and UBS109 are structurally related to curcumin with modifications made to the aromatic rings, the beta-diketone moiety, and the two flanking double bonds conjugated to the beta-diketone moiety.

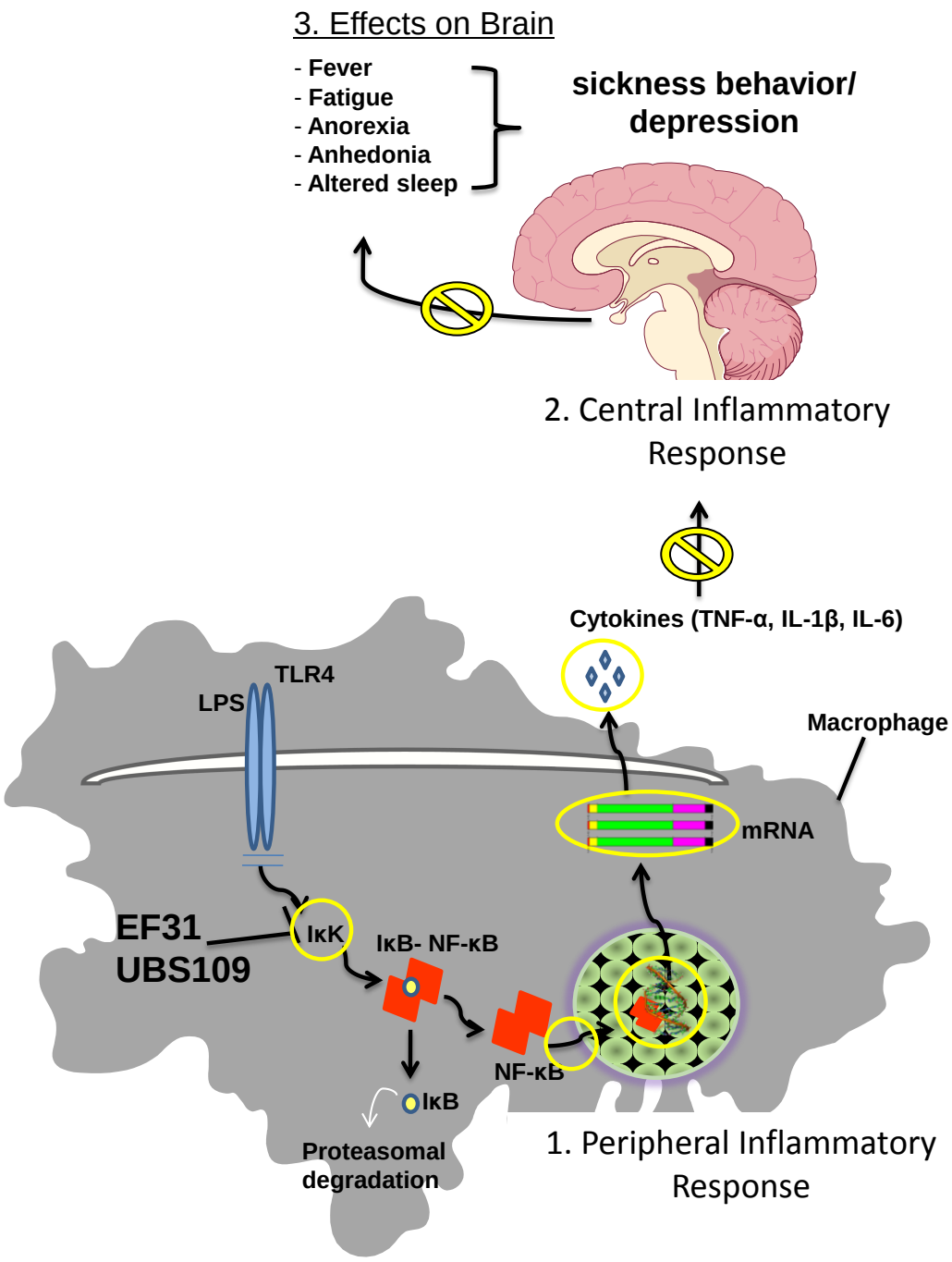


Figure 4-2. Effects of curcumin analogs on the NF-κB pathway. EF31 and UBS109 are potent inhibitors of the NF-κB pathway. By targeting the activity of IκK, these compounds block translocation of NF-κB to the nucleus, NF-κB DNA-binding, cytokine mRNA expression, and release of cytokine proteins. Given the role that NF-κB plays in the peripheral and central inflammatory response to LPS, it is hypothesized that they have the potential to block the development of LPS-induced sickness and depressive-like behaviors.

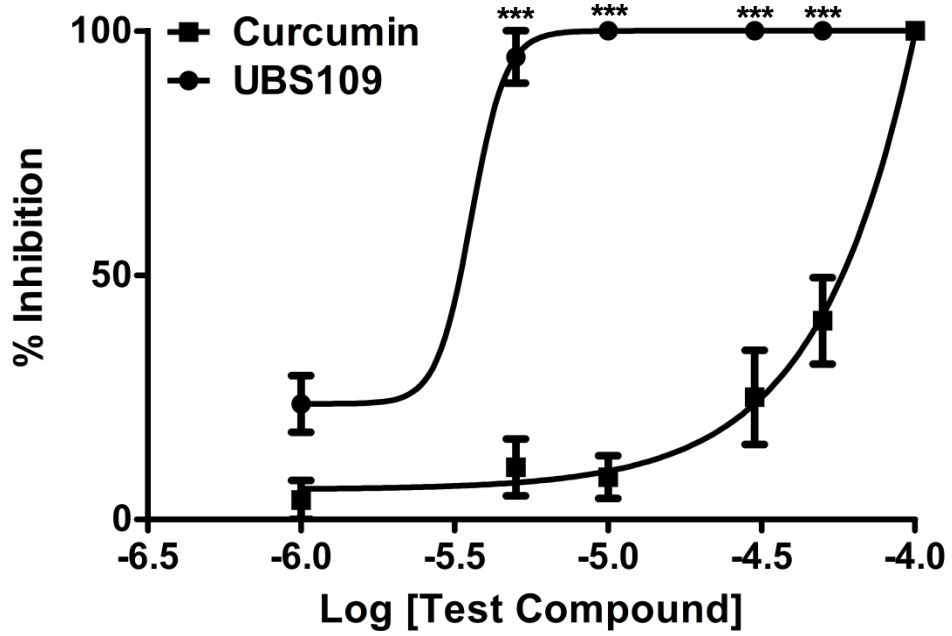


Figure 4-3. UBS109 is a potent inhibitor of LPS-induced NF-κB DNA-binding activity in mouse RAW264.7 macrophages. Mouse RAW264.7 macrophages were pre-treated with curcumin, UBS109 (1, 5, 10, 30, 50, or 100 µM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 µg/mL) for 15 minutes. Values shown are means (±SEM) expressed as percent inhibition from vehicle. *** $p < 0.001$ vs. curcumin at same concentration.

