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Andrea I. Liatis Date

NOREPINEPHRINE REGULATION OF INFLAMMATORY RESPONSES DURING MONOCYTE ACTIVATION AND DIFFERENTIATION AND DURING ACUTE STRESS IN PATIENTS WITH MAJOR DEPRESSION

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Abstract

NOREPINEPHRINE REGULATION OF INFLAMMATORY RESPONSES DURING MONOCYTE ACTIVATION AND DIFFERENTIATION AND DURING ACUTE STRESS IN PATIENTS WITH MAJOR DEPRESSION

By Andrea I. Liatis

Activation of the sympathetic nervous system and release of catecholamines, such as norepinephrine (NE), regulate inflammation through interactions with the innate immune system. Although crosstalk between these two systems is clear, the role of NE in mediating inflammatory responses is less clear, with evidence of both pro- and anti-inflammatory effects. To further understand these paradoxical effects of NE, we examined the impact of cellular activation and differentiation on inflammatory cytokine production after NE administration to a human monocyte cell line. Using lipopolysaccharide (LPS) to induce cellular activation, we showed that NE inhibits interleukin (IL)-6 protein release from the monocytic form of the cell, when administered before LPS. However, treating the cells with NE after LPS-induced activation led to a decrease in NE's inhibitory effects. NE inhibition of LPS-induced IL-6 was shown to be mediated by the beta 2- adrenergic receptor (B2-AR) as well as cAMP. Interestingly, the decreased sensitivity to NE following monocyte activation by LPS was found to be mediated by a PKA-dependent decrease in B2-AR mRNA. Indeed, blocking PKA reversed B2-AR mRNA downregulation and restored cell sensitivity to NE. Differentiation of cells from the monocyte state to the macrophage state also resulted in decreased NE sensitivity, likely a result of increased mRNA expression of beta arrestin-2 (BARR-2), which can lead to B2-AR desensitization. Finally, LPS-induced activation of cells in the macrophage state completely abolished NE's anti-inflammatory effects, in association with both decreased B2-AR mRNA as well as decreased mRNA for BARR-2, which can inhibit inflammatory responses. To study the impact of NE on inflammation in vivo, we examined stress-induced NE and IL-6 release in patients with major depression versus healthy controls. We found that patients with depression exhibited reduced sensitivity to NE, as depressed patients with high NE responses to stress exhibited the highest stress-induced IL-6 responses. Taken together, these studies indicate that NE primarily functions to inhibit inflammation. However, the extent of NE's anti-inflammatory action is dynamic and depends on the physiological state of the cell. Dysregulation of NE's anti-inflammatory effects may contribute to pathology in diseases involving inflammation, such as depression.

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CHAPTER ONE

INTRODUCTION: INTERPLAY BETWEEN INFLAMMATION AND THE SYMPATHETIC NERVOUS SYSTEM: ROLE OF ADRENERGIC RECEPTORS

I. Immune-nervous system interactions: A historical perspective on catecholaminergic immunomodulation

Although the areas of neuroscience and immunology developed separately and are still maintained as separate disciplines, in recent years there has been a growing number of researchers dedicated to studying the interaction between these two physiological systems. Indeed, over the last 50 years, there has been a wealth of data showing that these two systems communicate and influence each other's reactions to internal and external stimuli (Elenkov, 2000). Thus, the crosstalk between these two systems is considered an essential element for maintaining homeostasis (Elenkov, 2000). These interactions have also led to the exciting new field of neuroimmunology, where researchers have begun to study the extensive, and often complicated, interrelationships between these systems, both in terms of the maintenance of health and the development of disease.

Some of the earliest documented studies providing evidence for the crosstalk between the nervous and immune systems include anatomical studies, such as Tonkoff's study in 1899 (Elenkov, 2000; Oberbeck, 2006), showing that nerves were found to enter lymph nodes, independently of blood vessels. Around this same time, chemically oriented studies also laid the groundwork for this interaction, when an increase in blood pressure was observed following an injection of a preparation made from the adrenal gland (Oliver and Schafer, 1895). This preparation was later shown to contain the bioactive compound identified as "suprarenin", by Otto von Furth. "Suprarenin" later became known as epinephrine, a catecholamine involved in the sympathetic nervous system (SNS) (Elenkov, 2000; Oberbeck, 2006). The story between epinephrine and immune function evolved in 1904, when Louper and Crouzon described an increase in white blood cells, or

leukocytosis, in humans after an injection of epinephrine. This phenomenon was later determined to involve an increase in blood lymphocytes early after the injection, followed by a later increase in granulocytes (as reviewed in Oberbeck, 2006). Work continued in this area, which characterized in detail the idea of catecholamine-induced leukocytosis. For example, in the 1950's, through the use of more advanced cell sorting techniques, Dougherty and colleagues also observed this initial increase in lymphocytes, followed by an increase in a subset of cells, which they called "stress lymphocytes". They noticed that these cells were similar, if not the same as, the aforementioned second increase in granular cells after epinephrine injection, and identified them as having natural killer activity (Doughtery et al., 1953). This area is still being studied presently, with studies showing that epinephrine-induced leukocytosis generally leads to an increase in natural killer cells and CD8+ lymphocytes in part related to catecholamine effects on the expression of adhesion molecules (Sanders and Straub, 2002). Interestingly, in 1994, this catecholamine-induced leukocytosis was shown to occur with endogenously derived levels of catecholamines as well, when it was found that endogenous norepinephrine (NE) levels could also affect cytokine production in macrophages (Spengler et al., 1994). The area has continued to develop, and the effects of catecholamines on immune cell function have been studied in a variety of other areas. For example, in 1970, Hadden et al. reported that adrenergic agents could modulate lymphocyte proliferation, and thus indicated a functional hormone receptor on these cells. Other studies have focused on modulation of catecholamines on the production of different cytokines, expression of cell adhesion molecules, antibody release and phagocytosis (Benshop, 1996; Elenkov et al., 1996; Gan et al., 2002).

Early studies focused on the mechanisms by which the nervous system could affect and alter the function of the immune system. But, it wasn't until the 1970's and 1980's that it became clear that the interaction between the two systems was not one-way, but rather, bidirectional. Among many findings, Besedovsky and colleagues showed that immune responses could alter the nervous system through alteration of noradrenergic neurons in the hypothalamus (1983) and a decrease in the amount of NE in the spleen of mice (Besedovsky et al., 1979; Del Rey et al., 1982). Finally, in the 1970's through the 1990's, the idea of this interaction was strengthened, when Felten and colleagues provided some of the first in-depth evidence that lymphoid organs were anatomically connected to the nervous system, showing that noradrenergic fibers innervated primary and secondary lymphoid tissues, creating a direct neural connection between the SNS and the immune system (Felten et al., 1988; Felten et al., 1992).

The interplay between these two supersystems is still being studied extensively. Many researchers have now begun to focus on how catecholaminergic immunomodulation affects disease states and progression; studying its effect on neurodegenerative diseases, autoimmune disease, and mental illness (Eskandari and Sternberg, 2002; Heijnen and Cohen, 1999; Sternberg et al., 1992).

II. Innate Immune System

A. Biology of the innate immune response: Innate vs. adaptive

Prior to considering the interaction between the SNS and inflammation, it is important to review the general physiology of the innate immune system, as inflammation is one of its main physiological responses to any threats on homeostasis (Fujiwara and Kobayashi, 2005). In vertebrates, the immune system is split into two

divisions: the innate and the adaptive. The innate immune system is the first and most ancient line of defense that the body has against invading pathogens and trauma and can be found among all plants and animals, while the adaptive immune system evolved much later and is only found in vertebrate animals. A main difference that divides these two systems, other than evolutionary age, is that the innate immune system responds in a nonspecific manner to foreign substances, whilst the adaptive immune system uses specific recognition of antigens using antibodies. The characteristic of non-specificity in the innate division is what allows for a very rapid, immediate response to attack. On the other hand, the specificity of the adaptive immune response allows for greater selectivity and for the creation of immunological memory, which can elicit a faster and more vigorous response to a future attack. **Table 1-1** summarizes the main differences between the two divisions of the immune system (Mackay and Rosen, 2000; Janeway, 2001; Beutler, 2004; Kindt et al., 2007; Turvey and Broide, 2010). The rest of the section will focus solely on the innate immune system.

B. Components of the innate immune response

Anatomical barriers

The innate immune response begins externally with its anatomical barriers, the skin and mucosal surfaces that line the respiratory and gastrointestinal tracts (Kindt et al., 2007; Turvey and Broide, 2010). These anatomical features not only provide a physical barrier against attacking substances, but a chemical one as well (Janeway, 2001). For example, the skin contains antimicrobial peptides and proteins, such as the recently identified psoriasin, which has antimicrobial properties against E. coli (Glaser et al., 2004).

Receptors of the innate immune system

Once these barriers are breached, the innate immune system must respond to the invaders by detecting them in some manner that discriminates between the self and the foreign pathogen, and then attacking them. Recognition of pathogens occurs through pattern recognition receptors, which recognize broad structural motifs in pathogens that are highly conserved, called pathogen-associated molecular patterns (Medzhitov and Janeway, 2000; Janeway, 2001; Turvey and Broide, 2010). These can include soluble receptors that act to initiate the complement system and acute phase reactants, such as C-reactive protein (CRP). For example, the mannon-binding lectin is a protein composed of carbohydrate-binding lectin domains, which bind with high affinity to mannose or fructose on bacterial cell walls, and then stimulates the complement system (Beutler, 2004).

The innate immune system also contains membrane-bound receptors, such as the family of toll like receptors (TLR) (Medzhitov and Janeway, 2000). They were first described in Drosophila, where it was shown that the fruit flies required this protein, Toll, to defend against fungal infections (Lemaitre at al., 1996). TLRs are found on several immune cells and to date, ten have been described in humans, enumerated 1-10 (Beutler, 2004). Each TLR is thought to recognize a distinct set of ligands. For example, TLR4 detects bacterial lipopolysaccharide (LPS), and TLR2 can detect peptidoglycans and lipoproteins. Other TLRs can form heteromeric complexes with others to broaden their recognition ability. They exist as dimeric proteins, with an ectodomain composed of leucine-rich repeat motifs and a cytosolic component called the Toll/IL-1 receptor-like

domain (TIR), which is involved in the receptors signaling (Mackay and Rosen, 2000; Janeway, 2001; Beutler, 2004; Takeda and Akira, 2004; Kawai and Akira, 2006; Kindt et al., 2007). TLR's are one of the main signaling receptors to induce an innate immune response by activating several inflammatory signaling pathways. Their role in inflammation and the signaling mechanisms involved will be discussed in later sections with TLR4 and LPS as the example. Once pathogens are recognized, this sets into motion a variety of humoral and cellular components of the innate immune system.

Humoral barriers: Complement system

The complement system is the main humoral component of the innate immune response. It was first discovered as a component of normal plasma that helped in the opsonization of bacteria by antibodies, which is the process by which antibodies bind to antigens on bacteria to help signal that they should be engulfed, thus named 'complement'. The system is made up of a variety of plasma proteins that can be activated by pathogens directly, or by pathogen-bound antibodies. Three different pathways activate the system: the classical pathway, the mannin binding-lectin pathway and the alternative pathway. Although different molecules activate the pathways, their end result is the same. Antigen-antibody complexes trigger the classical pathway, the mannose binding-lectin pathway is triggered by lectin binding to the surface of pathogens, and the alternative pathway is triggered directly by pathogen surfaces. Activation of any of these pathways results in the initiation of a cascade of enzymatic activities of numerous complement proteins that in turn results in the three main functions of the complement system, which are the recruitment of inflammatory cells, the

opsonization of pathogens and the killing of pathogens. (Janeway et al., 2001; Rus et al., 2005)

Cellular barriers

Cells of the immune system originate in the bone marrow. Many of the cells mature there, and then migrate to peripheral tissues through circulation in the blood and the lymphatic system. Innate immunity largely involves myeloid progenitor cells, which are the precursors to the main cellular components of the innate immune response. The cells can be subdivided into mononuclear phagocytes and polymorphonuclear phagocytes. Mononuclear phagocytes include monocytes, which become macrophages in the tissues and dendritic cells. Polymorphonuclear phagocytes, also known as granulocytes, include, neutrophils, eosinophils, and basophils. There is a third kind of innate immune cell, the natural killer cell, which is actually differentiated from a lymphoid progenitor cell (Janeway, 2001; Beutler, 2004; Kindt et al, 2007).

*Cellular barriers***:** *Polymorphonuclear Phagocytes/Granulocytes*

These cells are so called due to densely staining granules in their cytoplasm, as well as oddly shaped nuclei. The granulocytes are very short lived and are produced in great number during an immune response. Neutrophils are the most numerous of this cell type. These cells are specialized killers and phagocytes, and the release of their granules contains a variety of toxic substances, including hydrogen peroxide and free oxygen and nitrogen radicals. Eosinophils and basophils are important during parasitic infection, and,

along with mast cells, are mediators of the allergic reaction, releasing products like histamine (Janeway, 2001; Beutler, 2004; Kindt et al, 2007).

*Cellular barriers***:** *Natural Killer Cells*

Although natural killer (NK) cells differentiate from lymphoid progenitor cells, they have very important functions in the innate immune response. They do not contain any antigen-specific receptors, and do not attack pathogens directly. Instead, these cells can destroy host cells such as tumors or virus-infected cells. They can also release a variety of molecules that stimulate the maturation of other phagocytic cells, such as macrophages and dendritic cells (Janeway, 2001; Beutler, 2004).

*Cellular barriers***:** *Mononuclear Phagocytes*

Mononuclear phagocytes include dendritic cells and macrophages. Dendritic cells are mostly found in tissues that are part of the anatomical barriers of innate immunity, including the skin and mucosal lining of the intestines. One of their main roles is to serve as a link between the innate and adaptive immune response through antigen presentation to T-cells. Along with this communicatory role, they can also release reactive oxygen species and nitric oxide to fight off invading pathogens, as well as a variety of other inflammatory mediators (Beutler, 2004; Kindt et al, 2007).

C. Macrophages

Development of macrophages

Of high relevance to the research contained in this dissertation are macrophages. Macrophages are derived from myeloid progenitor cells in the bone marrow. The first cell of this type to leave the bone marrow and move into the peripheral blood is the monocyte. Once monocytes migrate into tissue, they differentiate and become mature macrophages (Fujiwara and Kobayashi, 2005). The process of differentiation involves a variety of molecular mediators, including macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukins-6, -3, -1, and interferon-gamma (Metcalf, 1989; Janeway, 2001). Once inside the tissues, macrophages can then become activated, which results in larger cell size with increased production of inflammatory mediators and phagocytic ability. Activation of these cells also leads them to diverge in terms of morphology and function. For example, activated cells can fuse and form multinucleated giant cells, a cellular component of granulomas seen in chronic inflammation. Macrophages can differentiate and mature in a variety of tissues, and are named by their specialized location, such as Kupffer cell in the liver and microglial cells in the nervous system (Fujiwara and Kobayashi, 2005; Geissmann et al., 2010).

Macrophages have three main functions: phagocytosis, antigen presentation and immunomodulation (Fujiwara and Kobayashi, 2005). As phagocytic cells, macrophages ingest foreign pathogens and cellular debris into intracellular vesicles called phagolyzosomes (an integration of the phagosome and lysosomes inside the cell), where microbicidal activity takes place through the production of toxic products such as hydrogen peroxide and nitric oxide. Macrophages can detect these pathogens for phagocytosis through a variety of cell-surface receptors, including the TLRs. Binding of these receptors then leads to macrophage activation, phagocytosis and the production of

various inflammatory mediators (Ma et al., 2003; Fujiwara and Kobayashi, 2005). A more thorough discussion of macrophage activation through TLR4 will be addressed later.

Along with dendritic cells, macrophages are also involved in antigen presentation. This is one of the ways that the innate and adaptive immune mechanisms are bridged together. After phagocytosis, while pathogens are inside the phagolyzosyme, they are enzymatically degraded and this leads to the generation of antigenic peptides. These peptides can then adhere to peptide-binding clefts of the major histocompatabilty complex class II molecule and be presented on the cell surface. Once on the surface, the antigens can be presented to T-cells, initiating the cross-talk between the innate and adaptive immune response (Janeway, 2001; Fujiwara and Kobayashi, 2005).

One of the main immunomodulatory roles that they play involves their participation in the initiation, maintenance and resolution of the inflammatory response. This inflammatory process must be tightly-controlled; otherwise it could result in excessive cellular and tissue damage (Fujiwara and Kobayashi, 2005, Kindt et al., 2007).

Inflammation

One of the first responses to infection or damaged tissue is inflammation. Inflammation plays many roles in fighting infection. First, it helps in the recruitment and delivery of additional effector mediators, such as cytokines and macrophages, to the site of infection to aid in the attack (Kindt et al, 2007). Second, it helps provide a physical barrier preventing the spread of the infection. It also aids in the repair of damaged tissues (Janeway, 2005). The induction of an inflammatory response is linked to TLRs on

dendritic cells and macrophages, and it is one reason why macrophages are known as inflammatory cells (Fujihara et al., 2003). Once activated, these receptors can initiate inflammatory signaling pathways, such as activation of the transcription factor, nuclear factor kappa B (NFkB) and mitogen activated protein kinases (MAPK). The activation of these signaling molecules then leads to stimulation of the production of innate immune chemokines and cytokines (Ma et al., 2003). Chemokines are proteins that help direct other cells such as neutrophils and circulating monocytes to the site of infection or tissue damage. Cytokines are signaling proteins that induce a variety of cellular responses and alterations in cell function, such as induction and support of inflammation, expression of adhesion molecules, increases in permeability of blood vessels, or suppression of their own effects through feedback inhibition (Janeway, 2001). Among those released during an inflammatory response are interleukins (IL)-1, IL-6, IL-8, tumor necrosis factor-alpha (TNF-alpha), and interferon alpha (IFN-alpha). The release of these cytokines further orchestrates the inflammatory response. For example, IL-6 can stimulate the liver to produce a set of proteins known as acute phase reactants, among them being c-reactive protein (CRP), that function to promote phagocytosis and activate the complement system. IL-1, IL-6 and TNF-alpha are also endogenous pyrogens, capable of raising body temperature by influencing the hypothalamus and energy mobilization from fat and muscle. This leads to the characteristic fever seen during an inflammatory response, which can decrease bacterial replication (Janeway, 2001; Fujiwara and Kobayashi, 2003; Kindt et al., 2007).

It is important to note that undifferentiated monocytes themselves express chemokine receptors that are involved in the migration of cells from the blood to tissues,

and they can also produce proinflammatory cytokines, before being differentiated into dendritic cells or macrophages (Geissman et al., 2010).

Activation of Macrophages

In order for macrophages to be part of the inflammatory response, they must covert from a resting state to an activated one. Once entering the peripheral tissue, many undifferentiated monocytes and macrophages undergo apoptosis, while the remaining cells undergo differentiation and activation (Ma et al., 2003). Activation signals include macrophage-activating cytokines from T-cells and NK cells, such as interferon-gamma and TNF-alpha, microbial products such as LPS, and a variety of other chemical mediators (Gordon, 2003; Fujiwara and Kobayashi, 2005). Once macrophages are exposed to inflammatory stimuli, such as LPS, they have the potential to activate themselves. For example, activated macrophages can release TNF-alpha, which can then activate other macrophages. Once activated the cells have an augmented capacity to kill microbes. They become larger, contain more pseudopods, and can produce a variety of chemical products involved in the inflammatory response, such as proinflammatory cytokines, chemokines and complement components (Ma et al., 2003; Fujiwara and Kobayashi, 2005). Since many experimental studies exploring the role of macrophages in immune regulation are done using LPS as the activating factor, it is important to consider how this specific type of activation works.

D. Role of LPS in Activation of Macrophages

Bacterial LPS is a major constituent of the outer wall of gram-negative bacteria that has long been known for its ability to induce septic shock. It is one of the most commonly used macrophage activators in experimental conditions as well. Lipid A is the substructure of LPS that is involved in the activation of macrophages (Akira et al., 2001). Once activated by LPS, macrophages release a variety of inflammatory mediators due to the upregulation of transcription factors that include NFkB and activator protein-1 (AP-1) (Akira et al., 2001; Guha and Mackman, 2001; Fujihara et al., 2003). The activation of macrophages by LPS is mediated by the specific toll like receptor, TLR4, and was discovered during positional cloning studies being done to elucidate the mechanism of LPS resistance in mice (Poltorak et al., 1998). In these studies, Poltorak et al. discovered that genetically mutated mice were unresponsive to LPS and did not suffer septic shock, although they had no defects in LPS-binding protein (LBP) and the receptor protein cluster of differentiation (CD) 14. CD14 is a protein expressed mainly by macrophages that recognizes LPS when it is bound to LBP. Through positional cloning, they discovered that TLR4 had been inactivated in these mice, and could be restored by inserting a transgene. It was later found that TLR4 binds to the CD14:LBP:LPS complex. In the human embryonic kidney 293 cell line, overexpression of TLR4 did not confer responsiveness to LPS, suggesting that yet another molecule may be required for TLR4-mediated LPS signaling (as reviewed in Guha and Mackman, 2001; Fujihara et al., 2003). This was identified as the secreted molecule, MD-2, when contransfection with both MD-2 and TLR4 imparted responsiveness to LPS in the cells. MD-2 does not form a complex itself with LPS like CD14 and LBP do, but rather, it is physically associated to the extracellular domain of TLR4 .The signaling pathway of this receptor is in itself quite

complex, and most of the molecules involved were discovered through the studies of knockout mice and their response, or lack thereof, to LPS in terms of inflammation.

As mentioned earlier, TLR signaling arises from its cytoplasmic TIR domain **(Figure 1-1).** TLR4 contains a TIR domain, which interacts with a TIR domain-containing adaptor protein known as myeloid differentiation primary response gene 88 (MyD88). MyD88 is essential for the inflammatory response mediated by TLR4 activation, evidenced by studies showing that MyD88 knockout mice showed no response to LPS activation in terms of production of inflammatory mediators. MyD88 contains a C-terminal TIR domain, which interacts with the TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) adaptor, which in turn promotes its association with TLR4, and a N-terminal death domain. This death domain recruits serine/threonine kinases such as IL-1 receptor-associated kinases (IRAK) to the cell membrane. IRAK1/IRAK4 associate with the receptor transiently, with IRAK1 dissociating after phosphorylation by IRAK4. In turn, IRAK1 activates TNF-receptor-associated factor 6 (TRAF6), by binding to it and forming the IRAK1/TRAF6 complex. This complex then dissociates from the receptor and associates with TGF-B-activated kinase 1 (TAK1) and TAK1 binding proteins. IRAK1 is degraded, but the complex including TRAF6/TAK1 and TAK binding proteins move into the cytoplasm, which goes on to activate the IKappaB kinase (IKK) complex and the MAPK cascade (Aderam, 2001; Akira et al., 2001; Takeda and Akira, 2004; Kawai and Akira, 2006).

The MAPK cascade can elicit proinflammatory responses through the activation of the AP-1 transcription factor. Evidence has shown that, acting through TLR4, LPS is a potent stimulator of all three MAPK pathways in human monocytic cells. These three

pathways end in the phosphorylation of three distinct MAPKs: the extracellular signal-regulated kinase (ERK) 1/2, C-jun N-terminal kinase (JNK) and p38. The kinases then phosphorylate AP-1 and initiate gene transcription. In terms of NFkB, activation of IKK is first needed in order to phosphorylate the inhibitor of kappa B (IkB). IkB is bound to NFkB, inhibiting its function. Phosphorylation of IkB causes it degradation and NFkB is now free to translocate into the nucleus, which leads to transcription of a variety of proinflammatory cytokine genes, such as the genes for IL-6 and TNF-alpha (Guha and Mackman, 2001).

There is now a line of evidence describing a different signaling mechanism that is MyD88-independent, and seems to be found only in TLR3 and TLR4, whereas the MyD88 pathway is common for most TLRs. This was discovered when MyD88 knockout mice still showed delayed activation of LPS-induced NFkB and MAPKs, although they did not produce any inflammatory cytokines in response to the stimulation. In this scenario, TIR-domain-containing adaptor inducing IFN-beta (Trif) substitutes for MyD88, with Trif-related adaptor molecule (TRAM) acting as the adaptor protein linking Trif to TLR4, much like the TIR domain does with MyD88. This signaling process also initiates a different series of molecules leading to the phosphorylation of interferon regulatory factor (IRF)-3. Although Trif itself can interact with TRAF6 to initiate NFkB and MAPK activation, IRF3 leads to the upregulation of Type 1 interferons, such as interferon-alpha, which can go on to activate signal transducers and activator of transcription 1 (STAT 1), involved in the induction of IFN-inducible genes (Takeda and Akira, 2004; Kawai and Akira, 2006). It is through these varies signaling pathways that

LPS induces cellular activation in undifferentiated monocytes, as well macrophages, thus initiating an inflammatory response cascade.

III. Innate immunity and stress

In 1919, Ishigami made an interesting discovery relating stress to the immune system and stress to disease progression. While treating patients with tuberculosis, Ishigami noticed that during periods of intense stress there was a decrease in phagocytic activity in the cells of the patients. The idea that stress can modulate the immune system is now more widely accepted as evidenced by numerous studies, both in laboratory animals, as well as in humans.

Stress has been defined in many different ways in the literature. Chrousos (1992) defined stressors as any disturbing force that threatened homeostasis, or the steady state of a physiological system. Thus, stress itself is a state of threatened homeostasis and can be induced by physiological, environmental or psychological stressors (Black, 2002). Cohen (2007) defined psychological stress as the occurrence of an environmental stressor that challenges the organism's adaptive capacity, or ability to cope. Stress can also be distinguished by the length of its stressors. Acute stressors occur rapidly, and have an obvious onset and offset, while chronic stressors are ongoing, and may not have a clear endpoint (Chen and Miller, 2007). Physiologically, stress results in the activation of the SNS and the hypothalamic-pituitary-adrenal (HPA) axis, triggering a response commonly known as the fight-or-flight response (Elenkov, 2000, Black, 2002). It is these reactions that allow the organism to adapt and attempt to overcome the negative impact of the stressor and return to a homeostatic state. Interestingly, initiating this reaction also leads

to the activation of the innate immune response in terms of the release of proinflammatory cytokines and transcription factors (Bierhaus et al., 2006). The intricacies of this mechanism will be discussed later, but it is important to describe some of the studies that have developed this relationship between stress and the inflammatory response.

There have been several studies to focus on the effects of psychological and physical stressors on the innate immune response in laboratory animals, showing an increase in proinflammatory cytokines and their signaling pathways. For instance, rodents exposed to stressors such as, restraint stress or social isolation, show increases in IL-1beta, IL-6 and TNF-alpha, both peripherally and in the brain (van Gool et al., 1990; Zhou et al., 1993; as reviewed in Pariante and Miller, 2000; O'Connor et al., 2003).

In human studies, both acute stressors, brought about by laboratory manipulations, and chronic stress, such as a lack of social support, have been associated with increases in both the release and the production of proinflammatory cytokines and other inflammatory signaling molecules. For example, subjects exposed to the laboratory-based stressor, the Trier Social Stress Test, (TSST), a commonly used public speaking and mental arithmetic stressor, exhibited increased DNA binding of NFkB (Bierhaus et al., 2003). Increased plasma levels of cytokines such as IL-6 and TNF-alpha have also been seen following psychological stressors including the TSST (Maes et al., 1998; Goebel et al., 2000; Steptoe et al., 2002; Kiecolt-Glaser et al., 2003, Pace et al., 1996). Acute stress has also been shown to affect the distribution and number of cells in the immune system, such as NK cells (Schedlowski et al., 1993; van der Pompe et al., 1998). Segerstrom and Miller (2004) published a meta-analysis including more than 300 articles

and over 30 years of inquiry describing the relationship between psychological stress and different parameters of the immune system in humans undergoing stress. Broadly, their findings showed that acute stressors were associated upregulation of innate immune responses, such as increases in cell populations like NK cells, as mentioned above, increases in the production of the proinflammatory cytokine IL-6, and increases in the production of macrophage stimulating cytokines (e.g. IFN-gamma). Interestingly, in reviewing studies involving chronic stressors, such as unemployment and dementia caregiving, they saw negative effects on almost all functional measures of the immune system, both innate and adaptive. They concluded that during times of acute stress, the immunological response of the body mimics that of the adaptive fight-or-flight response to stress, with an increase in innate immune parameters preparing the body possibly to fight infection or limit injury. In contrast, chronic stress may lead to global immunosupression, which would shift the stress response from an adaptive one to one that would be detrimental to the host. These conclusions are important to consider, because much of the literature involving stress and innate immunity, especially in terms of SNS modulation (as will be discussed in the following section), has a clear dichotomy regarding whether the modulatory effects of catecholamines (the endproducts of SNS activation) are proinflammatory or anti-inflammatory.

A. Link between stress, depression and inflammation

The detrimental effect of immune alterations due to chronic stress is evidenced by the co-morbidity seen in many diseases with stress. Increased stress has been linked with

various diseases, including cardiovascular disease, metabolic syndromes, arthritis, cancer, and major depression (Kiecolt-Glaser et al., 2002; Black, 2006; Cohen et al., 2007).

Major depression (MD) is a serious disease with an overall lifetime prevalence of 15% (Irwin and Miller, 2007). MD itself is frequently co-morbid with a variety of other medical ailments, including heart disease, diabetes and cancer and is often associated with a worse outcome in these diseases (Ader and Cohen, 1975; Evans et al., 1999; Raison and Miller, 2001; Raison and Miller, 2003; Irwin and Miller, 2007). Given emerging evidence that inflammation may play an important role in the pathophysiology of a number of medical disorders, this interaction between MD and activation of the innate immune response may serve as critical link between MD and medical disease. For example, cancer patients with MD were found to have increased concentrations of circulating IL-6, compared to both non-depressed cancer patients and healthy controls (Musselman et al., 2001b; Jehn et al., 2006). Patients who develop coronary heart disease are likely to have suffered from MD throughout their life or to become depressed after disease onset (Ferketich et al., 2005; Fenton and Stover, 2006). Increased levels of IL-6 have also been shown in patients with major depression and rheumatoid arthritis (Zautra et al., 2004) and cardiovascular disease (Lesperance et al., 2004).

Data has shown that MD is associated with an increase in a variety of inflammatory biomarkers including increases in plasma and cerebrospinal fluid concentrations of innate immune cytokines (eg. IL-6 and TNF-alpha), acute phase proteins, chemokines and cellular adhesion molecules (Maes et al., 1997; Lanquillon et al., 2000; Yirmiya et al., 2000; Anisman and Merali, 2003; Danner et al., 2003; Tuglu et al., 2003; Raison et al., 2006). In addition, administration of innate immune cytokines,

such as IFN-alpha, has been found to induce behavioral changes in laboratory animals (Felger, 2007) and humans (Musselman, 2001; Capuron and Miller, 2004) that resemble MD. Moreover, innate immune cytokines have been shown to interact with virtually every pathophysiologic domain known to be involved in depression including neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and regional brain activity (as reviewed in Raison et al., 2006). A decrease in proinflammatory cytokines such as IL-6, IL-1beta and TNF-alpha has also been seen with administration of antidepressants (Xia et al., 1996), which was shown to correlate with efficacy of the treatment (Lanquillon et al., 2000; Tuglu et al., 2003). Finally, inhibition of innate immune cytokines has been shown to reverse depressive symptoms in patients with inflammatory disorders (Tyring et al., 2006) as well as laboratory animals (Silverman et al., 2006; Koo and Duman, 2008).

Patients with MD have also been shown to react in an exaggerated manner to acute stress, compared to healthy controls. In a study comparing women with MD to those without, after going through a mock interview process, both groups showed increased mobilization of innate immune cells and CRP into circulation, and greater LPS-induced IL-6 and TNF-alpha in vitro. However, those with MD showed a decreased sensitivity to the anti-inflammatory effects of glucocorticoids, as seen in increased IL-6 and TNF-alpha production in the presence of dexamethesone (Miller et al., 2005). This would imply that under stress, those with MD have a greater resistance to substances that help terminate inflammatory responses. Interestingly, compared to healthy controls, patients with MD and early life stress also showed an exaggerated increase in plasma IL-6, as well as NFkB DNA binding, in response to the laboratory stressor, the Trier Social

Stress Test (TSST). The TSST involves subjects being challenged with a five-minute oral public speaking task, in which they are given ten minutes to prepare, followed by a five-minute mental arithmetic task. These patients also exhibited increased plasma concentrations of IL-6 at baseline (Pace et al., 2006) These data suggest that the occurrence of early life stress may lead to chronic activation of the innate immune response, which in turn may prime the immune system to react in a more robust manner during acute stress. Consistent with this notion is that childhood maltreatment (a form of early life stress) has been associated with increased peripheral blood biomarkers of inflammation including the acute phase protein, CRP (Danese et al., 2007, 2008). Elevated CRP has also been found in depressed subjects, and in one recent study, the increased CRP in depression was largely attributed to the co-occurrence of depression and childhood maltreatment (Danese et al., 2007, 2008). Taken together, these studies support the association between stress and activation of the innate immune system. Further, they show how this association can lead to the development of diseases, like MD.

IV. The Sympathetic Nervous System and Inflammation

The underlying mechanisms of the relationship among stress, inflammation and disease have yet to be elucidated. As mentioned earlier, physiologically, stress results in the activation of the SNS and the HPA (Elenkov, 2000; Black, 2002). The activation of these systems may, in turn, lead to the communication with the innate immune system, and thus link stress with inflammation and disease. The focus of this thesis will be on the interactions between the SNS and innate immune response.

