β_2-ADRENERGIC RECEPTOR MODULATION OF MACROPHAGE INFLAMMATORY MEDIATOR PRODUCTION

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Curriculum of Oral Biology.

Chapel Hill
2007

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ABSTRACT

E. AMANDA SNYDER: β_2- Adrenergic Receptor Modulation of Macrophage Inflammatory Mediator Production
(Under the direction of Dr. Patrick Flood)

Research has demonstrated that the complex interaction between the nervous system and the immune system plays a critical role in maintaining homeostasis. The nervous system is capable of modulating the immune response via activation of β_2-adrenergic receptors (β_2-ARs) present on immunocompetent cells. Because macrophages are major mediators of the immune response, several investigators have sought to determine the effect of β_2-AR stimulation upon inflammatory mediator production by these cells. Traditionally, scientists have regarded β_2-AR activity as anti-inflammatory since stimulation of these receptors inhibits LPS-induced production of inflammatory molecules. However, a thorough review of existing literature reveals several publications suggesting β_2-AR activation may actually have pro-inflammatory effects upon macrophage response. Importantly, β_2-AR drugs are often used to treat various diseases, including several diseases of inflammatory origin. As a result, recognizing the dual immunomodulatory potential of β_2-ARs is necessary to fully understand the inflammatory impact of β_2-AR drugs in therapy.

ACKNOWLEDGEMENTS

Though this thesis represents an individual work, I have benefited from many people who have served as a tremendous source of support, guidance and wisdom. First, I would like to thank the members of my committee, Dr. Patrick Flood, Dr. William Maixner and Dr. Luda Diatchenko for their guidance throughout the writing of this thesis. I would especially like to acknowledge my advisor, Dr. Patrick Flood, as I deeply admire his insight and supportive nature.

In addition to the academic support above, I am lucky enough to have received equally important assistance from my family and friends. I am particularly grateful to my wonderful family, James, Mary Ellen and Susan Snyder for their unconditional love, support and encouragement. I am also indebted to my friends, David Rouse and Sandra Shorter, for their generous support and remarkable proof-reading skills.

Together, each of these individuals provided me with valuable support and insight that has ultimately improved this thesis. And for that, I will be eternally grateful.

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LIST OF ABBREVIATIONS

α-AR	Alpha-Adrenergic receptor
Ab	Antibody
ANS	Autonomic Nervous System
AP-1	Activator protein-1
APC	Antigen presentation cell
AR	Adrenergic receptor/ Adrenoceptor
Arg	Arginine
Asn	Asparagine residue
Asp	Aspartate residue
ATF	Activating transcription factor
β-AR	Beta-Adrenergic receptor
bp	Base pairs
C/EBPβ	CCAAT/enhancer-binding protein beta
cAMP	Cyclic adenosine monophosphate
CAT	Cationic amino acid transporter
CBP	CREB-binding protein
CNS	Central Nervous System
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein

CSIF	Cytokine synthesis inhibiting factor
Cys	Cystine residue
Da	Daltons
DCs	Dendritic cell
eNOS	Endothelial nitric oxide synthase
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
ETS	E-twenty-six transcription factor
GEF	Guanine nucleotide exchange factor
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GPCR	G protein-coupled receptors
GRK	G protein-coupled kinase
h	Hour/hours
HPA	Hypothalamic-Pituitary-Adrenal
ICE	IL-1β converting enzyme
IFN-γ	Interferon gamma
IKK	IkappaB kinase
IL-1	Interleukin-1
IL-10	Interleukin-10

IL-1β	Interleukin-1 beta
IL-3	Interleukin-3
IL-6	Interleukin-6
Ile	Isoleucine
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinases
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF/CSF-1	Macrophage colony-stimulating factor
MD-2	myeloid differentiation protein-2
MHC	Major histocompatibility complex
min	Minute/minutes
MIP-α	Macrophage inhibitory protein-1 alpha
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NK	Natural killer cell
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMCs	Peripheral blood monocytes

PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
PNS	Peripheral Nervous System
RES	Reticulo-endothelial System
ROS	Reactive oxygen species
SCF	Stem cell factor
Ser	Serine residue
SNP	Single nucleotide polymorphisms
SNS	Sympathetic Nervous System
STAT 1	Signal transducers and activator of transcription 1
TCR	T cell receptor
Thr	Threonine
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor-alpha receptor
TRAF 6	TNF-receptor associated factor

CHAPTER I: IMMUNE – NERVOUS SYSTEM INTERACTION

For centuries, the complex interaction between the immune system and the nervous system has been a key area of interest for researchers and clinicians alike. Over time, an abundance of evidence has accumulated suggesting that these two systems are tightly integrated and communicate extensively with one another [18, 20, 34, 45]. This interaction results in a functional "cross-talk" between the immune and nervous systems that is critical for maintaining homeostasis [18, 34, 35, 40, 45, 50]. Indeed, numerous lines of research have demonstrated that these two systems communicate via a multifaceted network, and that this communication is not only important during periods of health but also during disease progression [19, 20, 33, 45, 50].

Historical Perspective of Immune – Nervous System Interactions.

Though the belief that the mind influences physical illness has existed since the earliest days of medicine, the disciplines of neuroscience and immunology developed independently for a number of years. As a result, the first reports directly addressing the interaction between the immune system and the nervous system were not published until the early twentieth century. During this time, several seminal papers were published that helped

establish the alleged relationship between these two super-systems. These publications demonstrated that certain immune organs, such as the lymph nodes and spleen, are innervated by the nervous system independently of blood vessels [45, 60]. Scientists suspected that, by innervating organs of the immune system, the nervous system could gain intimate access to immune cells and influence their activity. Adding to this preliminary evidence, Oliver and Schafer published a paper in 1895 characterizing a dramatic increase in blood pressure following the injection of adrenal medullary preparations [18, 45, 47]. Importantly, the bioactive component in these preparations was identified by Otto von Furth as "suprarenin," which later became known as epinephrine – an important catecholamine of the nervous system [18, 45, 62]. Taken together, these early papers provided the necessary groundwork to encourage the theory that these two systems are interconnected.

During the early twentieth century, Loeper and Crouzon published the first paper of its kind to demonstrate the modulatory effect of the nervous system, specifically catecholamines, on the immune response. In this landmark paper from 1904, Loeper and Crouzon describe a noticeable leukocytosis, or increase in white blood cells, following the subcutaneous injection of epinephrine to humans [18, 41, 45]. In 1914, Frey et. al. published a paper that characterized this epinephrine-induced leukocytosis in greater detail. According to this publication, the administration of epinephrine in humans resulted in an early increase of blood lymphocytes followed by a delayed increase in granulocytes [18, 26]. Not long after these initial reports, Ishigami advanced the field by exploring the effect of the nervous system on immune response as it pertains to disease progression. While treating patients suffering from chronic tuberculosis, Ishigami noted a decrease in phagocytic activity of

leukocytes during periods of intense stress [18, 36, 45]. This was the first of many papers to investigate the effect of the nervous system and stress on disease pathogenesis.

By the 1950s, improved scientific methods allowed for more advanced research involving these interactions. Due to more sophisticated techniques for separating and identifying leukocytes, scientists were capable of reporting more detailed accounts of catecholamine-induced leukocytosis [15, 24, 64]. For instance, these techniques led to the identification of a specialized subset of lymphocytes termed "stress-lymphocytes," which accompanied the previously recognized increase in "normal" lymphocytes following exposure to epinephrine. These "stress-lymphocytes" were later described as large, granular lymphocytes that possessed natural killer activity [15]. Improved separation techniques also allowed scientists to determine which leukocyte subsets were primarily responsible for this well-documented, catecholamine-induced leukocytosis. As it turns out, natural killer (NK) cells make up the largest subset of cells followed by CD8+ T cells. Catecholamine-induced increases in CD4+ T cells and B cells, though present, are not as dramatic [45].

As the characterization of catecholamine-induced leukocytosis advanced, the question of whether or not this phenomenon could be induced by endogenous sources received added attention. Early publications investigating the effect of acute physiologic and psychological stress upon the number and distribution of immune cells described a pattern similar to that of exogenously administered epinephrine. Based on these physiologically relevant observations, scientist gained confidence in the ability of endogenous mediators released by the nervous system to modulate the immune response [45, 53]. Additionally, advancements in scientific technology led to studies exploring the effect of catecholamines and other neuroendocrine molecules on cellular immune functions such as proliferation, apoptosis,

cytotoxic activity, cytokine production, antibody release, migration, phagocytosis, etc [17, 27, 30, 31, 63, 64].

Initially, the majority of the work exploring the immune-nervous system connection centered around the modulatory properties of the nervous system with respect to immune response. During the late 1970s and early 1980s, interest in the ability of the immune system to modulate the nervous system became more apparent. It was during this time that Besedovsky and colleagues published several important papers demonstrating that the immune and nervous systems communicate in a "bi-directional" manner [7, 9, 11, 12, 61]. As the field progressed, increasing amounts of evidence demonstrated that inflammatory mediators, such as cytokines, prostaglandins and chemokines, released by immunocompetent cells are able to influence various activities of the nervous system [7, 8, 10, 14, 61]. This collection of work established the concept that a biologically functional "cross-talk" exists between the nervous and immune systems.

As indicated by this brief historical review, there has been significant interest in understanding the complex interaction between the immune system and nervous system for many years. Currently, much of the work in this field is focusing on the effect of the nervous system upon immune response and disease progression. There is a great deal of attention directed toward investigating the effect of catecholamine-induced immunomodulation and the progression of various illnesses. These illnesses include not only neurodegenerative diseases such as Parkinson's and Alzheimer's Disease but also autoimmune diseases such as Rheumatoid Arthritis [19, 20, 33, 40, 45, 54, 56].

Nervous System Modulation of Immune Response.

Years of research have established several pathways to describe the complex communication network that exists between the nervous system and the immune system. As mentioned before, this integrative "cross-talk" is essential to maintaining homeostasis. If any of these pathways are interrupted, serious consequences may evolve. Indeed, disturbances in these routes of communication can directly influence various aspects of disease progression such as course, duration and severity [17, 19, 32, 52, 55].

There are many routes of communication linking the immune and nervous systems with one another as shown in Figure 1.1. This thesis, however, will focus primarily on the immunomodulatory effect of the nervous system upon immune response. More specifically, this paper will explore the effect of adrenergic modulation on the inflammatory response of macrophages. Nevertheless, it is important to recognize the multi-factorial communication network employed by the nervous system to modulate the immune response. There are two primary mechanisms by which the nervous system sends signals to the immune system: (1) neuroendocrine hormones released via the hypothalamic-pituitary-adrenal (HPA) axis and (2) adrenergic catecholamines released via the autonomic nervous system (ANS). These mechanisms allow the nervous system to communicate with the immune system not only on the local level at various sites of inflammation but also on a systemic level throughout the entire body [18, 32-34, 52].

For the past 50 years, scientists have recognized the effects of the HPA axis on the immune system. It is widely accepted that the HPA axis is the primary regulator of the hormonal stress response. It is also well known that glucocorticoids are the primary effector molecules of the HPA axis [17, 29]. These glucocorticoids interact with certain cells of the

immune system via glucocorticoid receptors [18-20, 34]. Research has shown that stimulation of glucocorticoid receptors can regulate a variety of immune cell functions including differentiation, proliferation, cytokine production, immune-cell trafficking and migration [3-5]. Although the importance of the HPA axis in modulating the immune system is undeniable and deserves a more detailed discussion, the remainder of this thesis will focus on catecholaminergic modulation of the immune response via the ANS.

The ANS, specifically the sympathetic division, is another well-known pathway of communication employed by the nervous system to modulate the immune system. In recent years, research has provided ample evidence to substantiate the role of the sympathetic nervous system (SNS) in modulating the immune response. Several studies have revealed that the SNS innervates both primary and secondary lymphoid organs, including the thymus, spleen and lymph nodes [1, 2, 23]. Upon stimulation, the SNS nerve fiber terminals release catecholamines directly into various lymphoid organs – allowing the nervous system to interact intimately with cells of the immune system. Importantly, existing data confirms that certain subsets of immune cells express receptors for and can be modulated by catecholamines [35, 38, 44, 51]. It is also known that catecholamines, circulating throughout the body, can stimulate and influence activity of immunocompetent cells [19, 45]. Based on the extensive communication network set forth by the SNS, the nervous system is capable of regulating the immune system locally at the site of inflammation, regionally within various lymphoid organs and systemically throughout the entire body.

Sympathetic Nervous System, Catecholamines and Adrenergic Immunomodulation.

The SNS is a division of the autonomic branch of the peripheral nervous system (PNS). As part of the ANS, the SNS and the majority of its activities are not under conscious control. To understand the actions of the SNS, it is imperative to have a basic understanding of its anatomic organization. Briefly, the SNS nerve fibers originate in nuclei located within the brain stem. These nuclei give rise to preganglionic efferent nerve fibers that exit the central nervous system (CNS) via the thracolumbar system. Most of the preganglionic fibers of the SNS terminate within the paravertebral chains, which lie on either side of the vertebral column. The postganglionic nerve fibers exit the paravertebral ganglia and go on to innervate peripheral tissues throughout the body [11, 18, 21]. The majority of postganglionic fibers release norepinephrine and are referred to as noradrenergic fibers [18]. A subset of preganglionic sympathetic nerve fibers terminates within the adrenal medulla, which house specialized cells known as chromaffin cells. These chromaffin cells, when stimulated by the preganglionic fibers of the SNS, release both epinephrine and norepinephrine [18].

As mentioned previously, catecholamines are important effector molecules of the nervous system. Epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine are the most abundant catecholamines of the nervous system [28, 45]. Dopamine is produced primarily by neuronal cell bodies located in the substantia nigra and acts as a neurotransmitter within the CNS [16, 49]. Epinephrine and norepinephrine can function as hormones or as neurotransmitters depending upon the situation. As hormones, both epinephrine and norepinephrine (to a lesser extent) are produced by chromaffin cells of the adrenal glands. During the stress response, epinephrine and norepinephrine are released into circulation by the adrenal gland in an approximate ratio of 4:1. Due to this epinephrine-

favored ratio, epinephrine is often considered the primary "stress" catecholamine of the "fight or flight" response [18, 28, 45]. Both epinephrine and norepinephrine act as neurotransmitters within the CNS. Norepinephrine is also a neurotransmitter of the PNS, specifically the SNS, and is released by noradrenergic nerve terminals. Norepinephrine found in the blood and tissues surrounding noradrenergic neurons is generally considered the result of its localized diffusion out of synaptic regions [21, 28].

As evidenced by the wealth of literature available, catecholamines, such as epinephrine and norepinephrine, are capable of modulating the immune response. These catecholamines are the endogenous ligands of specialized receptors known as adrenergic receptors (AR). These receptors are located on numerous cell types throughout the body, including cells of the immune system [19, 31, 45]. Studies have demonstrated that adrenergic stimulation of specific ARs by both endogenous and exogenous adrenergic agents can affect a variety of immune cell functions. The effect of catecholamines upon immune cell distribution and trafficking is the best known account of catecholaminergic modulation of immune cell function [18, 45, 64]. As the field has advanced, several publications have demonstrated the effects of epinephrine, norepinephrine and an assortment of adrenergic exogens on a variety of immune cell parameters. This is best illustrated by the recent increase in reports focusing on the ability of catecholamines and other adrenergic agonists to alter cytokine production [17, 18, 59]. However, contradicting reports have emerged with regard to this line of study as years of research have revealed that catecholaminergic modulation of cytokine production can be both pro- and anti- inflammatory in nature depending upon certain parameters. These parameters include not only the type of immune cell and

adrenergic receptor that is stimulated but also the duration of adrenergic exposure [32, 37, 45].