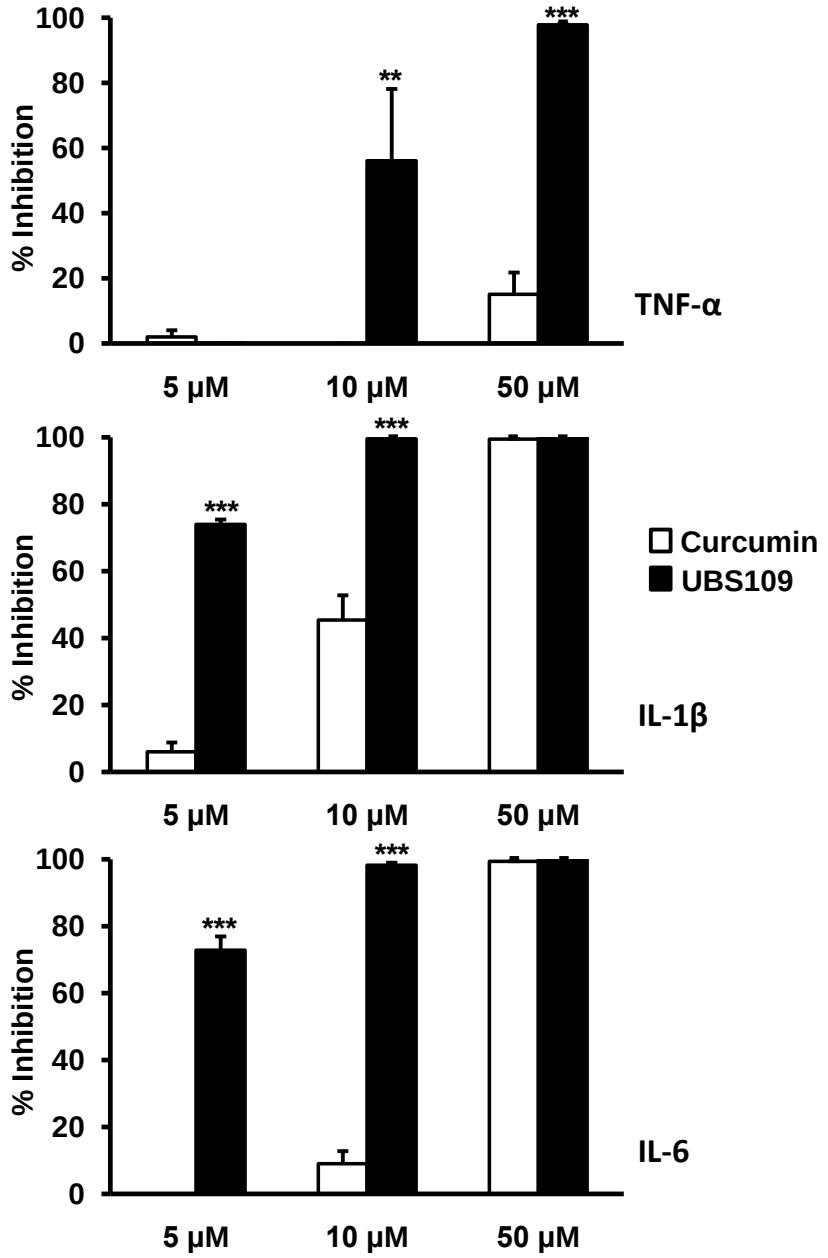


Figure 4-4. UBS109 inhibits LPS-induced pro-inflammatory cytokine mRNA expression in-vitro. Mouse RAW264.7 macrophages were pre-treated with curcumin, UBS109 (5, 10, or 50 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) for 3 hours. Whole cell mRNA was then collected and measured using RT-PCR. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent inhibition from vehicle. . ** $p < 0.01$, *** $p < 0.001$ vs. curcumin at same concentration.