A. Biology of the sympathetic nervous system

In order to fully appreciate the role that the SNS plays in stress and innate immunity, a basic understanding of the anatomy and physiology of the SNS is needed. The SNS is one of two subdivisions of the autonomic nervous system (ANS). The ANS contains three components. Originating in the central nervous system is the sympathetic component, driven largely by noradrenergic fibers, and the parasympathetic component, driven by cholinergic fibers. Originating in the gastrointestinal tract is the enteric component, which itself is modulated by the sympathetic and parasympathetic components. Sympathetic nerve fibers originate in nuclei within the brainstem, including the locus coeruleus (Kuhar et al., 1999). These fibers give rise to preganglionic efferent fibers that exit the central nervous system through the thoracic and lumbar spinal nerves, thus termed the thoracolumbar system. Many of these fibers terminate in ganglia on the paravertebral chains of the spinal column, which lie on the side of the spinal column, while the others terminate on prevertebral ganglia, in front of the spinal column. Postganglionic fibers then emanate from these ganglia to innervate peripheral tissues, such as the heart, gastrointestinal tract, blood vessels and lymphoid organs (Hasko and Szabo, 1998), terminating on sympathetic ganglion cells (Kvetnansky et al, 2009). These fibers are known as noradrenergic fibers, as they act by releasing the catecholamine, norepinephrine (NE). Along with noradrenergic fibers, the SNS also contains fibers that terminate within the adrenal medulla, which contain chromaffin cells that are homologous, both embryologically and anatomically to sympathetic ganglia. These cells are innervated by preganglionic sympathetic nerve fibers that release acetylcholine, and

when stimulated, release mainly epinephrine, another catecholamine. It should be noted that NE can also be released as a hormone from the adrenal gland, but the main product in this case is epinephrine, released in a 4:1 ratio with NE (Madden et al., 1995; Elenkov et al., 2000).

As previously mentioned, the main end products of the SNS are the catecholamines, NE (also known as noradrenaline) and epinephrine (also known as adrenaline). Along with dopamine, NE and epinephrine are the most abundant catecholamines in the nervous system (Oberbeck, 2006). The syntheses of catecholamines **(Figure 1-2)** rely on two enzymes: dopamine-beta-hydroxylase (DBH) and tyrosine hydroxylase (TH), which is the rate-limiting step in catecholaminergic synthesis. Briefly, catecholamines are initially synthesized from tyrosine. Tyrosine is then converted to dihydroxyphenylalanine (DOPA) by TH. DOPA is then converted into dopamine, which is transported into vesicles and converted into NE by DBH. Finally, in the adrenal medulla, NE is converted into epinephrine (Kuhar et al., 1999; Elenkov et al., 2000; Flierl et al., 2008). The highest concentration of these compounds is found in peripheral presynaptic nerve terminals, inside membrane-bound vesicles where they are protected from enzymatic destruction. Once nerve cells are stimulated, they release the stored catecholamines rapidly through the fusion of the vesicle with the cell membrane, which is then followed by termination of their activity by either neuronal reuptake, dilution into extracellular fluid, or metabolic inactivation by enzymes, such as monoamine oxidase and catechol-O-methyl transferase (Kuhar et al., 1999; Flierl et al., 2008). Once released, NE can act by neural communication, where it is released at the synapse and crosses the synaptic cleft to the postsynaptic cell, in a paracrine manner,

through nonsynaptic communication, by diffusing away from its point of release to interact with receptors expressed on adjacent and distant target cells, or in an endocrine fashion, in which it reaches cells via blood circulation (Hasko and Szabo, 1998; Bellinger et al., 2008).

B. Adrenergic Receptors

The effects of NE are mediated through adrenergic receptors (AR). Briefly, ARs can be characterized into two groups: alpha-AR and beta-ARs. Among the alpha-ARs, there are three subtypes of the alpha-1 receptor (A1-AR): alpha-1D (A1d-AR), alpha-1B (A1b-AR) and alpha-1A (A1a-AR) and three subtypes of the alpha-2 receptor (A2-AR): alpha-2A, alpha-2B and alpha-2C (A2a-AR, A2b-AR and A2c-AR). Among the beta-ARs, there are also three subtypes: beta-1, beta-2 and beta-3 (B1-AR, B2-AR, B3-AR). B1-ARs are found predominantly in the heart and cerebral cortex and B2-ARs are widely expressed, and also found in the lung and cerebellum, and the B1-AR and B2-AR may also coexist in the same tissue. B3-ARs are found in adipose tissue (Kuhar et al., 1999; Michelotti et al., 2000; Hall, 2004).

All subtypes of ARs are G-protein-coupled receptors (GPCR). So called, because all GPCRs mediate their effects through the coupling of heterotrimeric G-proteins upon agonist-induced activation **(Figure 1-3).** These seven-transmembrane domain receptors are among the largest known superfamily of receptors and are widely studied and targeted for drug discovery and development (Lattin et al., 2007). There are three subunits that make up the heterotrimeric G-protein components of these receptors, G-alpha, G-beta and G-gamma, which essentially function as dimers, since signaling

involves either the G-alpha subunit or the G-beta/gamma subunit. In the absence of agonists, the G-beta/gamma subunit associates with the G-alpha subunit. In general, the specificity of the receptor is modulated by the alpha subunit. Agonist binding stimulates the release of the G-alpha subunit from the G-beta/gamma subunit, and allows it to regulate effector enzymes, which in turn lead to the generation of intracellular mediators termed second messengers, in a positive or negative manner. Sequentially, these second messengers, such as cyclic adenosine monophosphate (cAMP), calcium and phospoinositides, activate or inhibit certain kinases, such as protein kinase A (PKA) and protein kinase C (PKC), ultimately leading to a biological response through activation of relevant transcription factors (Neer, 1995; Gether and Kobilka, 1998; Wettschureck and Offermanns, 2005; Lattin et al., 2007). The specific subunits of the G-alpha protein determine the type of signal generated by the receptor. Signaling via the G-alpha-s (Gs) subunit results in stimulation of adenylyl cyclase (AC), signaling via the G-alpha-i (Gi) subunit results in inhibition of AC, and the G-alpha-q (Gq) subunit results in stimulation of phospholipase C (PLC) (Lohse et al., 1996).

Interest in these receptors does not solely lie in their signaling mechanism, but also, on their ability to control overstimulation by agonists through feedback mechanisms that attenuate their responsiveness. Termed desensitization, this process involves several regulatory molecules, including second-messenger kinases, such as PKA and PKC, GPCR kinases (GRKs) and arrestins (Lefkowitz, 1993; Ferguson et al., 1996). There are two forms of desensitization, heterologous and homologous (summarized in **Table 1-2**). Heterologous desensitization involves the inhibition of other receptors as well, through stimulation of one GPCR, and the receptor being downregulated does not need to be

bound to an agonist. This type of desensitization involves PKA or PKC, since these kinases can phosphorylate a variety of receptors (Lohse et al., 1996; Wallukat, 2002; Kohout and Lefkowitz, 2003). Homologous desensitization occurs only when the agonist-stimulated receptor undergoes inhibition of response, and it is carried out by the serine/threonine kinases, GRKs. There are currently seven known GRK subtypes, GRK1-7, of which four are expressed ubiquitously, GRK2, -3, -5, and -6). In the case of arrestins, two are expressed ubiquitously, beta-arrestin-1 (BARR-1) and beta-arrestin-2 (BARR-2) (Ferguson, 2001; Kohout and Lefkowitz, 2003). This process of desensitization involves many steps **(Figure 1-4).** First, GRKs phosphorylate the activated GPCR either at the third intracellular loop or the carboxyl-terminal tail. This increases the affinity of the receptor for arrestins, and it promotes the translocation of the adaptor arrestin proteins to the membrane, where they bind to the phosphorylated receptor. It should be noted that GRK phosphorylation alone does little to affect the receptor-G-protein coupling, thus it is the arrestins that are involved in the actual decrease in receptor signaling (Lutrell and Lefkowitz, 2002). This interaction then sterically inhibits the interaction of the receptor with its corresponding G-protein, and reduces or prevents further signaling. Finally, the GRK-arrestin system can promote internalization of the inactivated receptor through the interaction with endocytic machinery (Hall and Lefkowitz, 2002). Eventually, the receptor is either degraded to continue downregulation of signaling, or recycled back to the membrane for further signaling, termed resensitization (Ferguson, 2001; Laporte et al., 2002; Kohout and Lefkowitz, 2003; Lattin et al., 2007).

The scope of this thesis will be on the A1-AR and the B2-AR subtypes.

A1-AR

The physiological agonists for the A1-AR are epinephrine and NE, which bind to the hydrophilic ligand pocket created by the seven transmembrane domains of the GPCR. Since both A1-ARs and A2-ARs have identical agonist potencies with regard to epinephrine and NE, they were indistinguishable pharmacologically until selective antagonists were discovered, such as prazosin for A1-AR and yohimbine for A2-AR (Michelotti et al., 2000). Agonist mediated activation of this receptor is thought to first involve the disruption of an ionic salt bridge that maintains the receptor in an inactive, basal state. As stated earlier, among the A1-ARs there are three subtypes, A1a, A1b and A1d, with each subtype distributed in a tissue selective manner (Price et al., 1993; Kavelaars. 2002). Furthermore, it appears that their localization in the cell is subtype-specific as well, with A1d found predominantly intracellularly, A1b on the cell surface, and A1a both intracellularly and on the cell surface (Piascik and Perez, 2001).

Physiologically, postsynaptic stimulation of the A1-AR leads to vasoconstriction, smooth muscle contraction, and cardiac contractility and hypertension (Michelotti et al., 2000). They can also regulate the growth of cardiomyocytes and smooth muscle cells (Kavelaars, 2002).

The A1-AR is Gq-coupled **(See Figure 1-3),** which, upon agonist stimulation, activates PLC, leading to an increase in intracellular inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These second messengers then act to mobilize calcium from intercellular stores and activate PKC, respectively (Elenkov et al., 2000; Michelotti et al., 2000). Interestingly, A1-AR signaling has been shown to stimulate the MAPK pathway

as well, leading to stimulation of ERK1/2, JNK and p38, which are elements of the inflammatory response (Michelotti et al., 2000). It is thought that activation of this pathway is what mediates the cellular growth promoting abilities upon activation of these receptors (Piascik and Perez, 2001).

Although most studies involving receptor desensitization of GPCRs have focused on the B2-AR (discussed below), results from this model have been extended to include the A1-AR. For example, GRK2 and -3, along with BARR-1 and -2, have been shown to phosphorylate the A1b-AR and cause agonist-induced desensitization and dampening of signaling, and PKC phosphorylation is involved in heterologous desensitization of this receptor (Diviani et al., 1996). However, it seems that the A1a subtype does not undergo desensitization, but instead has the ability for continuous signaling, which is likely to be one of the contributing factors to myocardial hypertrophy (Michelotti et al., 2000).

B2-AR

The physiological ligands for the B2-AR are also epinephrine and NE (Kohm and Sanders, 2001). As mentioned, B2-ARs are seven-transmembrane GPCRs (Kohm and Sanders, 2001; Zheng et al., 2005), and they share around 65-70% homology with the other two beta-AR receptor subtypes, B1 and B3 (Johnson, 1998). Agonist binding to the B2-AR generally occurs within the hydrophobic core of the protein, inserted among the transmembrane helices, and anchored by molecular interactions between the receptor and the agonist (Johnson, 1998; Liggett, 1999). The current concept of how agonists trigger B2-ARs is no longer a simple lock-and-key mechanism, but rather, the receptor toggles between an active conformation and an inactive conformation, favoring the inactive

conformation in the absence of agonist. When agonists bind to the spontaneous active conformation, they stabilize it there, and shift the equilibrium (Liggett, 2002). Stimulation of these receptors results in bronchodilatatory effects, such as relaxation of vascular and airway smooth muscle (Sitkauskiene and Sakalauskas, 2005), as well as cardiac effects (Michelotti et al., 2000), such as myocardial contractility, and glucose and lipid metabolism (Zheng et al., 2005).

Since the B2-AR is mainly Gs-coupled (**See Figure 1-3**), stimulation of this receptor by catecholamines activates AC, leading to increases in intracellular cAMP from the conversion of adenosine triphosphate. In turn, cAMP goes on to activate PKA (Elenkov et al., 2000: Benovic, 2002). Through phosphorylation of PKA, the B2-AR receptor has also been shown to be able to 'switch' its coupling from Gs to Gi, and in turn, activate the MAPK pathway (Daaka et al., 1997).

Almost all studies examining the mechanisms and effects of GPCR desensitization have been done using the B2-AR as the model receptor. In the specific case of the B2-AR, receptor desensitization through uncoupling of the Gs-alpha subunit prevents signaling to adenylyl cyclase. This can occur through PKA or PKC, or GRK2 and GRK3 (also known in this case as beta adrenergic receptor kinase 1 and beta adrenergic receptor kinase 2), depending on the type of desensitization involved. As mentioned previously, PKA can directly phosphorylate the receptor, whether it is agonist-bound or not, since any mechanism that increases cAMP will activate PKA (Liggett, 1999). This, in turn, impairs G-protein coupling, causing heterologous desensitization. On the other hand, GRK phosphorylation involves the recruitment and increased affinity of arrestins, BARR-1 and BARR-2, only to the agonist-bound receptor, with subsequent

blocking of the receptors interaction with its G protein, thus inhibiting its ability to signal to adenylyl cyclase (Lohse et al., 1996; Benovic, 2002; Wallikat, 2002). Overexpression of either GRK2 or GRK3 in cultured cells shows an increase in beta-AR phosphorylation and desensitization (Benovic et al., 1991). Interestingly, studies have shown that overexpression of GRK2 can also increase the rate and extent of internalization, showing a possible involvement of this kinase in downregulation of the receptor, in addition to receptor phosphorylation (Ruiz-Gomez and Mayor, 1997). The next step in regulation of signaling is receptor internalization, where the receptor is removed from the cell surface by endocytosis. BARR-1 and BARR-2 are key elements to this step, as they are known to interact and associate with a variety of proteins that are involved in the process of endocytosis (Hall and Lefkowitz, 2002). The final mechanism involved in B2-AR desensitization is downregulation of the receptor, seen as a decrease in mRNA expression and stability, and degradation of receptor protein. The mechanism of downregulation requires several hours of exposure and appears to be highly cell-type dependent (Liggett, 2002). Arrestins have also been implicated in regulating internalization, as they are known to facilitate endocytosis, recycling, resensitization and downregulation of the receptor (Benovic, 2002; Hall and Lefkowitz, 2002). The final two steps in this process, internalization and downregulation, ultimately lead to a reduction in the total number of receptors on the plasma membrane, whereas the initial steps modulated by PKA and GRK/arrestins, result in decreased signaling, without necessarily affecting expression. Thus, although the end result is less signaling, the expression of the receptor depends on which mechanism of desensitization is activated (Benovic, 2002; Wallukat, 2002).

C. SNS and stress: Catecholamine response

As mentioned previously, stress is a state of threatened homeostasis induced by environmental, physiological, or psychological triggers (Black, 2002). The role of the SNS is to maintain homeostasis in the body, both during basal and stress states. Catecholamines maintain many bodily functions during basal states, such as fuel metabolism, thermogenesis, heart rate and blood vessel tone (Elenkov et al., 2000). During times of stress, biosynthesis and concentrations of NE are increased due to the activation of the SNS, with up to 70% of it being released from sympathetic nerve terminals, and the rest from the adrenal medulla (Kvetnansky et al., 2009). Activation of the locus coeruleus (LC) in the brain stem leads to the release of NE, which then acts centrally and peripherally. Centrally, it activates areas of the brain responsible for arousal and vigilance (Elenkov et al., 2000) Interestingly, studies have shown that a single exposure to an extreme stressor in rodents, such as severe cold exposure or inescapable shock, leads to impairment in performance on avoidance-escape tasks, which seems to come about from a reduction in brain NE activity caused by the stressor (Weiss and Glazer, 1975), yet repeated exposure to a stressor led to habituation in both NE levels and behavior (Weiss et al., 1975), with decreased uptake of NE by cortex slices in vitro. These studies demonstrated that changes in behavior following stress are partly due to changes in central NE activity. Peripherally, LC activation increases the output of NE from sympathetic nerve terminals (Elenkov et al., 2000). This increase in peripheral NE then leads to a number of stress-induced physiological responses including increased energy metabolism, changes in cardiac and vascular function and thermoregulation (Kavelaars, 2002). Commonly termed as the ‘fight-or-flight’ response, NE interacting

with adrenergic receptors leads to increases in heart rate and cardiac contractility, and changes in vasomotor tone (Wong et al., 2007), as well as mobilization of metabolic reserves to provide energy (Flierl et al., 2008).

D. Communication Between the SNS and the Innate Immune System

Studies have examined the possibility that stress-induced inflammatory responses are mediated, in part, by the SNS, which can mediate the stress response through the release of NE from nerve endings and epinephrine from the adrenal medulla. As early as the late 1920's, a study showed that acute psychological stress, as well as physical exercise, induced leukocytosis similar to that seen with epinephrine injection. This led to the assumption that catecholamine-induced activation of the immune system might be a necessary mechanism during the flight or flight response (Oberbeck, 2006). Indeed, there are many lines of evidence that now show that the SNS and the innate immune response can communicate and affect one another.

Immune system signals to the central nervous system

As early as the 1970-1980's, studies showed that products of the immune system could signal to the central nervous system. For example, administration of IL-1 in animals was shown to alter the activity of hypothalamic noradrenergic neurons and decrease the content of NE in the spleen (Besedovsky et al., 1983, 1986). Several studies have followed examining the effects of immune activation, showing that a variety of cytokines can signal to the brain and activate the SNS (Elenkov et al., 2000)., Intracranial injections of IL-1beta were shown to activate the SNS through an increase in the turnover

rate of NE in the spleen (Vriend et al., 1993). Injections of this cytokine into the LC led to an increase in activity of LC neurons, which could be abolished by injection with the IL-1 receptor antagonist (Borsody and Weiss, 2002). Furthermore, central and peripheral injection with LPS also led to a prolonged increase in LC neuronal activity, which also seemed to be driven by IL-1, as blocking IL-1 with the IL-1 receptor antagonist blocked the effect (Borsody and Weiss, 2002, 2004). Peripheral administration of IL-1 was also shown to increase turnover of NE in the hypothalamus (Dunn et al., 1999), indicating that the communication between the two systems is bidirectional, with the peripheral and central components able to influence one another. The previously mentioned studies show that systemic administration of cytokines increase NE turnover, centrally and peripherally; however, the local effects of cytokines may be different. TNF-alpha has been shown to inhibit stimulation-evoked NE from the rat median eminence (Elenkov et al., 1992) and the rat hippocampus (Ignatowski and Spengler, 1994).

SNS signals to the immune system: Innervation of lymphoid organs

Noradrenergic fibers are known to innervate primary lymphoid organs, such as the bone marrow and thymus, as well as secondary lymphoid organs, such as the spleen and lymph nodes, thus creating a direct neural connection between the SNS and the immune system (Felten, 1993; Friedman and Irwin, 1997; Bellinger et al., 2008). One manner in which the presence of this type of innervation was confirmed was through the use of immunohistochemistry for TH and DBH, two enzymes responsible for the synthesis of catecholamines (Felten and Olschowka, 1987). These fibers innervate the vasculature where they act in controlling blood flow to these organs, as well as the

parenchyma, where immune cells reside and thus, may act to affect cell function (Felten et al., 1985; Friedman and Irwin, 1997). The innervation also appears to be regional, where zones of T cells, macrophages and plasma cells of the spleen are highly innervated (Madden et al., 1995; Felten et al., 1998; Benarroch, 2009) It should be noted that neural-immune connections are not restricted to lymphoid organs alone, but are encountered in almost every somatic and visceral tissue (Weihe et al., 1991). Denervation of the SNS in rats was shown to enhance LPS-induced IL-1beta and IL-6 release, providing evidence of the ability of this SNS innervation to modulate immune profiles (De Luigi et al., 1998). It also meets the criteria for neurotransmission with immune cells, as it has been demonstrated that there is release of NE from these nerve fibers and that functional ARs are present on lymphoid cells (Felten et al., 1993; Stevens-Felten and Bellinger, 1997; Bellinger et al., 2008).

SNS signals to the immune system: Neural release of NE and its effects on immunity

Through the use of various techniques measuring TH and DBH (such as immunohystochemistry, studies in animals have shown that NE and other sympathetic mediators, such as neuropeptide Y (NPY) are released from nerves in the spleen and other lymphoid organs under physiological conditions (Weihe, 1991; Vizi et al., 1995; Elenkov et al., 2000; Friedman and Irwin, 1997). Moreover, using the noradrenergic neurotoxin 6-hydroxydopamine (6-OHDA), an up to 95% reduction in splenic NE was seen, indicating its neural origin (Felten and Olschowka, 1987). NE is also released from lymphoid organs in a nonsynpatic manner, where it can affect immune cells in a more remote manner, by diffusing away from the organ, without the need for synaptic contact

(Vizi et al., 1995). Catecholamines have been identified in human peripheral blood mononuclear cells using mass spectrometry techniques (Bergquist and Silberring, 1998), and have now been shown to be produced de novo by immune cells, which contain the necessary enzymes, TH and DBH, for their production (Brown et al., 2003; Flierl et al., 2007; Flierl et al., 2008; Ley et al., 2010). Interestingly, a study showed that administration of beta-AR antagonists increased LPS-induced IL-1beta in mice peritoneal macrophages, while alpha-AR antagonists decreased its production, providing functional evidence that macrophage-derived catecholamines may have autocrine actions on inflammatory responses (Engler et al., 2005). While beta-AR stimulation in macrophages decreased TNF-alpha production, and alpha-AR stimulation increased it, beta-AR antagonists led to an increase in LPS-induced TNF-alpha production, while alpha-AR antagonists decreased it. NE was found in these cells using chromatography techniques, and LPS stimulation was shown to decrease their content, further showing that NE may act in an autocrine manner in these cells (Spengler et al., 1994).

NE and epinephrine have been shown to transiently increase lymphocytes and NK cells in humans, an effect mimicked by acute psychological stress and exercise (Benschop et al., 1996; Elenkov et al., 2000; Sanders and Straub, 2002; Oberbeck, 2006), but may actually reduce their activity in some cases (Friedman and Irwin, 1997, Elenkov and Chrousos, 1999). For example, mental stress in humans activated the SNS and resulted in a concomitant increase in the number of circulating NK cells, which was inhibited by propranolol, suggesting that the stress-induced increases are mediated by the beta-AR (Benschop et al., 1994). But catecholamines can also suppress NK cell activity, through their suppression of cytokines such as IL-12 and IFN-gamma, which are essential

for NK cell activity (Elenkov and Chrousos, 1999). Chronic catecholamine release, however, decreases the number of lymphocytes and NK cells in peripheral blood (Elenkov et al., 2000; Sanders and Straub, 2002), and repeated stress was shown not to alter immune cell redistribution, as has been found from a single bout of stress (Imrich et al., 2004). These contradictory findings may be a result of receptor desensitization or downregulation (Sanders and Straub, 2002). NE released from lymphoid organs can also alter immune cell function (Elenkov et al., 2000; Nance and Sanders, 2007). In regard to the adaptive immune response, NE seems to shift the response from cellular to humoral immunity, by inhibiting T-helper 1 cell, which are more pro-inflammatory in nature, and stimulating T-helper 2 cells, which are more anti-inflammatory in nature. These influences in turn, may be a mechanism by which the body helps protect itself from 'overshooting' with a type 1-mediated pro-inflammatory response (Elenkov and Chrousos, 1999; Kohm and Sanders, 2001; Sanders and Straub, 2002). In fact, dosing animals with 6-hyroxy-dopamine, which degenerates NE-containing neurons and thus removes NE, before inducing hemorrhagic shock, caused an increase in the inflammatory response through a rise in lung TNF-alpha production, further showing that NE is partly responsible for suppressing pro-inflammatory responses in vivo (Molina, 2005).

NE can also affect clonal expansion, cytokine production, chemokine production and responsiveness of cells to antigens by altering receptor expression, shifting the balance between innate and adaptive responses and enhancing or inhibiting the inflammatory response (Elenkov et al., 2000; Bellinger et al., 2008). For example, NE was shown to inhibit LPS-induced TNF-alpha and IL-6 release in human blood (Van der Poll, et al., 1994). Macrophage phagocytic activity in wounds, for example, was shown to

be decreased in a NE-dependent manner (Gosain et al., 2007; Ley et al., 2010). NE has also been shown to upregulate IL-6, IL-8 and tumor-mediating molecules in tumor cell lines, implicating stress and SNS activation in tumor progression (Yang et al., 2009).

Among the innate immune system specifically, the literature is split between whether NE plays an inflammatory or anti-inflammatory role. For example, NE administered to murine macrophages showed an increase in TNF-alpha production, an effect that was blocked by an A2-AR antagonist (Spengler et al., 1990). Furthermore, NE modulated stress-induced elevations in IL-1beta in the spleen and hypothalamus of rats experiencing footshock (Blandino Jr et al., 2006). NE has also been shown to induce IL-6 production in neonatal rat astrocytes, however, microglia were unresponsive to NE (Norris and Benveniste, 1993). Most studies, however, indicate an anti-inflammatory role, such as inhibition of proinflammatory cytokines (Oberbeck, 2006; Sternberg, 2006), especially in macrophages (Elenkov et al., 2000). Both NE and epinephrine were shown to inhibit LPS-induced release of IL-6 and TNF-alpha in human monocytes isolated from whole blood (Rontgen et al., 2004), and NE also inhibited TNF-alpha production from LPS-stimulated rat spleen macrophages (Hu et al., 1991). TH-positive cells were found in inflamed tissue samples from patients with arthritis, but not in control tissue, and experimental increases in NE through blockage of the monoamine transporter, showed decreases in TNF-alpha (Capellino et al., 2010). These contradictory actions on the effect of catecholamines may depend on the receptor subtype that is being stimulated (Bellinger et al., 2008), on the experimental conditions (mouse versus human cells), the presence or absence of antigens, the presence of proinflammatory mediators, and the state of

activation or differentiation in the cell (Elenkov et al., 2000). Many of these factors will be discussed below, within each receptor subtype response.

SNS signals to the immune system: Expression of receptors on lymphoid cells

After anatomical studies supported the findings of the location of sympathetic nerves and the release of NE near immune cells, studies started focusing on the expression of ARs on immune cells. Indeed, alpha-ARs and beta-ARs are found on immune cells, including cells of the adaptive immune response (T- and B-cells), and, of relevance to this thesis, cells of the innate immune response, such as macrophages and NK cells (Bellinger et al., 2008). However, these receptors are not equally expressed on all types of immune cells (Friedman and Irwin, 1997). The B2-ARs are the most commonly expressed of the adrenergic receptors, found in virtually every immune cell, except T-helper 2 cells (Madden et al., 1995; Elenkov et al., 2000; Nance and Sanders, 2007). Expression of the alpha-AR is more controversial. It seems that alpha-ARs (both of the A2 and A1 subtypes), are not expressed in lymphocytes and monocytes under normal conditions, but may be in certain lymphoid compartments (such as alveolar macrophages) or under certain pathological conditions (Elenkov et al., 2000). For example, expression of the A1-AR subtype has also been reported on monocytes and macrophages of patients with arthritis (Heijnen et al., 1996; Kavelaars, 2002). Thus, the expression and activity of ARs in immune cells varies greatly among cells and can be regulated by factors such as cell activation, cytokines, neurotransmitters and hormones (Elenkov et al., 2000; Nance and Sanders, 2007; Benarroch, 2009). Both these concepts are important in considering the contradictory nature of studies in concluding whether

SNS, and, in turn, NE activation, will be pro- or anti-inflammatory in nature (as discussed below).

E. Adrenergic Receptor Subtypes and Inflammation

Both A1- and B2-AR subtypes have been shown to mediate the effects of the SNS and catecholamines on the inflammatory response (Sanders and Straub, 2002; Nance and Sanders, 2007). Furthermore, the functional effects of stimulation of AR on macrophages and other innate immune cells are dependent on the subtype of the receptor stimulated (Bellinger et al., 2008). For example, the increase in NK cell number and activity seen after NE administration is driven by the B2-AR (Oberbeck, 2006), whereas lymphocyte formation in the bone marrow is driven by the alpha-ARs (Elenkov et al., 2000). Beta-AR stimulation can inhibit lymphocyte proliferation, antibody secretion, and the production of proinflammatory cytokines, whereas alpha-AR stimulation seems to have the opposite effect (Madden et al., 1995). NE can bind to B2-ARs and stimulate the cAMP-PKA pathway, or it may bind to A1-ARs, thus stimulating the PKC and MAPK pathways (Heijnen, 2007). Thus, the response to the same ligand, can be completely different depending on which receptor subtype is stimulated. The following section will review the functional consequences of activation of the A1-ARs and B2-ARs on the inflammatory response.

A1-AR

Although the majority of studies have concluded that the immunoregulatory effects of SNS activation are mediated by the beta-AR, some studies now show that the

alpha-AR plays a role in immunomodulation as well. First, A1a-, A1b- and A1d-AR mRNA were found in human peripheral blood lymphocytes, along with radioligand detection of binding sites and antibodies for each receptor subtype, with A1b representing the most, and A1d the least (Ricci et al., 1999). Using RT-PCR techniques, mRNA for these receptors has also been found in the spleen, thymus and bone marrow, as well as their corresponding cell types, with some subtypes dominating others in a tissue-specific manner (Kavelaars, 2002). Furthermore, it seems that expression of these receptors are regulated during cell maturation and lymphocyte development, since their expression is typically not found on mature, but resting, peripheral blood mononuclear cells, and they reappear when these cells become activated and differentiate during inflammation (discussed further below) (Kavelaars, 2002). Second, A1-ARs have the ability to stimulate proinflammatory pathways, such as the MAPK cascade (Michelotti et al., 2000). Although it is known that A1-ARs signal through the Gq-coupled pathway, ultimately leading to activation of PKC, they have also been shown to stimulate G protein-mediated phosphophatidylinositol 3 (PI3)-kinase, Ras/Raf, and the MAPK cascade (Michelotti et al., 2000; Kavelaars, 2002; Nance and Sanders, 2007), in particular ERK 1/2 (Rouppe van der Voort et al., 2000b), which in turn can activate inflammatory responses (Bierhaus et al., 2003).

In fact, A1-ARs can activate both NFkB and MAPK signaling molecules through the actions of NE, as stimulation of THP-1 cells with NE at physiological concentrations was found to increase the DNA binding activity of NFkB, an effect that was reversed by administration of alpha-AR antagonists. In addition, NE treatment of the cells was shown

to stimulate signaling through PI3-kinase and the Ras/Raf pathways, which are both components of the NFkB and MAPK cascade (Bierhaus et al., 2003).

Relevant to stress, work in both laboratory animals and humans suggests that stress-induced activation of innate immune cytokines is mediated in part by A1-ARs. For example, stress-induced increases in plasma IL-1beta and IL-6 in rats was blocked by pretreatment with the A1-AR antagonist, prazosin (Johnson et al., 2005). Rats that underwent immobilization stress also showed induction of NFkB gene expression, and this effect was blocked by pretreatment with prazosin (Bierhaus et al., 2003). A study looking at the effects of chronic high altitude stress in women showed that high altitude increased IL-6 levels, and blocking the A1-AR with prazosin resulted in inhibition of this IL-6 response. Furthermore, A1-AR blockade also reduced exercise-induced IL-6 levels in these women, both at sea level and at high altitude (Mazzeo et al., 2001).

Interestingly, under resting conditions, many cell types, including peripheral blood mononuclear cells (PBMC), fail to express A1-ARs. However, stress and inflammatory stimuli have been shown to induce and/or regulate the expression of A1-ARs. THP-1 cells, a human monocyctic cell line, treated with the cytokines, TNF-alpha and IL-1beta, were shown to decrease mRNA levels of A1d-AR, increase mRNA for A1a-AR, while levels for A1b-AR were unaffected. In the same study, stimulation of endothelial cells decreased both A1b- and A1d-AR mRNA levels, while the A1a-AR was undetected, showing that regulation of these receptors by inflammatory stimuli can be cell subtype- and tissue-specific (Heijnen et al., 2002). In another study using freshly isolated human monocytes, induction of A1b- and A1d-ARs, but not A1a-AR was seen after stimulation with the synthetic glucocorticoid, dexamethasone or terbutaline, a B2-

AR agonist, showing crosstalk between activation of B2-ARs and expression of A1-ARs. Using the human monocytic cell line, THP-1, it was shown that this increased level of A1-AR mRNA is also accompanied by increased levels of protein, and it was suggested that this upregulation in the A1-AR could be a stress marker since glucocorticoids and catecholamines both increase during stress (Rouppe van der Voort et al., 1999). When human PBMCs were activated by mitogenic stimuli in vitro, and isolated human monocytes by LPS, induction of A1a-, A1b- and A1d-ARs mRNA was found (Rouppe van der Voort et al., 2000b). Furthermore, stimulation of either of these cells with NE, after mitogenic activation, led to increased phosphorylation of ERK-2, which was absent in cells not expressing the A1-ARs, indicating that the induced receptors were functionally active, and could thus stimulate inflammatory pathways, since ERK-2 is known to stimulate, for example, the release of IL-6. Additionally, blocking the A1-AR, but not the A2-AR or beta-AR, completely inhibited the NE-induced phosphorylation of ERK-2 (Rouppe van der Voort et al., 2000b; Kavelaars, 2002).

Consistent with the notion that inflammatory mediators can activate the expression of A1-ARs, PBMCs isolated from children with juvenile rheumatoid arthritis expressed A1-ARs, whereas it was undetectable in healthy individuals (Heijnen et al., 1996; Rouppe van der Voort et al., 2000a). It is thought that this increase in expression of A1-ARs is mediated by increased NE from increased SNS outflow, and increased cytokines, often seen in this disease (Kavelaars, 2002). Moreover, despite similar increases in plasma catecholamines following exposure to the cold pressor test (a stressor known to stimulate NE release without stimulating epinephrine release), PBMCs of children with juvenile rheumatoid arthritis exhibited significantly greater increases in

LPS-induced IL-6 compared to healthy, age-matched controls (Rouppe van der Voort et al., 2000a; Kavelaars, 2002). In vitro, PBMCs isolated from these patients responded to A1-AR agonists with an increase in IL-6 production, unlike cells from healthy controls (Heijnen et al., 1996). This data seems to point to the A1-AR as a proinflammatory mediator of the innate immune response, which may act to enhance inflammation, even if detrimental, during times of stress. The role of the B2-AR, however, is not as clear.