Adrenergic Immunomodulation in Health and Disease.

Though many theories have evolved to explain the exact role of catecholaminergic modulation upon the immune response, most would agree that this line of communication is a vital part of maintaining homeostasis. Indeed, the tightly regulated interactions between the immune system and nervous system are required for maintaining health as well as for preventing disease. Disturbances in this regulatory system lead to severe changes in the susceptibility and pathogenesis of a whole host of illnesses. As expected, research in this area has become increasingly important as understanding this interaction can lead to improved management of numerous diseases.

The initial studies exploring the effect of adrenergic immunomodulation took place in healthy subjects. As described earlier, this work focused primarily upon characterizing the effect of catecholamines upon immune cell mobilization during epinephrine-induced leukocytosis [18, 19]. Researchers also used immune cells from healthy subjects to identify and loosely characterize the effect of adrenergic stimulation on proliferation, differentiation, cytokine production and expression of various surface molecules [18, 25, 35, 38]. More revealing information was collected when scientists turned their attention toward identifying the physiologic significance of catecholaminergic modulation during pathological conditions such as systemic inflammation, hemorrhagic shock and sepsis.

Aside from their immunomodulatory activities, research has demonstrated that adrenergic agonists possess a wide range of physiologically relevant effects within the human body. For years, scientists have known that epinephrine and norepinephrine are involved in

regulating autonomic activities within the body, especially during the "fight or flight" response. It is also widely accepted that adrenergic stimulation of the appropriate AR can influence blood pressure, airway reactivity, metabolic function, etc. Based on these facts, adrenergic drugs have been used to selectively treat conditions such as asthma, hypertension, cardiac disease, severe burns, shock and sepsis [19, 45]. The idea of studying adrenergic immunomodulation during disease pathogenesis originally arose from the fact that adrenergic drugs are commonly used to treat chronically ill patients [33, 40]. The idea behind these studies was to determine whether or not exposing the body to adrenergic agents administered to treat other conditions would alter the inflammatory response and/or exacerbate disease state. For instance, many investigators have focused on defining the immunomodulatory effect of adrenergic stimulation during shock and sepsis. Aside from the fact that circulating catecholamine levels rise during instances of shock and sepsis, adrenergic drugs are often administered to stabilize certain conditions such as irregular cardiac function and blood pressure. Data show that, in septic patients, adrenergic stimulation is capable of modulating the release of both pro- and anti- inflammatory cytokines [18, 48]. In fact, it has been suggested that the anti-inflammatory nature of β-AR stimulation may be the cause of immune response dysregulation that is often noted late in septic shock [6, 48]. This research, along with research investigating other conditions that require the use of adrenergic therapy, has promoted awareness of the immunomodulatory consequences of administering exogenous sources of adrenergic agonists and/or antagonists during disease.

It did not take long for the field to recognize the importance of understanding the role of adrenergic modulation in regard to disease susceptibility. Early research demonstrated that aberrant sympathetic activity affects immune response, thus influencing the outcome and

susceptibility of various disease processes. Studies have shown that unregulated ANS function is associated with certain autoimmune diseases such as rheumatoid arthritis [13, 42]. For example, a study by Felten and colleagues demonstrated that chemical denervation of noradrenergic lymph node fibers resulted in increased inflammation and enhanced arthritic disease severity [22]. The systemic depletion of noradrenergic activity, on the other hand, led to decreased joint destruction in addition to decreased inflammation [19, 42, 46]. According to existing literature, noradrenergic innervation by the SNS has a dual modulatory effect on inflammation and subsequent disease progression [43]. Studies have also been done exploring the effect of exogenously administered catecholamines upon the initiation, progression and severity of various disease processes. Staub and colleagues explored the immunomodulatory effects of catecholamines upon inflammatory joint disease. Results from this research demonstrated that catecholamines released by the SNS exhibited a dual immunomodulatory profile. During the early stages of joint disease, catecholamines appeared be pro-inflammatory in nature. However, during late stage joint disease, catecholamines are capable of reducing inflammation. These data, in conjunction with other reports, suggest catecholamines and exogenously administered adrenergic drugs have varying effects on inflammation and disease progression [57, 58]. Therefore, recognizing the dual capabilities of adrenergic agents is very important when considering the efficacy of using adrenergic drugs as a form of therapy for treatment of any disease.

Summary.

Understanding the complexity of the "bi-directional" communication network that nature has established between the nervous and immune systems has been a topic of interest

for many years. The nervous system has several mechanisms by which it communicates with cells of the immune system. The immune system, in return, employs a variety of inflammatory mediators to communicate with the nervous system. Research has demonstrated repeatedly that this interaction is a vital part of maintaining homeostasis. This thesis will focus on the interaction between the SNS and the immune system. More specifically, this thesis will address the role of catecholamines in modulating the inflammatory response as it pertains to macrophage response. Nevertheless, it is important to acknowledge the complex interactions that exist between these two super-systems, and how these interactions influence the immune system during periods of health and disease.

FIGURE 1.1

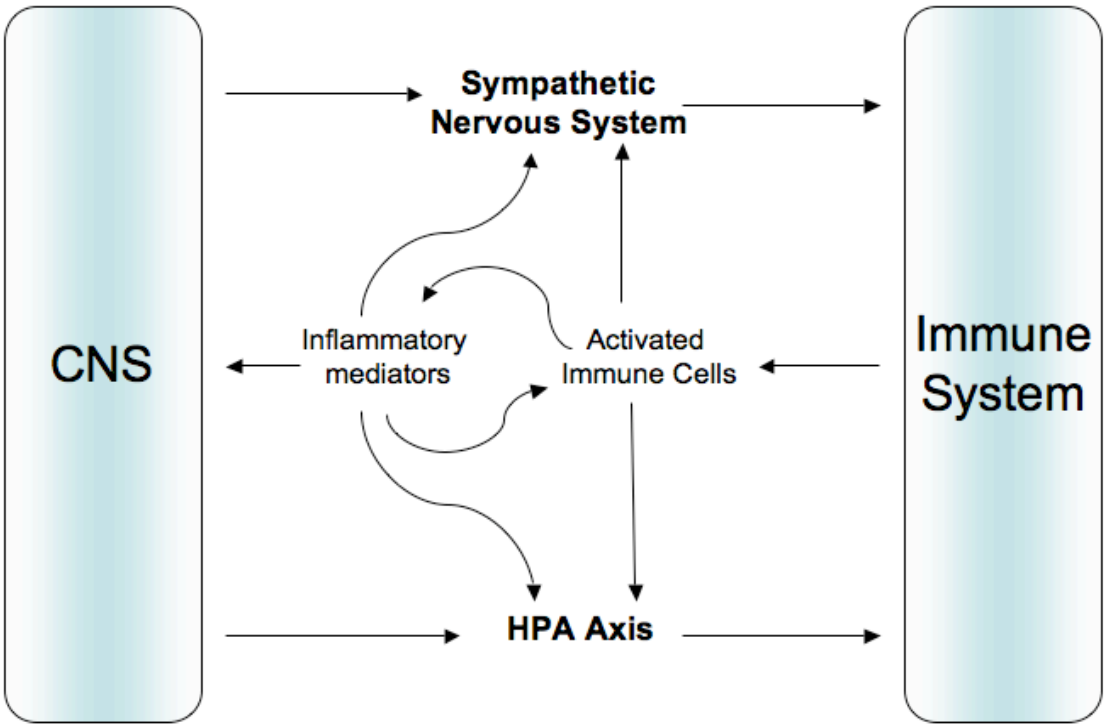

FIGURE 1.1 – Bi-directional Communication Network of the Immune and Nervous Systems. As shown by this diagram, these two super-systems interact via a complex, multi-faceted communication network. This biologically relevant "bi-directional" interaction is essential to maintain homeostasis [18, 39].

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CHAPTER II: β_2 – ADRENERGIC RECEPTORS

Adrenergic receptors (AR), also referred to as adrenoceptors, are a class of membrane-bound receptors that are widely dispersed throughout the human body. These receptors, whose endogenous ligands are epinephrine and norepinephrine, are found on a variety of cell types including cells of the immune system [5, 9, 22, 45]. These receptors are often considered the reactive components of the effector tissues in which they reside due to their response to signals received from postganglionic fibers of the SNS. In the case of immune cells, adrenergic stimulation can modulate cellular immune function, which ultimately influences the immune response [22, 71]. The contents of this chapter will provide a general overview of adrenoceptors, specifically β_2 -ARs.

Adrenergic Receptor Overview.

Adrenoceptors are a type of G protein-coupled receptors (GPCR) that bind to and are activated by catecholamines in addition to numerous exogenously administered adrenergic drugs. Aside from rhodopsin, ARs are the most extensively studied group of GPCR and are often used as a model to investigate GPCR signaling mechanisms [20, 76]. In 1948, Ahlquist

differentiated the AR family into two major classes, alpha (α) and beta (β) adrenoceptors, based on their pharmacological response to various adrenergic activating and blocking agents [1, 7]. Twenty years later, Lands and colleagues used techniques similar to those of Ahlquist to subdivide β-ARs further into β_1 and β_2 subtypes [41]. More recently, in the mid- to late- 1980s, a third subtype of β-AR was identified. This subtype is now known as the β_3-AR [35, 81]. During the 1970s, research demonstrated that α-ARs could also be broken down into two distinct subgroups. The initial distinction between α-AR subtypes was based entirely on anatomical location. Based on this classification scheme, α–ARs located on the pre- junctional synapse were termed α_2-ARs while α–ARs located on post-junctional synapses were called α_1-ARs. Experiments using adrenergic agonists and antagonists extended the division of α–ARs to include functional differences as well [8, 74]. As time progressed, advancements in the pharmacological tools available for studying drug-receptor interactions, such as radioligand-binding assays, led to the identification of additional subtypes of both α_1-ARs and α_2-ARs. The development of certain molecular biology techniques has also influenced the classification and description of the adrenergic subtypes. For instance, recombinant DNA and cloning techniques have been highly instrumental in identifying functional characteristics of AR subtypes. Additionally, the use of homologous mRNA hybridization techniques has advanced the field by aiding in the identification of AR subtypes located throughout the body [7, 12, 44]. Ultimately, as illustrated by Figure 2.1, the original classification system, as described by Ahlquist in 1948, has been refined and additional subtypes have been identified [43, 45]. The current consensus regarding the nomenclature of the AR family is as follows: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3 [7, 8].

Although primarily characterized by differences in function and distribution, research has demonstrated that members of the adrenergic family of receptors share several similarities with respect to structural properties. All of these receptor subtypes are composed of a single polypeptide chain that is approximately 400 to 500 amino acid residues in length. The primary structure of all AR receptors contains seven stretches of hydrophobic amino acid residues. These highly conserved hydrophobic stretches correspond to seven alpha-helical transmembrane regions that span the lipid bilayer of the cell upon which the receptor resides [9, 43, 44]. As a result, all ARs have three intracellular and three extracellular loops. Each AR possesses an extracellular N-terminal domain and an intracellular C-terminal domain. Both of these terminal domains are of variable length and sequence depending on AR subtype [44, 77]. The extracellular and transmembrane domains are responsible for forming and stabilizing the ligand-binding pocket. The intracellular regions associate with G-proteins, which are coupled to different signaling cascades [45, 63]. Activation of these second messenger systems can lead to a variety of outcomes including intracellular Ca^{2+} release, ion channel/pump activation, kinase activation, protein phosphorylation and gene transcription [31]. It is important to note, although similar in structure, each AR subtype varies not only in their specificity for certain ligands but also in their coupling to G-proteins and subsequent second messenger signaling systems. Moreover, recent data suggests that individual AR subtypes are not limited to one specific G-protein but can bind to multiple G-proteins or none at all to activate different signaling cascades [30, 79]. Based on their diverse nature, the adrenergic family of receptors is capable of mediating a wide range of physiologically relevant activities throughout the human body including the inflammatory immune response.

Distribution and Physiologic Relevance of Adrenergic Receptors.

As mentioned previously, ARs mediate the highly complex biologic functions of both epinephrine and norepinephrine. To do this, ARs are distributed extensively throughout the body [7]. Historically, each subclass of adrenoceptor has been primarily associated with a prototypic tissue as shown in Figure 2.2 [44, 77]. For instance, while β_1-ARs are considered adrenoceptors of the heart, β_2-ARs and β_3-ARs are most often associated with lung and adipose tissue respectively [77]. However, adrenoceptor subtypes are not limited to the tissues with which they are most often recognized. This fact is very important, especially for the purpose of this thesis, as ARs can be found on various subsets of immunologically competent cells [63].

Physiologically, the activation of these receptor subtypes is important in both health and disease. For instance, ARs are involved in maintaining proper blood pressure, myocardial contractile force and rate, and airway reactivity [9, 63]. In a very general sense, α-ARs mediate excitatory functions, such as vasoconstriction and pupil dilation. β-ARs, on the other hand, typically regulate inhibitory functions including vasodilation and bronchodilation. These excitatory and inhibitory guidelines do not always hold true. A prime exception to this rule is the excitatory role of β-ARs in regulating cardiac function [66]. During times of illness, adrenergic responses can be manipulated via the administration of adrenergic drugs to alleviate symptoms. Adrenergic therapy is most notably used for conditions such as asthma, angina and hypertension [35, 43]. Although manipulation of AR activity can be an effective means of treating many illnesses, recent research has shown that adrenergic therapy may exacerbate certain disease processes including rheumatoid arthritis,

chronic obstructive pulmonary disease and asthma [53, 79]. As a result, AR activation must be tightly regulated and carefully manipulated at all times.

Years of research have focused on exploring the role of ARs during periods of health and disease. In the past, due to limitations surrounding the availability of subtype-specific ligands, scientists were hindered in their ability to determine the precise physiologic role and therapeutic potential of each AR subtype. With the advent of gene-targeted mice, however, investigators are better able to identify the physiologic functions of each subset [13, 66, 74]. By combining the use of gene-targeted mice and available subtype-selective ligands, the ability to determine the biological effects of AR subtype stimulation has improved drastically. For example, because of these techniques, α_1-ARs are now recognized as the primary "cardiovascular regulators" of catecholamine activity. Indeed, members of the α_1-ARs subtype are involved in regulating cardiac growth and contractibility [58, 74]. Studies have also shown that α_1-AR subtypes are found throughout the vasculature of the body and are involved in regulating contractibility of blood vessels in response to catecholamines [14, 66]. Gene-targeted mice and subtype-specific adrenergic ligands have also been used to study the physiological role of α_2-ARs and β-ARs. Research investigating the physiologic role of β_2-ARs located throughout the respiratory tract has demonstrated that these receptors are highly instrumental in regulating airway reactivity. Based on the knowledge that β_2-AR activity results in dilation of the bronchial passages, β_2-AR agonists are often used to treat patients with asthma [2, 16, 80]. A detailed account of each subtype and its physiologic relevance is beyond the scope of this work and has been reviewed thoroughly by other authors [7, 14, 66]. Nevertheless, it is important to acknowledge the fact that ARs participate in a wide array of physiologically relevant functions. By understanding their role in health, it

is possible to manipulate the appropriate AR subtype in an attempt to treat and/or prevent certain disease processes.

β_2 – Adrenergic Receptor Overview.

Since understanding the β_2-ARs is a critical component of this thesis, the remainder of this chapter will address this particular subset of AR. The β_2-AR is perhaps the most studied member of the adrenergic family of receptors. With regard to physiological relevance, the β_2-AR is known to influence a variety of biological functions. Most often, β_2-ARs are associated with their role in regulating the smooth muscle of the airway and vasculature. Over the past three decades, the immunomodulatory properties of β_2-ARs have gained added attention. Importantly, adrenoceptors of the β_2 subtype have been identified on several immunocompetent cell types and are known to influence the inflammatory immune response of these cells.