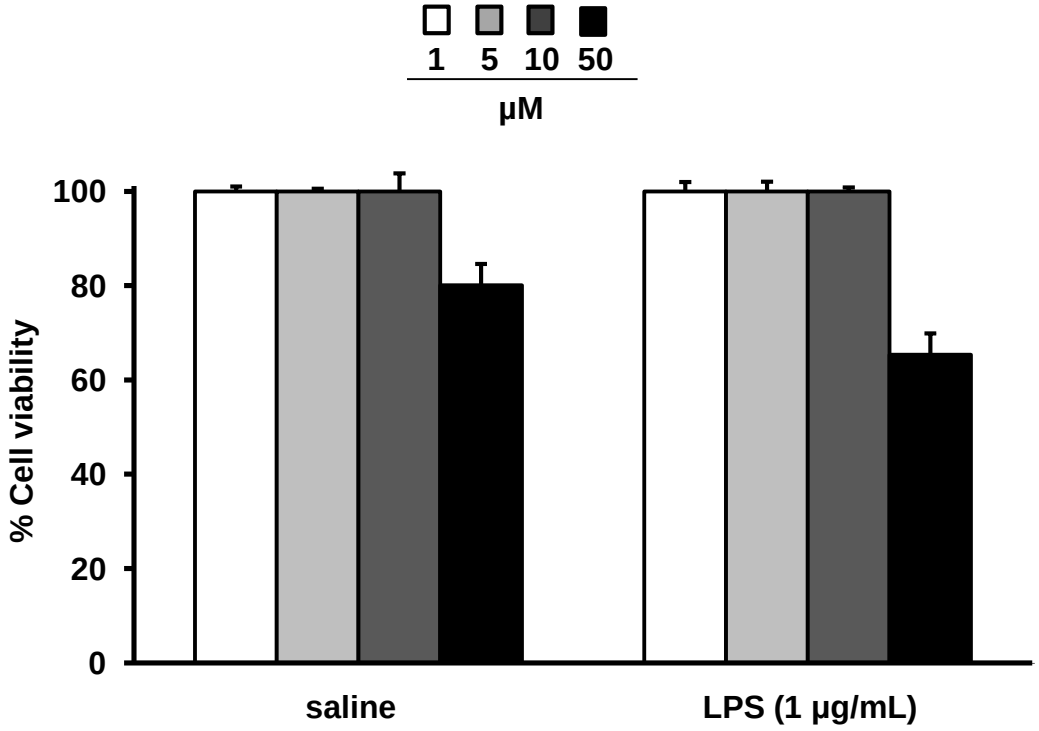


Figure 4-5. UBS109 shows no reduction in cell viability at concentrations that inhibit NF-κB DNA-binding activity. Mouse RAW264.7 macrophages were pre-treated with UBS109 (1, 5, 10, or 50 μM), or vehicle (DMSO 1%) for one hour prior to treatment with LPS (1 μg/mL) or saline for 15 minutes. Cell viability/ proliferation was then measured as described in Methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent of vehicle.

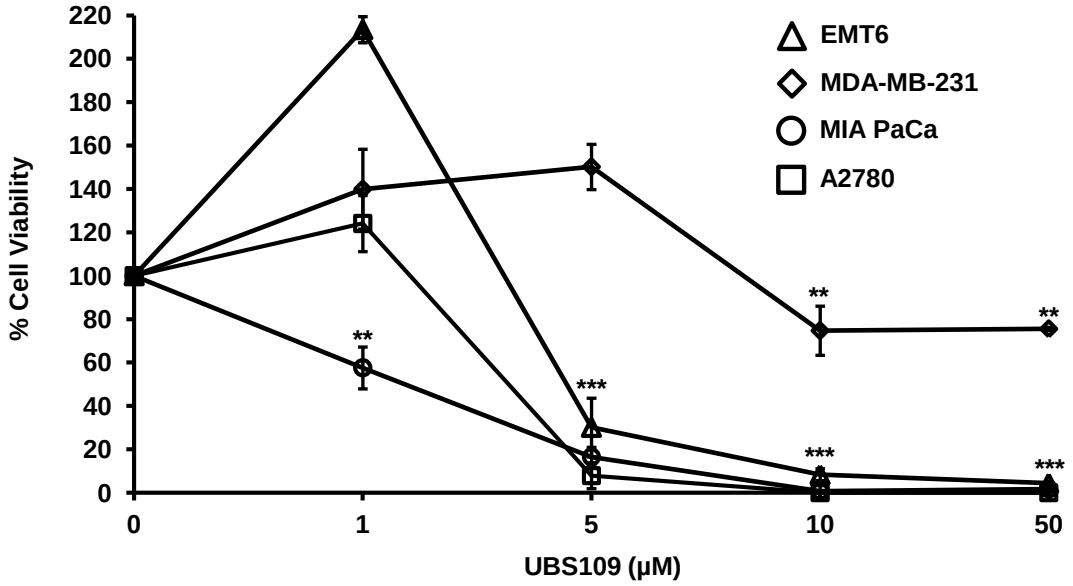


Figure 4-6. UBS109 shows potent toxicity on NF-κB-dependent cancer cell lines in-vitro. Cancer cell lines were treated with UBS109 (1, 5, 10, or 50 µM), or vehicle (DMSO 1%) for 48 hours. Cell viability/ proliferation was measured as described in methods. All conditions were run in triplicate, and values shown are means (±SEM) expressed as percent of vehicle. ** $p < 0.01$, *** $p < 0.001$ vs. vehicle.