B2-AR

Radioligand binding studies have shown that macrophages express B2-ARs. The density of the receptor on these cells ranges from about 1000 to 23,000 binding sites per cell (Bellinger et al., 2008). Other cells of the innate immune system, such as NK cells, mast cells and neutrophils, express these receptors as well (Barnes, 1999; Elenkov et al., 2000). In contrast to the A1-AR, activation of the B2-AR by catecholamines has been shown to inhibit innate immune responses, although there are instances where B2-ARs have been shown to play a role in immune activation. For example, stress induction by tail-shock, as well as peripherally-induced immune challenge by bacteria, led to activation of IL-1beta expression in the brain of rats, which was blocked by pretreatment with a beta-AR antagonist (Johnson et al., 2005; Johnson et al., 2008). In addition, stimulation of cultured murine pituicytes with epinephrine or IL-1beta-induced increases in IL-6 release, which was blocked by coincubation with B2-AR antagonists (Christensen et al., 1999). Both these studies are focused on the central effects of catecholaminergic stimulation, but proinflammatory functions can be seen in the periphery as well. B2-AR agonists were shown to increase LPS-induced IL-8 levels in isolated human monocytes,

as well as the in the human premonocytic cell line, U937, in a cAMP-dependent manner (Kavelaars et al., 1997). Rat renal resident macrophage cells stimulated with LPS and the B2-AR agonist, terbutaline, showed an increase in IL-6 at high doses of the agonist, but a decrease in the cytokine at low doses (Nakamura et al., 1999). Bronchial epithelial cells stimulated with rhinovirus also showed an increased IL-6 response after administration of salmeterol, a long acting B2-AR agonist (Edwards et al., 2007). Mouse cardiac fibroblasts also showed an isoproterenol-induced, B2-AR dependent, increase in IL-6, which involved cAMP and phosphorylation of the MAPK, p38, but not of PKA (Yin et al., 2006). A few things to note in the last few studies cited, however, is that they were predominantly, although not all, done in rodent cells, and many of them did not involve the use of an inflammatory stimulus along with B2-AR activation. In accordance with this, Tan et al. (2006), showed that in the absence of any inflammatory stimuli, B2-AR activation led to increases in IL-1beta and IL-6, mRNA and protein, in a mouse macrophage cell line, not through NFkB or PKA pathways, but rather, through the MAPK pathway, by activation of ERK1/2 and p38.

Despite evidence of immune activation through B2-ARs, it is generally considered that catecholamine-induced stimulation of this receptor inhibits innate immune responses (see section on '**SNS signals to the immune system: Neural release of NE and its effects on immunity'** for more examples). Infusion of the beta-AR agonist, isoproterenol, in healthy humans inhibited TNF-alpha release (Goebel et al., 2000). Interestingly, in the same study, activation of the SNS through acute psychological stress using a performed speech, or exercise, showed an increase in TNF-alpha, suggesting that the type of activation of the SNS may give different results, with beta-AR

activation mediating anti-inflammatory effects only. Isoproterenol administered to mouse macrophages inhibited LPS-induced TNF-alpha and nitric oxide (Hasko et al., 1998a), while terbutaline, another beta-AR agonist, suppressed TNF-alpha and INF-gamma production in LPS-stimulated mouse splenocytes and peritoneal macrophages (Haerter et al., 2004). Formoterol and salmeterol, both long acting B2-AR agonists, inhibited LPS-induced TNF-alpha release in monocyte-derived macrophages (Donnelly et al., 2010), and salmeterol also inhibited TNF-alpha release in LPS-stimulated THP-1 cells, a human monocyte cell line (Sekut et al., 1995). Isoproterenol also increased IL-10, an anti-inflammatory cytokine, release from LPS-stimulated mouse macrophages, and this increase in IL-10 was partly responsible for a decrease in TNF-alpha, thus inhibiting inflammation even further, indirectly (Suberville et al., 1996). PMA-differentiated human macrophages also showed decreases in LPS-induced TNF-alpha and IL-6, along with an increase in IL-10, after beta-AR agonist stimulation, which was blocked by B2-AR antagonists, but not B1-AR antagonists (Izeboud et al., 1999a). LPS-stimulated bone marrow derived macrophages isolated from sepsis-induced mice also showed a B2-AR mediated reduction in TNF-alpha, with an increase in IL-10 production (Muthu et al., 2005).

In addition to inhibiting proinflammatory cytokines, B2-AR activation can inhibit other mediators of the inflammatory response. For example, epinephrine was shown to decrease LPS-induced macrophage inflammatory protein-1alpha (MIP-1alpha), a chemokine involved in trafficking of cells to sites of inflammation, in a B2-AR specific manner in human monocytes (Li et al., 2003). MIP-1alpha was also decreased in LPS-stimulated mouse macrophages by NE and cAMP-elevating agents (Hasko et al., 1998b).

Other chemokines, such as chemokine CXC ligand 9 (CXCL9) and eotaxin-1, were also reduced in PBMCs dosed with NE (Torres et al., 2005). Salmeterol administration was shown to decrease proliferation of lymphocytes, along with inhibiting IL-4 release, after activation (Mohede et al., 1996). Isoproterenol in IFN-gamma-activated THP-1 cells also induced increases in cathepsin B, a proteinase present in macrophages that plays a role in the pathophysiology of multiple sclerosis (Li and Bever, Jr, 1998). Finally, B2-AR stimulation of dendritic cells downregulated expression of various chemokines, leading to inhibition of their migration and reduced T helper 1 cell priming (Maestroni, 2006).

Interestingly, activation of B2-ARs on human airway macrophages did not lead to inhibition of an inflammatory response (Barnes, 1999), and it is thought that this decrease in B2-AR function may be a result of cell differentiation. Baker and Fuller (1995) showed that stimulation of beta-ARs in freshly isolated human monocytes led to inhibition of thromboxane B2 (TXB2), an inflammatory mediator. This inhibition was lost when they tested the cells after maturing them in culture. Whereas some studies showed that monocytic cells expressed less B2-ARs compared to PMA-induced differentiation of these cells (Izeboud et al., 1999a), others have shown the opposite effect (Radojcic et al., 1991). Although contradictory, these studies show that studying cells at different states of differentiation is necessary due to changes in receptor expression and, thus, the responsiveness of a cell to the inhibitory effects of beta-agonists may depend on the differentiation state of the cell.

The B2-AR is coupled to the Gs-pathway and its inhibitory effects may be a result of its increased cAMP signaling, as evidence has shown that activation of B2-ARs in immune cells result in cAMP accumulation (Fedyk et al., 1996). For example,

administration of the beta-agonist, isoproterenol, inhibited LPS-induced TNF-alpha production in human blood and THP-1 cells, while the agonist increased cAMP levels at the same concentration that inhibited TNF-alpha (Severn et al., 1992). Beta-agonists administered to LPS-stimulated human PBMCs also decreased TNF-alpha and IL-1beta production, which was mimicked by administration of dibutyryl cAMP (Yoshimura et al., 1997). Human monocyte cells lines, THP-1 and U937, dosed with B2-AR agonists, as well as a variety of cAMP-elevating agents, such as prostaglandins, forskolin and cAMP analogs, showed decreases in TNF-alpha and IL-8 release (Farmer and Pugin, 2000), and in TNF-alpha and IL-6, (Izeboud et al., 1999b), respectively. Izeboud et al.'s study (1999b) also mimicked their results in vivo, using rats dosed with the beta-AR agonists and cAMP elevating agents one hour before LPS administration. Farmer and Pugin (2000) blocked the decrease in the inflammatory mediators by inhibition of PKA (Farmer and Pugin, 2000). Furthermore, elevated levels of cAMP are known to inhibit activation of NFkB, thus inhibiting the transcription of proinflammatory cytokines (Elenkov et al., 2000). PKA activation brought about by increases in cAMP has been shown to inhibit NFkB transcription by phosphorylating cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (Parry and Mackman, 1997).

One way cAMP is regulated in the cell is through degradation by phosphodiesterases (PDE). PDE4 is the most abundant of these enzymes in monocytes (Manning et al., 1996), and can be induced by LPS stimulation (Wang et al., 1999). Studies have found that inhibition of this enzyme by pharmacological agents such as rolipram, prevents LPS induced increases in TNF-alpha, IL-12 and IFN-gamma,

suggesting a role for cAMP in modulating inflammation (Hasko et al., 1998c). Interestingly, inflammation and increased SNS outflow, as seen in patients with juvenile rheumatoid arthritis, may change the ability of cAMP to act in an anti-inflammatory manner. For example, leukocytes from these patients showed a lower cAMP response to B2-AR stimulation, compared to healthy controls, which was reversed when the cells were co-incubated with an inhibitor of a cAMP-degrading enzyme (Kuis et al., 1996), suggesting that the lower response to the B2-AR agonist was due to increased cAMP-phosphodiesterase activity in the cells. However, some studies have also shown that inhibition of proinflammatory mediators by B2-AR agonists can act in a cAMP-independent manner as well. For example, salmeterol decreases LPS-induced TNF-alpha in monocyte-derived macrophages without increasing cAMP levels (Donnelly et al., 2010).

The mechanism by which B2-AR stimulation exerts its anti-inflammatory effects is still being investigated. Some studies suggest the anti-inflammatory effects result from the receptor activation's ability to downregulate TLR signaling, thus downregulating inflammatory signals (Kuroki et al., 2004; Wang et al., 2009). Inhibition of membrane-bound CD14, which is a co-factor of TLRs and known to regulate LPS signaling, was seen in LPS-stimulated monocytes after B2-AR agonist administration (Kuroki et al., 2004). Wang et al., (2009) suggested that inhibition of inflammatory cytokine production by the B2-AR agonist, fenoterol, in LPS-stimulated THP-1 cells was also due to downregulation of TLR signaling, potentially resulting from BARR-2 mediated redistribution of CD14 and the CD14/TLR4 complex. Using flow cytometric techniques, they labeled TLR4 and CD14 in these cells and then stimulated them with the B2-AR

agonist and LPS. Along with inhibition of TNF-alpha and IL-8 release, addition of the agonist resulted in a decrease of LPS-induced membrane-bound CD14 and CD14/TLR4 complex expression, although total protein expression was not affected. Using confocal microscopy, they showed that B2-AR stimulation redistributed these proteins and increased membrane-bound BARR-2. Finally, using siRNA, they silenced BARR-2 and were able to reverse the anti-inflammatory effects and redistribution of the CD14/TLR4 complex.

In a different study, it was suggested that beta-AR agonists exerted their anti-inflammatory effects through their regulation of NFkB, which is a transcription factor activated by TLR4 signaling (Farmer and Pugin, 2000). Specifically, activation and translocation of LPS-induced NFkB in THP-1 cells was inhibited by long-term treatment with a beta-AR agonist. The mechanism of NFkB inhibition is thought to occur through increased stabilization of IkB-alpha, thus not allowing IkB to degrade so it cannot dissociate and release NFkB, rendering it inactive. Indeed, after three hours of LPS and isoproterenol treatment, IkB-alpha protein levels increased, accompanied by an increase in IkB-alpha cytoplasmic half-life. Furthermore, stimulation with prostaglandin E2, a cAMP-elevating agent, mimicked the effects of the beta-AR agonist, and were blocked by H89, a PKA inhibitor, suggesting that the increased levels of IkB-alpha are due to elevations in cAMP brought about by the agonist (Farmer and Pugin, 2000).

Since B2-ARs are known to be tightly regulated in their signaling, it is important to note the role of desensitization and downregulation in the inflammatory response. Human lymphocyte beta-ARs have shown the characteristic downregulation of response to chronic treatment with B2-AR agonists, with a difference between cell types affected

(Elenkov et al., 2000). For example, chronic treatment with the B2-AR agonist, terbutaline, in healthy volunteers, resulted in a decrease in the number of B2-ARs in T-cytotoxic cells, but not as much of a decrease was seen in NK and T-helper cells. This downregulation was also related to a decreased cAMP response to isoproterenol, another beta-AR agonist (Maisel et al., 1989). More related to innate immunity, a study using murine macrophages in vitro showed the often seen decrease in LPS-induced TNF-alpha mRNA and protein after B2-AR agonist administration. However, when the cells were pre-exposed to the agonist, then washed and re-exposed to the agonist and LPS, it shifted the agonist concentration-effect curve to the right, suggesting a desensitized beta-AR (Ignatowski and Spengler, 1995).

B2-AR signaling regulation and inflammation

Since the extent of agonist-induced signaling and desensitization in GPCRs like the B2-AR, is modulated by the expression levels of GRKs and beta-arrestins, then one of the ways of regulating the effect of GPCRs on inflammatory responses is through the expression of these proteins (Vroon et al., 2006). Interestingly, GRK2, GRK3, GRK5 and GRK6 are highly expressed at the mRNA level in monocytes and macrophages (Elenkov et al., 2000; Lattin et al., 2007). Furthermore, GRK6 protein levels were shown to be induced during monocytic differentiation in HL-60 cells, whereas stimulation with PMA led to a reduction in GRK6 and GRK2. Conversely, in lymphocytes, activation resulted in the increase of both GRK2 and GRK6 (Loudon et al, 1996). Chuang et al. (1992) cloned and sequenced cDNA of human GRK2, and found GRK2 mRNA to be highly expressed in peripheral blood leukocytes, and in several immune cell lines. Additionally, they

showed that administration of the beta-AR agonist, isoproterenol, induced translocation of the protein from the cytosol to the plasma membrane, where it acts to desensitize the receptor. This suggests a role for the kinase in modulating immune responses brought about through stimulation of the beta-AR.

Inflammation has also been shown to regulate expression of GRKs. In humans, IL-6 and IFN reduced GRK2 protein levels in T-cells (Lombardi et al., 2002) and peripheral blood mononuclear cells (PBMC) (Lombardi et al., 1999). Additionally, PBMCs from patients with rheumatoid arthritis and multiple sclerosis also show a decreased level of GRK2 and GRK6 protein expression (Lombardi et al., 1999; Vroon et al., 2005). GRK2 knockout mice showed an increase in T-cell and macrophage infiltration, associated with earlier onset of experimental autoimmune encephalitis (Vroon et al., 2005). Thus, this reduction in GRK2 during inflammatory processes may enhance leukocyte infiltration and, in some cases, disease progression (Lattin et al., 2007).

Many of these studies, however, were focused on immune-based GPCRs, such as chemokine receptors, and their effect on GRK regulation during inflammatory processes. The effect of modulating GRK and arrestin expression during inflammation in terms of the B2-AR may be different, and has not been well studied. Of interest, in a study on airway responsiveness in rats, treatment with IL-1beta resulted in a decrease in B2-AR density in the lungs, a decrease in isoproterenol- and forskolin-induced cAMP accumulation, an increase in the activity of cytosolic GRK, and elevated expression of GRK2 and GRK5 in lung macrophages (Mak et al., 2002). The authors concluded that IL-1beta treatment led to increased pulmonary B2-AR desensitization, likely mediated by

the increase in GRKs and the decrease in cAMP activity. Furthermore, a recent study by Loniewski et al. (2008) showed that the expression of GRKs, as well as arrestins, could be altered by inflammatory stimuli in primary mouse macrophages. In the specific case of the TLR4 receptor, LPS stimulation resulted in increased levels of GRK2 protein and mRNA, but decreased levels of GRK5 and GRK6. Thus, changes in expression of GRKs due to an inflammatory stimulus or cell differentiation, specifically in relation to the B2-AR, may lead to changes in receptor desensitization, but it seems that their regulation is different depending on the GRK involved, and the model system.

Interestingly, GRKs may also affect inflammatory signaling pathways outside of GPCR desensitization. For example, GRK5 knockdown resulted in enhanced LPS-induced ERK phosphorylation in the mouse macrophage cell line, RAW264.7, suggesting negative regulation of LPS-induced, MAPK-mediated inflammatory activation (Parameswaran et al., 2006). On the other hand, GRK2 and GRK5 knockdown decreased TNF-alpha induced NFkB signaling, suggesting a proinflammatory role for these kinases (Patial et al., 2009). This suggests the action of these kinases in inflammation, outside of receptor desensitization, may depend on the specific kinase involved, on the ligand used to stimulate inflammation, and the corresponding signaling pathway.

Since GRK phosphorylation alone does little to reduce B2-AR signaling, and its main function is to increase the receptors affinity to BARR-1 and BARR-2, then regulation of these proteins may be more relevant in studying changes in receptor signaling (Lutrell and Lefkowitz, 2002; Lefkowitz and Whalen, 2004). Increases in these proteins leads to increased receptor desensitization (Lefkowitz and Whalen, 2004), which may thus lead to decreases in B2-ARs ability to inhibit inflammation. Similar to GRKs,

mRNA for BARR-1 and BARR-2 is also highly expressed in mouse and human macrophages (Lattin et al., 2007), so it has the ability to affect these cells.

However, like the GRKs, arrestins can signal and function outside of receptor desensitization as well, and are known to associate with a variety of other proteins and receptors (Hall and Lefkowitz, 2002). Knockdown of BARR-2 has been shown to result in increased LPS-induced ERK1/2 phosphorylation, suggesting it can also negatively regulate ERK1/2 (Paramswaran et al., 2006). Knockdown of BARR-2 also enhanced inflammatory-induced mRNA and protein expression of IL-6 and IL-8, while its overexpression inhibited TNF-induced NFkB DNA binding (Gao et al., 2004), suggesting its ability to act in an anti-inflammatory manner. Furthermore, BARR-1 and BARR-2 can inhibit TLR4-mediated activation in macrophages, through its interaction with TRAF6, preventing it from activating IKK, which acts to degrade IkB and activate NFkB (Wang et al., 2006). A recent study has revealed its possible role in mediating the anti-inflammatory effects of B2-AR activation, apart from its role in desensitizing the receptor. As mentioned earlier, one mechanism that may mediate B2-AR effects is downregulation of TLR signaling. BARR-2 was shown to mediate this downregulation in THP-1 cells stimulated with LPS and a B2-AR agonist (Wang et al., 2009). In this study, membrane-bound BARR-2 increased with stimulation, while silencing of BARR-2 using siRNA attenuated the inhibition of inflammatory cytokine production, as well as the reduction in membrane-bound CD14 and CD14/TLR4, after agonist and LPS administration. This study suggests that BARR-2 may be needed for B2-ARs to exert their anti-inflammatory effects, which would contradict their role in desensitizing the receptor.

Conversely, in the mouse macrophage cell line, RAW264, TLR4 stimulation by LPS resulted in downregulation of B2-AR protein and mRNA levels, and BARR-2 levels (Kizaki et al., 2008). To determine the consequence of this downregulation, the cells were transfected with a B2-AR expression vector, RAWar, and LPS stimulation resulted in decreased nitric oxide production, decreased activation of NFkB and decreased degradation of IkB, supporting the findings from Farmer and Pugin (2000) in the human THP-1 cell line (see above), that B2-AR activation leads to decreased NFkB activation. Interestingly, BARR-2 expression was decreased in the downregulated cell line after LPS stimulation as well, but not in the B2-AR transfected RAWar cells. Overexpression of BARR-2 was shown to interact and stabilize cytosolic IkB, and it resulted in inhibition of nitric oxide. These findings further support the Wang et al. (2009) study that B2-ARs and BARR-2 act together to inhibit LPS-induced inflammation. The authors concluded that TLR4 signaling suppressed B2-AR expression, which led to downregulation of BARR-2 expression, and thus decreased its ability to degrade IkB, leading to increased activation of NFkB, providing a mechanism for 'escaping' B2-ARs anti-inflammatory signaling. It is interesting that these studies show that B2-AR activation may downregulate TLR4 signaling, while TLR4 signaling may downregulate B2-AR expression, so the timing of signals may make a difference in the effects seen on inflammation. These studies also suggest that BARR-2 is needed for B2-AR stimulation to exert anti-inflammatory effects, however inflammation itself may decrease the ability of it to act in an anti-inflammatory manner through decreases in its expression.

Another mechanism used to regulate the responsiveness of a cell to a specific GPCR ligand, like NE, is regulation of the number of receptors the cell expresses

(Heijnen, 2007), and B2-ARs are known to undergo internalization and downregulation as another mechanism for desensitization (as described previously). Administration of salmeterol, a long acting beta-AR agonist, for seven days in rats reduced B2-AR density in the lungs and impaired cAMP signaling (Finney et al., 2001). One factor that may change the level of receptor expression may be cellular activation. Indeed, studies have shown that the level of B2-AR on immune cells decreases as a function of inflammation. The study described above by Kizaki et al. (2008) showed that LPS could induce downregulation of mRNA for the B2-AR, as well as BARR-2, in a mouse cell line. Furthermore, Baerwald et al. (1992) described decreased density of this receptor on the peripheral immune cells of patients with rheumatoid arthritis, a chronic inflammatory disease. They later showed that this decrease in receptor density also reduced the inhibitory effects of NE and epinephrine on lymphocyte proliferation in a beta-AR-dependent manner (Baerwald et al., 1999). Furthermore, decreased B2-AR mRNA expression was found in the context of chronic stress and asthma, another chronic inflammatory disease (Miller and Chen, 2006). Children with asthma that had simultaneously experienced chronic or acute stress had a 9.5-fold reduction in B2-AR mRNA from isolated leukocytes. In a sample of healthy children, however, the direction of this effect was reversed. These data suggest that the combination of a chronic inflammatory state and increased stress, may lead to decreased B2-AR expression, perhaps through the receptor's capacity to undergo desensitization and downregulation, thus potentially contributing to a decreased response to the inhibitory effects of catecholamines. Interestingly, as discussed in a previous section, A1-AR expression seems to be induced and increased during stress and/or inflammation (Kavelaars, 2002),

suggesting that these factors may further attenuate cathecholamine's ability to inhibit inflammation, since A1-ARs have been shown to stimulate inflammatory responses.

Aside from whether or not the receptor is desensitized or downregulated, the ability of the B2-AR to modulate inflammatory mediators can also differ depending of the type of inflammatory stimulus used, even among the same cell types. Szelenyi et al. (2006), showed that stimulation by LPS or PMA produced opposite effects on isoproterenol's actions on cytokine production and MAPK phosphorylation in murine macrophages, isolated human monocytes and a differentiated monocyte/macrophage cell line. Specifically, LPS-induced TNF-alpha and nitric oxide production was inhibited by pretreatment with the beta-AR agonist, whereas the agonist potentiated their release in PMA-stimulated cells. This response was mimicked in the phosphorylation status of the MAPKs, ERK1/2 and p38. A later study suggested that this may involve activation of different signaling pathways. B2-ARs have been shown to be able to switch its signaling mechanism (Daaka et al., 1997). Although predominantly Gs-coupled, this receptor can also switch to a pertussis toxin-sensitive, Gi-coupled signaling pathway upon stimulation and phosphorylation by PKA, and thus signaling is now switched to a decrease in adenylate cyclase activity and an increase in MAPK activation (Daaka et al., 1997; Magocsi et al., 2007). This switch is of interest in the immune response, as Gi-signaling is more likely to be proinflammatory in nature, since it can activate the MAPK pathway, as well as inhibit cAMP signaling. Interestingly, B2-AR stimulation on murine peritoneal macrophages using isoproterenol was found to increase ERK1/2 and p38 phosphorylation in a pertussis toxin-sensitive manner, suggesting that this switch was occurring in these cells (Magocsi et al., 2007). Furthermore, this same group showed that the stimulatory

effects of isoproterenol on PMA-induced TNF-alpha production was inhibited by pertussis toxin, further showing the proinflammatory actions of stimulating this specific G-coupled signaling pathway. However, pertussis toxin did not affect the inhibitory response of the agonist on LPS-induced TNF-alpha production, suggesting that the opposite effects of the agonist with different inflammatory stimuli may be a result of differences in G-coupled signaling pathways.

V. Summary

Although this chapter has summarized numerous studies, a better understanding is still needed in describing the interaction between stress-induced activation of the SNS, release of NE, and the inflammatory response, as the amount of contradicting information is vast, specifically in regards to whether NE acts in a pro- or anti-inflammatory manner. To further explore the relationship between NE stimulation/release and inflammatory responses, we first examined the effect of cellular activation and differentiation on inflammatory cytokine production induced by NE administration **(Chapter 2)**. THP-1 human monocytic cells were dosed with NE, as well as A1-AR and B2-AR agonists and antagonists, either before or after LPS-mediated cellular activation, and IL-6 protein concentrations were measured to determine inflammatory response. THP-1 cells were also differentiated into macrophages using PMA, and the inflammatory response to NE was further examined during LPS-mediated activation. The roles of potential adrenergic receptor signaling pathways were also elucidated. To study the role of NE release and inflammation in vivo **(Chapter 3)**, we examined the effect of an acute stressor on inflammatory and NE responses in patients suffering with MD and healthy controls. IL-6

and NE were measured and compared pre- and post-stressor in both groups, and participants were also evaluated in terms of the extent of their NE-mediated stress response in relation to their IL-6 response to stress. Finally, **Chapter 4** summarizes the data presented in this dissertation and attempts to relate Chapters 2 and 3 with discussion of limitations and potential future directions to further this area of study (See **Figure 1-5** for a schematic summary).

Characteristics	Innate Immune System	Adaptive Immune System
Response Time	Immediate	Slow, lasting hours to days
Memory	No memory of prior exposure	Memory of prior exposure; enhances and quickens response to future exposures
Physiochemical Barriers	Skin, mucous membranes	Cutaneous and mucosal immune system
Humoral Elements	Complement proteins, C-reactive protein, acute phase reactants	Antibodies, immunoglobulins secreted by B cells
Cellular Elements	Phagocytes (macrophages, neutrophils) dendritic cells, mast cells, natural killer cells	Lymphocytes (B and T cells)
Receptors	Pattern recognition receptors: Toll like receptors, narrow specificity	T- and B-cell receptors; high specificity
Soluble Mediators that affect other cells	Macrophage-derived cytokines: interleukin-6, interferons, tumor necrosis factor-alpha	Lymphocyte-derived cytokines: interleukin-2, interleukin-4, interferon-gamma

Table 1-1. Properties of the innate and adaptive immune systems. The immune system is made up of two divisions: the innate immune system and the adaptive immune system. The innate immune component is evolutionarily older and acts as the first line of defense for organisms. The adaptive immune component, on the other hand, evolved much later and is only found in vertebrates. Although both components work together to rid organisms of foreign pathogens and to fight off infection and other ailments, there manner of functioning is quite different, with the innate immune system acting in a rapid and non-specific manner, and the adaptive immune system functioning slower, yet in a highly specific and selective manner.

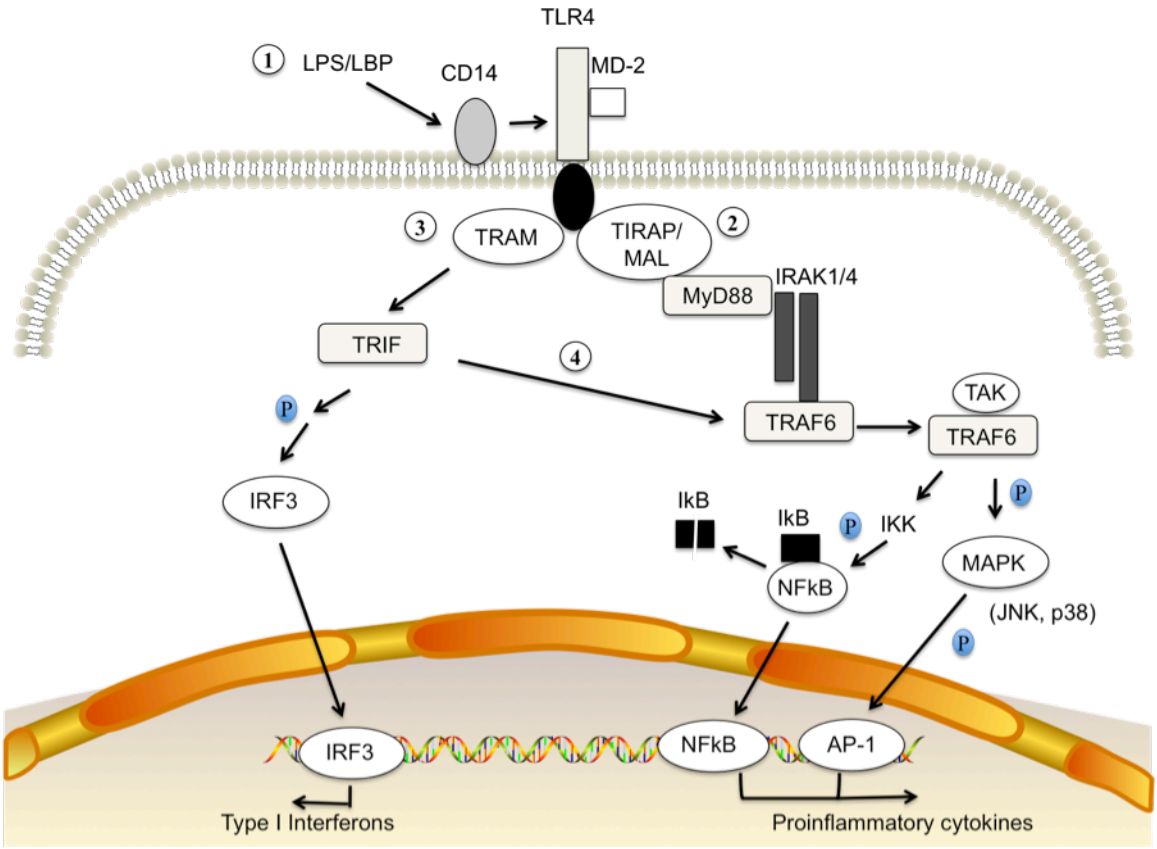

Figure 1-1. Toll-like receptor 4 (TLR4) signaling induced by lipopolysaccharide (LPS). 1.) LPS, bound to LPS-binding protein (LBP), interacts with CD14 and activates the TLR4 receptor signaling pathways, 2.) activation of the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway results in activation of the transcription factors, nuclear factor kappa B (NFkB) and mitogen activated protein kinase (MAPK) activated activator protein-1 (AP-1), which leads to the release of proinflammatory cytokines, such as interleukins-1, -6, and tumor necrosis factor, 3) activation of the MyD88-independent pathway replaces MyD88 with the TIR-domain-containing adaptor inducing IFN-beta (TRIF), and results in activation of the interferon regulatory factor (IRF)-3 transcription factor, which leads to type 1 interferon release, 4) the MyD88-independent pathway can also interact with the MyD88-dependent pathway, leading to proinflammatory cytokine release, as well. Please see text for other abbreviations.

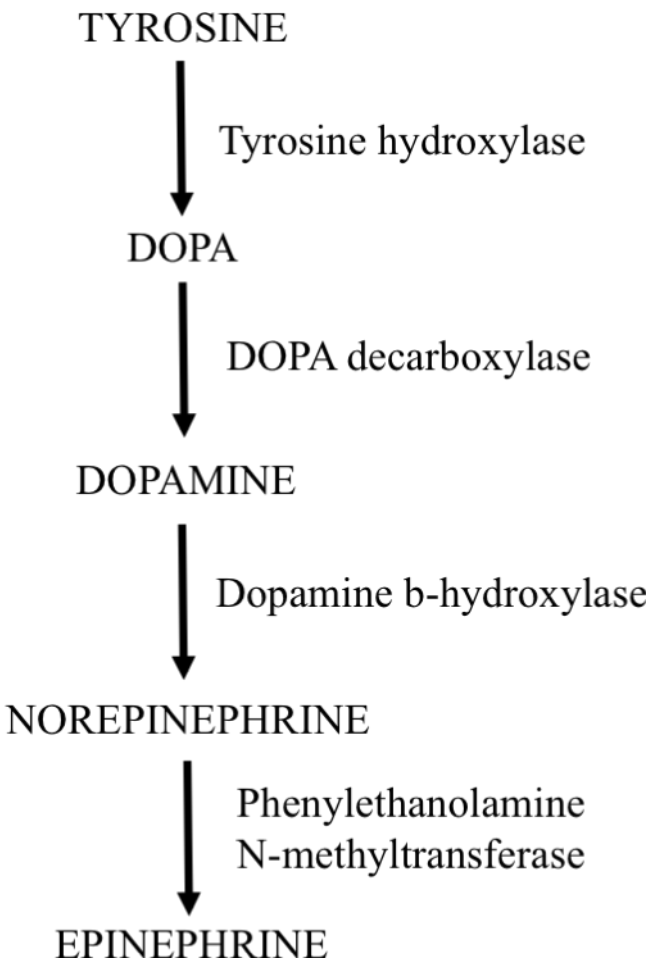

Figure 1-2. Norepinephrine and epinephrine synthesis. Synthesis of catecholamines dopamine, norepinephrine and epinephrine, begins with tyrosine and involves the use of numerous enzymes to convert one catecholamine into another, with tyrosine hydroxylase acting as the rate-limiting step in the process.