Over time, research has determined that the gene for the human β_2-AR is located on the long arm of chromosome 5 (5q31 - 32) and codes for an intron-less gene product of approximately 1,200 base pairs (bp) in length [35, 47]. Upon translation, the β_2-AR is made up of 413 amino acid residues with a mass of approximately 46,500 Daltons (Da) [35, 42]. As shown in Figure 2.1, there are three major β-AR subtypes (β_1, β_2 and β_3); each of which has been identified through a combination of biochemical and pharmacological techniques [7]. More recently, evidence has accumulated suggesting there may be a fourth subtype (β_4), but additional research must be done to confirm these reports [35, 38]. Some studies suggest the β_4-AR is actually an artifact that represents a specialized state of the cardiac β_2- AR [2].

β_2 – Adrenergic Receptor Structural and Functional Domains.

The β_2-AR, like all GPCRs, is often referred to as a serpentine receptor because of the way they "snake" through the plasma membrane for a total of seven times. As illustrated in Figure 2.3, the β_2-AR possesses seven α-helical transmembrane-spanning domains, which give rise to three extracellular and three intracellular loops. Moreover, the amino-terminus of the β_2-AR is located extracellularly while the carboxyl terminus is found in the intracellular compartment of the cell [35].

There are several post-synthetic modifications that are important to note with regard to β_2-AR structure. The human β_2-AR possesses three sites of N-linked glycosylation located at amino acids 6, 15 and 187. Though the role of these N-linked sugars has not been fully defined, research has indicated these sugars are not involved in ligand-binding [18, 75]. Several lines of research have demonstrated the presence of these carbohydrate moieties are important for receptor trafficking as their absence results in reduced β_2-AR expression at the cell surface [10]. These post-translational modifications are believed to be important not only for proper insertion of the receptor into the membrane but also for receptor trafficking following agonist exposure [35, 59, 67]. Another important modification of the human β_2-AR is the palmitoylation of the cysteine residue located at position 341. It is believed that this palmitoylated cysteine residue serves to anchor the carboxy-terminus to the plasma membrane [62]. Furthermore, research has demonstrated that palmitoylation of this residue contributes to the ability of the receptor to stimulate adenylyl cyclase following ligand-receptor interaction [60, 62]. The region located between the palmitoylated cysteine and the final transmembrane-spanning region is α-helical in structure and is often referred to as the fourth intracellular loop [35]. Disulfide bonds are the final post-synthetic modification of

interest. Studies have revealed that four extracellular cysteine residues ($Cys^{106, 184, 190, 191}$) are capable of participating in disulfide bond formation. These disulfide linkages are thought to play an important role in stabilizing the ligand-binding pocket of the β_2-AR. Research employing amino acid residue substitution and site-directed mutagenesis techniques has demonstrated that alterations in any one of the four extracellular cysteine residues mentioned above result in lowered ligand binding and/or agonist-receptor specificity [19, 24, 68].

Many of the structural properties of the β_2-AR mentioned above are important contributors to receptor function. One of the major functional domains of the β_2-AR is the ligand-binding pocket, which is formed by the seven α-helical transmembrane domains. As indicated earlier, the cysteine residues of the extracellular domains help stabilize this binding pocket via the formation of disulfide bonds. Other than aiding in stability, the extracellular domains play a minimal role in ligand binding [9, 10, 13, 30]. Furthermore, the interruption or removal of the amino- and/or carboxy- terminal domains has little effect upon receptor-ligand binding [18, 70]. The current model describing the active site of the ligand-binding domain has it located approximately one-third of the way into the receptor core [35]. The use of site-directed mutagenesis has identified several important residues critical for ligand binding within the active site. As shown in Figure 2.3, these residues are as follows: the aspartate residue located in the third transmembrane domain (Asp^{113}); the two serine residues found in the fifth transmembrane spanning region (Ser^{204} and Ser^{207}); the asparagine residue in the sixth domain (Asn^{293}) [35, 51]. These four amino acid residues are thought to form bonds with the functional groups of the β_2-AR ligand and anchor the ligand within the receptor core [68, 78]. At this point, it is important to remember that ligand-receptor interaction is also influenced by the structure of the β_2-AR ligands themselves [35, 68].

The intracellular domains of the β_2-AR give also rise to several important functional domains. These domains are involved in G-protein coupling, phosphorylation reactions and receptor desensitization. As shown in Figure 2.3, the amino acid residues of the amino- and carboxy- regions of the third intracellular loop and the amino-portion of the carboxyl terminus are involved in coupling to G-proteins [35, 68]. The cytoplasmic regions are also involved in functional regulation of β_2-ARs signaling. Certain residues located throughout these intracellular regions are involved in receptor desensitization. Receptor desensitization, in this case, refers to the process by which the functional interaction between the β_2-AR and important molecules of the signaling cascade becomes impaired. The purpose of receptor desensitization is to prevent overstimulation of the β_2-AR in the presence of excessive receptor ligand. This process occurs via receptor phosphorylation, receptor internalization and/or receptor uncoupling. Importantly, receptor uncoupling refers to the uncoupling of the β_2-AR and its second messenger system. Phosphorylation of β_2-AR can occur at various serine and threonine residues located throughout the third intracellular loop and the carboxy-terminal domain [15, 35]. Several kinases are involved in β_2-AR phosphorylation and desensitization. Protein kinase A (PKA) is perhaps the most recognized kinase involved in this process. G protein-coupled receptor kinases (GRKs) can also play a role in β_2-AR desensitization [4, 52]. It is important to note that, while both PKA and GRKs lead to receptor desensitization, they each do so in a different manner. For instance, GRKs can only phosphorylate ligand-bound receptors and require an accessory protein, known as β-arrestin, for desensitization [46, 52]. PKA, on the other hand, can phosphorylate the β_2-AR in the absence of ligand. Moreover, PKA does not require an accessory molecule and can directly impair β_2-AR activity [31]. Increased phosphorylation by PKA or GRK plays an important

role in agonist-induced receptor uncoupling. This receptor uncoupling initially leads to rapid desensitization and translocation of the β_2-AR into endocytic vesicles. Within the endocytic vesicles, the receptors are typically dephosphorylated and recycled back to the cell surface. With time, some receptors fail to be recycled and are sorted into lysozomal vesicles where they are degraded. This, in conjunction with decreased gene transcription, leads to a noticeable downregulation of β_2-ARs on the cell surface [4, 31, 37].

Signaling mechanisms of the β_2 – Adrenergic Receptor.

To better understand the effects of β_2-AR stimulation on macrophage response, it is important to have a basic understanding of β_2-AR signaling mechanisms. The accepted dogma surrounding β_2-AR signaling states that β_2-AR activation leads to the increase in cyclic adenosine monophosphate (cAMP). As shown in Figure 2.4, this increase is due to the activation of adenylate cyclase by the α-subunit of the receptor-associated G_s-protein. Once activated, adenylate cyclase catalyzes the conversion of adenosine triposphate into cAMP. At this point, the increase in cAMP leads to the stimulation of PKA [35, 51, 68]. Though the majority of β_2-AR mediated signaling occurs via G_s-proteins and subsequent cAMP-dependent mechanisms, there is evidence of other signaling schemes [30, 51, 77, 79]. The most notable alternative signaling pathway is the G_i-dependent pathway that results in the activation of the mitogen-activated protein kinase (MAPK) pathway [3, 55, 79]. This G_i-dependent pathway requires the phosphorylation of the β_2-AR by PKA and is mediated by the $\beta\gamma$-subunit of the associated G-protein. This subunit, along with β-arrestin, serves as a scaffold for other signaling molecules such as SOS, cSrc, RAS and Raf [17, 31]. Recent data suggests that the MAPK pathways can also be activated by G_s-dependent mechanisms. This

signaling pathway is complex but ultimately leads to MAPK activation via the B-raf signaling cascade [21, 33, 79]. Additionally, some studies have shown that β_2-AR signaling can occur via G-protein independent mechanisms [30, 79]. Without a doubt, the complexity of the β_2-AR signaling mechanisms is mirrored by the diverse role of these receptors. Additional discussion regarding β_2-AR signaling that is specific to macrophage immunomodulation will be discussed in chapters four and five.

β_2 – Adrenergic Receptor Ligands: Agonist, Antagonists and Inverse Agonists.

Traditionally, the "lock and key" theory of receptor-ligand binding was used to describe agonist activation of β_2-ARs. This theory proposed the β_2-AR agonist would bind the receptor in such a way that the receptor would adopt a conformation that is better suited to associate with G_s [51, 57, 61]. Recent research, however, suggests that the receptor actually "toggles" between different conformational states in the absence of β_2-AR ligand [64]. Indeed, several lines of research indicate that GPCRs, like β_2-ARs, may be "active" even in the absence of receptor agonist [73]. Under resting conditions, the active and inactive states are in equilibrium with the inactive state predominating. β_2-AR agonists are believed to exert their effects by binding to and stabilizing the active form of the receptor. Antagonists of the β_2-AR, on the other hand, preferentially bind to the inactivated form of the receptor – thus moving the equilibrium further away from the active form of the β_2-AR [36, 57]. Technically, β_2-AR agonists and antagonists should not be thought of as competing for the same receptor. Instead, these ligands bind to different forms of the β_2-AR and shift the receptor confirmation equilibrium in their favor.

As described earlier, studies using site-directed mutagenesis techniques have identified important regions of the β_2-AR protein for ligand-binding. Based on these studies, a model has emerged that places the ligand-binding domain within the hydrophobic core of the β_2-AR protein [33, 48 64, 74]. The molecular structure of the β_2-AR ligand determines the way in which it interacts with the receptor and its binding domain. For instance, hydrophilic agonists are able to access the β_2-AR binding site directly from the aqueous extracellular region. These hydrophilic agonists are often referred to as "short-acting" agonists due to their direct access and rapid onset of action. Those agonists that are referred to as "long-acting" are typically lipophilic and are readily taken up into the cell membrane. Once within the cell membrane, the β_2-AR agonist slowly leaches out into the active site of the receptor [35, 36]. Furthermore, like most biological systems, β_2-ARs are stereo-specific. In recent years, several studies have shown that stereo-specificity influences ligand-binding and functional responses of β_2-ARs. According to most data, activity of β_2-AR ligands lie primarily in the R-enantiomer [34, 37]. Importantly, current research suggests that stereoselectivity is an influential determinant regarding the immunomodulatory effect of β_2-AR stimulation upon macrophage response [34, 36].

As the concept of a spontaneously active β_2-AR evolved, so did the ideas surrounding classification of receptor ligands. Adrenergic drugs were originally classified as full agonists, partial agonists or antagonists. Based on the idea that there are multiple states of receptors, this classification system seemed over-simplified [39]. As a result, the idea of inverse agonism developed. Inverse agonism is defined as the ability of a ligand to reduce the basal level of signaling activity following receptor-ligand binding. Prior to the introduction of inverse agonists, antagonists were believed to have no effect of their own.

Instead, these ligands functioned only to prohibit agonist activation of the receptor. As techniques advanced, scientists were able to show that some adrenergic agents are capable of reducing basal β_2-AR activity. Subsequently, these drugs were placed in the inverse agonist category of β_2-AR ligands [11, 65]. With the increased knowledge regarding inverse agonists, the classification system used to identify receptor ligands has been modified as follows: full agonist, partial agonist, full inverse agonist, partial inverse agonist and antagonists. Based on the current classification system, a β_2-AR antagonist is one that is unable to modify constitutive receptor activity alone but is able to block β_2-AR agonist-induced activity [65]. Finally, it is important to note that the concept of inverse agonism is relatively new. As a result, very little research has explored this concept with respect to the immunomodulatory properties of this class of adrenergic drugs.

β_2 – Adrenergic Receptor Gene Polymorphisms.

As mentioned earlier, β_2-ARs modulate numerous physiologically relevant events in the human body. In recent years, research has identified several polymorphisms of β_2-ARs. Studies have shown that some of these polymorphisms affect the biological responses of β_2-ARs [49]. With regard to clinical relevance, these polymorphisms are believed to influence airway responsiveness and bronchodilation following exposure to β_2-AR agonist. It is also possible that these polymorphisms influence the modulatory properties of β_2-AR with regard to immune response.

Because there are two genes for the β_2-AR, an individual can be either heterozygous or homozygous for a particular polymorphism. A total of nine different single base substitutions have been identified in the coding region of the β_2-AR. Due to the redundancy

of the genetic code, five of these polymorphisms are degenerate and are often considered "clinically silent" [25, 48, 69]. However, several papers have been published in recent years suggesting that these silent (or synonymous) mutations may actually influence receptor function [6, 48, 49, 50]. Research has shown that the remaining four polymorphisms result in alterations of the β_2-AR amino acid sequence. Three of these polymorphisms are known to influence β_2- AR functionality both *in vivo* and *in vitro* [6, 48]. These polymorphisms are located at position 46 (Arg16Gly), 79 (Gln27Glu) and 491 (Thr164Ile) within the coding region of the β_2-AR [6, 50]. The fourth SNP located at position 100 (Val34Met) is extremely rare and is regarded as having minimal (if any) effect upon receptor function. As a result, very little research has been done exploring this particular polymorphism [6, 48, 49].

Early studies investigating β_2-AR polymorphisms focused on the single nucleotide polymorphism (SNP) located within codon 16. As shown in Figure 2.3, this polymorphism is found in the extracellular amino terminus of the β_2-AR and results in either arginine or glycine (Arg → Gly) at residue position 16. *In vitro* studies using site-directed mutagenesis of the wild type (wt) β_2-AR have demonstrated that receptors with the Arg → Gly polymorphism do not exhibit altered binding of receptor ligand or activation of adenylate cyclase [28]. However, this polymorphism appears to influence receptor desensitization and trafficking. Following exposure to receptor agonist, the Gly 16 form of β_2-AR is downregulated to a greater degree than the Arg 16 variant [69]. Clinically, the Gly 16 receptor polymorphism has been linked to increased airway reactivity [32]. Furthermore, several studies have focused on examining the effect of the Arg16Gly polymorphism on cardiac responsiveness. For years, it has been accepted that stimulation of human β_2-ARs increases hearth rate and contractility. Data exploring this topic indicates that there is no

difference with respect to heart rate and contractility between individuals with wt β_2-ARs and those with Gly 16 β_2-ARs [48, 49]. Although the Arg 16 variant is considered the wt receptor, the occurrence of the Gly 16 form is higher in Caucasians. The allelic frequency is 35% and 65% for the Arg 16 and Gly 16 variants respectively [27, 35].

The second SNP of interest is positioned within codon 27 of the β_2-AR. This polymorphism results in a receptor possessing either glutamine or glutamate (Gln → Glu) as the 27th amino acid residue [35]. The Gln27Glu polymorphism is located within the N-terminal domain as shown in Figure 2.3. The allelic frequencies of the Gln 27 (55%) and Glu 27 (45%) variants are quite similar. *In vitro* studies have demonstrated that the Gln → Glu polymorphism, like the Arg → Gly polymorphism, does not affect ligand binding or adenylate cyclase activity [27, 28]. Like the Gly 16 variant, the Glu 27 form appears to influence receptor downregulation. However, studies indicate that the effect of the Glu 27 variant is opposite to that of the Gly 16 variant. Research comparing the Gln 27 and Glu 27 variants of the β_2-AR has shown that the Glu 27 form protects against downregulation of β_2-AR expression following exposure to antigen [29, 35]. Clinical studies have demonstrated that subjects with the Glu 27 β_2-AR variant possess 4 times less reactive airways than those with the Gln 27 form [35].