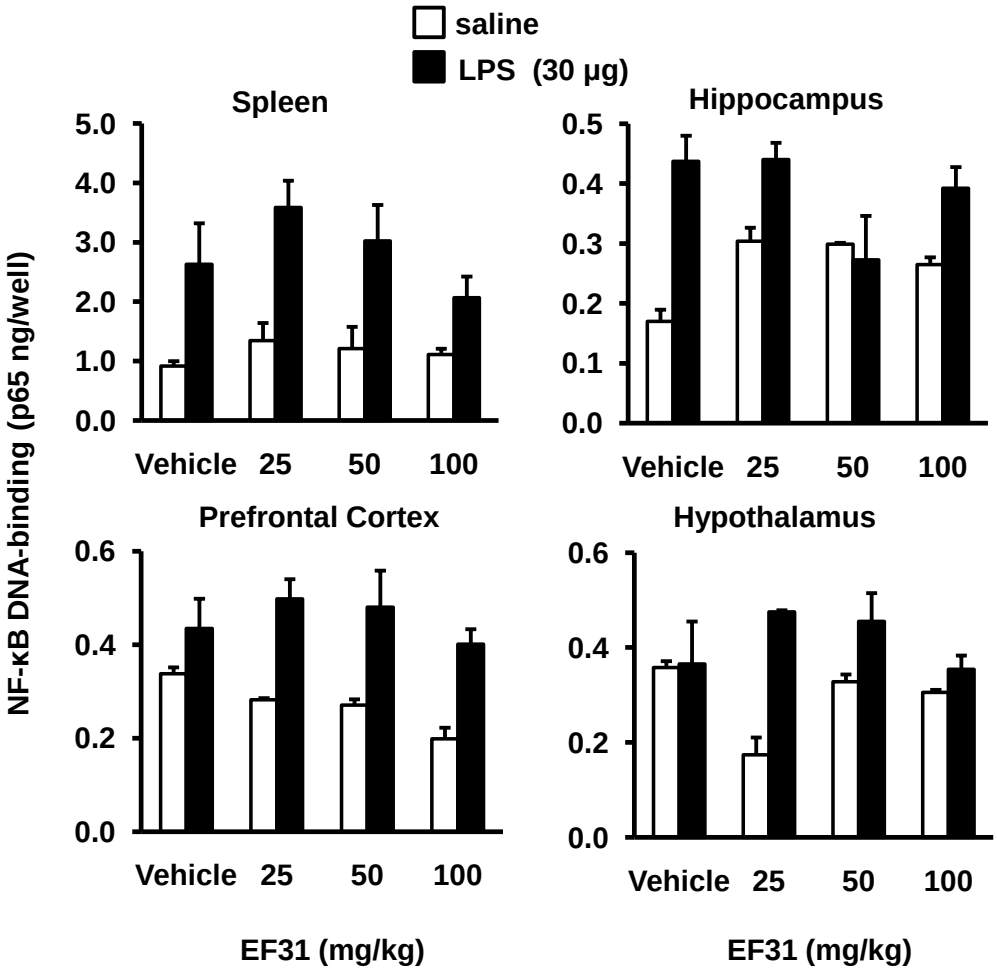


Figure 4-7. Oral treatment with EF31 did not significantly reduce LPS-induced NF-κB DNA-binding activity in the spleen or brain of C57BL/6 mice. Mice (n= 5/ treatment group) were pre-treated orally with either EF31 (25, 50, or 100mg/kg/255 µL), or vehicle [PEG300/DMA (85%/15%)] one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Spleen, hippocampus, prefrontal cortex, and hypothalamus were dissected one hour post LPS (n= 5 mice/treatment group) and used to generate nuclear extracts. The nuclear extracts were analyzed using a NF-κB DNA-binding ELISA. Values shown are means (±SEM).

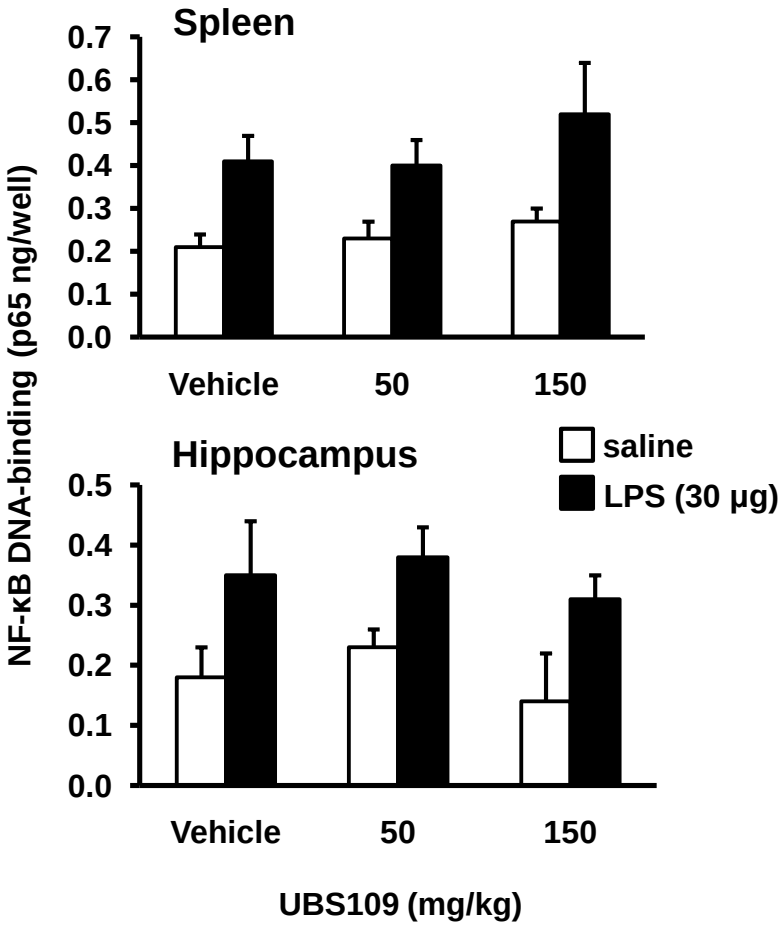


Figure 4-8. Oral treatment with UBS109 did not significantly reduce LPS-induced NF-κB DNA-binding activity in the spleen or brain of C57BL/6 mice. Mice (n= 5/ treatment group) were pre-treated orally with either UBS109 (50 or 150mg/kg/255 µL), or vehicle [PEG300/DMA/Cyclodextrin (28%/5%/30%)] one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Spleen and hippocampus were dissected one hour post LPS (n= 5 mice/treatment group) and used to generate nuclear extracts. The nuclear extracts were analyzed using a NF-κB DNA-binding ELISA. Values shown are means (±SEM).