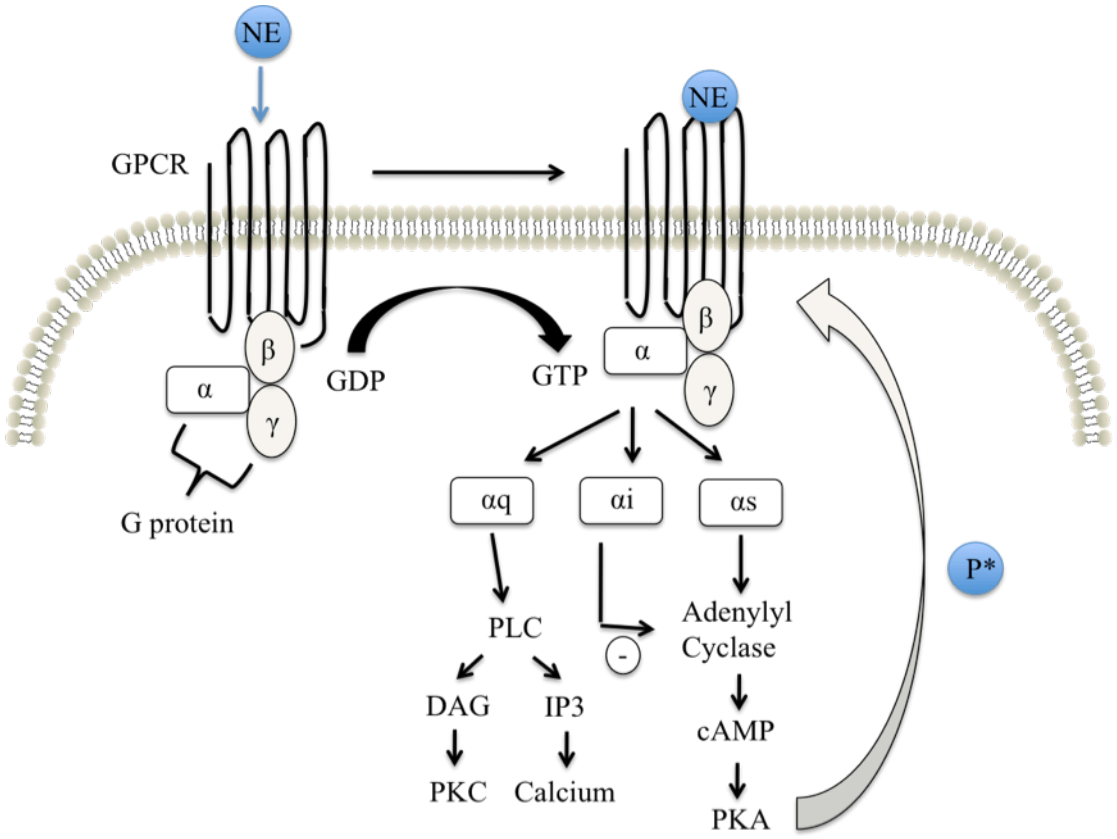

Figure 1-3. G-protein coupled receptor (GPCR) signaling pathways. Agonist binding of GPCRs by norepinephrine (NE) results in activation of GPCR-mediated signaling pathways, modulated by different G-alpha subunits. The catalysis of guanine diphosphate (GDP) to guanine triphosphate (GTP) leads to dissociation of the G-alpha subunit from the G-beta/G-gamma subunit of the G-protein. Downstream effectors are then induced, depending on the type of G-alpha subunit being released. Binding of NE to the alpha 1 adrenergic receptor results in activation of the G-alpha-q subunit, which involves activation of phospholipase C (PLC), which ultimately results in activation of protein kinase C (PKC) by diacylglycerol (DAG) and release of calcium by intracellular inositol 1,4,5-triphosphate (IP3). On the other hand, activation of the beta 2 adrenergic receptor leads to release of the G-alpha-s subunit, which activates adenylyl cyclase, leading to cyclic adenosine monophosphate (cAMP) accumulation and activation of protein kinase A (PKA). Of note, activation of PKA can also lead to phosphorylation of the GPCR, which can lead to receptor desensitization and inactivation, resulting in decreased signaling.

Heterologous Desensitization	Homologous Desensitization
• Independent of agonist occupancy • Inducible by processes other than receptor activation (ie: cAMP elevation) • Targets both occupied and unoccupied receptors • Mediated by phosphorylation by PKA or PKC • Decreases coupling to G-protein subunit • Can lead to receptor sequestration and downregulation	• Dependent on agonist occupancy • Induced by conformational change caused by agonist binding and receptor activation • Targets occupied receptors • Mediated by phosphorylation by GRKs • Requires recruitment and binding of beta-arrestin proteins • Decreases coupling to G-protein subunit • Can lead to receptor sequestration and downregulation

Table 1-2. Properties of heterologous and homologous desensitization. NE-mediated binding of adrenergic receptors, such as activation of the beta-2 adrenergic receptor, can lead to activation of desensitization mechanisms. Two forms of desensitization are known for GPCRs like adrenergic receptors, heterologous and homologous. Although they involve different mechanisms, both forms ultimately lead to regulation of receptor signaling in a negative fashion in order to avoid overstimulation.

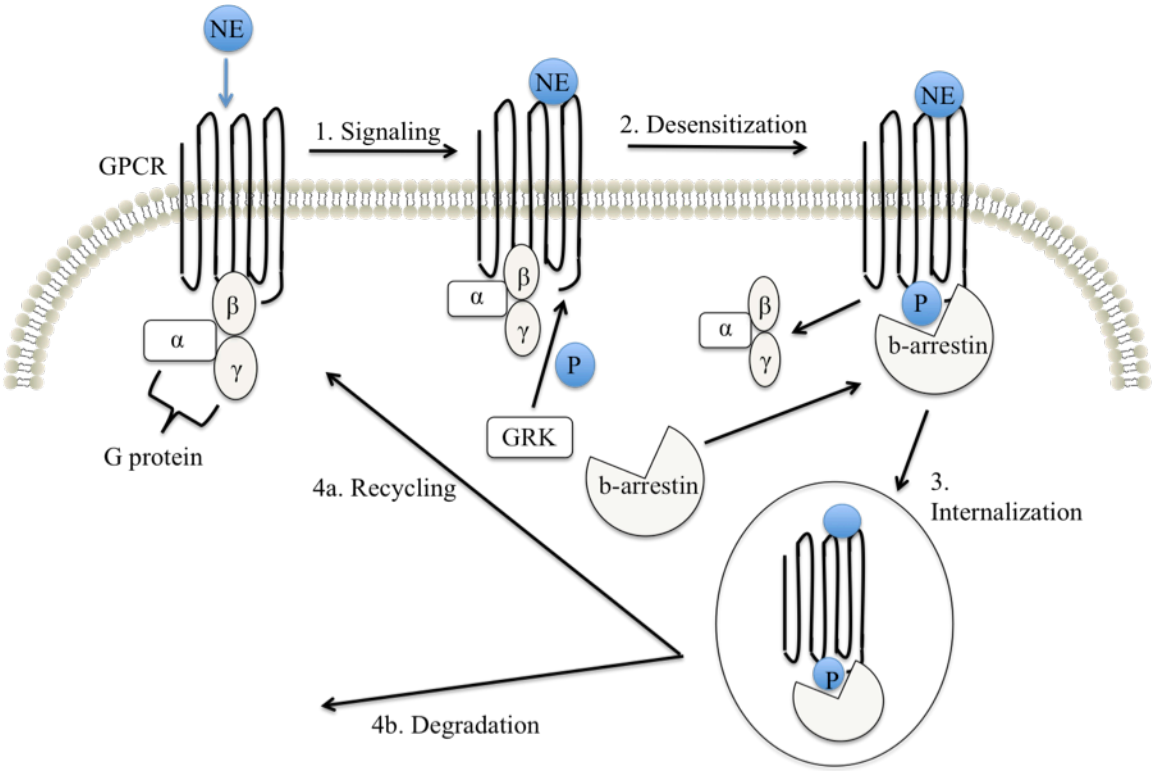

Figure 1-4. Schematic of homologous desensitization of GPCRs. Briefly, agonist-induced binding of GPCRs, such as NE binding of the beta-2 adrenergic receptor, leads to 1) GPCR-mediated signaling. Activation of the receptor can then lead to 2) desensitization of the receptor involving the phosphorylation of the receptor by GPCR kinases (GRK), and the recruitment and binding of beta-arrestins to the receptor, functionally uncoupling the receptor from its G-protein subunit. This can further lead to 3) receptor internalization into an endosome, which will ultimately result in 4a) recycling of the receptor back to the cell membrane, or 4b) degradation and downregulation of the receptor.

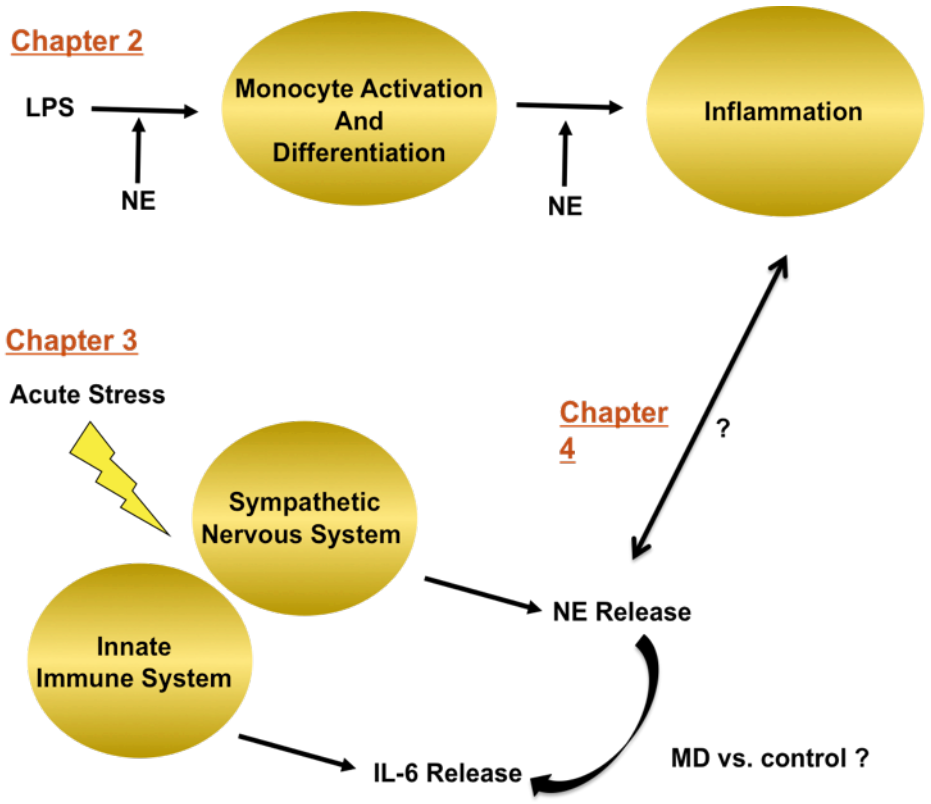

Figure 1-5. Summary of studies. The scope of this dissertation involves understanding the interplay between stress-induced activation of the SNS, release of NE, and the inflammatory response. Chapter 2 examines the effect of in vitro NE administration to a human monocytic cell line, before and after cellular activation and monocyte-to-macrophage differentiation, and how it relates to NE's ability to inhibit inflammatory responses. Chapter 3 takes a preliminary look at the effect of acute stress on inflammation and NE release, and studies the relationship between stress-induced NE responses and inflammatory markers in patients with major depression (MD) and controls. Finally, chapter 4 attempts to tie results from chapters 2 and 3 together, and offer future directions for questions still unanswered.

CHAPTER TWO

BETA-2 ADRENERGIC RECEPTOR REGULATION OF INFLAMMATORY RESPONSES IN RESTING AND ACTIVATED MONOCYTES AND DIFFERENTIATED MACROPHAGES

I. Introduction

Evidence has shown that activation of the sympathetic nervous system (SNS), along with endogenous or exogenous stimulation of adrenergic receptors (AR) can influence the innate immune inflammatory response (Elenkov et al., 2000; Kohm and Sanders, 2001; Oberbeck, 2006). Sympathetic nerve fibers innervate both primary and secondary lymphoid organs (Felten, 1993; Friedman and Irwin, 1997), and norepinephrine (NE), the main sympathetic neurotransmitter, has been shown to influence a variety of innate immune responses (Elenkov et al., 2000; Sanders and Straub, 2002; Oberbeck, 2006; Nance and Sanders, 2007; Bellinger et al., 2008), including inhibition of endotoxin-induced pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-alpha (Hu et al., 1991) and interleukin (IL)-6 (van der Poll, 1994; Rontgen et al., 2004). However, the literature describing the relationship between SNS activation and its regulation of the inflammatory response is paradoxical. For example, activation of immune cells by catecholamines has been described to have both pro-inflammatory and anti-inflammatory properties (Elenkov et al., 2000; Kohm and Sanders, 2001).

NE released from activation of the SNS mediates its effect through ARs, including the beta 2 (B2)-AR (Caron and Lefkowitz, 1993). The B2-AR is a G-protein coupled receptor (GPCR), which is commonly associated with modulating the inflammatory response (Liggett, 1999; Sitkauskiene and Sakalauskas, 2005; Wong et al., 2007), and is found on several immune cell types, including T-cells, B-cells, mast cells and macrophages (Madden et al., 1995; Elenkov et al., 2000; Nance and Sanders, 2007). Stimulation of the B2-AR on immune cells has been shown to regulate cytokine secretion, lymphocyte trafficking and proliferation, and antibody secretion (Madden et

al., 1995; Mohede et al., 1996; Barnes, 1999; Hanania and Moore, 2004; Wong et al., 2007), primarily through generation of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA) (Yoshimura et al., 1997; Farmer and Pugin, 2000; Wong et al., 2007).

Furthermore, as a GPCR, B2-AR signaling is also regulated by arrestins, such as beta-arrestin 2 (BARR-2) (Lefkowitz and Whalen, 2004). Recently, it has been demonstrated that B2-AR stimulation may also mediate inflammatory responses though its interaction with BARR-2 and the endotoxin stimulated toll-like receptor 4 (TLR4) (Kizaki et al., 2009; Wang et al., 2009), followed by modulation of downstream nuclear factor kappa B (NFkB) signaling (Farmer and Pugin, 2000; Kizaki et al., 2009).

Although commonly associated with an anti-inflammatory response, B2-AR activation has also been shown to induce pro-inflammatory cellular changes (Kavelaars et al., 1997; Tan et al., 2006; Yin et al., 2006). These B2-AR mediated immune responses may differ depending on the cell type being studied, the experimental conditions, and the developmental stage of relevant inflammatory immune cells (Elenkov et al., 2000). Monocyte-derived macrophages form a major group of cells involved in the innate immune response and are main sources of cytokine release. These cells also undergo both activation and differentiation (Adams, 1994). Monocytes undergo differentiation into macrophages once they enter tissues (Adams, 1994; Fujiwara and Kobayashi, 2005), while activation of either cell type involves movement from a resting to an activated state, usually induced by inflammatory cytokines, such as IL-1beta, or microbial products, such as lipopolysaccharide (LPS) which bind to TLR4 (Gordon, 2003; Fujiwara and Kobayashi, 2005). Activation enhances cellular functions relevant to

immunomodulation (Fujihara et al., 2003; Ma et al., 2003). Both undifferentiated monocytes and macrophages can undergo activation to enhance their immunomodulatory functions (Geissman et al., 2010).

Stimulation of B2-ARs on monocytes and macrophages has led to changes in a variety of innate immune responses, including chemotaxis and expression of adhesion molecules (Hasko et al., 1998b) and inflammatory mediator production, such as inhibition of endotoxin-induced release of IL-8 (Farmer and Pugin, 2000), TNF-alpha (Hu et al., 1991; Hasko et al., 1998a; Szelenyi et al., 2006) and IL-6 (Izeboud et al., 1999b; Nakamura et al., 1999). However, studies have also shown that dosing monocytes or macrophages with a B2-AR agonist alone, without an inflammatory stimulus or activation of the cell, actually results in an increase of proinflammatory mediators, such as IL-6 and IL-1beta (Tan et al., 2006), and an increase in NFkB signaling (Bierhaus et al., 2003; Tan et al., 2006). Furthermore, B2-AR density and expression has been shown to be different in differentiated versus undifferentiated cells (Radojcic et al., 1991; Baker and Fuller, 1995; Izeboud et al., 1999a). Therefore, the state of activation, as well as the differentiation state of the cell, may affect how the cell responds to NE and B2-AR stimulation. However, many of these studies only have examined effects on either monocytes or macrophages, and only stimulate with B2-AR agonists either before or concurrently with activation.

Thus, in the present study we sought to determine the effects of NE administration on inflammatory cytokine production before and after LPS-induced cellular activation in both monocyte and differentiated macrophages. Using the human monocytic cell line, THP-1, as an in vitro model of monocyte to macrophage differentiation (Tsuchiya et al.,

1980; Auwerx, 1991), we first examined the role of NE administration on the IL-6 response of the cell in its monocytic form. Monocytes were dosed with NE, both prior to and after stimulation with LPS, in order to establish any differences in NE modulation of IL-6 release due to the activation state of the cell. In order to characterize the role of the B2-AR in any NE-mediated effects, cells were dosed with specific agonists and antagonists for the receptor under each experimental condition, and relevant signaling pathways for the receptor were assessed. Next, we examined the role of NE administration on IL-6 release in the differentiated form of the cell, and investigated any differences in IL-6 responses to NE administration and B2-AR stimulation in the inactivated versus activated macrophage.

II. Methods

Chemicals

LPS, norepinephrine bitartrate salt, ICI 118,551, forskolin, prazosin, methoxamine, fenoterol, phorbol-myristate-acetate (PMA), and H-89 were purchased from Sigma (St. Louis, MO).

Cell Culture

Cell Maintenance

The THP-1 human monocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI-1640 medium with 2mM L-glutamine (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone), and antibiotics in 75-cm2 flasks at 37°C with 5% CO2 in ambient air. Cells

were passed once they reached 80% confluency. For experiments involving the measurement of cytokine protein, 5 X 10^5 cells/mL were plated in flat-bottomed 6-well plates. For mRNA experiments, 5 X 10^5 cells/mL were cultured in 12-well plates.

Differentiation of Cells

THP-1 cells were differentiated by dosing with 100 nM PMA for 48 hours (Tsuchiya et al., 1982). Cells were then washed three times and resuspended in fresh complete medium. Cells rested for 24 hours before activation with LPS and/or AR stimulation.

Cell Activation and Stimulation

To activate the cells as either monocytes or macrophages, THP-1 cells were dosed with LPS at 1 ug/mL or 5 ug/mL for 6 hours for RNA collection and 24 hours for protein collection. To determine the effects of AR stimulation before activation, cells were dosed with the corresponding agonist, NE, methoxamine for the alpha 1 (A1)-AR, or fenoterol for the B2-AR, for one hour prior to LPS activation. To determine the effects of AR stimulation after activation, agonists were added to the culture 6 hours after LPS activation. To determine AR subtype specificity, cells were preincubated with the specific AR antagonists, ICI 118,551 for the B2-AR and prazosin for the A1-AR, for 45 minutes prior to AR stimulation, either before or after LPS-induced activation as described above. Finally, to determine the signaling pathways involved, cells were dosed with the cAMP-elevating agents forskolin or Sp-cAMP, one hour prior to and six hours after LPS activation, or with the PKA inhibitor, H-89, for 45 minutes prior to AR stimulation, either before or after LPS-induced activation as described above.

IL-6 Protein Determination

IL-6 protein concentrations were measured from the supernatant using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Minneapolis, MN; sensitivity mean = 0.16 pg/ml). Intra- and inter-assay coefficients of variability were 3.8% and 4.9%, respectively.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Directly after treatments, cells were collected and total RNA was isolated using the RNeasy Mini Kit from Qiagen (Valencia, CA) following manufacturer's instructions. RNA samples were dissolved in RNAse-free water, and their concentrations and A260/280 ratio were determined using the MBA 2000 System (PerkinElmer, Shelton, CT). Samples of RNA were either frozen at -80 degrees Celsius or used directly for cDNA synthesis. cDNA was synthesized on 1 μg denatured total cellular RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. Changes in gene transcription were analyzed using real-time polymerase chain reaction (PCR) techniques. Primers for each specific gene, B2-AR, CD14 receptor, IL-6, and BARR-2 were purchased from Qiagen (Valencia, CA) using the Quantitect Primer Assay. PCR reactions were carried out in the Applied Biosystems 7500 Fast System Cycler using QuantiFast SYBR Green PCR Master Mix from Qiagen according to manufacturer's instructions. The relative quantification of each specific gene was analyzed using the included software against the standard and normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), serving as the internal reference

value. The comparative delta-delta Ct method was used to compare relative gene expression in each experimental condition. Negative controls were included in each cycle by omitting the addition of the cDNA template.

Statistical Analysis

Experiments were replicated in triplicate, and all dependent factors (IL-6 protein concentrations and CD14, IL-6, B2-AR and BARR-2 mRNA expression) were first analyzed using descriptive statistics, and reported as the mean and standard error of the mean of one representative experiment. Two-way analysis of variance (ANOVA) was used to assess all main effects and interactions. Specifically, the factors tested included differences between drug treatments (NE, cAMP elevating agents or AR agonist doses) and time of activation (Pre-LPS vs. Post-LPS), between NE treatment and AR antagonist administration, and between NE treatment and cell type (monocyte vs. macrophage). In the case of significant main effects or interaction, post-hoc comparisons of specific means was conducted by the Student-Newman-Keuls test. One-way ANOVAs were used to assess dependent variables when only one treatment factor (agonist administration in the monocytes, H89 administration in both monocytes and macrophages) was present. Differences in mRNA gene expression levels for CD14, B2-AR and BARR-2 were statistically evaluated using the Student's t-test. The level of significance was set at $p < 0.05$, and all tests were two-tailed.

III. Results

A. Monocytes

Dose and time effect of NE on LPS-induced IL-6 concentrations in activated THP-1 monocytes

To determine the effect of timing of NE administration on IL-6 production by LPS-mediated stimulation of THP-1 monocytes, NE was administered 1 hr prior to and 6 hrs into 24-hrs of LPS (1 ug/ml) treatment. The 24-hr LPS-induced IL-6 concentrations in cultured supernatants were similar for all experiments and were 70-80 pg/ml. The 6 hr time point into LPS administration was chosen because IL-6 concentrations were elevated (~20 pg/ml), but CD14 was not yet expressed at the mRNA or protein level, indicating that the cells were activated, but had not yet differentiated into the macrophage phenotype. Administration of NE **(Figure 2-1)** at both time points significantly inhibited the release of LPS-induced IL-6 protein concentrations in a dose-dependent fashion when compared to cells treated with LPS alone, (treatment, $F[5,24] = 245.7$, $p < 0.0001$; time, $F[1,24] = 541.5$, $p < 0.0001$). However, a significant treatment and time interaction, ($F[5, 24] = 30.04$, $p < 0.0001$), indicated that the extent of inhibition by NE on IL-6 protein concentrations differed between the two time points within each NE dose. Post hoc tests revealed that all doses of NE were significantly more inhibitory on IL-6 protein concentrations when given before LPS, compared to NE given 6 hours into 24 hour LPS treatment ($p < 0.001$).

Role of the A1- and B2-AR subtypes on the NE effects on LPS-induced IL-6 concentrations

To examine whether the effects of adrenergic stimulation on IL-6 production were mediated via the A1-AR versus the B2-AR, specific antagonists and agonists were administered prior to and after LPS-stimulation. Administration of the selective A1-AR antagonist, prazosin, given prior to NE at the 1 hr pre-LPS time point **(Figure 2-2A)** showed no effect on NE-mediated IL-6 inhibition (NE treatment, $F[5,24] = 251.7$, $p < 0.0001$; AR blocker, $F[1,24] = 0.2202$, $p = 0.6431$; interaction, $F[5, 24] = 1.518$, $p = 0.2214$). However, administration of the selective B2-AR antagonist, ICI 115,881, **(Figure 2-2B)**, significantly reversed the inhibition IL-6 by NE (NE treatment, $F[5,24] = 115.3$, $p < 0.0001$; AR blocker, $F[1,24] = 175.6$, $p < 0.0001$; interaction, $F[5, 24] = 18.28$, $p < 0.0001$). Similarly, administration of the B2-AR agonist, fenoterol, decreased LPS-induced IL-6 protein concentrations ($F[3, 11] = 193.2$, $p < 0.0001$), whereas the A1-AR agonist, methoxamine, did not have as significant of an effect ($F[3,11] = 4.6$, $p = 0.0375$) **(Figure 2-2C).**

Results for the 6 hr time point were similar to those of the 1 hr pretreatment time point. Administration of prazosin given before the 6 hr NE dose **(Figure 2-3A)** did not affect the inhibitory impact of NE (NE treatment, $F[5,24] = 10.62$, $p < 0.0001$; AR blocker, $F[1,24] = 0.1437$, $p = 0.7079$; interaction, $F[5, 24] = 0.3113$, $p = 0.9013$), whereas administration of ICI 118,551 **(Figure 2-3B)** reversed NE-induced inhibition on IL-6 protein concentrations (NE treatment, $F[5,24] = 7.2441$, $p < 0.0003$; AR blocker, $F[1,24] = 16.62$, $p < 0.0004$; interaction, $F[5, 24] = 2.979$, $p < 0.313$). Agonist effects showed that the inhibition on LPS-induced IL-6 concentrations at the 6 hr time point

(Figure 2-3C) were also B2-AR driven (methoxamine, $F[3,11] = 4.893$, $p = 0.0323$; fenoterol, $F[3, 11] = 48.02$, $p < 0.0001$).

Effect of cAMP-elevating agents on LPS-induced IL-6 levels in activated THP-1 monocytes

Since stimulation of the B2-AR leads to signaling involving elevations in cAMP, the timing of administration of cAMP-elevating agents were also examined, both prior to and after LPS stimulation. Compared to LPS alone, forskolin and Sp-cAMP, both cAMP-elevating agents, **(Figure 2-4)** significantly inhibited LPS-induced IL-6 protein concentrations ($F[2,12] = 758.2$, $p < 0.0001$) at the 1 hr pre-and 6 hrs into-LPS time points ($F[1,12] = 173.1$, $p < 0.0001$). However, the treatment effect differed between the two time points, ($F[2,12] = 43.38$, $p < 0.0001$), such that forskolin and Sp-cAMP were both significantly more inhibitory on IL-6 protein if given 1 hr before LPS-induced cellular activation ($p < 0.001$).

Effect of LPS-induced cellular activation on B2-AR mRNA expression and the effect of PKA inhibition on mRNA expression and cytokine release in THP-1 monocytes

B2-AR mRNA was measured after LPS treatment to determine if the change in NE sensitivity after cellular activation was due to a change in receptor expression. Compared to vehicle, LPS-induced activation of THP-1 monocytic cells showed a

significant decrease ($t = 9.676$, $df = 4$, $p = 0.0006$) in B2-AR mRNA expression as detected by RT-PCR **(Figure 2-5)**.

Given that activated PKA can downregulate the B2-AR, we dosed cells with the PKA inhibitor, H89, and measured B2-AR mRNA expression. Administration of H89 significantly reversed the LPS-induced downregulation of B2-AR mRNA expression ($F[2,8] = 63.02$, $p < 0.0001$), as detected by RT-PCR **(Figure 2-6A)**. Administration of H89 prior to LPS treatment was also shown to significantly enhance the inhibitory effects of NE given 6 hrs into LPS treatment **(Figure 2-6B)**, when compared to the effects of NE alone, 6 hrs into LPS treatment ($F[2,8] = 125.7$, $p < 0.0001$). H89 alone had no effect on B2-AR mRNA expression or IL-6 protein concentrations (data not shown).

B. Macrophages

Dose effect of NE on LPS-induced IL-6 protein concentrations and mRNA in PMA-differentiated THP-1 cells

First, using PMA**,** THP-1 monocytes were differentiated into macrophages, as reflected by a significant increase ($t = 6.840$, $df = 4$, $p = 0.0024$) in CD14 mRNA gene expression **(Figure 2-7)**.

To determine the timing effect of NE administration in relation to macrophage activation, NE was administered 1 hr prior to and 6 hrs into a 24-hr LPS treatment of PMA-differentiated THP-1 cells. The 24-hr LPS-induced IL-6 values were similar at both time points, reaching 7000-8000 pg/ml. Administration of NE **(Figure 2-8)**, did not affect

the release of LPS-induced IL-6 protein in THP-1 macrophages when compared to LPS alone, at either time point of administration (treatment, $F[3,16] = 2.863$, $p = 0.0695$; time, $F[1,16] = 0.1301$, $p = 0.7230$; interaction, $F[3,16] = 0.01904$, $p = 0.9963$).

To determine whether the absence of NE's inhibitory effects on IL-6 protein concentrations in PMA-differentiated THP-1 cells was due to excessive stimulation by LPS, cells in their monocytic and macrophage forms were matched for LPS-induced IL-6 stimulation by varying the dose of LPS used for activation**.** Dosing of monocytes with 5 ug/ml of LPS, and macrophages with 0.1 ng/ml of LPS led to matching of LPS-induced IL-6 protein concentrations in THP-1 monocytes and PMA-differentiated cells **(Figure 2-9)** showed a significant inhibition of IL-6 protein by NE when given prior to the LPS stimulus ($F[1,8] = 155.3$, $p < 0.0001$), in both cell types ($F[1,8] = 149.8$, $p < 0.0001$). However, a significant treatment by cell type interaction ($F[1,8] = 346.0$, $p < 0.0001$), indicated that the extent of inhibition by NE on IL-6 protein concentrations differed between the two cell forms (monocyte versus macrophage). Post hoc tests revealed that NE was significantly more inhibitory on IL-6 concentrations in monocytes compared to PMA-differentiated macrophages, when given before a matched LPS-induced activation of IL-6 ($p < 0.001$).

To determine if NE administration was only affecting de novo synthesis of IL-6 protein, we also measured the expression of IL-6 mRNA. Stimulation by the same LPS concentrations used to activate matching levels of IL-6 protein in THP-1 monocytes and macrophages also showed a difference in the inhibitory effect of NE on IL-6 mRNA gene expression levels **(Figure 2-10)**. NE significantly inhibited IL-6 mRNA expression when given prior to the LPS stimulus ($F[1,8] = 65.94$, $p < 0.0001$) in both cell types ($F[1,8] =$

13.35, p = 0.0065), however a significant interaction effect (F[1,8] = 12.37, p = 0.0079) indicated that the inhibition by NE on IL-6 mRNA gene expression differed among the cell forms with NE inhibiting IL-6 mRNA in monocytes to a significantly greater extent than in PMA-differentiated THP-1 cells ($p < 0.01$).

Effect of PMA-induced differentiation on B2-AR and BARR-2 mRNA expression in THP-1 cells

To determine the mechanism by which monocyte differentiation to the macrophage phenotype leads to reduced sensitivity to NE**,** mRNA expression for the B2-AR, as well as BARR-2, was examined.. Compared to vehicle, PMA-induced differentiation of THP-1 cells into macrophages showed a significant increase (t = 17.32, df = 4, $p < 0.0001$) in B2-AR mRNA gene expression **(Figure 2-11A)**, and a significant increase (t = 12.61, df = 4, p = 0.0002) in BARR-2 mRNA gene expression **(Figure 2-11B)**, as detected by RT-PCR.

Dose and Time effect of NE and the role of the A1- and B2-AR subtypes on lower dosed, LPS-induced IL-6 concentrations in PMA-differentiated cells

To determine the AR subtype responsible for the effects of NE on LPS-induced IL-6 release without excessive stimulation in PMA-differentiated macrophages, cells were dosed with NE, or respective AR antagonists and agonists, prior to and 6 hrs into LPS treatment. Administration of NE given to PMA-differentiated cells induced by the

lower LPS concentration showed a significant decrease in IL-6 protein concentrations ($F[3,16] = 22.40$, $p < 0.0001$), which differed among the pre- and post-LPS administration time points ($F[1,16] = 19.53$, $p = 0.0004$), with NE being significantly inhibitory only when given 1 hr prior to an LPS stimulation, as compared to 6 hrs into LPS treatment ($F[3,16] = 8.543$, $p = 0.0013$). The inhibitory nature of NE on IL-6 at the pre-LPS time point was reversed by the B2-AR antagonist, ICI, 118,551 ($p < 0.001$), but not by the A1-AR antagonist, prazosin **(Figure 2-12).** Similarly, administration of the B2-AR agonist, fenoterol, significantly inhibited LPS-induced IL-6 levels, but only when given 1 hr prior to LPS treatment. Administration of the A1-AR agonist, methoxamine, had no effect (agonist treatment, $F[2,12] = 18.65$, $p = 0.0002$; time, $F[1,12] = 6.311$, $p = 0.0271$; interaction, $F[2,12] = 5.239$, $p = 0.0231$) **(Figure 2-13)**.

Effect of a cAMP-elevating agent on LPS-induced IL-6 levels in activated PMA-differentiated THP-1 cells

To determine the role of cAMP on LPS-induced IL-6 levels in macrophages, cells were dosed with forskolin prior to, and after, cellular activation. Compared to LPS alone, forskolin **(Figure 2-14)** significantly inhibited IL-6 protein concentrations (treatment, $F[1,8] = 19.30$, $p = 0.0023$; time, $F[1,8] = 7.369$, $p = 0.0265$). However, the treatment effect differed between the two time points, ($F[1,8] = 7.369$, $p = 0.0265$). Forskolin significantly inhibited IL-6 protein only when given before LPS-induced cellular activation ($p < 0.001$), and not when given 6 hrs into LPS treatment, as determined by post-hoc analysis.

Effect of LPS-induced cellular activation on B2-AR mRNA expression and the effect of PKA inhibition on mRNA expression and cytokine release in PMA-differentiated THP-1 cells

To assess the effect of cell activation on receptor expression in macrophages, mRNA for the B2-AR was measured. Compared to PMA alone, LPS-treated PMA-differentiated THP-1 cells showed a significant decrease ($t = 10.06$, $df = 4$, $p = 0.0005$) in B2-AR mRNA expression as detected by RT-PCR **(Figure 2-15)**.