The third, and final, polymorphism of significance is located at amino acid site 164, which can be either the threonine (Thr) or isoleucine (Ile). This polymorphism, unlike the Gly 16 and Glu 27 variants, is very rare. In fact, the allelic frequency of the Thr → Ile polymorphism is approximately 1% [48]. As shown in Figure 2.3, the Thr164Ile polymorphism is located within the fourth transmembrane domain. Notably, the Thr → Ile polymorphism lies adjacent to the serine (Ser) residue located at site 165. Because this Ser

residue is believed to interact with the hydroxyl group (OH) of β_2-AR ligands, the Thr164Ile polymorphism is thought to influence ligand binding [35]. Studies comparing the Thr 164 and Ile 164 variants demonstrated that Ile 164 receptors exhibit a four-fold lower affinity for various β_2-AR ligands when compared to Thr 164 variants [25]. Additionally, the Thr164Ile polymorphism appears to have an effect on adenylate cyclase activation. Research has shown that the Ile 164 variant exhibits a decreased level of basal and ligand-induced adenylate cyclase activity [25, 26, 35]. Unfortunately, there is very little data exploring the physiological effect of this polymorphism due to the scarcity of the Ile 164 variant within the human population [29, 35].

It is also important to recognize that though these polymorphisms have received much attention, there is a great deal of conflicting data regarding their physiological relevance. This inconsistency is believed to be the result of the linkage disequilibrium that exists with respect to the β_2-AR polymorphisms. It has become evident these polymorphisms are linked and give rise to certain haplotypes. For instance, subjects that are homozygous for Glu 27 are almost always homozygous for Gly 16. Indeed, the Arg16Arg/Glu27Glu haplotype exists in nature but makes up less that 1% of the population [29, 72]. Many of the *in vitro* studies investigated only one polymorphism at a time. As a result, these studies did not account for the combined effect of multiple polymorphic loci. Also, certain haplotypes are more commonly associated with specific races [6, 29].

As indicated above, the β_2-AR polymorphisms are capable of influencing receptor activity. Without a doubt, these polymorphisms affect β_2-AR function *in vitro*. The *in vivo* effects of the various β_2-AR polymorphisms, on the other hand, have been more difficult to determine. To date, the majority of the research focusing on the physiological relevance of

these SNPs indicates that β_2-AR polymorphisms do not alter disease susceptibility and are, therefore, not "disease-causing." Instead, it is believed that these polymorphisms modify disease progression [6]. It is also believed that β_2-AR polymorphisms influence receptor response to various adrenergic agents. Understanding the effect of these polymorphic variants upon adrenergic therapy is vital to improving current therapeutic techniques. Nevertheless, due to the variation associated with β_2-AR haplotypes, large populations must be studied to fully assess the physiological relevance of these polymorphisms [48].

Inflammatory Role of β_2 – Adrenergic Receptors.

Stimulation of ARs via endogenous and exogenous ligands can influence immune response. Over the years, research has indicated that the β_2-ARs, in particular, play a major role in adrenergic immunomodulation [37, 63, 71]. Importantly, β_2-ARs have been identified on several immune cell subsets including T cells, B cells, mast cells and macrophages [40, 54, 56, 63]. As shown in Figure 2.5, stimulation of β_2-ARs on these immune cells are capable of influencing cellular function and ultimately the immune response. Historically, β_2-AR immunomodulation was considered to be anti-inflammatory in nature. However, most of the data exploring the role of β_2-ARs in adrenergic immunomodulation has been *in vitro* and *in vivo* data remains inconclusive [63, 71]. As indicated by the sheer complexity of β_2-AR functionality, it is possible that β_2-AR activation has a pleiotropic effect on immune response and inflammation. Figure 2.5 indicates that the immunomodulatory effect of β_2-AR with regard to macrophage function remains debatable. Later, in chapters four and five, this thesis will explore the dual immunomodulatory property of β_2-AR stimulation of macrophages.

Summary.

The adrenergic family of receptors is quite complex and is composed of several receptor subsets. These receptors are ubiquitously expressed and are involved in a variety of physiologically relevant functions. The β_2-AR is the most studied member of the adrenergic family of receptors. In fact, the β_2-AR is often considered the prototypical adrenoceptor with regard to receptor structure. Studies exploring β_2-AR function have demonstrated the diverse nature of the receptor. Signaling and functional responses of the β_2-AR vary in response to a several factors including cell type, ligand structure, duration of ligand exposure, etc. In the past three decades, increasing amounts of evidence have suggested that β_2-ARs play a major role in adrenergic modulation of the immune response. The ultimate outcome of β_2-AR mediated immunomodulation remains debatable, especially with regard to macrophage function. In later chapters, this thesis will address the discrepancy surrounding the impact of β_2-AR immunomodulation on the inflammatory response of macrophages.

FIGURE 2.1

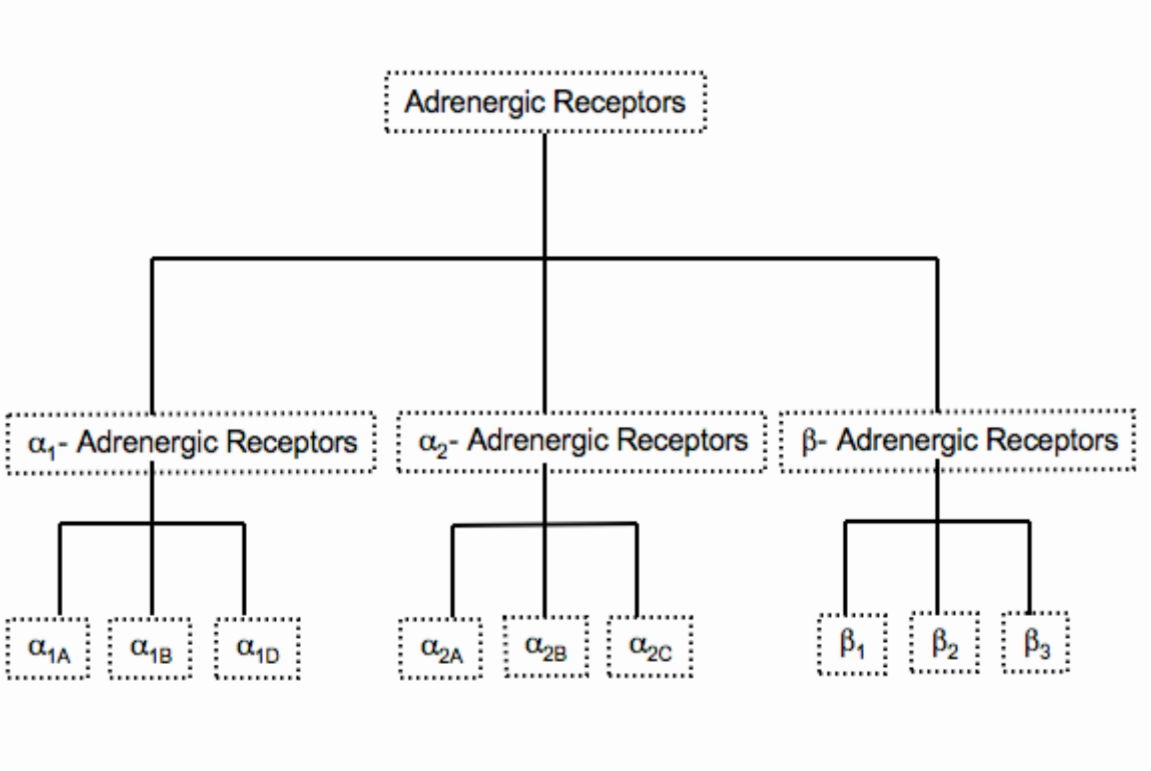

FIGURE 2.1 – Classification of Adrenergic Receptors. Adrenergic receptors can be broadly classified into two major categories – alpha (α) and beta (β) adrenoceptors. These receptor classes can be broken down further into several subtypes as illustrated above [7, 8, 40, 79].

FIGURE 2.2

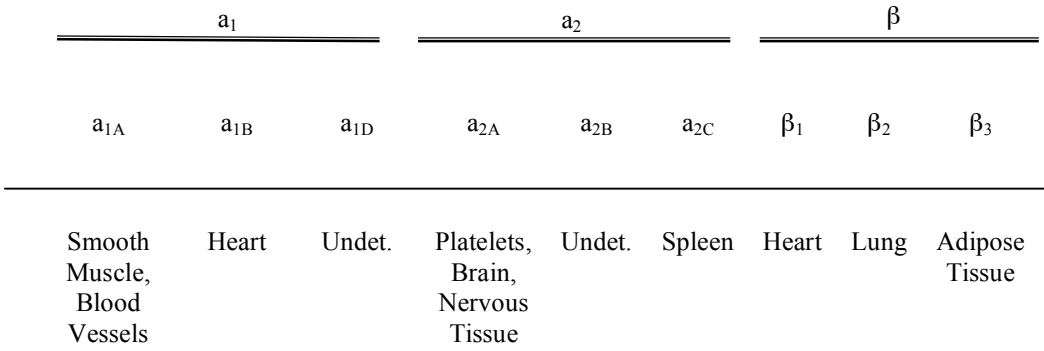

FIGURE 2.2 – Prototypic Tissue Distribution of Adrenoceptors. Historically, adrenergic receptors have been associated with a prototypic tissue distribution. However, it is well known that adrenoceptors are not limited to a certain tissue or cell type and can be found on a variety of cells, including cells of the immune system [9, 63, 77, 79]

FIGURE 2.3

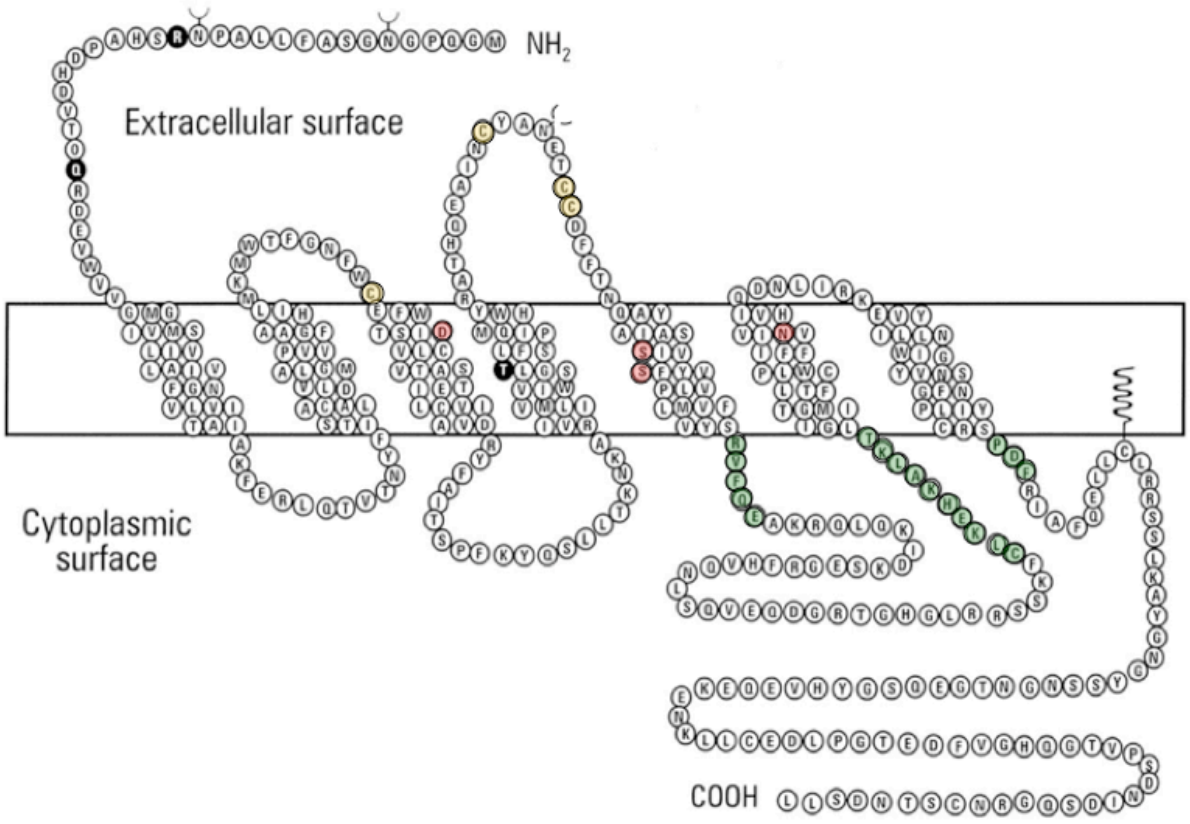

FIGURE 2.3 – β_2-Adrenergic Receptor Structure. The β_2-AR is a member of the GPCR family of receptors. As shown above, the β_2-AR has seven transmembrane-spanning regions that give rise to three intracellular and three extracellular loops. The N-terminal domain is located extracellularly, and the C-terminal is found intracellularly. The polymorphic sites are indicated in black. The yellow cysteine residues are involved in the formation of disulfide bonds, which are important for stabilizing the ligand-binding pocket. The four amino acid residues involved in ligand-binding are indicated in red. Finally, the amino acid residues involved in G-protein coupling are shown in green [23, 37, 63, 77].

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FIGURE 2.4

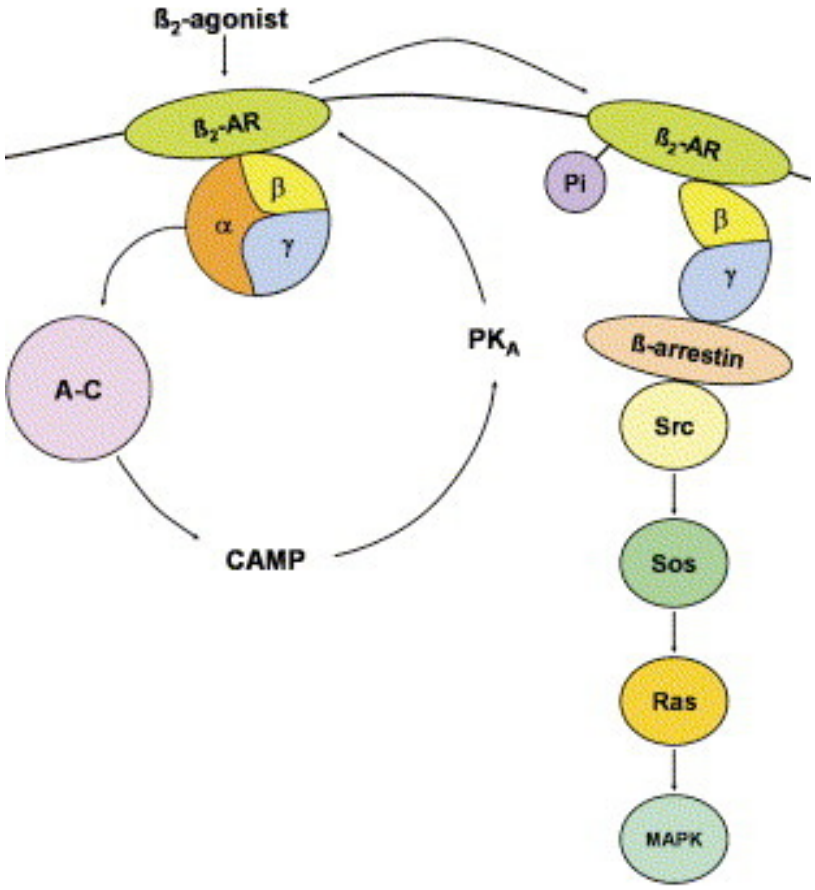

FIGURE 2.4 – β_2-Adrenergic Receptor Signaling. The accepted dogma of β_2-AR signaling results in the increase of cAMP. As illustrated above, research has demonstrated that signaling can occur via G_s or G_i mechanisms. The α-subunit of the G_s protein couples with adenylate cyclase (A-C). This leads to the upregulation of cAMP, which results in the activation PKA. PKA can go on to activate other signaling molecules leading to changes in cellular activity. PKA also phosphorylates the β_2-AR and causes the uncoupling of the receptor to G_s. The receptor then couples with the $\beta\gamma$-subunit of the G_i protein. The G_i pathway requires an accessory molecule (β-arrestin), which serves as a scaffold for the other molecules in the signaling pathway. It is important to note, that although not shown here, research has shown that β_2-AR signaling can occur via G-protein independent mechanisms as well [37, 40, 68, 79].