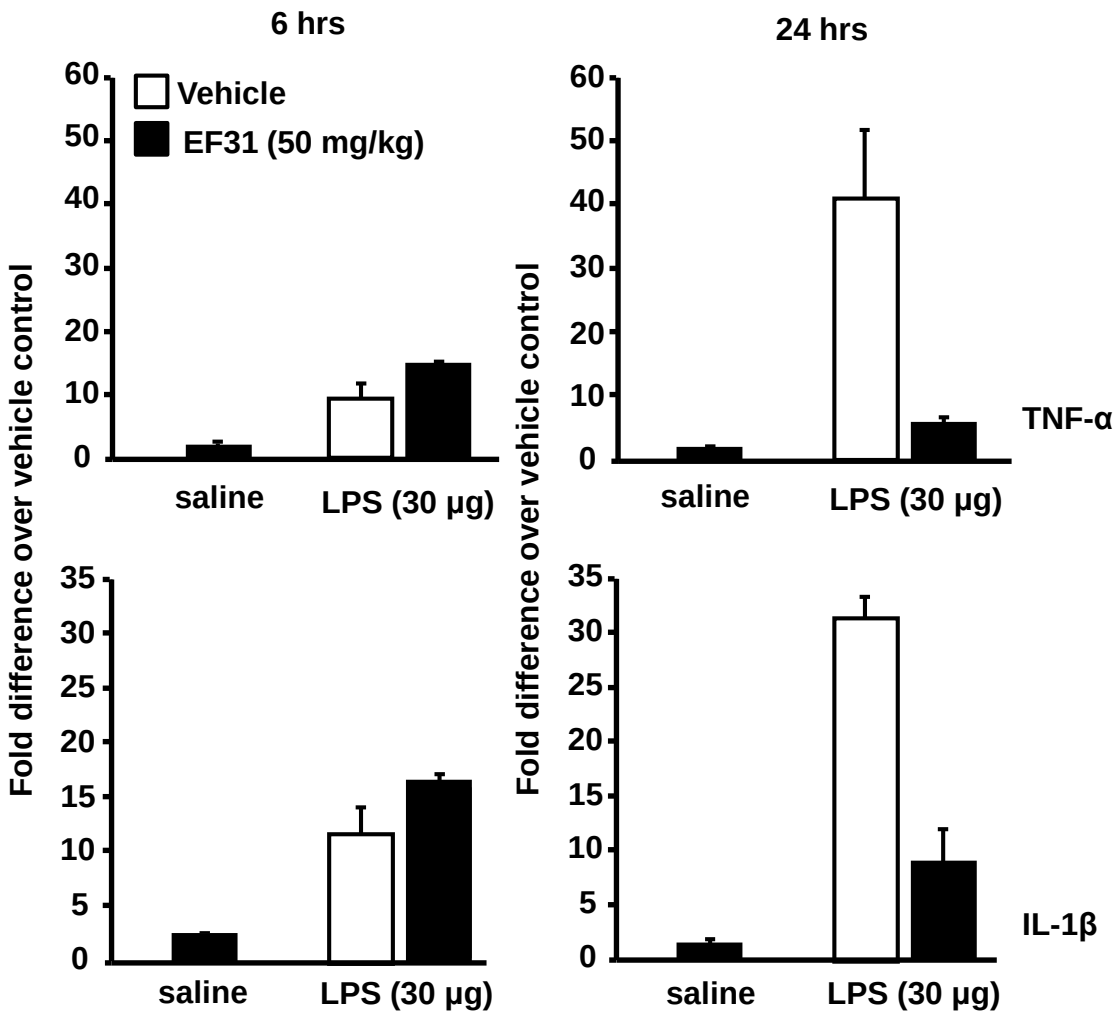


Figure 4-9. Oral treatment with EF31 did not reliably attenuate LPS-induced expression of TNF-α and IL-1β in the brain of C57BL/6 mice. Mice (n= 3 /treatment group) were treated orally with EF31 (150mg/kg/255 µL) or vehicle [9 parts Methyl cellulose (.5%) + 1 part PEG200/255 µL], for two consecutive days, followed by treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL) 6 or 24 hours before sacrifice. Total mRNA was extracted from the spleen and hippocampus and the expression of TNF-α and IL-1β was measured using RT-PCR. Results are expressed as fold difference over the saline + vehicle treated group. Values shown are means (±SEM).

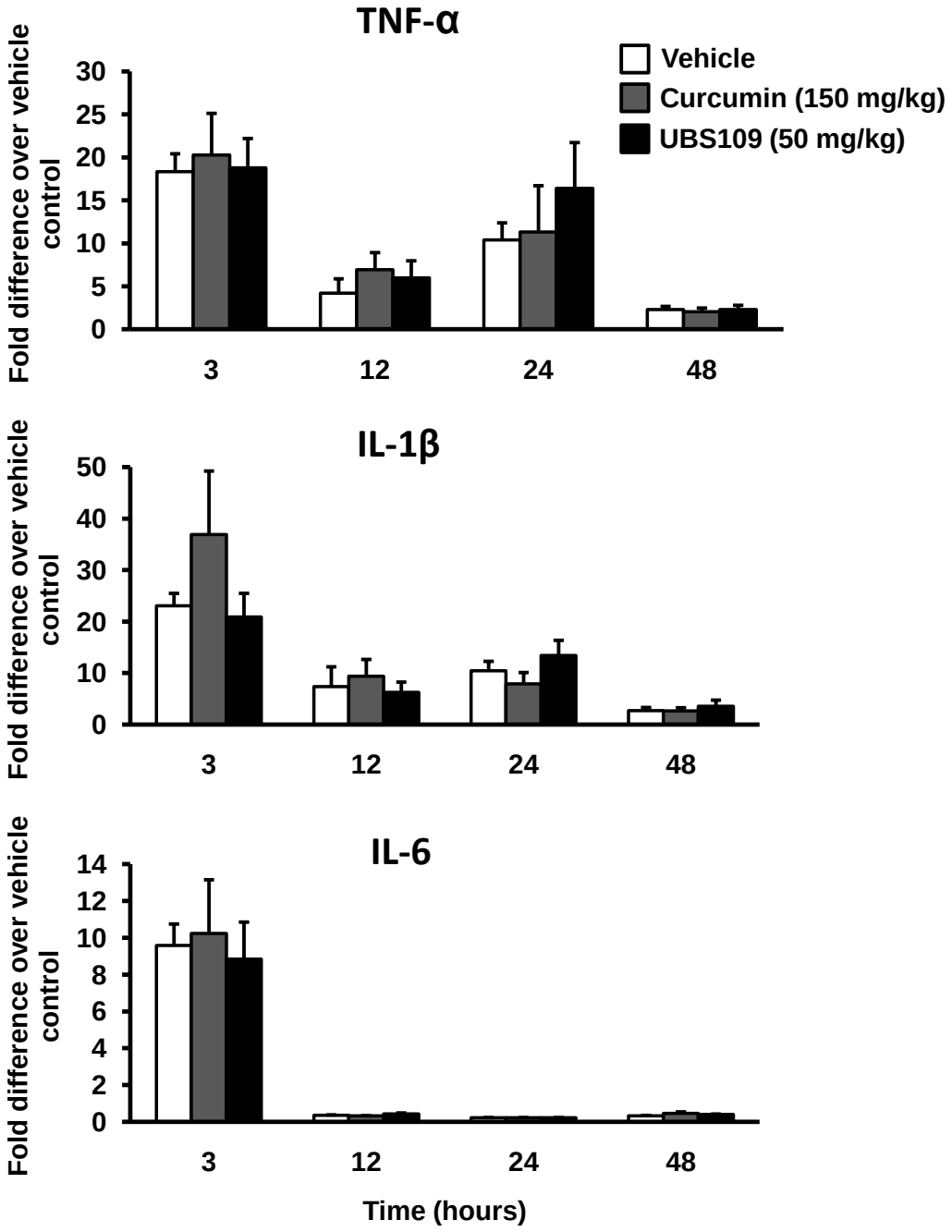


Figure 4-10. Oral treatment with UBS109 did not attenuate LPS-induced expression of TNF-α, IL-1β, and IL-6 in the brain of C57BL/6 mice. Mice (n= 6 /treatment group) were pre-treated orally with either UBS109 (50mg/kg/255 µL), curcumin (150mg/kg/255 µL), or vehicle [PEG300/DMA/Cyclodextrin (28%/5%/30%)] one hour prior to treatment with LPS (i.p./30 µg/ 100 µL/mouse) or saline (i.p./100 µL). Total mRNA was extracted from the hippocampus and the expression of TNF-α, IL-1β, and IL-6 was measured using RT-PCR. Results are expressed as fold difference over the saline + vehicle treated group. Values shown are means (±SEM).