To further characterize the role of B2-AR signaling and cAMP elevation on IL-6 production in the macrophages, cells were dosed with the PKA inhibitor, H89, and B2-AR mRNA expression and NE-mediated IL-6 production was measured. Administration of the PKA inhibitor, H89, to PMA-differentiated THP-1 cells did not affect the LPS-induced downregulation of B2-AR mRNA expression ($F[2,8] = 26.09$, $p = 0.0011$), as detected by RT-PCR **(Figure 2-16A)**. Furthermore, administration of H89 prior to LPS-induced activation of PMA-differentiated THP-1 cells did not affect the inhibitory effect of NE given 6 hrs into LPS treatment **(Figure 2-16B)**, when compared to the effects of NE alone given 6 hrs into LPS treatment ($F[2,8] = 0.8772$, $p = 0.4633$). H89 given alone had no effect on B2-AR mRNA expression or IL-6 protein concentrations (data not shown).

Effect of LPS-induced cellular activation on BARR-2 mRNA expression in PMA-differentiated THP-1 cells

To determine the effect of cell activation on BARR-2 expression in the differentiated cells, mRNA expression levels of the protein were measured following a 6 hr LPS dose. Compared to PMA alone, LPS-induced activation of PMA-differentiated THP-1 cells showed a significant decrease ($t = 7.709$, $df = 4$, $p = 0.0015$) in BARR-2 mRNA as detected by RT-PCR **(Figure 2-17)**.

IV. Discussion

Results from this study show that NE, the main SNS neurotransmitter, exerts an inhibitory effect on LPS-induced IL-6 levels in THP-1 cells. Although other studies have also provided evidence for NE's inhibitory effect on inflammation (Van der Poll, et al., 1994; Rontgen et al., 2004), some studies have shown that NE can also act in a stimulatory manner (Bierhaus et al., 2003; Tan et al., 2006; Yang et al., 2009). However, these studies were done in the absence of any inflammatory stimuli. This suggests that the inflammatory activation state of the cell may have an effect on the inhibitory nature of NE and other AR agonists. The present study demonstrates that the inhibitory nature of NE on LPS-induced IL-6 production in monocytes may depend on the activation state of the cell, suggesting a possible reason for the contradicting literature. Monocytes have the capacity to become activated by LPS (Adams, 1994; Gordon, 2003; Osterud and Bjorklid, 2003; Geissman et al, 2010), which allows the cell to become more reactive to stimuli and enhances its release of immune factors, such as the proinflammatory cytokine, IL-6 (Ma et al., 2003; Osterud and Bjorklid, 2003; Fujiwara and Kobayashi, 2005). Administration of NE in THP-1 monocytes given prior to LPS-induced cellular

activation was shown to inhibit the release of IL-6 protein, indicating an anti-inflammatory role for NE. This data supports numerous studies that also show NE's inhibitory effect on LPS-induced inflammatory markers such as TNF-alpha and IL-6 (Hu et al., 1991; Van der Poll, et al., 1994; Rontgen et al., 2004). However, our data indicates that the extent of this inhibitory role seems to depend on the timing of NE administration in relation to cellular activation. When NE was administered after monocyte activation, it was less capable of inhibiting IL-6 release, with the amount of NE-modulated inhibition dropping from 80% to approximately 20% of LPS control, suggesting that the cell was less sensitive to the inhibitory or anti-inflammatory role of NE. This change in the sensitivity of monocytes to NE's anti-inflammatory capacity may explain some of the contradictory literature that shows that NE can also induce inflammation (Elenkov et al., 2000).

Immune cells have been shown to contain both A1 (Kavelaars, 2002) and B2 (Bellinger et al., 2008) adrenergic receptors, which could mediate the effects of NE. To determine the adrenergic receptors involved in NE's inhibition of IL-6, specific antagonists and agonists were used. Using the selective B2-AR antagonist, ICI 115,881, prior to NE administration, the inhibition on IL-6 was reversed, whereas the selective A1-AR antagonist, prazosin, showed no effect on IL-6 inhibition. These results were replicated in cells exposed to NE after LPS-mediated activation. Similarly, administration of the B2-AR agonist, fenoterol, decreased LPS-induced IL-6 in the same manner as NE, whereas the A1-AR agonist, methoxamine, showed no effect. Thus, the inhibition of IL-6 described in the monocytes, was mediated by the B2-AR and not the A1-AR. The inhibitory role of B2-AR activation on LPS-induced inflammatory mediators has been

shown in many studies (Sekut et al., 1995; Izeboud et al., 1999a; Szelenyi et al., 2006; Wang et al., 2009; Donnelly et al., 2010)

The B2-AR is a GPCR that is typically Gs-coupled and classically signals through the cAMP-PKA pathway. The inhibitory effects of NE have been shown to be a result of cAMP accumulation. For example, activation of the cAMP-mediated pathway by B2-AR stimulation has been shown to mediate the anti-inflammatory effects of the receptor on TNF-alpha in THP-1 cells (Farmer and Pugin, 2000). We thus wanted to determine if the change in sensitivity of NE's inhibitory effects due to LPS stimulation could be mimicked with cAMP-elevating agents. In accordance with these studies, we showed that forskolin and Sp-cAMP, both cAMP-elevating agents, inhibited LPS-induced IL-6 release in monocytes, in the same manner as NE. Specifically, both agents greatly inhibited IL-6 when given before the LPS-induced activation, but this inhibition was greatly reduced when they were given after LPS-induced activation. Thus, the change in the cells' sensitivity to NE due to cellular activation appears to be B2-AR and cAMP-dependent.

Since the inhibitory role of NE is cAMP and B2-AR dependent, then one mechanism that may explain the cells' shift in sensitivity to NE's inhibitory ability after LPS-induced activation may involve a change in the B2-AR's ability to signal. The B2-AR is commonly known to undergo receptor desensitization and downregulation with continued agonist exposure (Lefkowitz, 1993; Ferguson et al., 1996). Desensitization and downregulation of the B2-AR occurs in order to prevent overstimulation of signaling, and downregulation of the B2-AR is thought to be the additive endpoint of receptor desensitization (Bohm et al., 1997; Liggett, 2002). Indeed, administration of salmeterol, a

long acting beta-AR agonist, in rats reduced B2-AR density in the lungs and impaired cAMP signaling (Finney et al., 2001). Inflammation itself may also down regulate the receptor, as decreased density of the B2-AR was shown in the immune cells of patients with rheumatoid arthritis, a chronic inflammatory disease (Baerwald et al., 1992), which was later shown to reduce the inhibitory effects of NE on cell proliferation (Baerwald et al., 1999). Interestingly, our results show that activation of the THP-1 monocyte with LPS alone caused a reduction of B2-AR mRNA expression at six hours, indicating downregulation of the receptor. The timing of this reduction in B2-AR mRNA by LPS corresponded to the decrease in NE's inhibitory action on IL-6 protein levels in the cells as well, since giving NE six hours after the LPS stimulus was found to inhibit IL-6 by only 20% compared to the 80% seen when NE was given before LPS. Thus, the lack of inhibition seen after activation may be a result of decreased B2-AR mRNA expression, and thus decreased signaling by NE, in these cells, once they are activated by LPS.

A variety of mechanisms exist mediating B2-AR desensitization (Lohse et al., 1996; Bohm et al., 1997; Wallukat, 2002). PKA is one of the most recognized kinases involved in receptor desensitization and downregulation. Although G-protein receptor kinases (GRKs) also play a major role in this process, these two kinases desensitize the receptor in a different manner relevant to this study. While GRKs only phosphorylate ligand-bound receptors and require beta-arrestins as their accessory proteins in this process, PKA can phosphorylate and impair the receptor directly, in the absence of ligand, a mechanism termed heterologous desensitization, and without the need of an accessory protein (Lefkowitz et al., 1992; Hein and Kobilka, 1995; Bohm et al., 1997; Kohout and Lefkowitz, 2003). Direct phosphorylation of the receptor by PKA can occur

by any mechanism that increases cAMP and activate PKA (Liggett, 1999), which then leads to mRNA downregulation (Bouvier et al., 1989) and our data has shown that the change in sensitivity to NE's inhibitory nature on IL-6 is cAMP-dependent. Furthermore, in regards to the LPS-induced downregulation of the B2-AR, it is likely to be following a PKA-dependent heterologous desensitizing mechanism, since no agonist, in this case, NE, is present. We indeed showed that by dosing the cells with H89, a PKA inhibitor, before LPS-induced activation, the downregulation of B2-AR mRNA expression was reversed. Furthermore, subjecting the cells to H89 prior to LPS-activation, followed by administration of NE at the 6 hr post-LPS time point, returned NE's inhibitory actions on IL-6 protein levels, indicating that the reversal of LPS-induced B2-AR downregulation by H89 functionally returned NE's capacity to act in an anti-inflammatory fashion when administered after LPS-induced activation. Thus, LPS itself may be stimulating PKA and causing heterologous desensitization, leading to receptor downregulation. The mechanism by which LPS is stimulating PKA, however, still remains to be elucidated.

Since inflammatory-induced activation of monocytes changed the cell's sensitivity to NE's anti-inflammatory actions, we next tested to see if monocyte to macrophage differentiation also affected NE's ability to inhibit LPS-stimulated IL-6 levels. The human monocytic THP-1 cell line (Tsuchiya et al., 1980) has been employed in numerous studies as a useful model system to study monocyte-to-macrophage differentiation (Auwerx, 1991). This particular cell line can be differentiated into macrophage-like cells, which mimic native monocyte-derived macrophages in several aspects, such as morphology, behavior, and expression of macrophage-specific genes (Auwerx, 1991). Thus, the use of this cell line allowed for easy comparison between

monocytes and macrophages in terms of NE-mediated inflammatory outcomes. THP-1 cells were differentiated into macrophages using PMA, and expression of CD14 mRNA validated that the cells had successfully transformed (Tsuchiya et al., 1982; Martin et al., 1994).

Administration of NE in PMA-differentiated THP-1 cells prior to LPS stimulation showed no effect on the release of IL-6. However, once these cells were differentiated, they became more responsive to LPS stimulation (Martin et al., 1994) and the relative amount of IL-6 released from LPS stimulation was much higher than that seen in the non-activated cells. Thus, the absence of an inhibitory effect of NE could have been due to the increased stimulation of these cells by LPS, causing a ceiling effect on IL-6 protein levels. Indeed, when the concentration of LPS used to stimulate the PMA-differentiated cells was decreased, NE regained some inhibitory function on IL-6 release, when given prior to cellular activation. However, the degree of NE-mediated IL-6 inhibition was still much weaker than that seen in the monocytic form of the cell. Untreated THP-1 monocytes were then activated with a higher concentration of LPS, in order to match the amount of IL-6 released from the PMA-differentiated cells activated by the lower dose of LPS, to determine if the magnitude of LPS-induced activation was the reason NE was less inhibitory. Although LPS-stimulated IL-6 levels were matched in the two cell forms, NE inhibited IL-6 to a significantly lesser extent in the macrophages (30% inhibition) compared to the monocytes (80% inhibition).

Since macrophages are primed to release more proinflammatory proteins than their monocytic counterparts, it is possible that NE was only inhibiting de novo synthesis of IL-6 in the PMA-differentiated cells, and that is why there was less inhibition at the

protein level compared to the inactivated monocytes. We thus measured LPS-induced IL-6 mRNA expression in the two cell types after NE administration. IL-6 mRNA expression, however, recapitulated the pattern seen with IL-6 protein levels in the two forms of the cell, where NE administration one hour prior to the LPS stimulation greatly inhibits IL-6 mRNA in the monocytes, and does so to a lesser extent in the macrophages. Thus, it seems that the macrophage form of these cells are less sensitive to the inhibitory effects of NE on IL-6, at both the protein and mRNA level, compared to the monocytic form. Some studies have suggested a reduced number of B2-ARs in macrophages compared to monocytes (Radojcic et al.,1991; Barnes, 1999; Sitkauskience and Sakalauskas, 2005). However, we found that mRNA expression for the B2-AR was increased in the PMA-differentiated cells compared to the undifferentiated cells, suggesting that the differentiated cells should be more sensitive to NE than their monocytic counterparts. As mentioned previously, the B2-AR can undergo different forms of desensitization, other than downregulation. Homologous desensitization of the receptor involves phosphorylation by GRK's, with beta-arrestins acting as necessary accessory proteins (Lutrell and Lefkowitz, 2002), when the receptor is bound by agonist. BARR-2 is a main beta-arrestin involved in dampening of B2-AR signaling (Lefkowitz and Whalen, 2004), and interestingly, we show that BARR-2 mRNA expression also markedly increased with PMA-induced differentiation. When NE is being administered prior to the LPS-induced stimulation of IL-6 in these cells, then the B2-AR is bound by the agonist before any IL-6 is being released, and the receptor is susceptible to homologous desensitization. The increase in B-ARR-2 mRNA expression due to differentiation may thus dampen the receptor's signaling ability after NE administration,

explaining the decrease in NE's ability to inhibit LPS-induced IL-6 levels as compared to the monocytes, even though B2-AR mRNA is also increased.

Interestingly, once the PMA-differentiated cells were activated with LPS with a decreased concentration in order to avoid overstimulation of IL-6, the cells were not only less sensitive to NE's inhibitory effects when given prior to LPS-induced activation compared to the monocytes, but the inhibition on IL-6 by NE was completely lost if NE was administered 6 hours after LPS activation. The NE-mediated inhibition on IL-6 prior to activation was still B2-AR driven, as ICI 118,551 reversed the inhibition and prazosin did not. Furthermore, administration of fenoterol also showed IL-6 inhibition if given prior to LPS, although not when given after activation, and methoxamine had no effect at either time point. Since the slight inhibition in the differentiated cells was still B2-AR driven and likely signaling through cAMP, forskolin was also administered to the cells. Again, forskolin was able to inhibit IL-6, to a lesser extent than in the monocytes, but only when given prior to LPS-induced activation. When administered after LPS activation, it did not have any effect on IL-6 levels, following the same pattern as NE. Thus, LPS-induced activation of PMA-differentiated THP-1 cells completely abolishes any inhibitory ability of NE on the release of IL-6 in these cells.

Like its monocytic counterparts, PMA-differentiated THP-1 macrophages showed decreased B2-AR mRNA expression after 6 hours of LPS-induced activation, suggesting downregulation of the receptor and potentially explaining why NE loses its inhibitory effect on IL-6 if given after activation. However, unlike the THP-1 monocyte form of the cell, PKA inhibition by H89 did not reverse the LPS-induced downregulation of the B2-AR, nor did it restore any of NE's inhibition of IL-6 when administered post-activation.

Thus, another mechanism may be mediating the loss of NE's anti-inflammatory properties upon activation of the macrophages. Aside from its role in receptor desensitization, BARR-2 can interact with nuclear factor kappa-B (NFkB) signaling, a transcription factor involved in LPS-mediated inflammatory signaling and the release of proinflammatory cytokines, such as IL-6 (Gao et al., 2004). Specifically, it was shown to interact and stabilize cytosolic IkappaB, an inhibitor of NFkB, which leads to an increased inhibition of NFkB and decreased inflammatory signaling. These findings further support Wang et al.'s (2009) study that B2-ARs and BARR-2 act together to inhibit LPS-induced inflammation. Thus, BARR-2 can act in an anti-inflammatory manner on its own, along with activation of the B2-AR.

Interestingly, our results show that LPS activation in the PMA-differentiated THP-1 macrophages also reduced BARR-2 mRNA expression, along with B2-AR mRNA expression. Our results are in accordance with a study by Kizaki et al. (2008) showing that stimulation by LPS in a mouse macrophage cell line, RAW256, resulted in downregulation of B2-AR protein and mRNA levels, and preventing this downregulation resulted in inhibition of LPS-stimulated NFkB activation. They also found that LPS stimulation reduced BARR-2 mRNA levels in the mouse macrophage cells. A decrease in BARR-2 mRNA was shown to decrease its ability to stablize IkappaB, which led to increased activation of NFkB. The authors conclude that this may result in a mechanism for escaping the anti-inflammatory signaling effects of B2-AR. Our results showing decreased LPS-induced BARR-2 mRNA expression may also explain why PMA-differentiated THP-1 macrophages due not respond to NE's anti-inflammatory effects on IL-6 after LPS activation, since less BARR-2 may cause less inhibition of NFkB, which

could also translate to less inhibition of IL-6 release, thus abolishing NE's inhibition on IL-6 after LPS-induced activation.

In summary, the present study suggests that the catecholamine responsiveness of monocytes and macrophages may be dependent on both the state of inflammatory activation and the state of differentiation of the cells, potentially explaining paradoxical stimulatory and inhibitory effects of catecholamine's on inflammatory responses. Both inflammatory activation and differentiation seem to cause cells to become less sensitive to the inhibitory effects of NE and cAMP-elevating agents. Specifically, differentiated macrophages are less sensitive to the inhibitory actions of NE on LPS-induced IL-6 release, compared to monocytes, both at the transcriptional and translational levels. The mechanism involving the difference in sensitivities between undifferentiated and differentiated cells remains to be elucidated, but may involve homologous receptor desensitization by BARR-2. In addition, NE administration after inflammatory-mediated activation of either monocytes or macrophages causes the cell to become less sensitive to NE's inhibitory actions on IL-6 release. Although LPS-induced cellular activation leads to downregulation of the B2-AR in both cell types, potentially explaining the decrease in NE's inhibitory action, the mechanism leading to this downregulation seems to differ between the two cell states. Downregulation of B2-AR, and decreased sensitivity to NE after activation in the monocytes seems to be PKA-mediated and may involve heterologous desensitization of the B2-AR. On the other hand, receptor downregulation and decreased NE inhibition in PMA-differentiated macrophages may involve the joint interaction of downregulation in BARR-2, along with the decrease in the B2-AR. Given the contradicting nature of the literature involving SNS activation and inflammation, it is

important to further elucidate the role of different states of immune cell activation and maturation, and how they may affect the signaling capacity and signaling mechanisms of SNS mediators, such as NE, providing possible mechanisms mediating these changes.

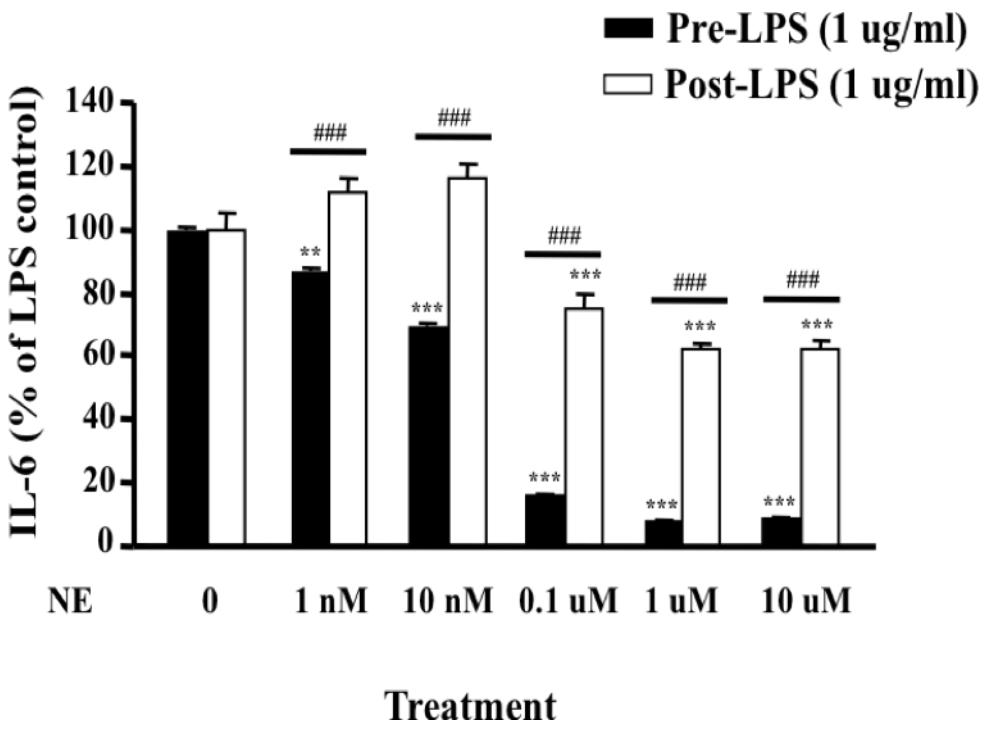

Figure 2-1. LPS-induced stimulation of IL-6 protein levels following NE administration in THP-1 human monocytic cells. THP-1 cells were exposed to varying doses of NE either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls).

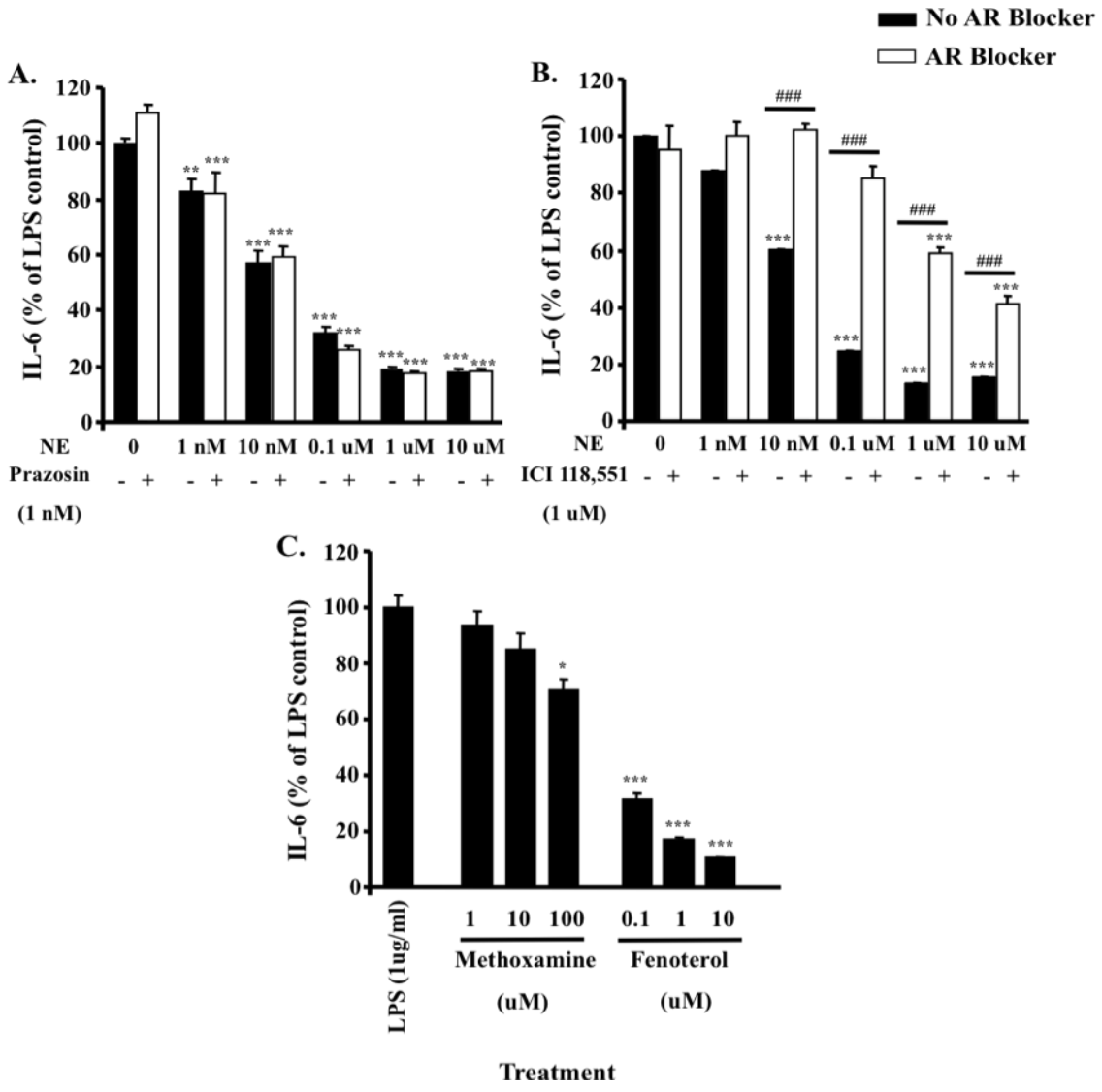

Figure 2-2. LPS-induced stimulation of IL-6 protein levels following NE administered prior to LPS, with and without the alpha-AR antagonist, prazosin (A), or the B2-AR antagonist, ICI 118,551 (B), and LPS-induced IL-6 levels after administration of the alpha-AR agonist, methoxamine, or the B2-AR agonist, fenoterol (C). THP-1 cells were dosed with respective antagonists for 45 minutes, followed by administration of NE (A-B), or with respective agonists alone (C), for 1 hr prior to a 24-hr LPS stimulation. Data is represented as % of LPS alone and presented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – indicate significant treatment effects compared to LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represent significant differences within exposure to antagonist (Newman-Keuls).

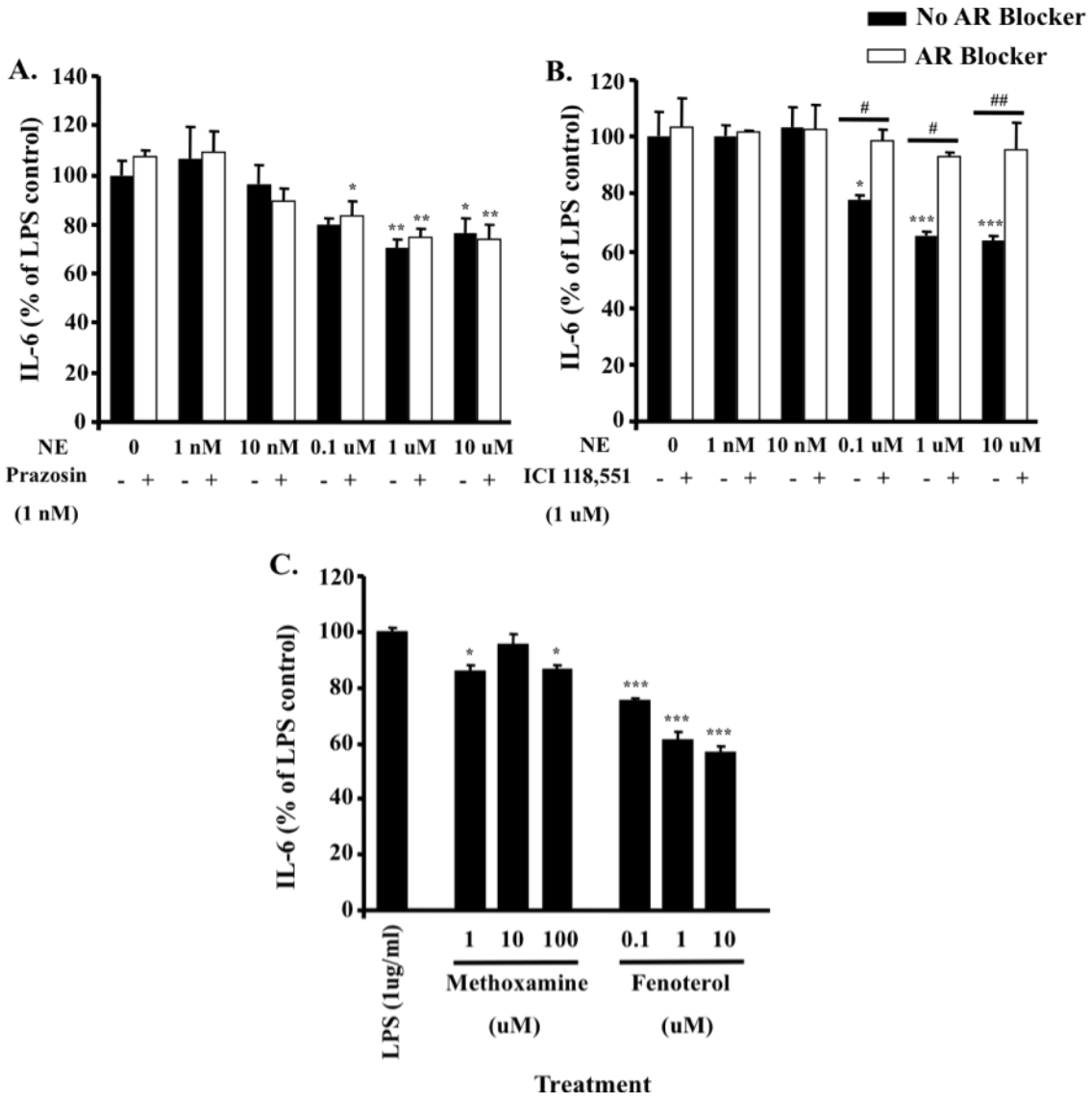

Figure 2-3. LPS-induced stimulation of IL-6 protein levels following NE administered after LPS, with and without the alpha-AR antagonist, prazosin (A), or the B2-AR antagonist, ICI 118,551 (B), and LPS-induced IL-6 levels after administration of the alpha-AR agonist, methoxamine, or the B2-AR agonist, fenoterol (C). THP-1 cells were dosed with respective antagonists for 45 minutes, followed by administration of NE (A-B), or with respective agonists alone (C), 6 hrs after a 24-hr LPS stimulation. Data is represented as % of LPS alone and presented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – indicate significant treatment effects compared to LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represent significant differences within exposure to antagonist (Newman-Keuls).

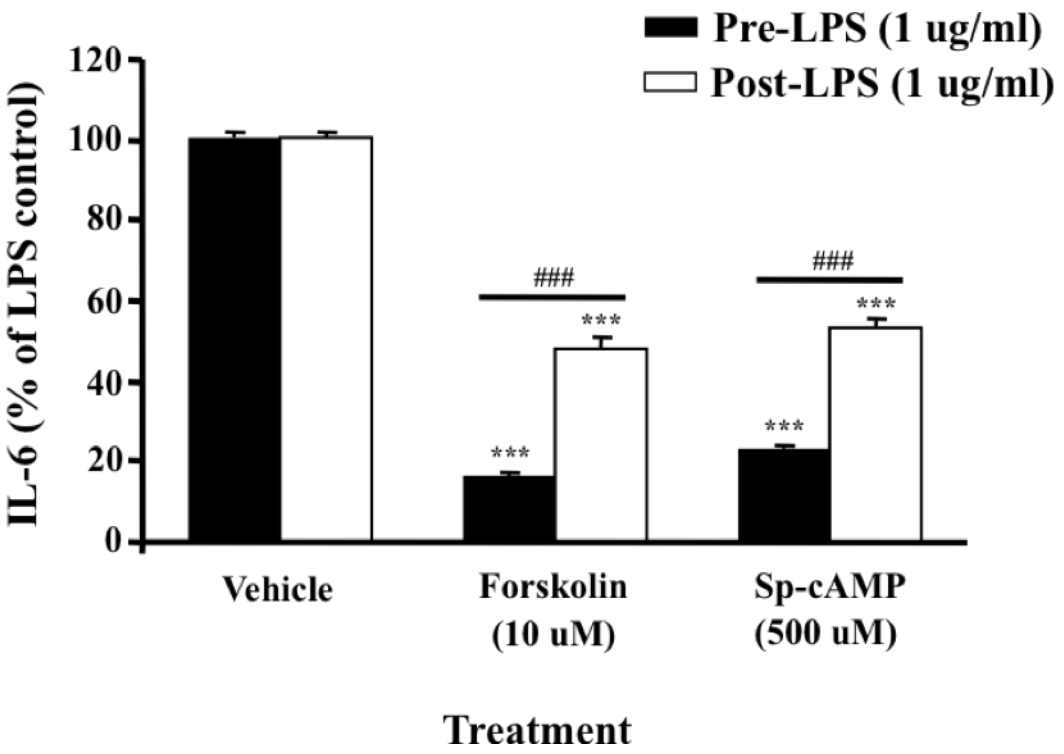

Figure 2-4. LPS-induced stimulation of IL-6 protein levels following administration of cAMP-elevating agents in THP-1 monocytic cells. THP-1 cells were exposed to forskolin or Sp-cAMP, either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls).

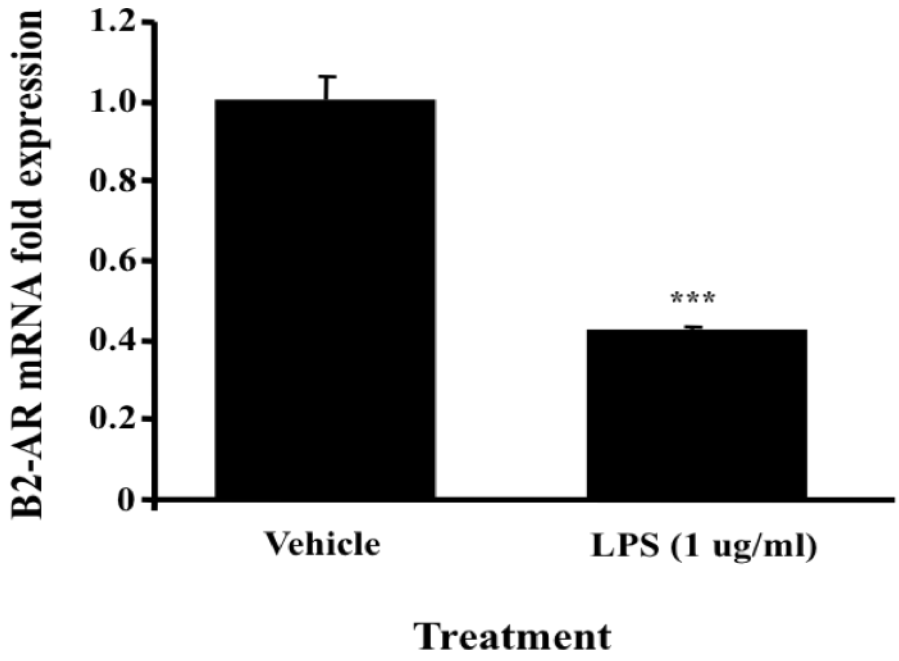

Figure 2-5. B2-AR mRNA expression in LPS-stimulated THP-1 monocytic cells. THP-1 cells were exposed to media alone (vehicle) or to a 6-hr LPS stimulation, followed by measurement of B2-AR gene expression. Data is represented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly lower than vehicle control.