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FIGURE 2.5

Cell Type	Immunomodulatory Function
Mast Cells	↓ Release of histamine, leukotrienes, etc.
T Lymphocytes Type 1 (Th1) Type 2 (Th2)	 ↓ Synthesis and release of INF-γ, IL – 12 ↓ Release of IL – 3, GM – CSF
B Lymphocytes	↓ Secretion of IgE (?)
Macrophage	↓/↑ Secretion of IL – 1β, IL -6, etc (?)

FIGURE 2.5 – Immunomodulatory Properties of β_2-Adrenergic Receptor Stimulation of Immune Cells. The β_2-AR is commonly associated with its role in airway reactivity. However, in recent years, the role of β_2-AR with regard to adrenergic immunomodulation has gained a great deal of interest. Many immune cells are known to express β_2-ARs. Moreover, research has demonstrated that stimulation of these receptors can alter immune cell function, which influences immune response and inflammation [54, 71].

CHAPTER II: REFERENCES

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CHAPTER III: MACROPHAGES

To fully explore the effect of adrenergic modulation on macrophage inflammatory immune response, it is imperative to have a basic understanding of cells belonging to the mononuclear phagocyte system. Macrophages and macrophage-like cells are derived from a common precursor and are capable of carrying out a variety of cellular functions including phagocytosis, antigen presentation and inflammatory mediator production [14, 19]. Recently, numerous lines of research have focused on determining the effect of β_2-AR stimulation upon macrophage response. However, there is some debate over whether β_2-AR immunomodulation of macrophage function results in a pro- or anti- inflammatory response [6, 12, 30, 42]. By providing the background necessary to understand macrophage function, the contents of this chapter are designed to enhance comprehension of β_2-AR modulation of macrophage response during inflammation.

Macrophages and the Immune System.

The immune system can be divided into two components based on the degree of specificity. These two divisions are known as the innate and adaptive immune systems. Though these divisions vary with respect to specificity, both systems are capable of

distinguishing between self vs. non-self. Innate immunity is present at birth and serves as the body's first line of defense against foreign pathogens. The innate immune response is often referred to as "non-specific" immunity as it does not rely on antigen-specificity to mount a productive response. The adaptive immune response, on the other hand, is capable of recognizing, responding to and remembering specific pathogens. Unlike adaptive immunity, the innate immune response is unable to confer long-term, immunologic memory. The innate and adaptive immune systems interact extensively via cell-to-cell contact and through various inflammatory mediators. In fact, many cells and molecular components of the innate immune system are also employed by adaptive immunity. By working together, these two divisions provide valuable protection against numerous pathogens [14, 19, 32].

Macrophages belong to the mononuclear phagocytic family of immune cells, which was formerly known as the reticulo-endothelial system (RES). The mononuclear phagocytic family is made up of peripheral blood monocytes and tissue macrophages in their many forms. These cells share both a common precursor and similar morphology. As shown in Figure 3.1, macrophages arise from pluripotent haematopoietic stem cells located in the bone marrow. These pluripotent precursors further differentiate into monoblasts, monocytes and eventually mature to become tissue macrophages [14, 27, 32]. After leaving the bone marrow, the first cell of this family to enter peripheral blood is the monocyte. Upon migration into tissues, these monocytes differentiate and mature to become macrophages. It is important to note that this differentiation process involves the presence of an important combination of polypeptide growth factors including macrophage colony-stimulating factor (M-CSF or CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-3 (IL-3), stem cell factor (SCF), interleukin-1 (IL-1) and

interferon-γ (IFN-γ) [24, 27, 28, 44]. However, studies indicate that M-CSF is the only colony-stimulating factor that is absolutely necessary for macrophage differentiation and proliferation *in vivo*. Furthermore, *in vitro* experiments have shown that M-CSF alone is enough to successfully direct macrophage differentiation from bone marrow progenitors [16, 17, 32].

After settling in various tissues throughout the body, macrophages have the potential to become activated. Activation results in larger cell size, increased production of inflammatory mediators and enhanced phagocytic ability [15, 19, 21]. Following activation via external stimuli, macrophages can exhibit divergent morphology and function. For instance, a subset of activated macrophages may differentiate into epithelioid cells. These specialized macrophages get their name due to the noticeable increase in cytoplasm and similar appearance to squamous epithelial cells. Additionally, activated macrophages may fuse together and form multinucleated giant cells. These two cell types are the primary cellular constituents associated with the formation of granulomas [23, 29].

Macrophages are ubiquitous and can be found throughout the body. Those macrophages residing in different tissues possess distinctive functional properties and vary in their expression of surface molecules [14, 15, 19]. Over time, two major theories have developed to explain these variations. The first theory suggests that different precursors exist for each issue type. Based on the second theory, the microenvironment in which the cell resides has a powerful impact upon macrophage differentiation. Several lines of research support the latter theory as macrophage cultures derived from a single stem cell progenitor can exhibit heterogeneity based on variation of culture conditions [14, 32]. As shown in Figure 3.1, these cells been given explicit names based on their specific location. For

example, macrophages of the CNS are called microglia while those located in the liver are called Kupffer cells. Moreover, macrophages residing in the bone are called osteoclasts, and alveolar macrophages are those macrophages found in the lung [14, 19].

Macrophage Function.

Macrophages play a dual role in host defense by contributing to both the innate and adaptive immune responses. Indeed, these cells function not only as important effector cells of the innate immunity but also as accessory cells in the adaptive immune response. Macrophages are responsible for three major functions. These functions include phagocytosis, antigen presentation and immunomodulation [19, 21]. Macrophages contribute to host defense via their participation in the initiation, maintenance and resolution of the inflammatory immune response [14, 21, 32]. Furthermore, the functional responses of macrophages are quite complex and are tightly regulated under normal circumstances. If not tightly controlled, the inflammatory response of macrophages can contribute to exacerbated cellular and tissue damage [19, 32].

Macrophages were originally identified based on their phagocytic properties. As phagocytic cells, an important function of macrophages is to ingest cellular debris and foreign pathogens. Once engulfed, the microbicidal activity takes place within intracellular vesicles known as phagolysozomes. These phagolysozomes are formed by the fusion of lysozomal vesicles with phagosomes, which contain the ingested materials [14, 19, 21]. To physically ingest these pathogens, macrophages must recognize harmful microbes via cell-surface receptors [14, 19, 33, 39]. Importantly, these receptors are capable of discriminating between the surface molecules of the host and those of foreign pathogens. These receptors

include mannose receptors, scavenger receptors, receptors for opsonins and toll-like receptors (TLRs). Ligation of these receptors results in a variety of outcomes including macrophage activation, phagocytosis and the production of various inflammatory mediators [1, 2, 21]. For instance, TLR activation can lead to the upregulation and secretion of various inflammatory mediators, which help direct both the innate and adaptive immune responses. To date, eleven TLRs have been identified [2, 9, 10, 41]. Due to limited space, a detailed discussion of each TLR and their functional responses cannot be addressed. For the purpose of this thesis, TLR 4 activation will be discussed in more detail later in this chapter.

Antigen presentation is another important function of macrophage activity. In fact, macrophages, along with dendritic cells (DCs) and B cells, are often referred to as professional antigen presenting cells (APCs). With regard to the adaptive immune system, antigen presentation is often considered the most important function of the macrophage [14, 26]. Following phagocytosis, ingested pathogens are degraded enzymatically within intracellular vesicles to generate antigenic peptides. Some of these peptides possess structural properties that allow them to adhere to the peptide-binding clefts of the major histocompatibility complex (MHC) class II molecule [14, 19, 32]. As a result, these peptides are presented in the context of MHC II on the macrophage cell surface. The peptide-MHC II complex is presented to the T cell receptor (TCR) of CD4+ T lymphocytes. Then, the CD4+ effector T cells and the macrophages engage in a beneficial "cross-talk" that is critical to the development of a productive immune response against intracellular pathogens [14, 19, 26].

The immunomodulatory character of macrophages is yet another valuable function of these multi-purpose cells. This role is perhaps the most important function with regard to β_2-AR modulation of macrophage response. It is well known that activated macrophages

secrete various cytokines capable of modulating the immune response [14, 19, 21, 26]. Additionally, activated macrophages are known to release other inflammatory mediators including various chemoattractants and short-lived lipid mediators such as prostaglandins and leukotrienes [14, 32]. It is also known that activated macrophages are capable of releasing reactive oxygen species into the extracellular space, which not only influence inflammatory response but also led to tissue destruction [7, 18]. Together, these inflammatory molecules represent another functional bridge between the innate and adaptive immune systems [21, 32].

Macrophage Activation.

For years, it has been accepted that macrophage function greatly influences the quality, duration and extent of inflammatory reactions. In order to exert these effects, the macrophage must undergo conversion from a resting state to one of activation. The term "activated macrophage" refers to a macrophage that has enhanced phagocytic, antigen presentation and immunomodulatory capabilities. It is important to note that, following entry into peripheral tissues, the majority of monocyte/macrophage cells die via apoptosis. Those cells that survive can differentiate and become activated with or without the need of a priming event. Stimuli for activation include T cell-derived cytokines, microbial products, immune complexes and various chemical mediators [14, 19, 21]. Activated macrophages have an altered phenotype and cellular morphology. They appear larger, possess more pseudopods and exhibit increased ruffling of the plasma membrane [14, 19]. As illustrated in Figure 3.2, the process of macrophage activation is quite complex and cannot be covered in great detail within the scope of this thesis [15]. Since many of the studies exploring β_2-AR

immunomodulation were done in conjunction with lipopolysaccharide (LPS)-costimulation, LPS-induced macrophage activation will be discussed in greater detail in the following section.

LPS Activation of Macrophages.

Bacterial LPS is a major constituent of the outer wall of Gram-negative bacteria and is known to be a potent activator of macrophages. More specifically, lipid A, a substructural component of LPS, is responsible for mediating macrophage activation [8, 25, 35]. Upon activation by LPS, a variety of inflammatory mediators are expressed in macrophages following the upregulation of various transcription factors including nuclear factor κB (NF-κB) and activator protein – 1 (AP-1). These signaling pathways are very complex and will be summarized below and in Figure 3.3 [22].

The LPS receptor complex is comprised of CD14, TLR 4 and the myeloid differentiation protein -2 (MD-2) [1, 13]. The LPS-TLR 4 signaling pathway arises from cytoplasmic Toll/IL-1 receptor (TIR) domains of the TLR 4 receptor. These TIR domains interact with a TIR domain-containing adaptor protein known as MyD88. While MyD88 has a C-terminal TIR domain that promotes its association with TLR 4, it also has an N-terminal death domain. The MyD88 death domains serve to recruit serine/threonine kinases such as IL-1 receptor-associated kinases (IRAK) to the cell membrane. The IRAK 1/ IRAK 4 molecules associate with the receptor complex transiently. IRAK 1 dissociates from the receptor complex following phosphorylation by IRAK 4. At this point, IRAK is free to associate with and activate TNF-receptor-associated factor 6 (TRAF 6). TRAF 6 goes on to initiate two distinct signaling pathways by activating the IkappaB kinase (IKK) complex and

the MAPK signaling pathway. Activation of the IKK complex leads to the phosphorylation of IκB. Once phophorylated, IκB is degraded and NF-κB is liberated, which results in the transcription of numerous inflammatory cytokines. TRAF 6 activation of the MAPK pathway leads to the activation of the AP-1 transcription factor. Like NF-κB, activation of AP-1 leads to the transcription of a variety of inflammatory cytokines [1, 25, 26, 41].

Originally, TLR signaling was believed to be entirely MyD88-dependent. In recent years, however, research has demonstrated a MyD88-independent pathway in both TLR 3 and TLR 4 signaling pathways. As shown in Figure 3.3, LPS-mediated signaling through TLR 4 can lead to the activation of NF-κB and the phosphorylation of IRF 3 via a MyD88 independent pathway. This MyD88-independent signaling pathway leads to the upregulation of Type I interferons such as IFN-β. IFN-β goes on to activate signal transducers and activator of transcription 1 (STAT 1), which leads to induction of several IFN-inducible genes [13, 14, 19, 22, 25].

Activated Macrophages, Inflammation and β_2-Adrenergic Immunomodulation.

As mentioned earlier, activated macrophages undergo changes that enhance their microbicidal effectiveness and their ability to modulate the inflammatory immune response. These changes include enhanced phagocytic ability, microbial killing, antigen presentation and inflammatory mediator production. It is widely accepted that activated macrophages secrete pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 [14, 15, 19]. Activated macrophages also release nitric oxide (NO), which is produced by inducible nitric oxide synthase (iNOS) [14, 19]. Furthermore, macrophage activation leads to enhanced respiratory burst activity. The macrophage respiratory burst is characterized by the release of various

reactive oxygen species (ROS) including superoxide radicals and hydrogen peroxide [14, 18, 19]. Together, these chemical mediators function to recruit and activate a variety of immune cells, including other macrophages, at the site of inflammation. As a result, these inflammatory molecules are highly effective mediators of the inflammatory response. If left unchecked, however, these inflammatory mediators, which are typically beneficial to the host, can exacerbate inflammation and lead to tissue and organ damage.

Due to their role in the inflammatory process, several lines of research have focused on exploring various ways to modulate macrophage activity. Based on the immunomodulatory potential of β_2-ARs, several investigators have turned their attention toward determining the effect of β_2-AR stimulation upon macrophage response [5, 34, 40, 42, 43]. Peripheral blood monocytes are known to express high levels of surface β_2-ARs. Though tissue macrophages express β_2-ARs, research has shown that they possess fewer β_2-ARs than monocytes [3, 20, 37]. These receptors are known to interact with endogenous catecholamines and exogenously administered adrenergic drugs. Since macrophages express fewer β_2-ARs, they exhibit reduced sensitivity to adrenergic agonist compared to monocytes [20]. Although the major sources of these catecholamines are chromaffin cells of the adrenal medulla and noradrenergic neurons, reports have indicated that macrophages release epinephrine and norepinephrine following stimulation with LPS or IFN-γ. However, the source of these macrophage-associated catecholamines is unclear. While some studies suggest that macrophages acquire epinephrine and norepinephrine from extracellular fluids, others indicate that macrophages actually synthesize these catecholamines. Currently, the consensus is that both uptake and synthesis are responsible for the presence of epinephrine and norepinephrine in macrophages [4, 11]. The novelty of these macrophage-derived

catecholamines lies within their ability to modulate macrophage immune response in an autocrine fashion [11, 38].

Over time, numerous reports have accumulated demonstrating the immunomodulatory effect of β_2-AR stimulation upon macrophage response. Research has shown that activation of β_2-ARs can influence a variety of macrophage functions including chemotaxis, expression of adherence molecules and inflammatory mediator production [31, 36]. For the purpose of this thesis, β_2-AR-mediated regulation of inflammatory mediator production by monocytes and macrophages is of chief interest. Stimulation of β_2-ARs on macrophages has been traditionally considered to have an anti-inflammatory effect upon inflammatory mediator release. However, recent reports suggest that β_2-AR may actually promote a pro-inflammatory response by macrophages [40, 42]. Indeed, as shown in Figure 2.5, studies investigating the effect of β_2-AR activation upon production of inflammatory cytokines by macrophages are inconclusive [40]. Understandably, this dual modulatory property of β_2-ARs with respect to macrophage activity is controversial and will be discussed in greater detail in the following chapters.