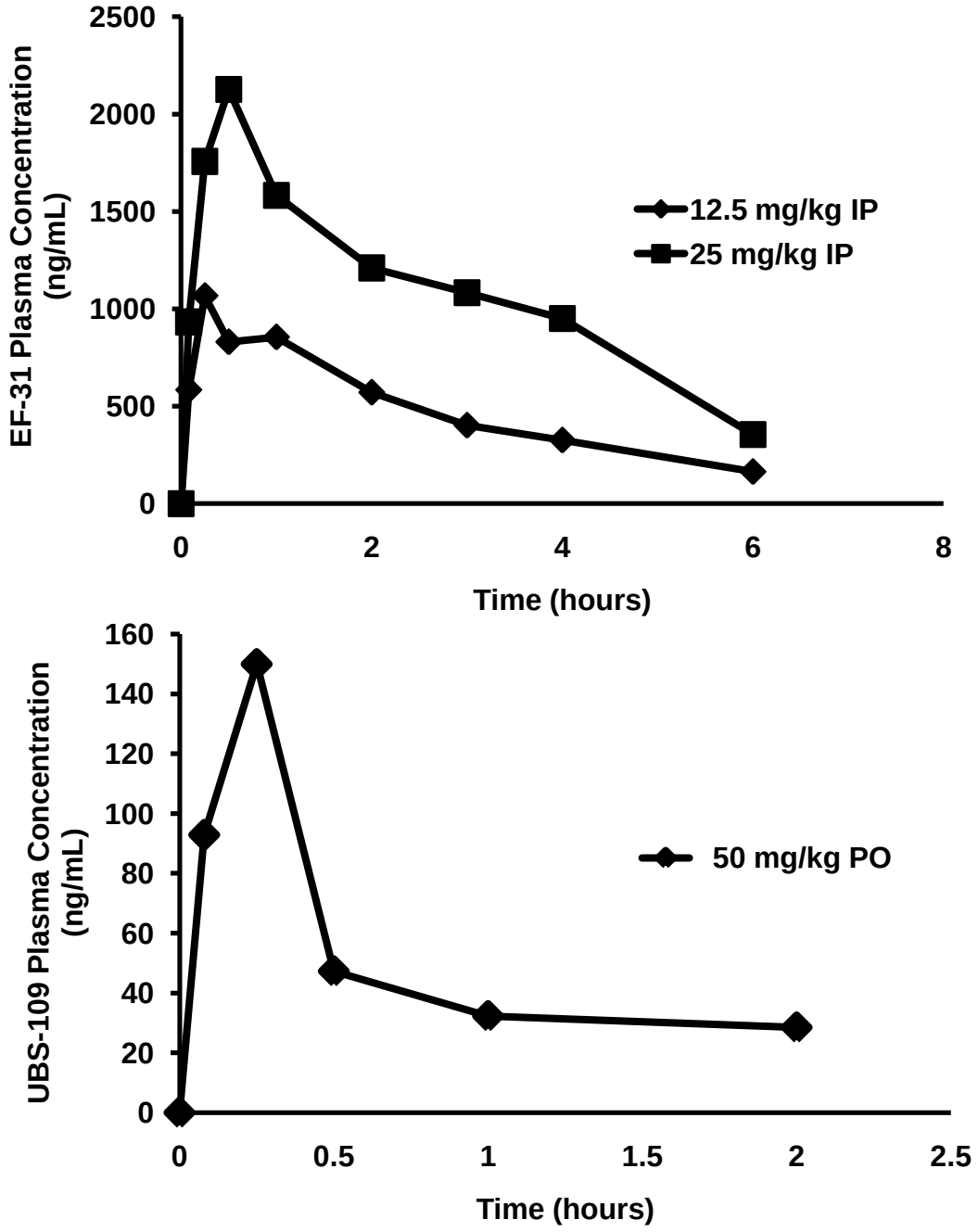


Figure 4-11. Preliminary pharmacokinetics for EF31 and UBS109. Mice (n= 1 /treatment group) were treated with either an i.p. injection of EF31 or orally with UBS109. Plasma was collected and the concentration of EF31 and UBS109 was determined using mass spectrometry.

Chapter 5

Conclusions, summary of findings, and other considerations

I. Introduction

Depression is a common disorder with a lifetime prevalence of over 15% (Kessler et al., 2003). The World Health Organization has identified depression as one of leading causes of disability worldwide (Lopez and Murray, 1998). Recently, it has become clear that the psychological symptoms of depression are not the only factors affecting the quality of life of the depressed patient. Major depression can also have notable immunological consequences (Irwin and Miller, 2007). Some depressed patients exhibit evidence of increased immune system activity, particularly increases in the innate immune (inflammatory) response (Raison et al., 2006). This increase in inflammation may have clinical implications in terms of cardiovascular disease, diabetes and cancer, where inflammation is increasingly being recognized as a common mechanism of disease (Black and Garbutt, 2002; Coussens and Werb, 2002; Dandona et al., 2004; Wellen and Hotamisligil, 2005; Libby, 2006). Inflammation may also directly contribute to depressive symptoms (Raison et al., 2006; Miller et al., 2009).