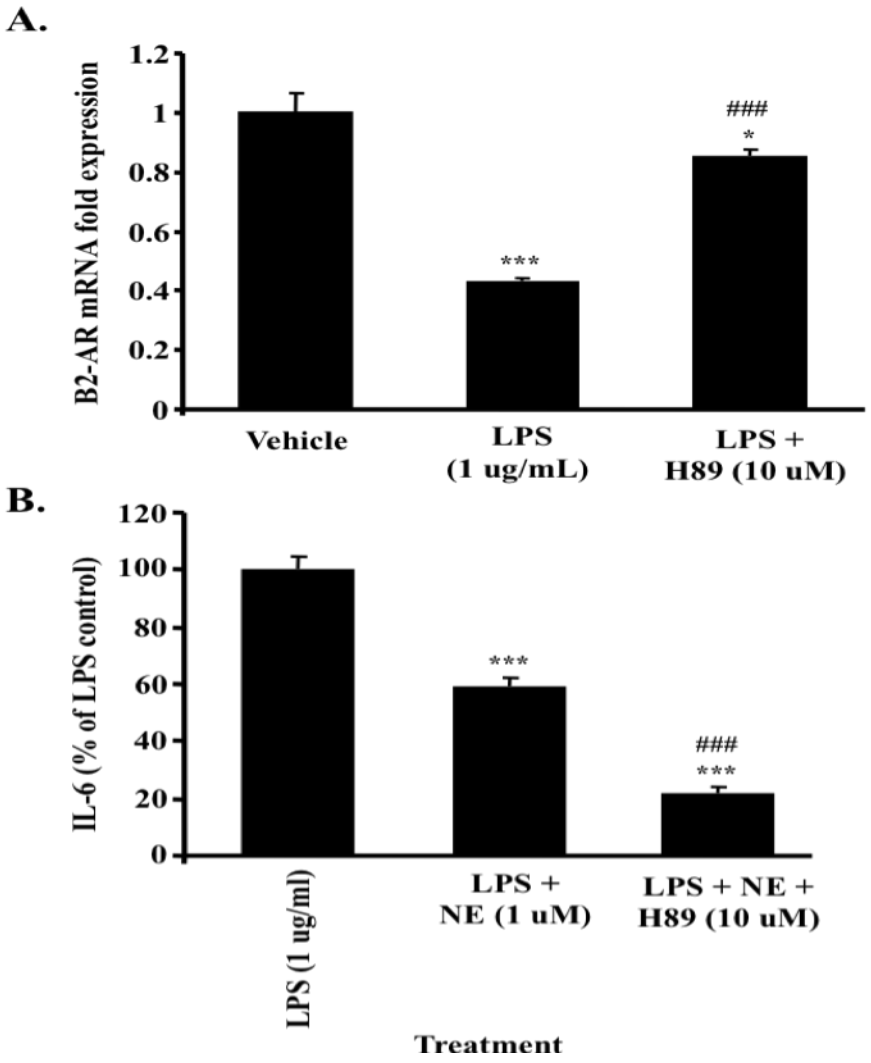

Figure 2-6. Effect of PKA inhibition on B2-AR mRNA expression in LPS-stimulated THP-1 monocytic cells (A) and on LPS-induced IL-6 protein levels following NE administration (B). THP-1 cells were dosed with the PKA inhibitor, H89, for 1 hr prior to a 6 hr LPS stimulation and collected for B2-AR mRNA measurement (A). THP-1 cells were also dosed with H89 for 1 hr, followed by NE, 6 hrs into a 24-hr LPS stimulation, and cell supernatant was collected and analyzed for IL-6 protein levels (B). Data is presented as the mean (+/- SEM) and is represented as % of LPS alone for IL-6 levels (B). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle (A) or LPS (B) control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between PKA inhibited and non-inhibited treatments (Newman-Keuls).

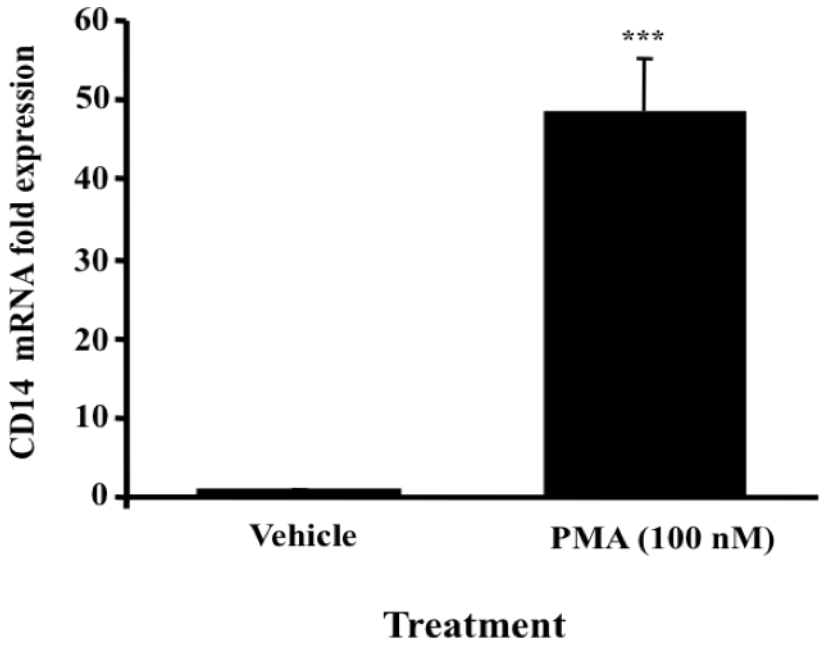

Figure 2-7. CD14 mRNA expression in PMA-differentiated THP-1 cells. THP-1 cells were exposed to PMA for 48 hrs and measured for CD14 mRNA expression to determine differentiation into macrophages. Data is represented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle control.

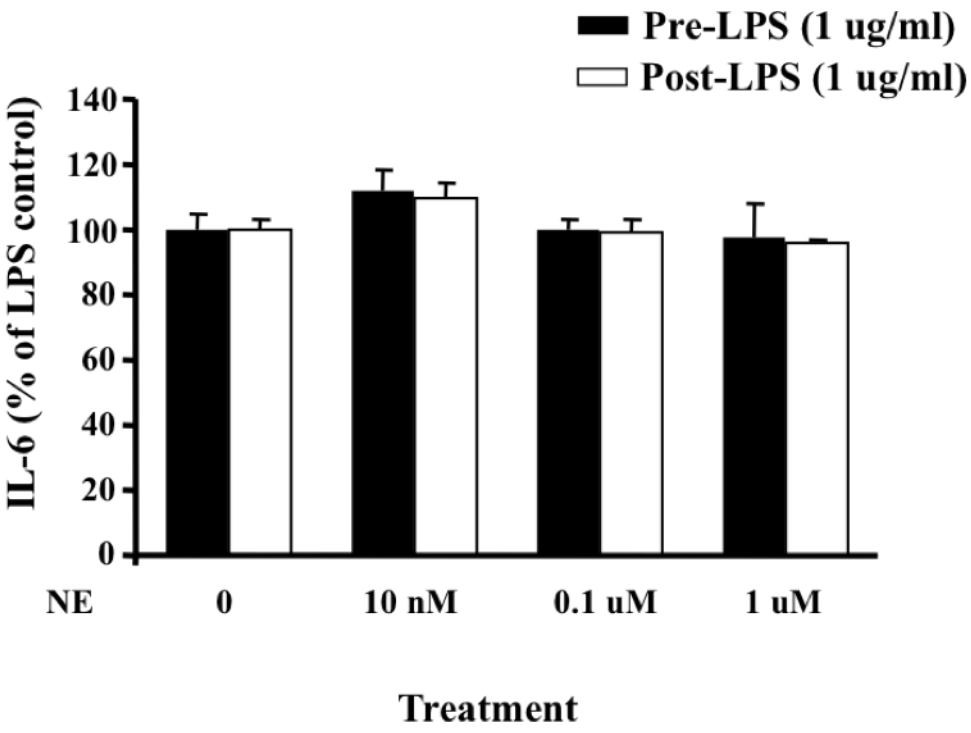

Figure 2-8. LPS-induced stimulation of IL-6 protein levels following NE administration in PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, followed by exposure to varying doses of NE either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls).

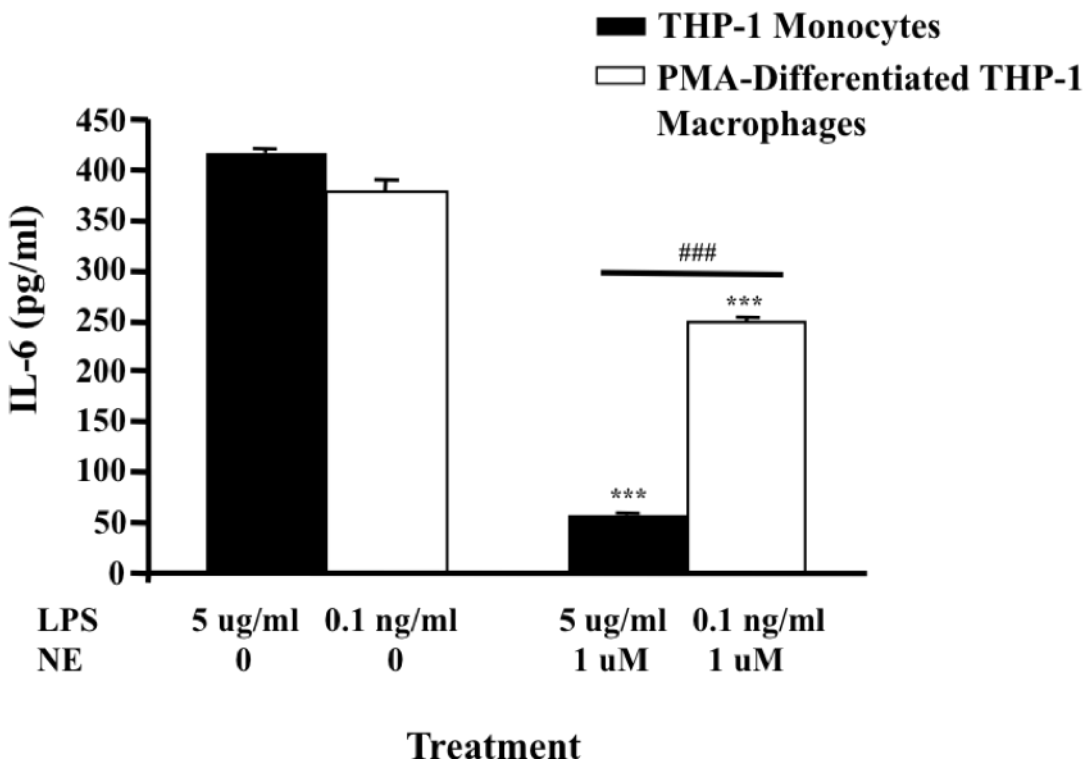

Figure 2-9. LPS-induced stimulation of IL-6 protein levels following NE administration in monocytic and PMA-differentiated THP-1 cells. THP-1 cells were either left untreated, or pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, and exposed to NE for 1 hr prior to a 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is presented as the mean (+/- SEM) for each cell type, and significance levels were determined using % of LPS control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than the respective LPS alone treatment (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the two cell forms among the same treatment (Newman-Keuls).

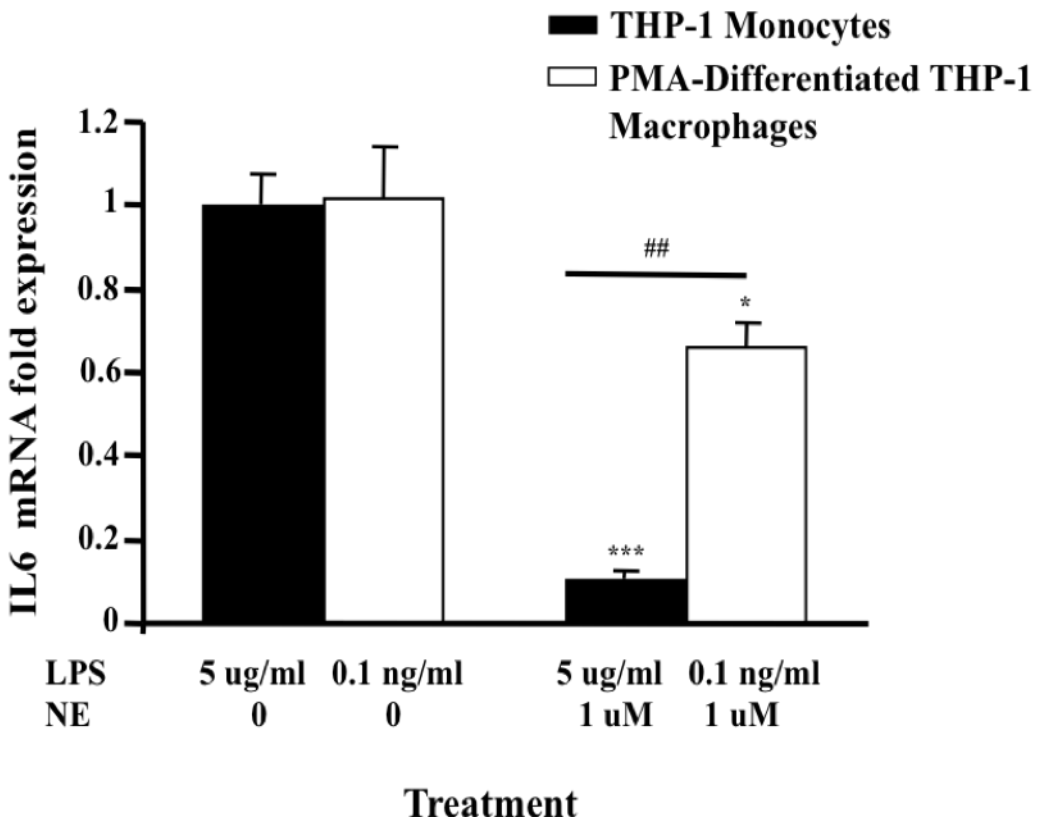

Figure 2-10. IL-6 mRNA expression in LPS-stimulated monocytic and PMA-differentiated THP-1 cells following NE administration. THP-1 cells were either left untreated, or pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, and exposed to NE for 1 hr prior to a 6 hr LPS stimulation and measured for IL-6 mRNA expression. Data is presented as the mean (+/- SEM) for each cell type. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than the respective LPS alone treatment (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the two cell forms among the same treatment (Newman-Keuls).

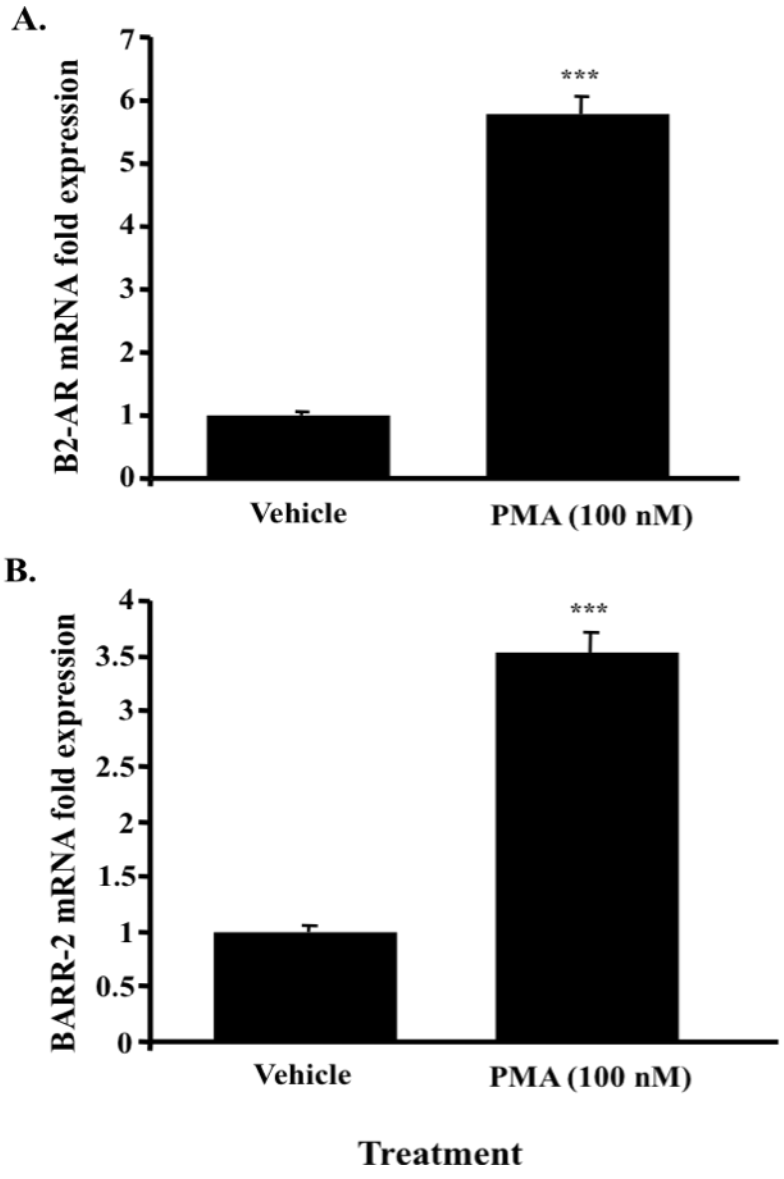

Figure 2-11. B2-AR mRNA expression (A) and BARR-2 mRNA expression (B) in PMA-differentiated THP-1 cells. THP-1 cells were either left untreated (vehicle control) or treated with PMA for 48 hrs to induce differentiation into macrophages. After 24 hrs of rest, cells were collected for measurement of B2-AR (A) and BARR-2 (B) mRNA expression. Data is represented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle control (Newman-Keuls).

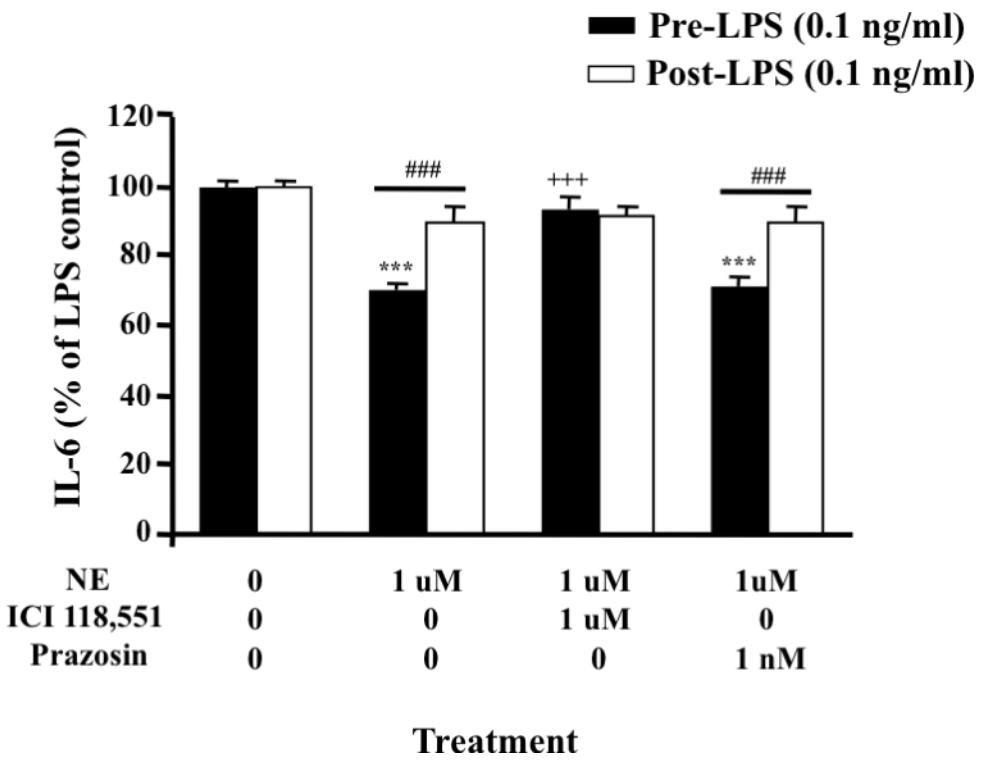

Figure 2-12. LPS-induced stimulation of IL-6 protein levels following NE and specific AR inhibitor administration in PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, followed by exposure to NE alone or following a 45 minute pre-treatment with the alpha-AR antagonist, prazosin, or the B2-AR antagonist, ICI 118,551, either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a low dose 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls). +++, $p < 0.001$ – represents a significant difference between NE and inhibitor treatments (Newman-Keuls).

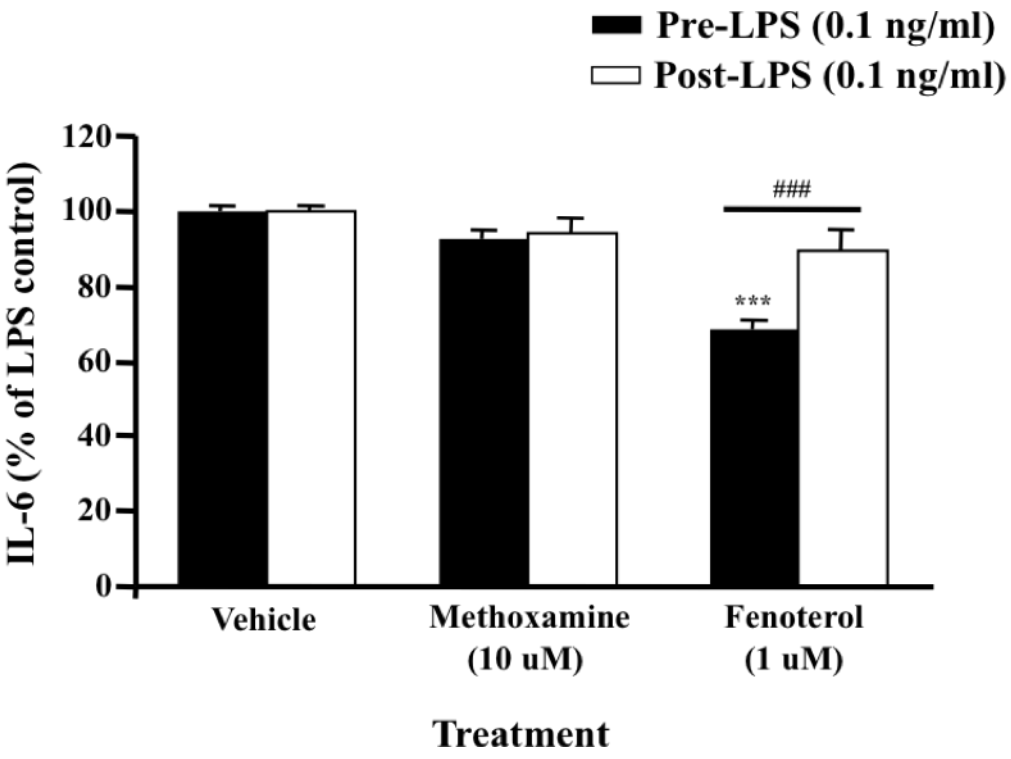

Figure 2-13. LPS-induced stimulation of IL-6 protein levels following specific AR agonist administration in PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, followed by exposure to the alpha-AR agonist, methoxamine, or the B2-AR agonist, fenoterol, either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a low dose 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls).

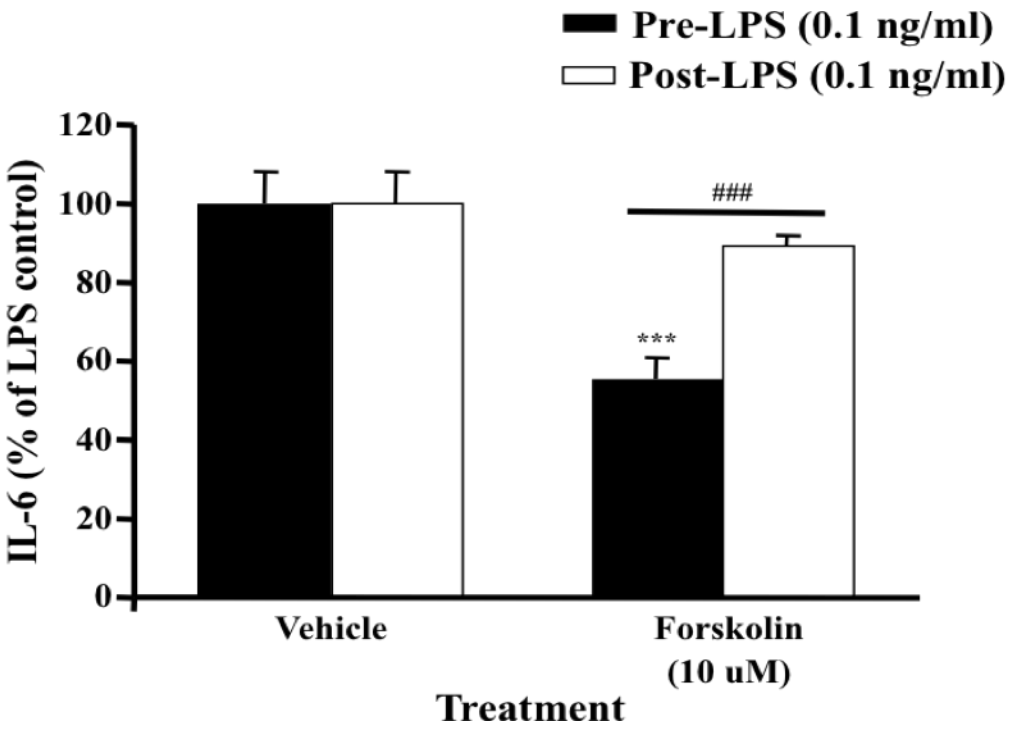

Figure 2-14. LPS-induced stimulation of IL-6 protein levels following administration of a cAMP-elevating agent in PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA (100 nM) for 48 hrs to induce differentiation into macrophages, followed by exposure to forskolin either 1 hr prior to (pre-LPS) or 6 hrs after (post-LPS) a low dose 24-hr LPS stimulation. Cell supernatant was collected at the 24-hr time point and analyzed for IL-6 protein levels. Data is represented as % of LPS alone and presented as the mean (+/- SEM) for each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents NE treatments that are significantly lower than LPS control (Newman-Keuls). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ – represents significant differences between the pre and post-LPS time points among the same treatment (Newman-Keuls).

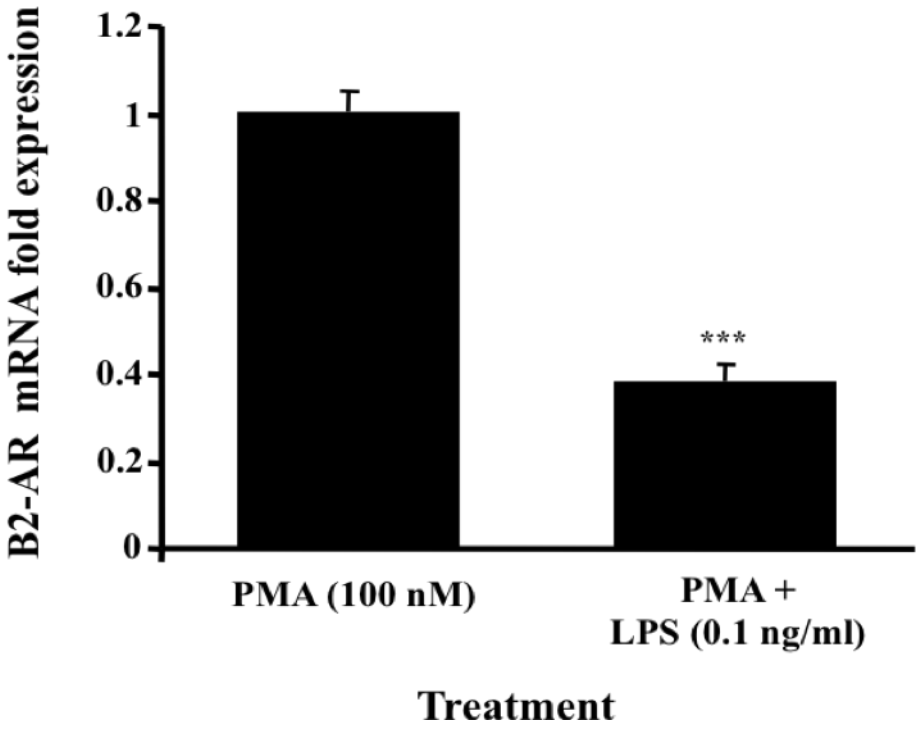

Figure 2-15. B2-AR mRNA expression in LPS-stimulated PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA for 48 hrs to induce differentiation into macrophages, followed by exposure to a 6-hr LPS stimulation and collected for measurement of B2-AR mRNA expression. Data is represented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle control.

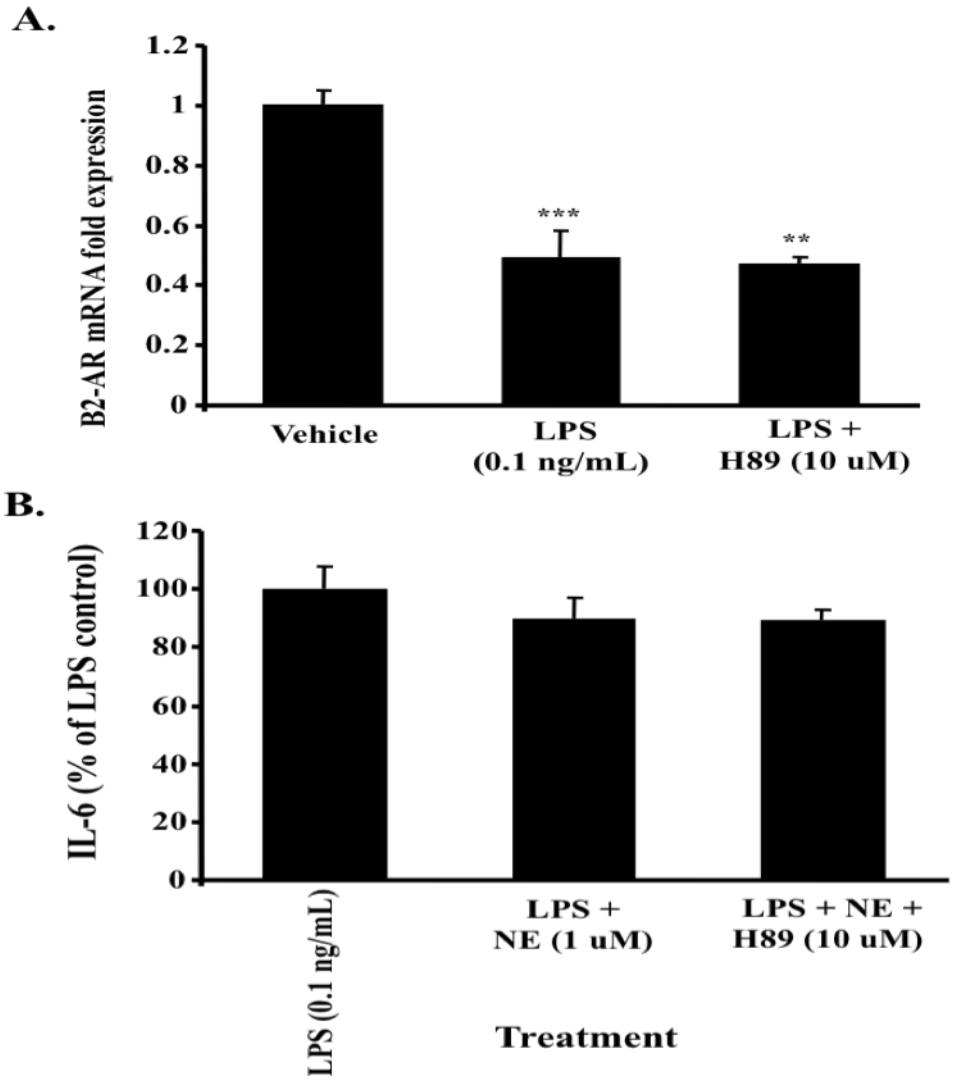

Figure 2-16. Effect of PKA inhibition on B2-AR mRNA expression in LPS-stimulated PMA-differentiated THP-1 cells (A) and on LPS-induced IL-6 protein levels following NE administration (B). THP-1 cells were pre-treated with PMA for 48 hrs to induce differentiation into macrophages, and then dosed with the PKA inhibitor, H89, for 1 hr prior to a 6 hr LPS stimulation and collected for B2-AR mRNA measurement (A). PMA-differentiated THP-1 cells were also dosed with H89 for 1 hr, followed by NE, 6 hrs into a 24-hr LPS stimulation, and cell supernatant was collected and analyzed for IL-6 protein levels (B). Data is presented as the mean (+/- SEM) and is represented as % of LPS alone for IL-6 levels (B). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle (A) or LPS (B) control (Newman-Keuls).