Summary.

Macrophages belong to the mononuclear phagocyte system. This system is defined as a family of cells including bone marrow progenitors, peripheral blood monocytes and tissue macrophages in their various forms. Macrophage development is complex and is composed of several phases for which there are distinct cell phenotypes. Briefly, macrophages are derived from a common precursor in the bone marrow, which differentiates to form blood monocytes. When monocytes exit the blood to enter peripheral tissues, these

cells differentiate further to become macrophages [14, 26, 32]. Once residing within tissues throughout the body, macrophages can become activated via a variety of mechanisms (Figure 3.2). Macrophages are much more than the "big eaters" that their name implies. Indeed, these cells are important inflammatory cells, which participate in both the innate and adaptive immune responses [14, 19]. Though phagocytosis of foreign material is a major function of the macrophage, these cells also function as professional APCs and potent immunomodulators of the inflammatory response [14, 19]. Because of the controversial role of β_2-ARs in modulating the inflammatory response of macrophages, the immunomodulatory potential of macrophages is of critical importance with regard to this thesis.

FIGURE 3.1

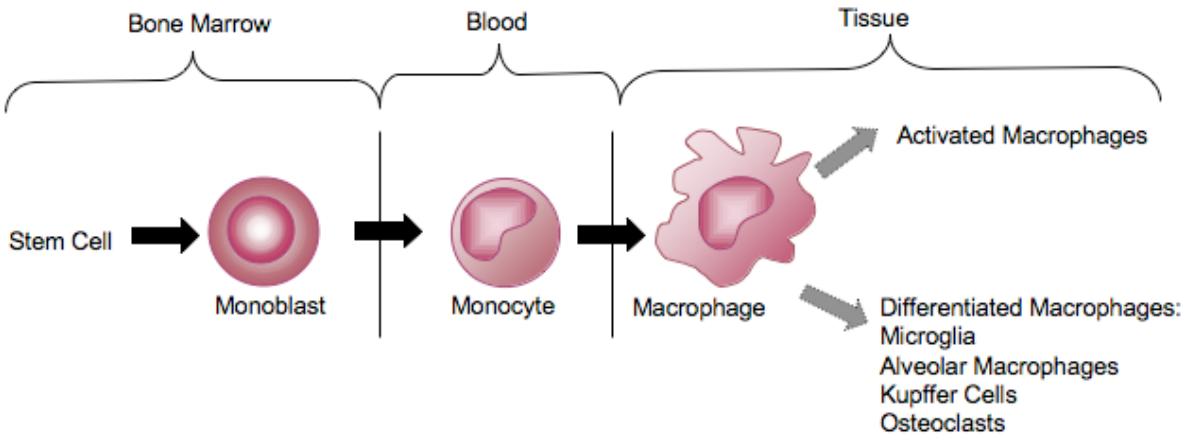

FIGURE 3.1 – Macrophage Development and Maturation. Macrophages are derived from pluripotent haematopoietic stem cells that reside in the bone marrow. While in the bone marrow, these cells differentiate into monoblasts and promonocytes. The first cell of this family to appear in peripheral blood is known as a monocyte. As these monocytes leave the blood and enter various tissues and organs the body, they become macrophages. Once distributed throughout the body, macrophages differentiate even further and are capable of becoming activated. Importantly, macrophages located in different organs of the body receive special names. For instance, macrophages of the CNS are known as microglia. Those found throughout the airways and the lung are known as alveolar macrophages. Kupffer cells are macrophages of the liver, and osteoclasts are located in bone [13, 19, 21, 26, 27].

FIGURE 3.2

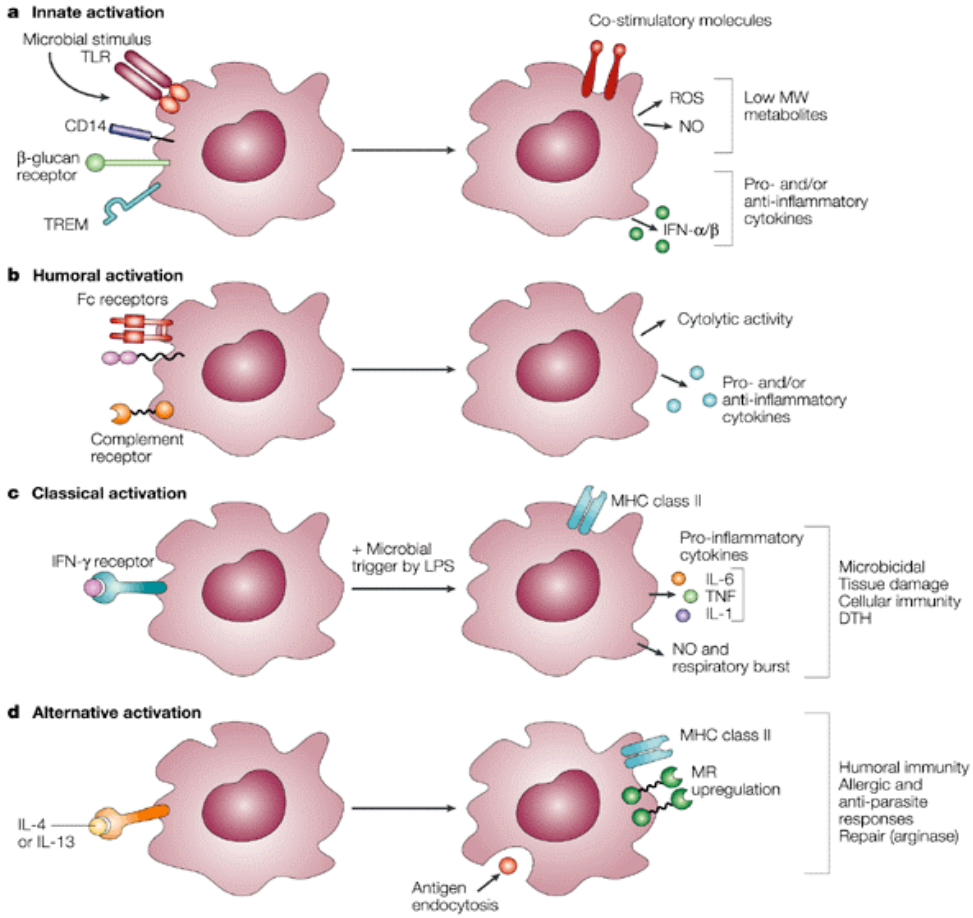

FIGURE 3.2 – Macrophage Activation. Macrophage activation is a complex process. Activating stimuli include T-cell derived cytokines, microbial products, immune complexes and various chemical mediators. Importantly, activated macrophages have enhanced phagocytic, antigen presentation and immunomodulatory capabilities [13-15, 19, 21].

Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35. © 2003.

FIGURE 3.3

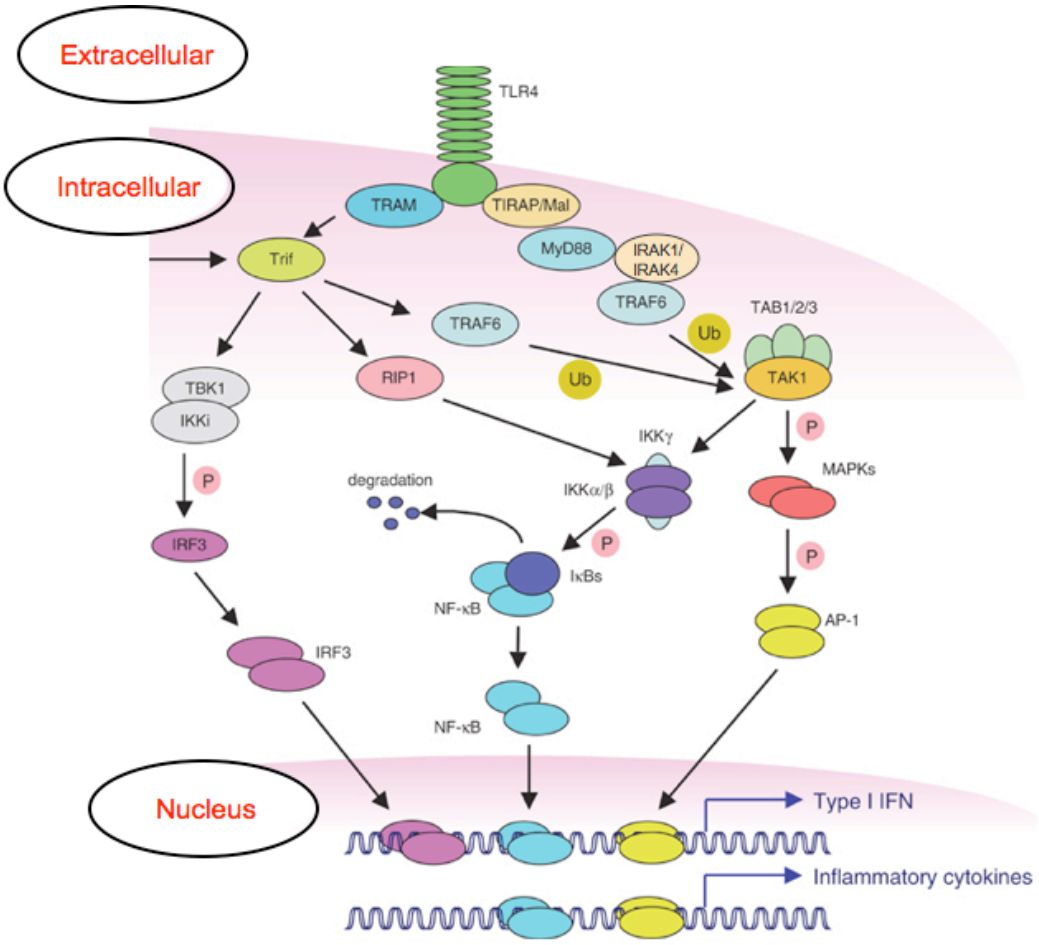

FIGURE 3.3 – LPS Activation via TLR 4 Stimulation. The LPS receptor complex is made up of CD14, TLR 4 and MD-2. As shown above, upon stimulation, TLR 4 initiates the intracellular signaling cascade via the recruitment of MyD88 and IRAK 1/ IRAK 4 to the membrane. IRAK 1/ IRAK 4 associates with the receptor complex transiently. Once phosphorylated, IRAK 1 dissociates from MyD88 and the receptor complex. At this point, IRAK activates TRAF 6. TRAF 6 then goes on to activate two signaling pathways via activation of IKK complex and the MAPK signaling pathway. Activation of the IKK complex leads to the degradation of IκB and liberation of NF-κB. Activation of the MAPK signaling pathway ultimately leads to the activation of the AP-1 transcription factor. Activation of both NF-κB and AP-1 lead to the transcription of inflammatory cytokines. It is important to note that there is also a MyD88-independent pathway that leads to IRF 3 activation and upregulation of Type 1 Interferons such as IFN-β [9, 19, 22, 26].

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CHAPTER III: REFERENCES

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CHAPTER IV: ANTI-INFLAMMATORY MODULATION OF MACROPHAGE RESPONSE BY β_2-ADRENERGIC RECEPTOR ACTIVITY

As multi-functional cells of the immune system, monocytes and macrophages play an instrumental role in modulating the inflammatory immune response. Because of this role, regulating macrophage activity can drastically alter the course of many inflammatory processes. In recent years, the ability of adrenergic drugs to modulate cells of the immune system, especially monocytes and macrophages, has gained interest. Accepted dogma indicates that β_2-AR stimulation has an anti-inflammatory effect upon monocyte and macrophage responses. To date, studies exploring the anti-inflammatory effect of β_2-AR activation have focused extensively on the production of numerous inflammatory mediators including cytokines, chemokines, NO and superoxide anions. The contents of this chapter are designed to provide a comprehensive review of existing literature with regard to the anti-inflammatory effects of β_2-AR modulation upon cells of the mononuclear phagocytic system.

β_2-Adrenergic Receptor Anti-inflammatory Modulation of Cytokine Production.

Cytokines are small, secreted proteins involved in mediating inflammation. Acting as signaling compounds, cytokines promote cell-to-cell communication during the inflammatory immune response [49, 72]. Activated macrophages produce a number of pro-

and anti-inflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and interleukin-10 (IL-10) [29, 31]. Research indicates that elevated cAMP levels are associated with reduced cytokine production [7, 53, 88]. Since β_2-AR activation is known to increase cAMP levels, several investigators have focused on determining the value of β_2-AR agonists as modulators of cytokine production. These studies have been conducted using both *in vitro* and *in vivo* model systems [22, 26, 34, 35, 37, 40, 42, 44, 46, 47, 66, 78, 85, 92, 94]. However, *in vivo* studies are often unable to isolate the effects of β_2-ARs on monocyte and macrophage function. This is due to the fact that monocytes and macrophages are not the only cells capable of producing cytokines *in vivo*. Indeed, cytokines, and other inflammatory mediators, are produced by a variety of cell types including (but not limited to) fibroblasts, endothelial cells, lymphocytes, and smooth muscle cells [49, 72]. As a result, much of the work done to explore the effects of β_2-AR stimulation upon monocyte/macrophage-mediated cytokine production has been conducted *in vitro*. Historically, research has demonstrated that β_2-AR stimulation of monocytes and macrophages downregulates pro-inflammatory cytokine production and potentiates the release of anti-inflammatory cytokines.

TNF-α:

TNF-α is a pleiotropic inflammatory cytokine produced primarily by monocytes and macrophages. This cytokine is involved in a wide array of biological processes including cellular proliferation, differentiation, apoptosis, lipid metabolism, coagulation and endothelial function [49, 72, 98]. Following LPS-activation of macrophages, TNF-α is the first cytokine to appear in a cascade of several inflammatory cytokines [28, 49]. In models of systemic inflammation induced by bacteria and/or bacterial by-products, the earliest cytokine

to emerge into circulation is TNF-α. Furthermore, animal studies have shown that neutralization of TNF-α activity during sepsis is important in protecting against lethality [28, 39, 41, 90]. Based on this knowledge, inhibition of TNF-α activity has become a major target of therapeutic exploration. Over the past two decades, several lines of research have focused on exploring the impact of β_2-AR agonists upon TNF-α production by LPS-treated monocytes and macrophages.

In 1992, Severn et. al. published one of the earliest papers addressing the anti-inflammatory potential of β_2-ARs with regard to monocyte production of TNF-α. These experiments were conducted *in vitro* using human whole blood and the THP-1 human monocytic cell line. Results demonstrated a dose-dependent reduction in LPS-induced TNF-α production following exposure to epinephrine. Treatment with isoproterenol, a β-AR specific agonist, had similar effects upon TNF-α levels. Moreover, the use of a β-AR antagonist prevented the effects of epinephrine/isoproterenol on TNF-α production. Use of an α-AR antagonist, on the other hand, had no effect. This paper also reported increased cAMP levels following β-AR activation in LPS-treated cells. As mentioned earlier, increased cAMP levels are believed to reduce the production of various cytokines, including TNF-α. Experiments designed to explore the mechanism of β-AR regulation indicated that LPS-induced TNF-α mRNA levels were not affected by exposure to epinephrine/isoproterenol. This, along with time-course data, prompted Severn and colleagues to suggest that β-AR mediation of TNF-α production occurs at the post-transcriptional level. Importantly, inhibition of TNF-α was only observed if epinephrine/isoproterenol was administered at the same time of LPS co-stimulation. A 24-hour (h) pretreatment with epinephrine/isoproterenol actually led to increased TNF-α

production following LPS exposure. This pro-inflammatory caveat was attributed to decreased cAMP levels (below basal levels) and receptor desensitization [78]. Though the report by Severn et. al. revealed that β-AR stimulation (and not α-ARs) influences TNF-α production by macrophages, the β-AR subtype was not directly identified by this paper. The general consensus supports the belief that β_2-ARs mediate the catecholamine-induced decrease in TNF-α production [35, 47, 64, 66, 77]. However, several papers have implicated β_1-ARs in this process. It is important to note that most of these studies used norepinephrine as the β-AR ligand. Since norepinephrine is known to preferentially bind α_2- and β_1-ARs, ligand-specificity could contribute to these claims [42, 94, 95].