There is a complex network linking the immune system with the CNS that communicates and modulates function in a bi-directional fashion. A high incidence of depression has been reported in patients with immune disorders such as infectious diseases, autoimmune disorders and cancer (Yirmiya, 2000; Yirmiya et al., 2000; Evans et al., 2005; Raison et al., 2006; Miller et al., 2009). There are also studies that demonstrate that immune activation or administration of inflammatory factors induce a depressive state in humans and laboratory

animals. For example, exogenous administration of several cytokines to humans including interferon (IFN)-α and interleukin (IL)-2 as well as the cytokine-inducer, lipopolysaccharide (LPS), has been associated with the development of depressive symptoms including anhedonia, fatigue, impaired sleep, anorexia, and decreased memory and concentration (Yirmiya et al., 2000; Reichenberg et al., 2002; Dantzer, 2006; Dantzer et al., 2008; Miller et al., 2009). In addition, the development of major depression in humans is associated with alterations in immune function including immune activation (Raison et al., 2006; Miller et al., 2009). There are several lines of evidence that indicate that this inter-dependant relationship between the immune system and depression involves neurotransmitters [e.g. serotonin (5-HT), dopamine (DA) and norepinephrine (NA)] and the hypothalamic-pituitary-adrenal (HPA) axis (Linthorst et al., 1995; Besedovsky and del Rey, 1996; Merali et al., 1997; Pauli et al., 1998; Dunn et al., 1999; Raison et al., 2006; Irwin and Miller, 2007). Overall, pathways known to be involved in the pathophysiology of depression can be influenced by cytokines, including neuroendocrine function, neurotransmitter function, and synaptic plasticity (Miller et al., 2009).

The release of innate immune cytokines during an inflammatory response is regulated largely by the inflammatory signaling molecule, nuclear factor κ B (NF-κB) in macrophages in the periphery and microglia in the brain (Baeuerle and Baltimore, 1996). NF-κB is a family of transcription factors known to exist in virtually all cell types, and is responsible for regulation of multiple target genes involved in inflammatory processes, cell proliferation, apoptosis, and also plays a

role in glial and neuronal cell function (Barnes and Karin, 1997; Giuliani et al., 2001; Tak and Firestein, 2001; Bharti and Aggarwal, 2002). LPS, a cell wall component of gram-negative bacteria induces NF-κB activation, which in turn regulates the production of cellular signaling molecules including cytokines, chemokines, adhesion molecules, acute phase proteins, inducible enzymes such as inducible nitric oxide synthase, major histocompatability proteins, and cyclooxygenase-2 (Chow et al., 1999). Treatment with LPS in mice results in the development of behavioral changes termed sickness behavior and depressive-like behaviors (Dantzer, 2006). These behaviors are comparable to the symptoms reported in depressed patients including fatigue, anhedonia, and reduced social interaction.

Turmeric is derived from the root of the plant *Curcuma longa*, and has been used for centuries in traditional Chinese medicine to treat inflammatory diseases (Jagetia and Aggarwal, 2007; Anand et al., 2008). Curcumin, a potent NF-κB inhibitor, is the active ingredient in turmeric and it has been shown in animal studies to exhibit anti-inflammatory and antidepressant properties (Anand et al., 2008). However, curcumin's use for clinical treatment is limited in part due to its reduced bioavailability, poor absorption and rapid metabolism (Anand et al., 2007). Structural modifications were made to enhance the potency of curcumin as well as improve its bioavailability. Curcumin analogues (EF31 and UBS109) with modifications made to the aromatic rings, the beta-diketone moiety, and the two flanking double bonds conjugated to the beta-diketone moiety are being developed.

The anti-inflammatory effects of these curcumin analogs were tested in-vitro in mouse RAW264.7 macrophages and in-vivo in male C57BL/6 mice using LPS as the inflammatory stimulus. The goal of the current studies was to develop novel drugs that target inflammatory signaling pathways, NF-κB in particular, and thereby lead to alternative approaches for treating patients with depression, especially those who are unresponsive to or intolerant of conventional antidepressant medications and the most likely to exhibit increased markers of inflammation.

II. Summary of findings

Chapter 2 presented a series of in-vitro experiments in which the activity of EF31 on the NF-κB pathway was examined in mouse RAW264.7 macrophages. EF31 (IC_{50} ~5 μM) exhibited significantly more potent inhibition of LPS-induced NF-κB DNA-binding compared to both EF24 (IC_{50}~35 μM) and curcumin (IC_{50} >50 μM). In addition, EF31 exhibited significantly greater inhibition of NF-κB nuclear translocation as well as the induction of downstream inflammatory mediators including pro-inflammatory cytokine mRNA and protein expression [tumor necrosis factor (TNF)-α, IL-1β, and IL-6]. Regarding the mechanism of these effects, EF31 (IC_{50}~1.92 μM) exhibited significantly greater inhibition of IκB kinase β compared to EF24 (IC_{50}~131 μM). Finally, EF31 demonstrated potent toxicity in NF-κB-dependent cancer cell lines while having minimal and reversible toxicity in RAW264.7 macrophages. These data indicate

that EF31 is a more potent inhibitor of NF-κB activity than either EF24 or curcumin while exhibiting both anti-inflammatory and anticancer activities.

In Chapter 3, a model of LPS-induced sickness and depressive-like behaviors was developed in male C57BL/6 mice. Results demonstrate that a single dose of LPS (30 μg/mouse/i.p.) induces sickness and depressive-like behaviors at 6 and 24 hours after treatment, respectively. Motor activity was decreased in the open field test at 6 hours and returned to baseline levels by 24 hours. However, immobility in the Porsolt forced swim task was significantly higher in the LPS treated group at 24 hours. Moreover, LPS treatment significantly reduced intake of a 2% sucrose solution in the sucrose preference for up to 72 hours. Finally, social behaviors were significantly reduced in the LPS-treated group at 6 hours and returned to baseline at 24 hours. These behavioral changes were accompanied by systemic and central innate immune/ inflammatory alterations including increases in NF-κB DNA-binding activity peaking at one hour in the spleen and the brain (including hippocampus, prefrontal cortex, and hypothalamus). Pro-inflammatory cytokine mRNA expression (TNF-α, IL-1β, and IL-6) peaked at one hour in the spleen and at 3 hours in the brain followed by a second peak of TNF-α and IL-1β expression at 24 hours in brain tissues only. Moreover, plasma MCP-1 protein peaked at 3 hours. A second peak of NF-κB DNA-binding was not present between 12 and 24 hours. Nonetheless, when mice were pre-treated with TAT-NEMO binding domain (NBD) peptide prior to LPS, a significant reduction in TNF-α mRNA expression in the hippocampus at 24 hours was evident. However, when the