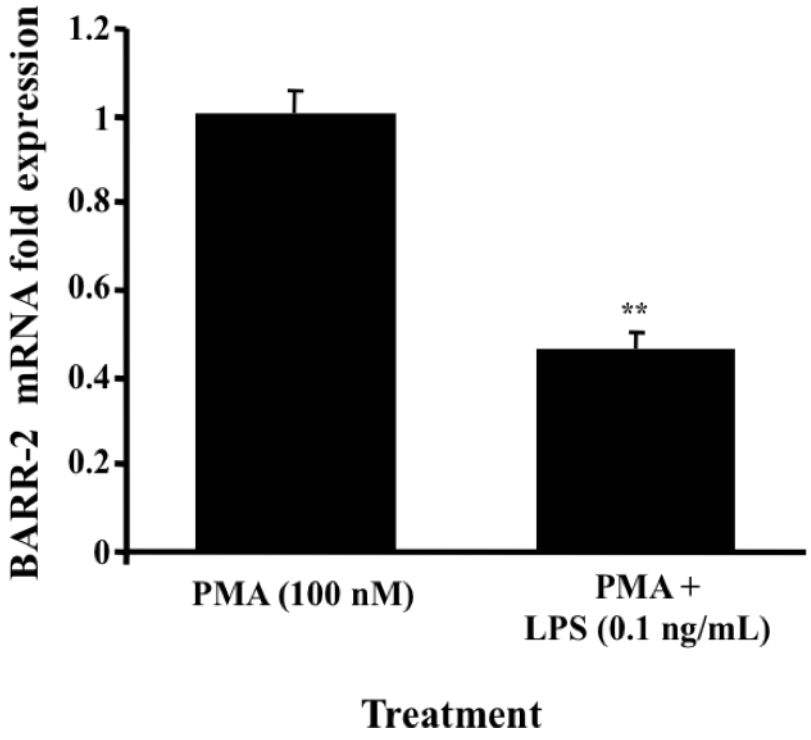

Figure 2-17. BARR-2 mRNA expression in LPS-stimulated PMA-differentiated THP-1 cells. THP-1 cells were pre-treated with PMA for 48 hrs to induce differentiation into macrophages, followed by exposure to a 6-hr LPS stimulation and collected for measurement of B-ARR2 mRNA expression. Data is represented as the mean (+/- SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – represents treatments that are significantly different than vehicle control.

CHAPTER THREE

DIFFERENTIAL NOREPINEPHRINE REGULATION OF STRESS-INDUCED INFLAMMATORY RESPONSES IN SUBJECTS WITH MAJOR DEPRESSION VERSUS HEALTHY CONTROLS

I. Introduction

Major depression (MD) is a common illness that is one of the leading causes of disability worldwide (Moussavi et al., 2007; Miller et al., 2009). In addition, MD is associated with other medical disorders, including heart disease, diabetes and cancer, and is often implicated as a risk factor for increased morbidity and mortality in these diseases (Ader and Cohen, 1975; Evans et al., 1999; Raison and Miller, 2001; Raison and Miller, 2003; Irwin and Miller, 2007; Redwine et al., 2010). The co-morbidity between MD and these medical illnesses, which are now recognized to involve inflammation, suggests an interaction between MD's etiology and the immune system. Many immunological changes have been shown to occur in patients with MD (Zorilla et al., 2001), including suppression of both the humoral and cellular arms of the adaptive immune response (Herbert and Cohen, 1993; Raison et al., 2006). However, increasing evidence suggests that MD is also associated with increased inflammation and activation of the innate immune response (Miller et al., 2005; Raison et al., 2006; Miller et al., 2009).

Activation of the innate immune response in depressed individuals has been shown to involve increases in inflammatory proteins, such as C-reactive protein (CRP), chemokines and cellular adhesion molecules, as well as proinflammatory cytokines, such as interleukin (IL)-6, IL-1beta, and tumor necrosis factor (TNF)-alpha (Maes et al., 1997; Yirmiya et al., 2000; Zorilla et al., 2001; Irwin, 2002; Miller et al., 2002;Anisman and Merali, 2003; Tuglu et al., 2003). Furthermore, many studies have shown increased inflammation, such as increases in IL-6 levels, in medically ill patients with MD. For example, increased levels of IL-6 were seen in patients with MD and cardiovascular disease (Lesperance et al., 2004) and rheumatoid arthritis (Zautra et al., 2004), both

inflammatory-related disorders. In addition, cancer patients with MD were found to have increased concentrations of circulating IL-6, compared to both non-depressed cancer patients and healthy controls (Musselman et al., 2001b; Jehn et al., 2006). This interaction between MD and inflammation may be a driving factor for the co-morbidity seen with MD and other medical disease.

One of the main risk factors leading to the development of MD is stress (Kendler et al., 2002). Interestingly, stress, both acute and chronic, has been shown to activate the inflammatory response (Segerstrom and Miller, 2004; Raison et al., 2006). Studies have shown that individuals going through an acute, stress-inducing task have increases in IL-6, both in medically healthy individuals and those suffering from MD (Pace et al. 2006). Chronic stress, such as childhood maltreatment, has also been associated with increased peripheral blood CRP concentrations in patients with MD later in life (Danese et al., 2007, 2008).In addition, increased stress has been linked with various diseases, including cardiovascular disease, metabolic syndromes, arthritis and cancer (Kiecolt-Glaser et al., 2002; Black, 2006; Cohen et al., 2007), thus providing a potential mechanism linking MD and activation of the innate inflammatory response, to its co-morbidity with other medical illnesses.

Stress is also known to lead to activation of the sympathetic nervous system (SNS), which mediates the stress response through the release of catecholamines including norepinephrine (NE) from nerve endings, and epinephrine from the adrenal medulla (Elenkov, 2000; Black, 2002). Recent studies have examined the possibility that stress-induced inflammatory responses are mediated, in part, by the SNS. The SNS is anatomically linked to the immune system, as shown by NE-containing nerve fibers

innervating primary and secondary lymphoid organs (Felten, 1993; Friedman and Irwin, 1997; Bellinger et al., 2008) as well as the expression of adrenergic receptors on various immune cells (Bellinger et al., 2008). Functionally, NE released in lymphoid organs can alter the behavior of immune cells (Elenkov et al., 2000). Moreover, studies have reported that IL-1 and IL-6 can activate the SNS, leading to an increased release of catecholamines, both peripherally and centrally (Besedovsky et al., 1986; Dunn et al., 1999). Finally, the increase in inflammatory markers measured after stress and SNS activation can be abolished by adrenergic receptor antagonists or sympathectomy (Rice et al., 2001; Bierhaus et al., 2003), thus providing further support for the role of the SNS in stress-induced inflammation. However, the role that NE specifically plays in inflammation (Elenkov et al., 2000) and MD (Ressler and Nemeroff, 1999) has been shown to involve both activation and inhibition.

Given the relationship between MD and stress, and the interaction between the SNS and inflammation, we sought to examine the possible role of stress-induced SNS activation and inflammation, in patients suffering from MD compared to healthy controls. Using the Trier Social Stress Test (TSST) as an acute, laboratory-based stressor, we measured NE and IL-6 levels in patients with MD and healthy controls before and after stressor challenge. We hypothesized that patients with MD would exhibit increased stress-induced levels of IL-6 and NE, compared to healthy controls. Based on observations from a previous pilot study, we also investigated the extent of stress-induced increases in NE on the inflammatory response to stress. We hypothesized that patients with MD and high NE responses to stress would exhibit higher concentrations of stress-induced IL-6 compared to controls with high or low stress-induced NE responses, and

patients with MD and low NE responses.

II. Methods

Study Participants

Participants for the current study were recruited and assessed as part of the National Institute of Mental Health-funded Emory Conte Center for the Neuroscience of Mental Disorders, which ended 08/2009. For the main study, male and female participants were recruited from the community using advertisements in newspapers and flyers. Participants were carefully prescreened for contraindications regarding all procedures. Participants were also compensated. For the diagnosis of MD, as well as for the diagnosis of exclusionary psychiatric disorders, the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (First et al., 1995) was used. Severity of MD was rated using the 21 Item Hamilton Depression Rating Scale (HAM-D 21) (Hamilton, 1960). The Childhood Trauma Questionnaire (CTQ), a self-report instrument covering 28 items, was utilized to rate early life stress, including the severity of emotional abuse and neglect, physical abuse, and sexual abuse. It has been validated in terms of psychometric test properties in samples of psychiatric patients, i.e. drug and substance abusers (Bernstein et al., 1994).

Participants were included in the current study if the following criteria were met: for participants assigned to the MD groups: presence of current MD according to DSM-IV diagnostic criteria and a HAM-D 21 score of greater than 18. For the healthy control group: subjects were defined as those with HAM-D 21 score of 7 or lower. **Table 3-1** shows demographics for the study participants included in this analysis.

Participants were excluded from the study if they met any of the following criteria: (1) significant medical illness, such as gastrointestinal, neurological, endocrine, cardiovascular, pulmonary, renal, hepatic, immunological or rheumatological disease, organic brain disease, or cancer as determined by history, physical examination, ECG, and laboratory tests; (2) past or current presence of psychotic symptoms or bipolar disorder; (3) current presence of psychoactive substance abuse/dependency or eating disorders; (4) hormonal medication; and (5)psychotropic medication in the two weeks prior to study entry (4 weeks for fluoxetine).

Stressor procedure and sample collection

Participants arrived at the study site the night before testing, and were provided a low-fat breakfast and lunch on the day of testing. The stressor challenge, the Trier Social Stress Test (TSST) began at 3 PM and followed procedures outlined previously (Kirschbaum et al., 1993). Briefly, participants were challenged with a 5-minute public speaking task (preceded by 10 minutes of preparation/ anticipation) and a 5-minute arithmetic task.

A total of 15 ml of blood was collected for the basic laboratory assessments that were performed to determine eligibility of the participants. Whole blood was collected throughout the TSST into EDTA-coated tubes and centrifuged immediately. Plasma was stored at -80°C until assay. Blood was obtained at time points before, during, and after TSST challenge (15 min before and immediately before the TSST, and then at 15, 30, 45, 60,75, and 90 min after the start of the TSST).

Determination of Plasma IL-6 Concentrations

IL-6 protein concentrations were measured in plasma using a high sensitivity sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The sensitivity of this assay ranges from 0.156 to 10 pg/ml, and intra- and inter-assay variability range from 7.0 to 7.8% and 6.5 to 9.6%, respectively.

NE Determination

Plasma concentrations of the catecholamine, NE, were measured by a core lab using high performance liquid chromatography, as described in detail in Weinstein et al., 2010. The intra-assay variability ranged from 6.1 to 7.1% and the mean inter-assay variability was 10.3%.

Statistical Analysis

Dependent factors were first analyzed using descriptive statistics, including the mean and standard error. Plasma IL-6 and NE concentrations were then compared between groups (control vs. MD) at each time point before and after the TSST using a 2-way ANOVA for repeated measures (group X time) with pairwise comparisons to check for mean differences of any main effect. To determine differences in the NE-mediated response to stress and IL-6 levels, the change (delta) in NE in response to the TSST was determined for each subject by taking the maximum NE response after the stressor and subtracting it from the baseline value. Once the delta NE was calculated, the subjects were split into high and low NE-responders by means of a median split on delta NE

concentrations. Plasma IL-6 concentrations 90 min after the start of TSST challenge were then compared among the high and low delta NE responders in the MD and control groups using a two-way ANOVA with depression status and delta NE as the fixed factors. Independent sample t-tests were used as post hoc tests to analyze specific means between the two groups. Covariates for this analysis included age, race, sex, weight and CTQ total scores. All tests of significance were 2-sided with an alpha level of $p <= 0.05$.

III. Results

Effect of acute stress challenge on plasma IL-6 and NE concentrations in depressed subjects and controls

To determine the effect of an acute stressor on plasma IL-6 and NE concentrations in subjects with MD versus controls, participants were challenge with TSST and blood samples were drawn before and after the challenge as described in the methods. All participants displayed a significant increase in both plasma IL-6 concentrations **(Figure 3-1)** and NE concentrations **(Figure 3-2)** during TSST challenge over time (IL-6, $F[4, 208] = 8.202$, $p < 0.001$; NE, $F[7, 343] = 43.335$, $p < 0.001$, respectively). However, no significant effect of group status or their interaction was observed. Pairwise comparisons indicated that plasma IL-6 concentrations were greater at 15, 30, 45 and 60 minutes after the TSST, compared to baseline (time 0) for both groups (at $p < 0.05$). Pairwise comparisons also indicated higher NE concentrations for both groups at 15 and 30 minutes after TSST onset, compared to 15 minutes prior to the TSST challenge, as well as compared to baseline concentrations (time 0) (at $p < 0.05$).

Role of NE-mediated stress response to TSST in depressed versus control subjects

To examine the role of the NE-mediated response to the TSST challenge in both groups, participants were divided into those that had a NE stress response below the median (low delta NE response) and those that had a response above the median (high delta NE response). Since the interest was in the response to the stress challenge, MD and controls were compared 60 minutes after TSST onset. Although no main effects were revealed for depression status or NE response groups (MD status, $F[1,34] = 0.063$, $p = 0.803$; NE group, $F[1,34] = 1.589$, $p = 0.216$), a significant interaction ($F[1,34] = 4.543$, $p = 0.40$) between delta NE response and group was found and indicated that IL-6 concentrations 60 minutes after TSST onset in groups was different depending on whether they had a low delta response or a high delta NE response **(Figure 3-3)**. These results were obtained controlling for age, race, sex, weight, and CTQ scores, which showed no significant effects in the model. Post hoc analysis revealed that subjects with MD and a high delta NE response to the TSST had significantly greater plasma IL-6 concentrations at time 6 ($t=2.5058$, $df = 5$, $p = 0.0242$), compared to subjects with MD and a low delta NE response, and controls at both NE response levels.

IV. Discussion

Preliminary findings from this study show that both patients with MD and healthy controls display an inflammatory response to an acute psychosocial stressor. Furthermore, patients with MD and a high NE-mediated response to the stressor show greater concentrations of IL-6 after the stressor, compared to controls with high NE-

mediated responses, as well as controls and depressed patients with low NE-mediated responses to stress. Numerous studies have provided evidence of increased inflammation in patients with MD and/or chronic stress (Pariante and Miller, 2000; Segerstrom and Miller, 2004; Bierhaus et al, 2006; Black, 2006). This study extends these findings by describing a potential relationship between NE and increased inflammation and MD in the context of acute stress challenge.

Previous studies have shown an increase in inflammatory markers in patients with MD. For example, Miller et al. (2002) showed increased baseline concentrations of IL-6 and CRP in patients suffering from MD, in the absence of an added stressor. Pace et al., (2006), extended these findings by showing that this increase in inflammation was not restricted to baseline levels, but that patients with MD also showed an exaggerated inflammatory profile, as measured by increased IL-6 and nuclear factor kappa-B (NFkB) DNA binding, in response to the acute stressor, the TSST, compared to healthy controls. Other studies have also shown increased inflammatory markers in response to acute stress, such as the TSST, in individuals with a history of moderate to severe childhood mistreatment without MD or other psychiatric illness (Carpenter et al., 2010), in women with a history of childhood abuse, with and without MD (Heim et al., 2000), and in patients, both male and female, with both MD and a history of childhood maltreatment (Danese et al., 2008). However, in the present study, although significant increases in IL-6 concentrations were seen in response to the acute stress challenge, there was no significant difference found in baseline or stress-induced IL-6 levels between those with MD and healthy controls. Numerous factors may have contributed to this, including the fact that the depressed patients in this study were not as severely depressed as subjects in

other studies (Pace et al., 2006). It may also be that there was a difference between the two groups in their inflammatory response to stress, but that it was not reflected in circulating concentrations of IL-6.

Stress is known to activate the neuroendocrine system by releasing stress hormones, such as glucocorticoids and NE, both of which can regulate inflammatory responses (Elenkov, 2000). Thus, the increase in IL-6 seen after stress may result from, or relate to, changes in neuroendocrine function and may be a differentiating factor between groups. However, the role of NE in regards to modulation of stress-induced inflammatory responses is not as clear as with glucocorticoids. Although abnormalities in the NE system have been reported in MD, it is not clear whether they are due to increases or decreases in NE availability or activity (Ressler and Nemeroff, 1999). In addition, NE has been shown to exhibit both pro-inflammatory and anti-inflammatory effects (Kavelaars et al., 1997; Elenkov, 2000). For example, activation of the SNS has been shown to enhance inflammatory responses both in vivo and in vitro (Bierhaus et al., 2003). Stress-induced increases in NE were seen along with stress-induced increases in NFkB binding in healthy volunteers exposed to the TSST, and administration of NE to cells in vitro led to increases in NFkB binding. However, in contrast to healthy volunteers, patients with MD and early life stress were shown to have increased autonomic activity in response to a stressor, as measured by increases in heart rate (Heim et al., 2000). Another study also showed that patients with MD had higher levels of both epinephrine and NE, compared to controls, after a mental arousal task, as well as higher concentrations of IL-6 and CRP (Weinstein et al., 2010). However, NE is more commonly thought of as having anti-inflammatory properties (Oberbeck, 2006; Sternberg, 2006; Hansel et al., 2010), and is

thought to provide an inhibitory feedback mechanism to shut off inflammation once it has begun(Elenkov, 2000; Hansel et al., 2010). Indeed, NE was shown to inhibit LPS-induced TNF-alpha and IL-6 release in human blood (Van der Poll, et al., 1994), as well as in human monocytes isolated from whole blood (Rontgen et al., 2004). Furthermore, activation of LPS-induced NFkB has also shown to be inhibited by long-term treatment with a beta-AR agonist, leading to decreases in inflammatory responses mediated by the transcription factor (Farmer and Pugin, 2000). Thus, potential alterations in NE feedback inhibition may result in increased inflammatory responses to stress, and results from the previous studies mentioned above may be explained by a lack of negative feedback regulation in patients with MD, even though they are showing increased levels of NE in response to stress (Weinstein et al., 2010). In the current study, we show that although there is no difference in the stress-induced NE response between MD patients and healthy controls, there appears to be a difference in how these groups respond to NE's inhibitory effects. Specifically, when we divided the groups by their stress-induced NE response (delta NE) into those that fell below the median delta NE and those that fell above the median delta NE, we saw that depressed patients with a high delta NE response to stress had higher stress-induced IL-6 concentrations (at time 6, after the TSST challenge), compared to controls and depressed patients with low delta NE responses. This suggests that the patients with MD and a high delta NE stress response have decreased sensitivity to the anti-inflammatory effects of NE, similar to what has been shown in the in vitro response to dexamethesone on IL-6 and TNF-alpha, in depressed subjects by Miller et al. (2005). Many factors could be contributing to this altered sensitivity, such as alterations in adrenergic receptor expression due to stress or inflammation, or changes in immune

cell phenotypes, that warrant further investigation. For example, data from Chapter 2 showed that LPS-induced activation of monocytes can lead to downregulation of the beta 2 adrenergic receptor, likely mediated by protein kinase A, which led to a decrease in NE's ability to inhibit LPS-mediated IL-6 release. Furthermore, when the cell phenotypically changed from a monocyte to a macrophage, NE lost even more of its inhibitory action on IL-6.Thus, although no changes were seen in stress-induced IL-6 concentrations between patients with MD and controls, we see that changes in the sensitivity to NE's anti-inflammatory abilities are apparent between the groups when divided by their NE response to stress and may contribute to increased stress-induced inflammation in patients with MD.

Of note, changes in sensitivity to anti-inflammatory mediators during stress or pathological conditions have been studied extensively in the context of glucocorticoids, and these findings may extend to NE. For example, although glucocorticoids are known to have an anti-inflammatory effect, alterations in glucocorticoid feedback inhibition, and reduced signaling, have been shown to modulate the inflammatory response in patients with MD (Raison and Miller, 2003b). Specifically, one study showed that although there were no changes in the cortisol response to stress between patients with MD and controls, when the cortisol response was divided into those that fell above or below the median response, only those with MD below the median showed higher stress-induced increases in IL-6 compared to controls and depressed patients above the median (Pace et al., 2006, abstract). This suggests that reduced stress-induced cortisol responses in MD patients may be associated with enhanced stress-induced IL-6 concentrations. In another study, women with MD and healthy controls both showed higher CRP levels in vivo and greater

IL-6 and TNF-alpha concentrations induced in vitro, after a mock interview process similar to the TSST (Miller et al., 2005). However, those with MD had increased IL-6 and TNF-alpha production in the presence of dexamethasone, a synthetic glucocorticoid, when administered in vitro, showing a decrease in the sensitivity to the anti-inflammatory effects of glucocorticoids. Interestingly, this so-called "glucocorticoid resistance" may be related to an inhibitory impact of inflammatory cytokines on glucocorticoid receptor function (Pace et al., 2007). Taken together, these studies, among others, indicate that the enhanced inflammatory responses seen in patients with MD, may be a result of blunted stress-induced cortisol production or responsiveness, or impaired sensitivity to the anti-inflammatory effects of glucocorticoids.

A similar mechanism of decreased responsiveness to the inhibitory actions of NE at the receptor level may be contributing to increased inflammation, even in the presence of high NE concentrations during times of stress. Furthermore, inflammation in depressed individuals may result in the innate immune system being constantly activated, which would result in constantly activated immune cells such as monocytes and macrophages. Results from Chapter 2 showed that these activated cells are less sensitive to the inhibitory actions of NE, even at high concentrations of the catecholamine due to decreased expression of the B2-AR and/or altered expression of factors that regulate B2-AR function. The decreased sensitivity seen in patients with MD and high NE responses to stress may be due to this type of B2-AR alteration. The implications and future directions for this concept are described further in Chapter 4. This would imply that under stressful conditions, those with MD have a decreased sensitivity, or a greater resistance, to hormones that help terminate inflammatory responses, such as NE, and this

lack of negative feedback regulation which is mediated by the receptors for these hormones may result in increased inflammation that may ultimately lead to exacerbation of inflammatory-based medical comorbidities in these individuals.

	MD	CON
Sample size, n	19	37
Gender, n		
Male	4	13
Female	15	24
Age, years	30 (1.44)	28 (1.11)
Weight, kg	79.67 (4.76)	71.27 (2.83)
Race, n		
Caucasian	6	4
African-American	12	21
Asian	1	2
Childhood Trauma Questionnaire (CTQ)	64.05 (6.12)	38.54 (2.25)
Hamilton Depressive Scale-21 (HAM-D 21)	22.95 (0.77)	2.46 (0.35)

Table 3-1. Demographics and psychometric characteristics of MD patients and healthy controls used in this analysis. Data for age, weight, CTQ scores and HAM-D 21 scores are given as mean (standard error). For this analysis, MD patients were chosen from the complete CONTE database (see methods) by selecting those with HAM-D 21 scores greater than 18. Controls were chosen by selecting those subjects with HAM-D 21 scores of 7 or lower.

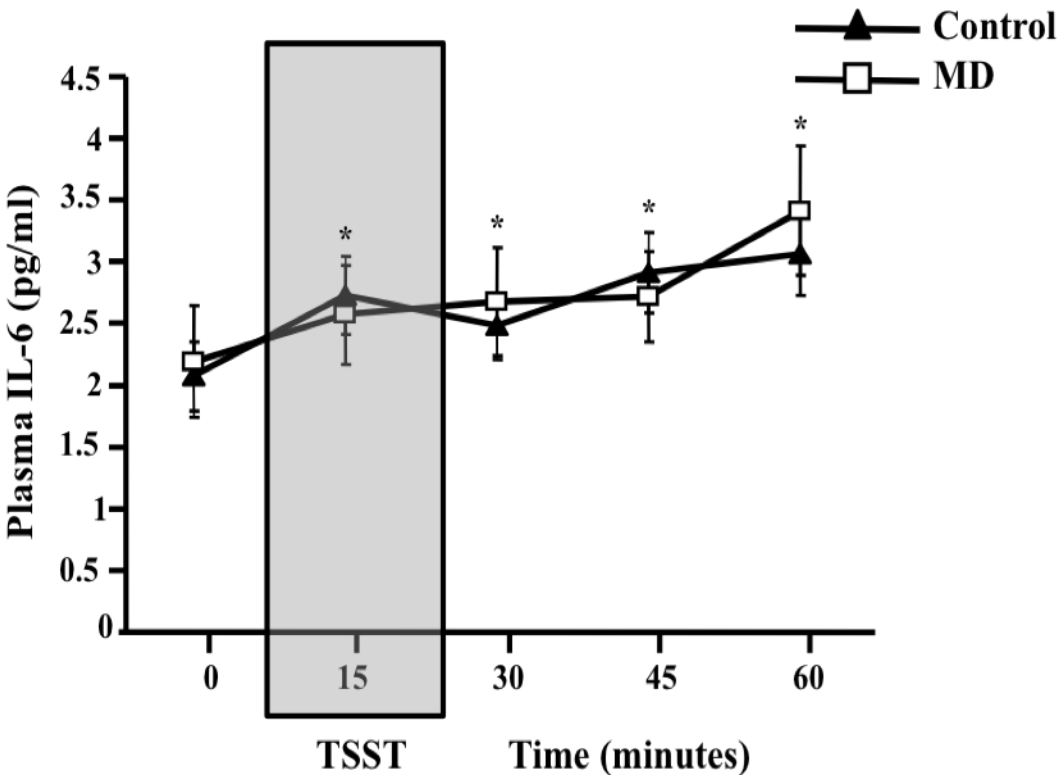

Figure 3-1. Plasma IL-6 concentrations in patients with MD and non-depressed controls before and after psychosocial stressor challenge. Blood was collected from subjects before onset of the TSST up to 60 minutes after onset of the TSST challenge, in 15-minute intervals. Plasma was collected from blood samples and IL-6 protein concentrations were measured at each time point. Data is represented as the mean (+/- SEM) for each time point. *, $p < 0.05$ - represents plasma IL-6 concentrations that are significantly higher than baseline (time 0) levels within the same group.

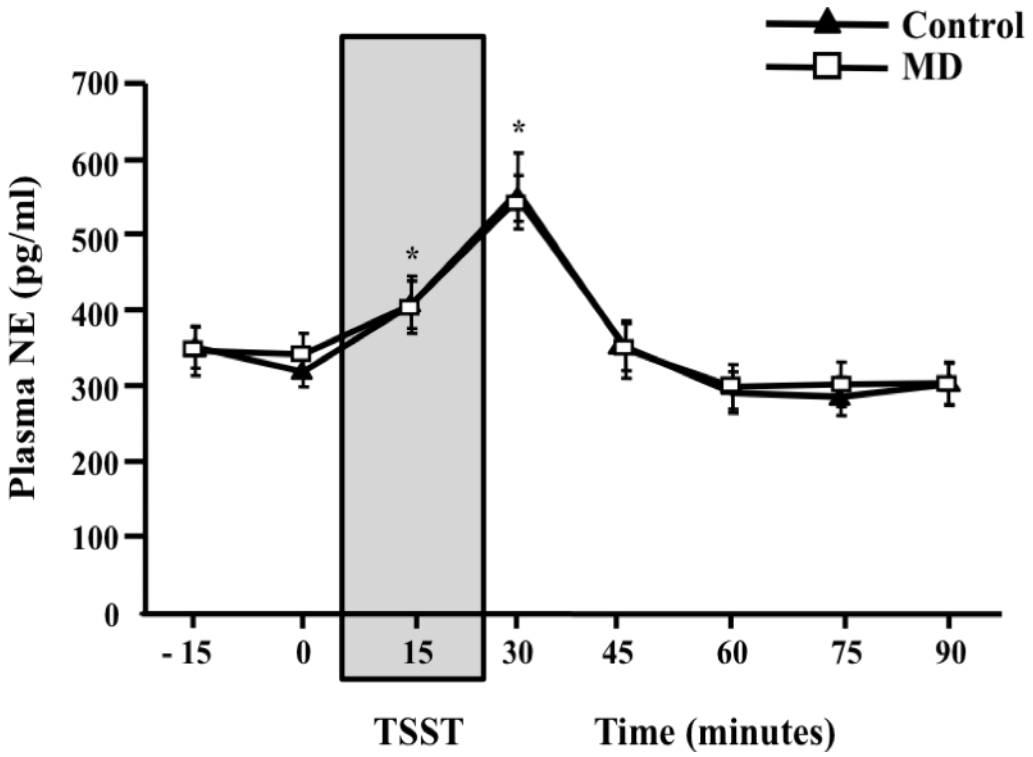

Figure 3-2. Plasma NE concentrations in patients with MD and non-depressed controls before and after psychosocial stressor challenge. Blood was collected from subjects before onset of the TSST up to 90 minutes after onset of the TSST challenge, in 15-minute intervals. Plasma was collected from blood samples and NE concentrations were measured at each time point. Data is represented as the mean (+/- SEM) for each time point. *, $p < 0.05$ - represents plasma NE concentrations that are significantly higher than both baseline levels (time -15 and time 0) within the same group.

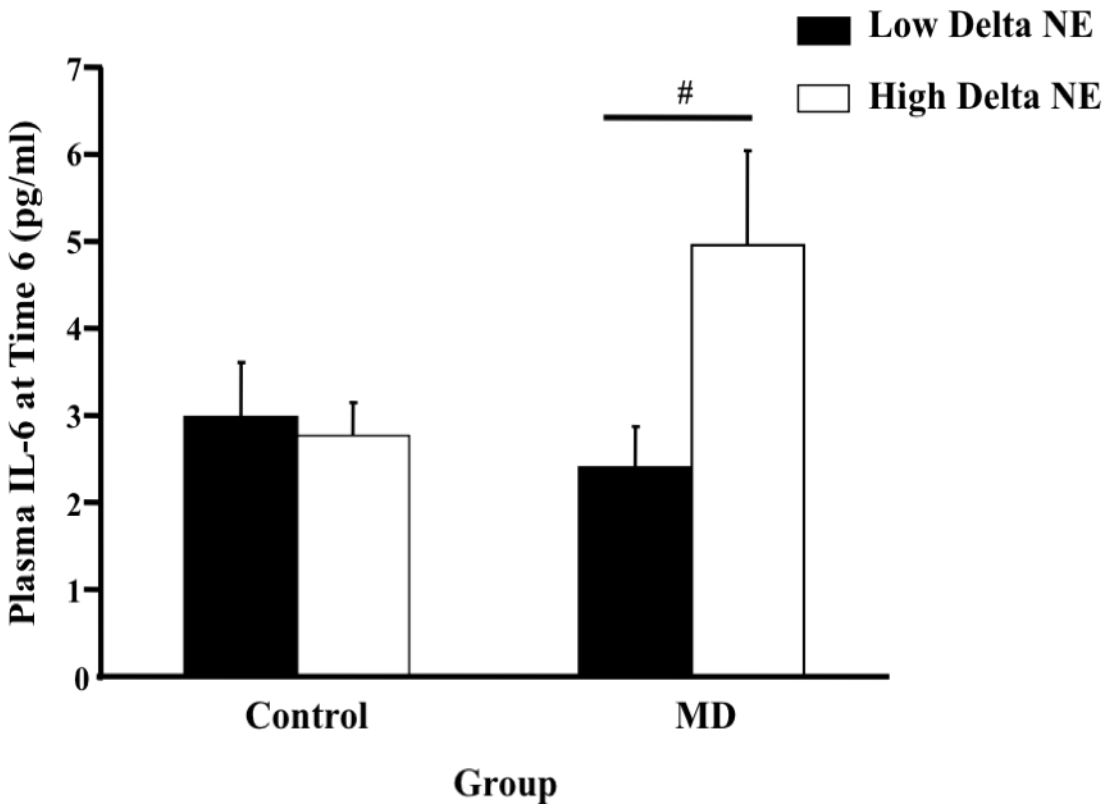

Figure 3-3. Plasma IL-6 concentrations in patients with MD and non-depressed controls split by their NE-mediated response after psychosocial stressor challenge. NE-mediated response to the TSST stressor was determined by taking the delta NE concentration (maximum NE response minus baseline NE concentration) for each subject followed by a median split to obtain low and high delta NE responders. Data is represented as the mean (+/- SEM) for each group. #, $p < 0.05$ - represents a significant difference in depressed subjects in their plasma IL-6 concentrations in response to TSST challenge (time 6: 60 minutes after TSST onset), when comparing low and high delta NE responses to stress.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

I. Summary of Results

Catecholamines help regulate inflammatory responses and maintain homeostasis through the intricate crosstalk between the immune system and the sympathetic nervous system (SNS) (Flier et al., 2008). Although this concept is well established, the specific function that norepinephrine (NE) plays in this regulation is not as clear, with inconsistencies in the data and different studies finding different roles for NE, specifically in terms of whether its main function is to inhibit inflammation or enhance it. A possible explanation for these inconsistencies are the numerous experimental conditions found in all the different studies examining this interaction, providing evidence that NE's role in regulating inflammation may be dependent on the physiological conditions of the cell at the time of challenge. Some of these differences include the specific adrenergic receptor (AR) subtype being stimulated, such as the beta-AR or the alpha-AR, or the origin of the cell being studied, such as mouse versus human, or the type of cell being studied, such as a macrophage versus a T-cell, or the absence or presence of other mediators, including immune mediators, such as antigens and cytokines.

The rationale for the in vitro studies presented in this dissertation was to elucidate the effect of monocyte activation and monocyte-to-macrophage differentiation on NE's ability to inhibit inflammation. Lipopolysaccharide (LPS) was used to induce activation in the human THP-1 monocytic cell line, and interleukin-6 (IL-6) protein concentration was measured as the primary endpoint of the inflammatory response. Generally, the data showed that NE does indeed inhibit inflammation in terms of reducing the amount of IL-6 protein released from the cell. However, it appears that the extent of this inhibition is

dependent upon the activation state of the cell. In the monocytic form of the THP-1 cell, during resting conditions, LPS-induced release of IL-6 was inhibited by the neurotransmitter by about 80%, compared to LPS alone. However, in the LPS-mediated activated form of the cell, NE only showed about a 20% reduction of IL-6. NE was shown to have its effects on IL-6 through activation of the beta-2 (B2)-AR, and not the alpha-1 (A1)-AR. LPS-mediated activation of THP-1 monocytes led to decreases in mRNA expression of the B2-AR, possibly explaining the decrease in NE-driven inhibition of IL-6 in the activated cell. Furthermore, the LPS-induced downregulation of the B2-AR may be a result of heterologous desensitization (Kohout and Lefkowitz, 2003), as it appeared to be mediated by protein kinase A (PKA). Addition of the PKA inhibitor, H89, before activation, reversed the downregulation of the receptor, and restored NE's ability to inhibit LPS-induced IL-6 in the activated monocyte **(Figure 4-1).**

Differentiation of THP-1 cells from the monocyte to macrophage state also affected NE's inhibition of LPS-induced IL-6. When LPS concentrations used to induce IL-6 were matched in the monocytes and macrophages, NE given prior to LPS was only able to inhibit IL-6 release by 30% in the macrophage state, compared to the 80% in the monocyte state, indicating that macrophages are less sensitive to NE's anti-inflammatory effects. This was seen at the protein level for IL-6, as well as at the mRNA level, showing that de novo synthesis of IL-6 was also less affected by NE in the activated, macrophage state compared to the monocyte state. Furthermore, when NE was given after LPS-mediated macrophage activation, it lost all its inhibitory effect on IL-6.