Following these early reports, several papers were published that confirmed the involvement of β_2-ARs in modulating LPS-induced TNF-α production by numerous cells of the monocytic lineage. These studies employed various monocyte/macrophage cell lines, human peripheral blood monocytes (PBMCs), microglia, alveolar macrophages, renal macrophages and Kupffer cells [4, 8, 25, 34, 35, 37, 40, 42, 44, 77, 101]. Many of these publications contradicted the idea put forth by Severn et. al. that β-AR modulation of cytokine production did not work at the level of transcription [4, 40, 44, 66, 67, 86]. For instance, a paper by Hetier and colleagues indicates that β_2-AR activity inhibits both the transcription and release of TNF-α by LPS-treated murine microglial cells [40]. In addition to the *in vitro* studies, numerous experiments were conducted using *in vivo* model systems [22, 59, 77, 84, 87]. Although TNF-α levels were lowered following exposure to β_2-AR agonists in many of these experiments, it is difficult to attribute the *in vivo* inhibitory effects of β_2-AR stimulation directly to monocyte and/or macrophage activity.

Though much of the work regarding this topic used exogenously administered catecholamines and/or β_2-AR drugs, several publications have explored the effect of macrophage-derived catecholamines upon inflammatory mediator production [23, 81]. As discussed in chapter three, macrophages are known to possess intracellular stores of epinephrine and norepinephrine [10, 81]. Based on this knowledge, a 1994 study by Spengler and colleagues investigated the effects of macrophage-derived norepinephrine upon TNF-α production. Results from this study demonstrated that macrophage-derived catecholamines are capable of regulating TNF-α production in an autocrine manner [44, 81]. While this phenomenon was attributed to β-AR activity, the particular subtype responsible for these results has yet to be defined.

In addition to exploring β_2-AR modulation of TNF-α production, Lowry et. al. investigated the effect of β_2-AR stimulation upon TNF-α receptor (TNFR) expression. Existing data suggests TNFR expression is linked to TNF-dependent apoptosis. As a result, it is believed that TNFR expression plays an important role in mediating inflammatory cell turnover [96]. It is well established that TNFR expression is lost during both experimental and clinical endotoxemia [12, 21, 91]. In septic patients, the loss of TNFR activity is associated with poor outcome. The re-establishment of these receptors, on the other hand, is associated with increased survival [12]. Importantly, agents that increase cAMP levels increase TNFR expression [12, 35, 92]. Since β_2-AR stimulation leads to the upregulation of cAMP, Lowry and colleagues published a set of papers exploring the effect catecholamines upon TNFR surface expression of LPS-treated monocytes. Results from these studies demonstrate that epinephrine prevents LPS-induced downregulation of TNFR expression on human monocytes. The use of AR-specific antagonists demonstrated that this effect was

primarily mediated through the activation of β_2-ARs [35, 92]. These studies, in conjunction with earlier studies, indicate that β_2-AR activity influences the bioavailability of TNF-α not only through TNF-α production but also through TNFR expression.

IL – 1β:

Like TNF-α, IL-1β is a potent inflammatory cytokine produced by monocytes and tissue macrophages in their various forms. IL-1β is initially translated as a pro-peptide. This immature form of IL-1β (pro-IL-1β) is the predominate intracellular form of the protein. Processing of pro-IL-1β to the mature form requires a cysteine-dependent protease known as IL-1β-converting enzyme (ICE) [3, 5, 61]. It is important to note that the release of IL-1β is often incomplete, even in macrophages. This results in the presence of both extracellular and cytoplasmic IL-1β [72]. The mature form of IL-1β is believed to play a key role in the inflammatory immune response and is involved in a multitude of biological activities [49, 72]. Based on what is known about β_2-AR modulation of TNF-α production, several lines of research have focused on identifying the effect of adrenergic modulation, if any, on IL-1β production by LPS-treated monocytes and macrophages.

In 1986, a paper published by Koff et. al. introduced the idea of catecholamine-induced modulation of IL-1 production by macrophages. This study demonstrated the ability of epinephrine and norepinephrine to inhibit LPS- or IFN-γ-induced IL-1 production by murine peritoneal macrophages. Analysis of intracellular and extracellular levels of IL-1 indicated that catecholamines are capable of blocking IL-1 synthesis without affecting IL-1 release. This was demonstrated by the fact that exposure to norepinephrine or epinephrine resulted in proportional decreases of both intracellular and extracellular concentrations of IL-

1. Although there was no data in this paper to prove the actual mechanism of action, Koff and colleagues hypothesized the decrease in IL-1 production was mediated by increased cAMP levels [55]. The paper by Koff and colleagues also failed to directly address the receptor subtype responsible for the observed effects. Fortunately, several papers followed aimed at identifying the subtype involved in modulating IL-1β production by monocytes/macrophages. According to current literature, the inhibitory effects of catecholamines and adrenergic drugs on IL-1β production by LPS-treated monocytes and macrophages are primarily mediated via β_2-AR activity [23, 40, 101, 102]. Furthermore, numerous lines of research have confirmed these early observations using a variety of cell types belonging to the mononuclear phagocytic system [4, 8, 23, 40, 102]. Notably, like TNF-α, existing data indicates that macrophage-derived catecholamines are capable of modulating IL-1β production in an autocrine fashion [10, 23, 81]. Although the impact of β_2-AR modulation varies based on cell type, this collection of papers indicates that adrenergic modulation is a relevant mechanism for regulating LPS-induced IL-1β production by monocytes/macrophages.

IL-6:

IL-6 is an important inflammatory mediator produced by a variety of cell types including monocytes and macrophages. IL-6 carries out a broad spectrum of biological activities, many of which overlap those of IL-1β and TNF-α. All three of these cytokines are deemed pro-inflammatory and are valuable players in the "acute-phase response." Occurring early during infection, the "acute-phase response" refers to the body's global response to foreign pathogens. Aside from inducing the release of various acute-phase proteins, IL-6 is

highly involved in mediating fever [49, 72]. Like TNF-α and IL-1β, the pro-inflammatory role of IL-6 has influenced many investigators to explore potential mechanisms of regulating IL-6 production including adrenergic immunomodulation.

In 1999, Izeboud and colleagues published a paper exploring the effects of the β-AR agonist, clenbuterol, upon pro-inflammatory cytokine production by LPS-induced monocytes and macrophages. Data from *in vitro* studies demonstrate a concentration-dependent inhibitory effect of β-AR agonists upon LPS-induced IL-6 production by macrophages. Izeboud also extended these experiments to include an *in vivo* endotoxemic rat model. In this model, plasma levels of IL-6 were lower in experimental groups (β-AR agonist) than in control groups (no β-AR agonist) [47]. This data supports the anti-inflammatory effect of β-AR agonists on IL-6 production. However, it is impossible to conclude these effects are due to β-AR agonist inhibition of macrophage function alone [47]. Because clenbuterol is not a β_2-AR-specific agonist, the receptor subtype mediating these effects is not fully addressed in this set of data. An additional paper published in 1999 by Izeboud and colleagues directly addresses this issue of β-AR subtype specificity. These studies used β_1- and β_2-specific antagonists to determine which β-AR subtype is responsible for the observed effects. Results from this set of experiments indicate that β_2-ARs, and not β_1-ARs, are involved in the adrenergic immunomodulation of IL-6 production by LPS-stimulated macrophages [46, 47]. Like with TNF-α and IL-1β, experiments exploring IL-6 activity were extended to include a variety of cell types belonging to the mononuclear family of phagocytes [47, 66, 84].

Over time, a select subset of papers has suggested molecular mechanisms to explain the effects of β_2-ARs on IL-6 production. In 1999, Nakamura and colleagues reported that the β-AR agonist, isoproterenol, is capable of altering LPS-induced IL-6 gene transcription

by monocytes and macrophages. However, this effect was not dose dependent and was only observed at high concentrations (10^{-5}M) of β-AR agonist. Again, β_2-AR-induced increases in cAMP levels were cited as a potential factor in regulating IL-6 production [47, 67]. Unfortunately, the exact signaling mechanisms (second messengers, transcription factors, etc) responsible for the β_2-AR-mediated reduction in IL-6 transcription were not addressed by these studies. Although there is substantial documentation of the anti-inflammatory effect of β_2-AR stimulation upon IL-6 production, several publications indicate that β_2-AR activating agents may actually increase IL-6 production by macrophages. These pro-inflammatory effects of β_2-AR stimulation will be described in more detail in chapter five.

IL-10:

IL-10 is a pluripotent cytokine produced by several cell populations including those belonging to the mononuclear phagocytic system. Unlike TNF-α, IL-1β and IL-6, IL-10 is classified as an anti-inflammatory cytokine. Due to the potent anti-inflammatory properties of IL-10, its main biological purpose appears to be the limitation and/or termination of the inflammatory immune response. With regard to macrophage activity, IL-10 functions to inhibit the production of pro-inflammatory cytokines [49, 72]. In fact, IL-10 was originally called cytokine synthesis inhibiting factor (CSIF) because of its ability to modulate cytokine production [72]. Recently, the potent anti-inflammatory activities of IL-10 have prompted numerous studies designed to understand and manipulate IL-10 production. Based on the ability of β_2-ARs to influence the production of other cytokines, several investigators have investigated the effect of β_2-AR stimulation on IL-10 production by monocytes/macrophages.

One of the earliest publications addressing β_2-AR modulation of IL-10 production was published by Suberville et. al. in 1996. In this paper, the experimental group consists of LPS-activated peritoneal macrophages, which were treated with the β-AR agonist, isoproterenol. According to data from these studies, β-AR agonist treatment significantly increased IL-10 production by macrophages in the experimental group. This increase in IL-10 production occurred in a dose-dependent manner with respect to isoproterenol concentration. Data revealed that the increase in IL-10 release was accompanied by an increase in cAMP and IL-10 mRNA levels in isoproterenol-treated groups. Furthermore, treatment with oxprenolol, a β-AR antagonist, inhibited the effects of isoproterenol on IL-10 production [83]. This paper also proposed the idea that β-AR-induced increases in IL-10 production may contribute to the well-documented catecholamine-induced reduction in TNF-α and IL-1β production [35, 55, 64, 83, 93]. However, several papers have provided data to contradict this theory [19, 64]. For instance, in 2005, Muthu and colleagues published a paper aimed at exploring the link between β_2-AR-induced IL-10 and TNF-α production. Results from these studies indicate that β_2-AR modulation of IL-10 and TNF-α are independent of one another. Indeed, experiments using anti-IL-10 antibodies (Ab) were not capable of blocking epinephrine-induced TNF-α production by LPS-stimulated macrophages [64].

Like with other cytokines, many of the original publications did not fully address the receptor subtype responsible for β-AR-induced increases in IL-10 production. Over time, publications have emerged suggesting these effects are mediated via the β_2-AR subtype [46, 64]. Furthermore, although there has been mention of cAMP involvement, the signaling pathways mediating the effects of β_2-AR activity have yet to be elucidated [82]. It is

important to recognize that though several papers have provided data supporting the β_2-AR-induced increase in IL-10 production by LPS-treated macrophages, there are several papers that indicate that stimulation of β_2-ARs has no effect upon IL-10 production.

β_2-Adrenergic Receptor Anti-inflammatory Modulation of MIP-1α Production.

Macrophage Inhibitory Protein-1α (MIP-1α) is a potent inflammatory mediator produced by monocytes and macrophages. As a member of the CC family of chemokines, MIP-1α is chemotactic for mononuclear phagocytes and lymphocytes [49]. Aside from its chemotactic properties, MIP-1α is also known to promote macrophage production of TNF-α, IL-1 and IL-6 [24, 38]. Furthermore, MIP-1α has been implicated as a contributing agent in the pathogenesis of a variety of inflammatory conditions including rheumatoid arthritis, asthma and glomerulonephritis [38, 54, 68, 82]. Over the years, due to the pro-inflammatory properties of MIP-1α, interest in modulating macrophage production of this multi-purpose mediator has increased. Importantly, with respect to this thesis, several investigators have considered adrenergic immunomodulation as a promising mechanism for regulating MIP-1α production by monocytes and macrophages.

The first paper to explore catecholamine-induced modulation of MIP-1α production was published in 1998 by Hasko and colleagues. In this landmark publication, the authors examine the effect of epinephrine and norepinephrine upon MIP-1α production by LPS-treated macrophages. This paper employed both the RAW 264.7 murine macrophage cell and thioglycollate-elicited murine peritoneal macrophages for experimentation. Results indicate that both epinephrine and norepinephrine inhibit MIP-1α production from LPS-induced macrophages in a dose-dependent manner. Use of β-AR antagonists inhibited the

anti-inflammatory effects of both epinephrine and norepinephrine. Additional experiments employing isoproterenol, a β-AR agonist, mimicked the effects of epinephrine and norepinephrine upon MIP-1α production. The use of an α-AR specific agonist, however, had no effect. Studies employing Northern blot analysis demonstrate that LPS-treated macrophages exposed to β-AR activating agents possess lower levels of MIP-1α mRNA than macrophages belonging to the control group (no β-AR treatment). This publication also explored the *in vivo* effects of β-AR stimulation upon MIP-1α production. In these experiments, Hasko et. al. observed lower MIP-1α plasma levels in endotoxemic mice exposed to β-AR agonists than in control mice (no β-AR agonist) [38]. In 2003, a paper by Li et. al. was published extending these studies to see if β-AR stimulation had the same effect upon MIP-1α production in human monocyte/macrophages. To do this, Li and colleagues exposed LPS-stimulated human monocytic THP-1 cells and human PBMCs to epinephrine (or isoproterenol) and measured MIP-1α production. Data from this publication demonstrates that epinephrine inhibits MIP-1α production by LPS-treated samples in a dose-dependent manner. This reduction in MIP-1α production occurred at both the protein and mRNA levels. While use of α-AR antagonists had no effect upon MIP-1α levels, β-AR-specific antagonists reversed the effects of epinephrine and β-AR agonists upon MIP-1α production [57]. In both papers, β-ARs were implicated in inhibiting MIP-1α production by LPS-stimulated macrophages and monocyte [38, 57]. However, neither publication determined which adrenoceptor subtype is responsible for the observed effects. As a result, additional studies are required to fully associate β_2-AR activity with catecholamine-induced inhibition of MIP-1α production.

β_2-Adrenergic Receptor Anti-inflammatory Modulation of Nitric Oxide Production.

Nitric Oxide (NO) is a multi-purpose molecule produced by many cells of the body including monocytes and macrophages. Aside from its role in regulating vessel tone, NO also plays an important role in mediating inflammation. NO functions not only as a valuable inflammatory signaling molecule but also as a fundamental microbicidal agent. As an inflammatory mediator, this molecule is implicated in the pathogenesis of sepsis, psoriasis, arthritis, type I diabetes and ulcerative colitis [16, 27, 43, 60, 73]. NO is synthesized from the oxidation of L-arginine by NOS. Currently, there are three known mammalian forms of this enzyme. Endothelial and neuronal NOS are constitutively expressed and are modulated by intracellular calcium levels. Inducible NOS (iNOS), which is found in macrophages, is not regulated by increases in intracellular calcium levels. Instead, iNOS expression is induced via interaction with various external stimuli (LPS, TNF-α, IL-1β, etc.). Once induced, iNOS is regulated by the inflammatory milieu present within the microenvironment [16, 58]. Moreover, several papers have demonstrated that increases in cAMP levels can inhibit iNOS induction [37, 58, 63, 69]. This idea is quite controversial as others have shown that increases in cAMP levels increase NO production [62]. Nevertheless, the possibility remains that β_2-AR stimulation may lead to a cAMP-mediated reduction in iNOS expression.