TAT-NBD peptide was given 12 hours after treatment with LPS, the expression of TNF-α was not attenuated at 24 hours. These results suggest that a second NF-κB peak (after 12 hours) does not appear to be responsible for the second peak of cytokine mRNA expression at 24 hours. Nevertheless, NF-κB has shown a bi-phasic response to LPS in previous studies (Han et al., 2002), with a second peak between 6 and 12 hours after LPS. Thus, the second peak in cytokine mRNA expression may be explained by an earlier second peak in NF-κB DNA-binding activity (prior to 12 hours) or the effect may be mediated by a complementary inflammatory pathway such as the mitogen activated protein kinase (MAPK) pathway. Taken together these results indicate that LPS-induced cytokine expression parallels the sickness behaviors and depressive-like behaviors in-vivo, whereas NF-κB appears to be responsible for the early initiation of the inflammatory cascade.

Results in Chapter 4 demonstrate that UBS109 (IC_{50} ~2 μM) exhibited significantly more potent inhibition of LPS-induced NF-κB DNA-binding compared to curcumin (IC_{50} >50μM) in mouse RAW264.7 macrophages. In addition, UBS109 exhibited significantly greater inhibition of downstream pro-inflammatory cytokine mRNA expression while maintaining potent anti-cancer activity in NF-κB-dependent cancer cell lines and no toxicity in mouse RAW264.7 macrophages. In-vivo experiments showed no effects of oral treatment with EF31 or UBS109 on LPS-induced NF-κB DNA-binding activity and no reliable effects were found on LPS-induced cytokine mRNA expression in the periphery or the brain of male C57BL/6 mice. Taken together these results indicate that UBS109

is a potent inhibitor of the NF-κB pathway in-vitro. However, in-vivo effects of curcumin analogs were inconsistent and may require a different treatment paradigm.

III. Conclusions and considerations

Given the link between inflammation and treatment resistant depression, novel anti-inflammatory agents could offer an alternative therapeutic option for the relief of depressive symptoms (Miller et al., 2009). NF-κB is a key mediator of the inflammatory immune response and has been shown to be involved in many of the cytokine-induced alterations in neuronal activity and monoaminergic transmission that are relevant to the pathophysiology of depression (Miller et al., 2009; Koo et al., 2010). Thus, NF-κB represents a good target for the development of novel anti-inflammatory agents. EF31 and UBS109 are two promising curcumin analogs with potent inhibition in the NF-κB pathway. Moreover, these compounds target multiple pathways which may be an advantage given that depression is a heterogeneous syndrome that affects multiple pathways and systems (Millan, 2006; Anand et al., 2008). In addition to the anti-inflammatory effects, these compounds also showed potent anti-cancer activity, and are therefore being developed as part of a series of curcumin analogs for therapeutic applications in inflammatory disorders and cancer.

One consideration for future development of these compounds are the possible side effects of NF-κB inhibition. Given the ubiquitous expression of NF-

κB throughout the brain and the periphery, an inhibitor could present undesirable side effects (Barnes and Karin, 1997; Nam, 2006). Previous studies have found that curcumin and curcuminoids (such as EF24) are relatively non-toxic in laboratory animals and humans (Cheng et al., 2001; Mosley et al., 2007). However, collaborative projects with the Emory Institute for Drug Discovery are being conducted to determine the bioavailability of EF31 and UBS109 in tissues including the spleen and the brain and determine plasma and tissue concentrations using mass spectrometry, as well as formal toxicity studies that will help to design an optimal dosing regimen for future experiments. These experiments will also inform the interpretation of future results. If EF31 or UBS109 effectively block cytokine expression in the brain, the interpretation of these results will depend on whether or not the compounds can cross the blood brain barrier. There are at least two possible outcomes: either a) the compounds can block central inflammation by targeting the peripheral inflammatory response, or b) the compounds can block central inflammation only by having access to the brain. Whether or not the compounds must be present in brain tissues to block LPS-induced inflammatory and behavioral responses remains to be determined.

A second consideration is that data interpretation of EF31 or UBS109-induced NF-κB inhibition may not be so straightforward. The effects of these compounds will likely be more complex than simple inhibition of NF-κB DNA-binding and the expression of genes with NF-κB responsive elements. Therefore, exploratory analyses of the effects of curcumin analogs on multiple immune,

neuroendocrine and monoamine signaling pathways (inferred by analysis of transcription factor activation from gene expression using DNA microarrays and the TELiS system) as well as their relationship with behavior should be conducted. Moreover, assessments of gene expression at early and late time-points will allow distinction between primary signaling pathways targeted by EF31 or UBS109 versus those that are more “downstream”.

One last consideration is the exclusive use of male mice in these experiments. The parametric studies presented in Chapter 3 were conducted in male mice for two major reasons. First, a number of studies in humans have found that the relationship between inflammatory markers [such as C-reactive protein (CRP) and IL-6] and depression is stronger in males than in females (Danner et al., 2003; Penninx et al., 2003; Ford and Erlinger, 2004). For example, in a sample of 6,149 individuals (2,981 males and 3,119 females) aged 17-39 years, Danner et al. (2003) found that a history of a major depression was associated with elevated CRP in men, while in women the comparable association was weak and not significant. In addition, in a sample of older individuals (3,024 persons, 70-79 years old, 51.5% women), Penninx et al. (2003) found that the association between depressed mood and serum concentrations of IL-6 was significantly stronger in men than in women. Second, administration of inflammatory stimuli such as the innate immune cytokine, IFN-α, to humans has not been shown to lead to a greater likelihood of depression in women than men. Indeed, in a study of 162 patients, gender was not found to be a significant predictor of the development of depression during IFN-α therapy

(Raison et al., 2005). Nevertheless, given the higher incidence of depressive symptoms in females versus males, as well as gender differences in immune responses (Whitacre et al., 1999), the potential therapeutic effects of curcumin analogs on depressive symptoms must be measured in female mice in future experiments.

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