The reduction in inhibition of IL-6 by NE in the macrophage state also seemed to be driven by the B2-AR. However, in the resting, differentiated macrophage, the lack of

the B2-AR's ability to inhibit IL-6 seems to be driven by an increase in beta arrestin-2 (BARR-2), not a decrease in B2-AR gene expression, because an increase was seen in B2-AR, which would result in increased inhibition. However, an increase in BARR-2 gene expression implies that the B2-AR may undergo increased homologous desensitization (Lutrell and Lefkowitz, 2002), through BARR-2 binding to the B2-AR and thus desensitizing its stimulation by NE **(Figure 4-2)**. Finally, PKA did not seem to mediate the decrease in NE-mediated inhibition in the activated macrophage, as administration of H89 had no effect on LPS-induced B2-AR downregulation of IL-6 release in these cells. However, BARR-2 gene expression decreased in the activated macrophage, and BARR-2 has been shown to interact with nuclear factor kappa-B (NFkB), a transcription factor also stimulated by LPS which leads to IL-6 release, by stabilizing its inhibitory proteins, and thus, inhibiting inflammation (Gao et al., 2004). Therefore, a decrease in BARR-2 may lead to increased inflammation since there is less inhibition of NFkB, which may potentially translate to greater release of IL-6 **(Figure 4-3)**.

NE's lack of inhibition of IL-6 under specific circumstances was also hinted upon in preliminary in vivo studies. In this case, although subjects with major depression (MD) had similar increases in IL-6 and NE after a psychosocial stressor compared to healthy controls, there was a difference seen in the IL-6 response to stress when the groups were studied in relation to their NE response to stress. Specifically, when the two groups (MD versus control), were split into high and low NE responders to stress, those with a high NE response and MD had significantly greater concentrations of stress-induced IL-6, compared to low NE responders with MD, and high and low NE responders in the control

group. This suggests that although they have a high NE response to stress, there is a lack in sensitivity to NE's inhibitory effects on IL-6 in these depressed subjects, which may be related to disease pathology. Interestingly, controls with a high NE response to stress also did not show much inhibition to stress-induced IL-6. A different analysis of this data may actually reveal that NE is acting in a stimulatory manner in individuals with high levels of NE in response to stress. Indeed, inflammation has been shown to increase A1-AR expression levels (Rouppe van der Voort et al., 2000b; Heijnen et al., 2002), when we dosed THP-1 cells with LPS, we not only saw a decrease in B2-AR mRNA expression, but an increase in A1-AR mRNA expression (data not shown.) Signaling through the A1-AR would lead to activation of a proinflammatory signaling cascade by NE (Heijnen et al., 1996; Kavelaars, 2002), potentially explaining why individuals with high NE responses to stress also show high IL-6 responses.

Taken together, these results indicate that NE functions to inhibit inflammation, however, the extent of its ability to inhibit depends on the physiological conditions, and perhaps even on any pathological conditions that may be present. The studies presented in this dissertation are only at the initial stages of exploring the regulation of the inflammatory response by the sympathetic nervous system. Although these data have started to tease apart the conflicting, and often contradictory, role of NE and its modulation of the inflammatory response, there are limitations to the in vitro and in vivo studies, with room for future studies in both areas to expand on the current findings.

II. Limitations and future directions: In vitro studies

The in vitro data presented in chapter two has some limitations in three main areas: 1) cell-based limitations, 2) NE-based limitations, and 3) receptor-based limitations. Although the data presented have indicated a clear shift in the sensitivity to NE's inhibitory effect on inflammation in regard to immune cellular activation and differentiation, one limitation in this study is that only one immune cell type was tested. Although monocytes/macrophages are important mediators of the inflammatory response, other immune cells that contribute to this physiological phenomenon, such as natural killer (NK) cells and T lymphocytes can also be affected by the SNS and NE. T lymphocytes are cells that make up the adaptive immune response. These cells have been shown to express the B2-AR exclusively (Nance and Sanders, 2007). T helper (Th) cells can be further split into two groups once they mature, the Th1 cells and the Th2 cells. Th1 cells release interferon gamma, IL-2 and tumor necrosis factor (TNF)-beta, and are responsible for cellular-mediated immunity, which is more pro-inflammatory in nature, while Th2 cells release other interleukins and are responsible for humoral mediated immunity involving the activation of B-cells and the antibody response, which is more anti-inflammatory in nature (Sanders and Straub, 2002). It appears that naïve T-helper cells and Th1 cells express B2-ARs, while Th2 cells do not (Sanders, 2006). Furthermore, NE stimulation seems to drive naïve T cells to shift more towards development of the Th2 cells, as it inhibits the production of IL-12, which is the main inducer of Th1 cells (Elenkov and Chrousos, 1999; Elenkov et al., 2000), shifting the adaptive immune response from cellular-mediated immunity to humoral-mediated immunity. A cell of the innate immune response that may be affected by this shift is the NK cell. NK cells become activated during cellular-mediated immunity, and NE can

inhibit the function of these cells directly by acting on B2-ARs on the cells themselves, as well as indirectly, through suppression of Th1 cells that induce their activity (Elenkov and Chrousos, 1999). Thus, future studies may involve conducting experiments in isolated NK cells, as well as T-cells, to determine if they react differently to NE in the absence or presence of an activating inflammatory stimulus. Additionally, since these studies were conducted only using monocytes from a cell line, a crucial next step would be to repeat these experiments using human monocytes freshly isolated from whole blood, to see if the results can be generalized. Furthermore, whole blood or peripheral blood mononuclear cells isolated from whole blood may also could be used to determine if cellular activation causes a change in NE's ability to affect inflammation in a system of cells, versus in just one cell type tested independently.

In addition to studying different cell types, it must also be noted that stimulation of the SNS and activation of the fight-or-flight response does not solely rely on NE, but on another catecholamine, epinephrine, as well. NE is converted into epinephrine in the adrenal medulla, where it is then released into the circulation (Elenkov et al., 2000). Administration of epinephrine, along with LPS, to human blood and THP-1 cells led to inhibition of TNF-alpha (Severn et al., 1992), which seemed to be mediated by an increase in cyclic adenosine monophosphate (cAMP) levels. Most studies involving epinephrine seem to point to a suppressive effect on inflammation (Friedman and Irwin, 1997). Furthermore, activation of the SNS can also activate the hypothalamic-pituitary-adrenal (HPA) axis, with the subsequent release of glucocorticoids (GC). GCs are also known to act as anti-inflammatory mediators (Eskandari et al., 2003), causing a shift from Th1 to Th2 mediated immune responses, and inhibition of proinflammatory

cytokines. Future studies involving administration of NE, along with epinephrine and GCs are needed to determine how all these mediators interact with one another to affect the inflammatory response of the cell. It may be that although cell activation and differentiation cause NE to become less inhibitory, these other mediators may not be as affected and may compensate for this lack of inhibition. On the other hand, all three mediators may lose their anti-inflammatory functions during cell activation and differentiation, specifically in monocytes.

Furthermore, stimulation of the autonomic nervous system (ANS) not only involves activation of the SNS, but also the parasympathetic nervous system (PNS) (Pavlov et al., 2003), with the two arms of the ANS rarely operating alone. Acetylcholine (ACh) is the main neurotransmitter of the PNS, and immune cells have been shown to contain choline acetyltransferase, a key enzyme in the production of ACh (Ley et al., 2010) Furthermore, human macrophages express five of the twelve nicotinic ACh receptors, as well as three of the muscarinic ACh receptors (Ley et al., 2010). The main role of ACh, as well as administered nicotine, is to function in suppressing the innate immune system (Tracey, 2009). Both agents, transmitted through vagus nerve stimulation, have been shown to decrease the production on inflammatory mediators, such as TNF-alpha, IL-1beta and IL-6 (Borovikova et al., 2000; Pavlov et al., 2003). The anti-inflammatory actions of ACh on the macrophage specifically, seem to be driven by the specific nicotinic ACh receptor subtype, nAChR-alpha7 (Pavlov et al., 2003; Ley et al., 2010). One mechanism used to inhibit inflammation through stimulation of this receptor on the macrophage is to inhibit nuclear translocation of NFkB by blocking degradation of the NFkB inhibitor, IkB (Yoshikawa et al., 2006).

Future studies in this area might focus on studying the effects of activation of the nAChR-alpha7, along with the B2-AR, in monocytes and macrophages. Since the PNS and SNS often operate together, it would be important to determine if the role of NE, and B2-AR activation, is enhanced or perhaps even changed, when ACh is also administered to monocytes/macrophages. Perhaps activated monocytes or macrophages that have lost sensitivity to the inhibitory effects of NE, may be more reactive when ACh is also present, in terms of inhibiting inflammation.

Other than the issues of cell types and systems activated, another main factor in the contradictory nature of NE on inflammation is the role of the adrenergic receptor subtypes involved. This study focused on the A1-AR and the B2-AR, with NE's action being mainly mediated by the B2-AR. However, another receptor subtype not investigated in this study that is also involved in the crosstalk between NE and inflammation is the alpha-2 (A2) AR.

Although the signaling cascade (ie: Gs, cAMP-PKA) activated by the B2-AR is anti-inflammatory in nature, the A2-AR is coupled to the Gi, which inhibits cAMP and PKA production, thus acting in a pro-inflammatory manner. Indeed, studies have shown that activation of the A2-AR on macrophages increases the production of proinflammatory mediators (Elenkov et al., 2000; Flierl et al., 2009), such as TNF-alpha (Spengler et al., 1990; Zhou et al., 2001). Pharmacological blockade of this receptor in rodents in vivo, was shown to reverse this pro-inflammatory response and inhibit TNF-alpha production (Hasko and Szabo, 1998). Although our results using pharmacological agents seemed to point to B2-AR as being the driving force in NE's actions on these cells, it would be interesting to use agents targeted to the A2-AR receptor, and to measure

the expression of this receptor , under the same experimental conditions presented in this dissertation, in order to determine if it plays a role in the modulation of NE's function in regard to monocyte activation and differentiation.

Finally, aspects of this study could be taken as preliminary results and investigated further. For example, only IL-6 was used as a dependent endpoint for inflammatory status. Although IL-6 is a commonly studied cytokine, and a major mediator in the inflammatory response, it would be noteworthy to determine if NE behaves similarly in these cells in terms of other inflammatory mediators. It does seem that NE also inhibits TNF-alpha production in the monocytes when given before LPS-mediated activation, with a decrease in the inhibitory actions of NE seen when given after activation (data not shown). Future studies could repeat these experiments involving the effect of THP-1 monocyte activation and differentiation with NE administration, looking at several other inflammatory mediators as the primary endpoint. Of interest would be other proinflammatory cytokines, such as TNF-alpha and IL-1B, anti-inflammatory cytokines, such as IL-10, and transcriptional regulators of the inflammatory response, such as NFkB and members of the mitogen activated protein kinases (MAPK) family, such as extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and p38.

Further examination could also focus on the signaling mechanisms involved in the changes of NE sensitivity in monocyte activation versus differentiation. Since our results indicate that the change in NE sensitivity and B2-AR expression during monocyte activation is driven by PKA, future studies in this area could examine molecules involved in the PKA pathway, particularly, cAMP. Data from this study showed that forskolin

mimicked the effects of NE, with a decrease in its inhibitory ability seen after monocyte activation, further indicating a role for cAMP in this process. Since NE-induced B2-AR activation results in stimulation of cAMP, it may be worthwhile to first examine the levels of cAMP accumulated by NE administration in these cells, and compare them at different stages of cellular activation (NE given prior to and after LPS) and differentiation (NE given to the monocyte versus the macrophage), to see if the decrease in NE sensitivity is also related to a decrease in cAMP accumulation. Phosphodiesterases (PDE) are the key regulators of cAMP, which function to degrade cAMP to prevent over accumulation and regulate intracellular signaling (Wang et al., 1999). LPS-induced production of TNF-alpha was suppressed in human peripheral blood monocytes using inhibitors of PDE4 (Seldon et al., 1995), indicating that cAMP functions in an anti-inflammatory manner, since blockade of PDE4 would block cAMP degradation and cause it to further accumulate. PDE inhibitors could also be used in the study presented in this dissertation to further assess the role of cAMP in mediating the inflammatory effects of NE and the B2-AR.

On the other hand, the effect of monocyte to macrophage differentiation on NE seemed to be driven by changes in BARR-2, not PKA. As discussed in Chapter 1, BARR-2 is a molecule involved in regulation of B2-AR signaling, primarily through a direct binding interaction between itself and the receptor, causing the receptor to uncouple from its G-protein, desensitizing it and turning off its signaling. The results from this study showed that B-ARR-2 gene expression was increased during differentiation, potentially explaining the decrease in NE's inhibitory actions since the B2-AR may now become more easily desensitized. Further studies should attempt to

reverse or block this increase in BARR-2 mRNA, to determine if this is really the driving force in the decreased sensitivity. A potential experiment may be to use siRNA targeted to BARR-2 to reduce its expression and measure whether B2-AR activation regains its anti-inflammatory ability in the macrophage. In terms of macrophage activation, BARR-2 expression decreased, but this protein is also known to interact with NFkB by stabilizing IkB, an inhibitor of NFkB, and a decrease in its expression can lead to less inhibition on the inflammatory transcription factor. Thus, in this case, it would be noteworthy to measure the levels of NFkB and IkB protein and mRNA expression, to see if they correlate with the decrease seen in BARR-2 gene expression. BARR-2 is not the only accessory molecule involved in B2-AR desensitization. G-protein coupled receptor kinases (GRKs) are recruited before beta-arrestins, and phosphorylation of the receptor by these proteins facilitates the binding of beta-arrestins (Kohout and Lefkowitz, 2002). GRKs are also expressed in immune cells (Heijnen, 2007). Studies have examined the role of GRKs, primarily GRK2, in inflammatory autoimmune diseases (Heijnen, 2007) and have found that the expression level of GRK2 decreases in patients with arthritis and multiple sclerosis (Lombardi et al, 1999; Vroon et al., 2005), but only at the protein level, while mRNA levels remained unchanged. Further in vitro studies involving NE's role in inhibiting inflammation in macrophages should thus also include regulation of the GRK family, as well as the beta-arrestins and adrenergic receptor at the mRNA, as well as, the protein level.

III. Limitations and future directions: In vivo studies

The in vivo results presented in chapter 3 are only preliminary findings that are meant to direct future questions and studies in relation to the effects of NE on inflammation, specifically in terms of MD versus healthy adults. One of the main limitations of this study was that it used data already obtained from a previous, closed study, so no new experimental manipulations could be conducted. However, results obtained from analysis of this database has led to numerous, yet directed questions, and experiments that can be done in order to further elucidate the role of NE in mediating the increased inflammatory response seen due to stress, in both healthy individuals and depressed patients.

The first observation from the study presented in this dissertation is the lack of a difference in the stress-induced increases in IL-6 between the control subjects and those with MD. Although an increase in IL-6 was seen due to stress in both groups, many other studies have provided evidence that individuals with MD will have an exaggerated increase in inflammation, both at baseline (Miller et al., 2002), and in response to stress compared to healthy controls (Pace et al., 2006). The lack of a difference here may have been due to a variety of factors, as discussed in chapter 3, but mainly, this would lead to a new study where the severity of MD in the individuals would be more robust, in order to provide for a more homogeneous sample of patients that could be more clearly differentiated from controls. Perhaps only sampling from a population that has treatment-resistant depression, a more severe form on the illness, or only sampling those with MD and increased baseline inflammation, may help in differentiating the two groups more distinctly, and make it easier to tease apart any inflammatory and/or SNS-driven differences.

Once a new study population has been established, the difference seen in the groups of high and low NE responders within the depressed population in terms of their stress-induced inflammatory response, can be re-evaluated and hopefully, replicated. If a difference between NE response and disease status on either baseline, or stress-induced inflammatory responses exists, then further experiments could be done to discover a potential mechanism. One such experiment would be to isolate peripheral blood cells, as well as isolating monocytes specifically from those cells, from patients with MD and healthy controls, and study the effects of NE administration and AR activation in vitro, to determine if cells isolated from the two groups also show different inflammatory profiles in response to adrenergic stimulation. Experiments similar to those presented in chapter 2 could be conducted on isolated monocytes taken directly from depressed patients. Since patients with MD are usually associated with increased inflammatory profiles at baseline, their cells may react to NE in a manner more related to the LPS-induced activated monocyte, or even the differentiated macrophage, with reduced sensitivity to NE's anti-inflammatory actions, thus explaining why patients with MD and a high NE response to stress still had an increased IL-6 response to stress. This shift in sensitivity may be due to their chronic inflammatory state, since we saw that activated monocytes are also less sensitive to NE's effects.

In accordance with testing NE's effect on cells taken directly from depressed patients and controls, measurement of adrenergic receptor gene expression on the isolated cells could be done to determine if a difference in expression of these receptors is driving the lack of NE's ability to inhibit the stress-induced inflammatory response. Indeed, studies done on populations with inflammatory disorders have shown differences in

receptor expression at the cellular level. Freshly isolated peripheral blood mononuclear cells from children with rheumatoid arthritis showed increases in A1-AR mRNA expression, while the receptor was detected in cells from control children, suggesting that upregulation of this receptor may be driving some of the inflammation seen in this cohort (Roupe van der Voort et al., 2000). Interestingly, when the same two groups were exposed to a cold pressor stressor, NE levels were similar in the arthritis patients and the controls, but LPS-induced IL-6 levels produced by isolated cells was higher in the children with arthritis, further showing that measurement of plasma NE alone is not enough to determine differences in the response to NE in regards to inflammation. More similar to the results we saw in chapter 2, where the driving force mediating the shift in NE's inhibitory functions seemed to be attributed to downregulation of the B2-AR, a study in children with asthma, a condition with a large inflammatory component, showed that chronic stress was associated with decreased mRNA expression of the B2-AR on leukocytes of these children (Miller and Chen, 2006). Another study done on a population free of psychiatric illness, showed a correlation between downregulation of B2-AR gene expression in lymphocytes isolated from these individuals and increased scores of tension and anxiety using the Profile of Mood States (Yu et al., 1999), indicating that psychological states may effect B2-AR expression in a population otherwise free of psychiatric disease. Thus, for patients with increased inflammation and MD, it would be interesting to see if isolated immune cells taken at baseline and after a laboratory-induced stressor, also show a decrease in expression of the B2-AR, or in the balance of expression between the anti-inflammatory B2-AR and the proinflammatory A1-AR and A2-ARs.

Another potential future direction for these studies would be to examine the role of adrenergic receptor signaling mediators, such as GRKs and beta-arrestins. As mentioned in the previous section, families of these two molecules are involved in regulating the signaling that arises from B2-AR activation by stimulants such as NE, and studies have shown that GRKs are differentially expressed, at the protein level, in immune cells isolated from patients with inflammatory conditions, such as arthritis and multiple sclerosis (Lombardi et al, 1999; Vroon et al., 2005). Since both GRKs and beta-arrestins work together to regulate adrenergic receptor signaling, studies measuring both of these mediators, at the protein and mRNA levels, could be done on isolated cells from MD patients, to determine if any dysregulation is seen that could potentially explain the NE-mediated inhibition of stress-induced inflammation in some of these patients.

Finally, future studies should also examine any potential interactions between cortisol and NE in patients with MD and inflammation. Activation of the stress response involves increases in plasma cortisol levels, along with NE, due to activation of the HPA axis (Elenkov et al., 2000). Administration of cytokines has also been shown to stimulate the expression of corticotropin-releasing factor, adenocorticotropic hormone, and cortisol, all mediators released during HPA activation, and all of which have been found to be elevated in patients with MD (Miller et al., 2009). However, although patients with MD have elevated levels of the glucocorticoid (GC), cortisol, a decreased responsiveness is seen in these individuals, termed GC resistance, which is manifested by increased cortisol concentrations seen after administration of dexamethesone, a synthetic GC, during the dexamethesone-suppression test (Raison and Miller, 2003b; Miller and Raison, 2008; Miller et al., 2009). Essentially, negative feedback signaling of the system is

impaired, as well as GC-receptor function and insufficient GC signaling (Raison and Miller, 2003b). Since abnormalities in GC function and signaling may be driving some of the inflammation, due to a lack of its inhibitory role on innate immune cell function (Irwin and Miller, 2007) in patients with MD, it would be noteworthy to determine if there is some sort of interaction between GC-receptor function and signaling, and B2-AR function and signaling, since both have the role of inhibiting the inflammatory response. Since both GC's and NE are activated and released during stress, it is unlikely that they function independently of one another, and an abnormality in one may relate to an abnormality in the other.

The final endeavor for these studies would be to tease apart the mechanisms that are contributing to increased inflammation in patients with MD, either at baseline or during stress, and to determine why the inhibitory systems in the body are not functioning as they should. Longitudinal studies following those specific patients with MD that show decreased sensitivity to NE and/or GC signaling, in terms of a lack in suppression of their innate immune response, could determine whether these patients are also ones that are most susceptible to becoming either treatment-resistant, or to develop a co-morbid disease with an inflammatory underpinning, such as cardiovascular disease, metabolic syndrome, or cancer. By identifying these patients, we may potentially reduce premature mortality due to these illnesses, as targeted therapies relating to altered NE (or GC) signaling and increased inflammation in the patients may be more appropriate and efficient.

IV. Concluding Remarks

Although inflammation is a necessary and adaptive physiological response used by organisms to fight off infection and heal wounds, if left unchecked and unregulated, it can become maladaptive and even harmful to the host. For this reason, it is crucial that mechanisms are in place to inhibit the inflammatory response once it has accomplished its goal. Activation of the SNS and release of NE is one of the mechanisms used to control inflammation, as NE mostly acts in an anti-inflammatory manner. Thus, dysregulation in NE's signaling abilities, or abnormalities in adrenergic receptors that NE signals through, could lead to further inflammation that goes unchecked. Furthermore, data presented here indicates that increased inflammation can lead to a decrease in sensitivity to NE's inhibitory signals, potentially creating a vicious cycle of increased inflammation and a lack of SNS-mediated inhibition of inflammation. Chapter 2 provided some evidence for mechanisms that may be causing this dysregulation at the cellular level, while Chapter 3 introduced a potential decrease in sensitivity to NE in patients with MD. The next step would be to see if the in vitro findings from Chapter 2 can help explain the abnormalities seen in vivo in Chapter 3, tying the two studies together. Taken together, the data presented provides potential mechanisms that may be involved in regulation of the inflammatory response by the SNS, specifically by NE and B2-AR, as well as how it may become disrupted or less functional. These findings may help target novel therapies in the treatment of MD and the treatment of other inflammatory-based disorders that also show SNS dysregulation.

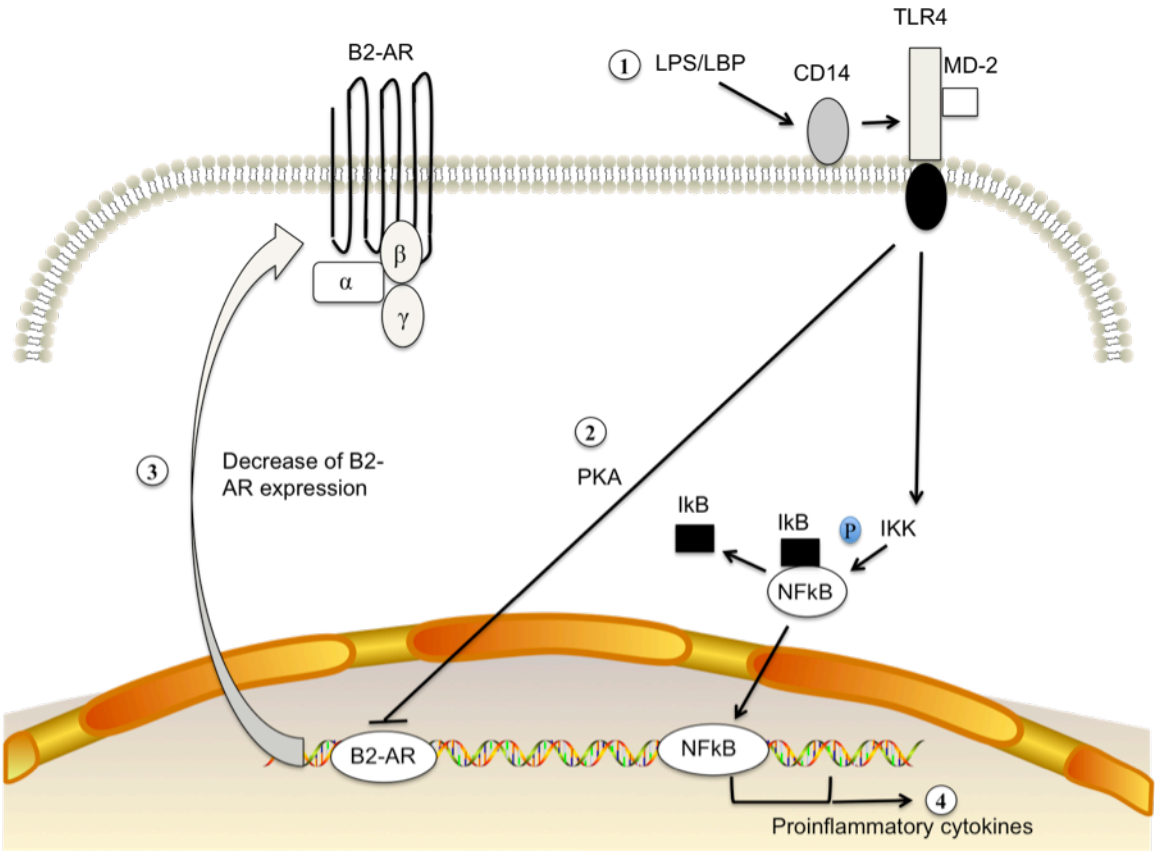

Figure 4-1. Potential mechanism for reduced sensitivity to norepinephrine in activated monocytes. 1) Activation of the monocyte by lipopolysaccharide (LPS) may lead to 2) increases in protein kinase A (PKA) activity. This increase in PKA activity leads to downregulation of the beta 2- adrenergic receptor (B2-AR) mRNA expression, which leads to 3) decreases in receptor expression and thus, norepinephrine's ability to signal through activation of the receptor. A reduction in the sensitivity to norepinephrine would lead to a decrease in its ability to inhibit inflammation, and LPS activation would lead to 4) more cytokine release. Since the process seems to be mediated by PKA, without the presence of norepinephrine, it is likely a result of heterologous desensitization of the B2-AR induced by LPS signaling.

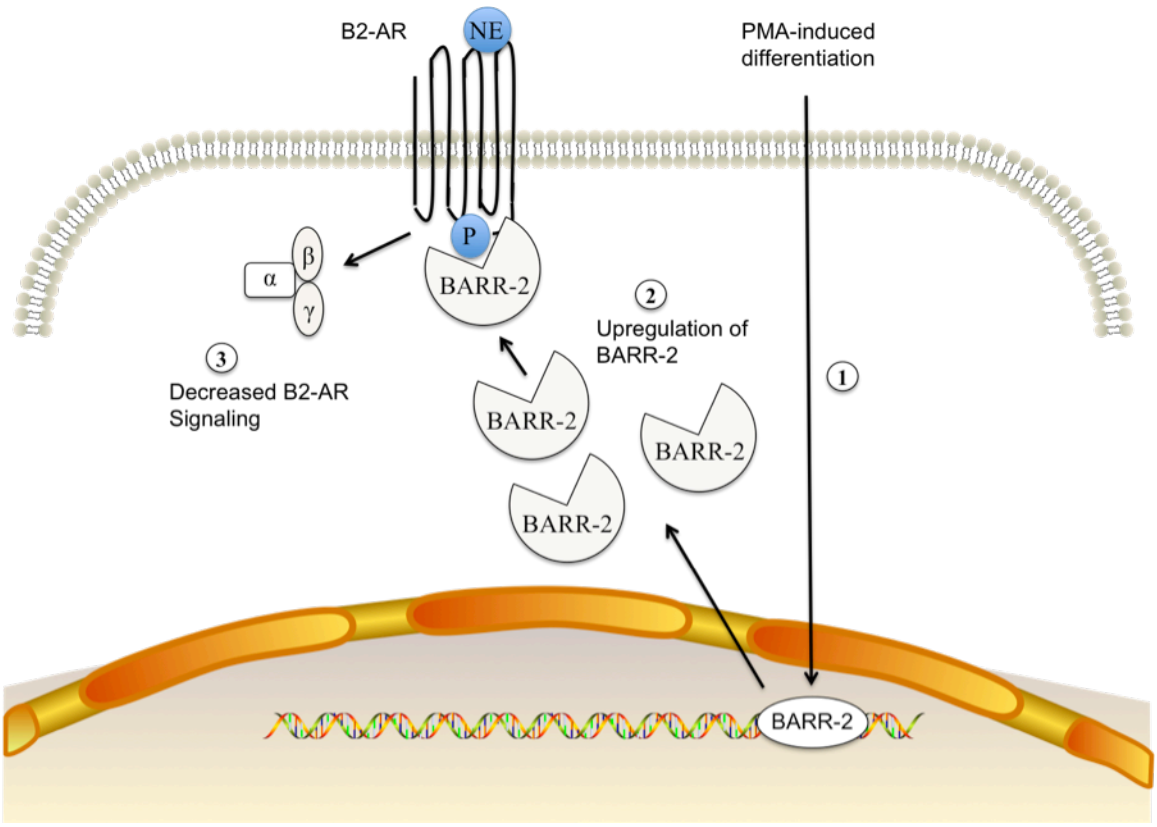

Figure 4-2. Potential mechanism for reduced sensitivity to norepinephrine due to cell differentiation. 1) Phorbol 12-myristate 13-acetate (PMA)- induced differentiation of a monocyte to a macrophage was shown to increase beta arrestin-2 (BARR-2) mRNA expression, which leads to 2) upregulation of BARR-2, increasing its ability to bind to the B2-AR, resulting in 3) decreased signaling by the receptor upon norepinephrine binding through the process of homologous desensitization.

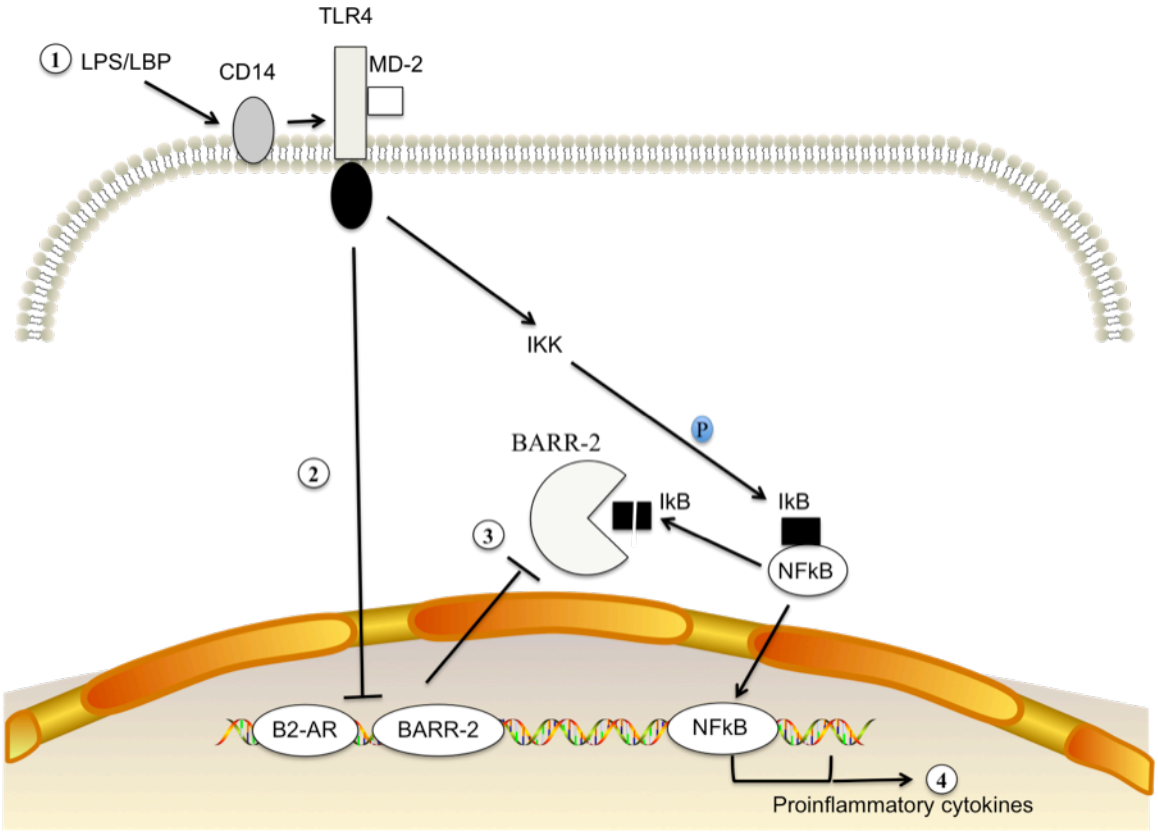

Figure 4-3. Potential mechanism for reduced sensitivity to norepinephrine in activated macrophages. 1) LPS-induced activation of a macrophage led to 2) decreases in mRNA expression of the B2-AR and BARR-2. Decreases in B2-AR mRNA expression can lead to decreases in receptor expression, thus decreasing norepinephrine's ability to signal through the receptor. A decrease in BARR-2 mRNA may result in less BARR-2 expression, which may lead to 3) a decrease in its ability to stabilize IkappaB (IkB), thus leading to increased degradation of IkB and activation of nuclear factor kappa B (NFkB), and 4) increased release of proinflammatory cytokines.

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