Early on, Szabo et. al. reported on the ability of isoproterenol, a β-AR agonist, to reduce plasma nitrite levels in LPS-challenged mice. Unfortunately, due to the numerous sources of NO *in vivo*, adrenergic modulation of macrophage activity may not be responsible for these results [84]. In 1999, Sigola and colleagues published a paper designed to explore the direct effects of β-AR agonists upon NO production by macrophages. Using an *in vitro* model system, this set of experiments investigated β-AR-mediated regulation of LPS-

induced NO production by primary murine peritoneal macrophages. Upon release from activated macrophages, NO rapidly reacts with oxygen to produce nitrite. As a result, this paper (and many others) used the Griess assay to measure nitrite levels as an indication of NO production. Data from these experiments demonstrate that epinephrine treatment results in a dose-dependent reduction of nitrate levels in the experimental group. These results could be blocked using β-AR antagonists, which indicates this phenomenon is mediated by β-AR activity.

Although very few studies were done to address the mechanism responsible for the observed reduction in NO, Sigola et. al. theorized about the involvement of cAMP and NF-κB in mediating the inhibitory effects of epinephrine [80]. Throughout time, there have been several reports supporting the involvement of cAMP and NF-kB in mediating β-AR-induced reduction of NO production [6, 37]. However, more research is required to confirm these reports and establish this as an accepted mechanism of action. In 2001, a paper by Zinyama and colleagues presented another mechanism to explain the β-AR-induced attenuation of NO production by macrophages. In this mechanism, the reduction of NO is secondary to the effects of epinephrine on TNF-α and IL-10 production. This theory rests on the idea that IL-10 and TNF-α can influence NO activity. Indeed, research has shown that while IL-10 inhibits NO production, TNF-α promotes iNOS activity [37, 103]. Data from this paper demonstrates that epinephrine-induced reduction of NO production can be abrogated by the addition of anti-IL-10 in a dose-dependent manner. The addition of exogenous TNF-α is also capable of a dose-dependent inhibition of epinephrine-induced reduction of NO production [14, 103]. Though this data provides some insight into how β-AR activity

modulates NO production, more work must be done to fully describe this phenomenon in a mechanistic manner.

Most reports exploring the effects of β_2-AR activity upon NO production by macrophages employed LPS as a means of macrophage activation [14]. However, a subset of publications demonstrated the ability of β_2-AR stimulation to inhibit NO generation by macrophages activated by alternative sources. A paper by Boomershine and colleagues explored the effect of β_2-AR activation upon NO production by *Mycobacterium avium* infected macrophages. Data from this paper indicate that β_2-AR agonists are capable of reducing NO production by IFN-γ-primed murine macrophages infected with *Mycobacterium avium*. Stimulation of β_2-ARs also results in the reduction of iNOS mRNA expression in these macrophages [6]. Data has also demonstrated that treatment with epinephrine and isoproterenol reduces NO production from macrophages activated *in vitro* by acetylated low-density lipoproteins [56].

β_2-Adrenergic Receptor Anti-inflammatory Modulation of Superoxide Production.

Reactive oxygen species (ROS), including superoxide radicals, are important molecules capable of promoting inflammation. Although small in size, ROS are highly reactive. This reactivity is due to the existence of unpaired valence shell electrons that rapidly interact with nearby molecules in an attempt to establish stability. Throughout nature, ROS are formed by a variety of mechanisms. With respect to the immune system, ROS are produced by neutrophils and macrophages during a process known as the respiratory burst. Once released, these reactive oxygen metabolites are not only capable of killing foreign pathogens but also harming valuable host tissues. As a result, the release of

ROS compounds must be regulated in an attempt to prevent destruction of "innocent," by-stander tissues of the host. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme involved in the production of ROS during the respiratory burst. This enzyme is made up of five components often referred to as "phox" (phagocytic oxidase) subunits – $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The NADPH oxidase complex catalyzes a reaction that uses electrons from the cytosolic side of the plasma membrane to reduce extracellular oxygen (O_2) to superoxide ($O_2^{\bullet -}$) in the following reaction:

$$NADPH + 2O_2 \xrightarrow{\text{NADPH Oxidase}} NADP^+ H^+ + 2O_2^{\bullet -}$$

Then, the superoxide anion can be spontaneously or enzymatically converted to other ROS such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and potentially even the hydroxyl radical (OH•) [45, 70, 97].

Although the antimicrobial properties of ROS produced by macrophages during the respiratory burst are well documented, the size of the respiratory burst is much smaller in macrophages than in neutrophils [45, 97]. Because of this fact, much of the work to identify and establish the mechanism of NADPH activation has been studied in the context of activated neutrophils [97]. Nevertheless, extensive research has been conducted to establish ways to regulate the production of ROS by macrophages [45]. Importantly, with respect to this thesis, the anti-inflammatory properties of β_2-AR activation have encouraged the idea of employing β_2-AR drugs as potential mediators of superoxide production by macrophages.

Unlike the previously described studies, LPS is not typically used to induce superoxide production by macrophages. In the studies discussed below, macrophages are either treated with phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan to induce superoxide production. Both of these treatments have been proven to induce superoxide

production by macrophages in the past [32, 33, 74]. The first paper to expand the anti-inflammatory activities of β_2-ARs to include superoxide production was published by Conlon et. al. in 1988. This paper used a chemiluminescence assay to determine the effect of adrenergic stimulation on superoxide production by bovine pulmonary alveolar macrophages previously challenged with opsonized zymosan. Results from this paper demonstrate the ability of the β-AR agonist, isoproterenol, to significantly reduce maximum chemiluminescence levels (i.e. superoxide production) in experimental groups compared to control groups (no β-AR treatment). Data from experiments using the β_2-AR-specific, ICI 118,551, suggest the β_2-AR subtype is responsible for the observed reduction in superoxide production following exposure to isoproterenol [18]. In 1991, a paper by Calhoun and colleagues extended these studies to include human PBMCs and alveolar macrophages. According to data from this publication, exposure to isoproterenol reduces PMA-induced superoxide production by human monocytes and macrophages *in vitro* [11]. Several papers followed confirming these early reports [13, 17, 34, 79]. One such paper explored the effect of isoproterenol on superoxide production by PMA-stimulated primary hamster microglial cells. Again, data from this paper demonstrated that isoproterenol is capable of inhibiting PMA-induced superoxide production in a dose-dependent manner. The addition of propranolol, a β-AR antagonist, abrogated the effect of isoproterenol on microglial superoxide production. Due to the ability of β-AR activity to raise intracellular cAMP levels, the authors of this paper proposed this as a potential mechanism of superoxide regulation. To test this hypothesis, PMA-treated microglia were exposed to forskolin, which is known to increase cAMP levels via adenylate cyclase activation. Upon exposure to forskolin, superoxide production was reduced in a manner similar to that of isoproterenol-treated

microglia. Results from these experiments suggest that cAMP levels may contribute to the observed effects. It was also proposed that these cAMP-mediated events may work by influencing NADPH oxidase activity [20, 30, 65]. However, these authors did not go further into this potential mechanism of superoxide production [17].

Mechanisms of β_2-Adrenergic Receptor Anti-inflammatory Activity.

The collection of papers discussed above demonstrates the anti-inflammatory properties of β_2-AR activity with regard to monocyte/macrophage function. Both endogenous and exogenous ligands of the β_2-AR are capable of reducing LPS-induced production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, etc. Research has also shown that β_2-AR activity promotes the production of IL-10, an anti-inflammatory cytokine. It is well established that the production of various inflammatory mediators, especially cytokines, can be regulated at transcriptional and post-transcriptional levels. As a result, there are several mechanisms by which β_2-AR agonists may regulate LPS-induced inflammatory mediator production. However, very little research has been done to fully explore the mechanisms by which β_2-AR agonists exert their anti-inflammatory properties. The most studied mechanism of β_2-AR immunomodulation focuses on the involvement of cAMP.

To understand the modulatory mechanisms of β_2-ARs, it is important to recall the canonical signaling pathway of β_2-AR activation. As shown in Figure 2.5, cAMP levels are elevated following activation of adenylate cyclase via the α-subunit of the G_s-protein that is coupled to the β_2-AR. Elevated cAMP levels lead to the activation of PKA [50, 52]. It is important to note that PKA is involved not only in the phosphorylation and desensitization of

the β_2-AR but also in the phosphorylation of the cAMP-responsive element binding protein (CREB). Phosphorylated CREB goes on to bind to cAMP-responsive element (CRE) sites present within the promoter region of several cAMP-responsive genes [75]. In addition to activating PKA, cAMP also stimulates other effector molecules including the guanine nucleotide exchange factor (GEF) known as exchange protein directly activated by cAMP (EPAC) [86, 89]. The involvement of EPAC in cAMP-mediated signaling will be discussed further in the next chapter since it is associated with pro-inflammatory activities of the β_2-AR [86].

As illustrated in Figure 4.2, LPS-stimulation of monocytes and macrophages via TLR 4 leads to the activation of NF-κB. NF-κB is an important molecular mediator of inflammation as it positively regulates the production of several pro-inflammatory cytokines. Research has demonstrated that elevated cAMP levels can inhibit NF-κB-mediated transcription via the PKA pathway [71]. In this pathway, PKA-mediated phosphorylation of CREB leads to the recruitment of CREB-binding protein (CBP). CBP is a known coactivator of NF-κB and interacts with the p65 subunit of NF-κB to promote effective transcription. As a result, the competition between p65 and phosphorylated CREB for CBP leads to reduced NF-κB activity [71, 99]. Since β_2-AR stimulation upregulates cAMP, the cAMP-dependent PKA pathway of NF-κB regulation is a potential mechanism of β_2-AR modulation (see Figure 4.2). However, additional research is required to fully confirm the involvement of this pathway in β_2-AR-mediated immunomodulation. Another potential mechanism of β_2-AR-mediated immunomodulation involves the regulation of IκB proteins. IκB serves to sequester cytoplasmic NF-κB molecules in their inactive form. The phosphorylation and degradation of IκB-α leads to nuclear translocation of NF-κB. Once within the nucleus, NF-

κB binds to responsive elements within promoter regions of various inflammatory genes. Several publications have demonstrated the regulation of pro-inflammatory cytokines at the level of IκB-α [1, 2, 26, 76, 100]. The following paragraph will review an article citing IκB-α regulation as a potential mechanism of β_2-AR-mediated immunomodulation.

Though β_2-AR-mediated increases in cAMP have been implicated in the transcriptional regulation of several pro-inflammatory molecules, the mechanisms surrounding TNF-α production are the most studied. In 2000, Farmer and Pugin published a landmark paper describing the molecular basis of TNF-α modulation by β-AR agonists in monocytic cells. In this paper, the authors demonstrated the ability of β-AR agonists to reduce TNF-α production via a NF-κB-dependent pathway. Treatment of LPS-induced THP-1 human monocytic cells with the β-AR agonist, isoproterenol, inhibited translocation of NF-κB (3 h post β-AR treatment). Isoproterenol did not alter IκB-α levels initially. However, 3 h after isoproterenol treatment, IκB-α protein levels were significantly increased. The use of H-89, an inhibitor of cAMP-dependent PKA, blocked the observed effects of isoproterenol. Data from this paper also demonstrated that isoproterenol treatment, in the absence of LPS-co-stimulation, did not increase IκB-α levels. These results indicate that LPS is responsible for some part of this signal. Previous reports have shown that the IκB-α gene includes a κB site. Therefore, LPS-induced NF-κB activity may auto-regulate IκB-α gene expression [9, 15]. Importantly, Farmer and Pugin noted that IκB-α exhibited an increased half-life in isoproterenol-treated THP-1 cells. Based on these results, the authors theorized that LPS and β_2-AR agonists work together to increase IκB-α levels and inhibit NF-κB activity. This theory proposes that while LPS may initially play a role in increasing IκB-α transcription, β_2-AR activity stabilizes these newly synthesized IκB-α molecules (see

Figure 4.2). As a result, IκB-α levels accumulate, and NF-κB activity is inhibited. The exact mechanism by which this occurs is not fully defined [26].

Biological Relevance and Future Directions.

As mentioned earlier, β_2-AR activity is involved in a variety of physiologically relevant processes during periods of health and disease. Of importance to this thesis is the involvement of catecholamines during various inflammatory conditions. It is widely accepted that endogenous catecholamine levels rise during systemic inflammation. Additionally, exogenous sources of catecholamines and adrenergic drugs are often administered to treat various disease processes including asthma, chronic obstructive pulmonary disorder and sepsis. Although adrenergic drugs are known influence the immune response, these drugs are not readily used as anti-inflammatory agents. The publications discussed throughout this chapter promote the anti-inflammatory properties of β_2-AR stimulation. Indeed, these papers provide the evidence necessary to suggest using β_2-AR drugs to treat various inflammatory conditions.

Though there is abundant evidence to support the anti-inflammatory role of β_2-AR agonists, additional studies must be done to fully characterize these effects. To begin, as with any collection of work, there are variations present throughout these reports. These variations may be due to several factors including ligand choice, cell type/source, experimental conditions, etc. For instance, studies have shown that ligand classification (short- vs. long- acting) and ligand-receptor stereoselectivity influence the anti-inflammatory activity of β_2-AR agonists [36, 48, 51]. The anti-inflammatory properties of β_2-AR stimulation are also influenced by cell type. According to several lines of research,

monocytes, when compared to macrophages, are often more responsive to the anti-inflammatory effects of β_2-AR stimulation. This variation is thought to be the result of decreased expression of β_2-ARs by macrophages in comparison to monocytes [51]. The effects of β_2-AR stimulation may also be influenced by cell source (primary vs. cell line/ human vs. other species). Furthermore, experimental conditions such as concentration, time course and duration of exposure (to β_2-AR agonists and the given co-stimulus) can greatly influence the anti-inflammatory effects of β_2-AR stimulation upon monocytes/macrophage response [51, 78]. As many (or all) of these factors may influence β_2-AR agonist activity, more research must be done to better define their impact upon the anti-inflammatory effects of β_2-ARs.

As indicated by the lack of publications, another area requiring additional research is identifying the signaling pathways responsible for the anti-inflammatory activities of β_2-AR agonists. Although many publications have suggested potential pathways of mediation, very few investigators have extended their theories into experimental evidence. The fact that cytokines and other inflammatory mediators can be regulated on a variety of levels (i.e. transcriptional, post-transcriptional, etc.) introduces multiple pathways of investigation. In 2000, Farmer and Pugin took existing research a step further by actually exploring the signaling pathway involved in β_2-AR modulation of TNF-α production. This paper confirmed earlier theories involving cAMP, PKA and NF-κB activity [26]. However, there are still areas that need clarification in this pathway – especially with respect to LPS/β_2-AR-mediated IκB-α regulation. Though Farmer and Pugin identified the signaling pathway involved in β_2-AR modulation of TNF-α production by monocytes, this pathway may not hold true for other inflammatory mediators. As a result, extensive research must be done to

fully define the various signaling cascades involved in β_2-AR-mediated modulation of inflammatory mediators including IL-1β, IL-6, IL-10, MIP-1α, NO and superoxide.

Summary.

For years, the immunomodulatory capacities of catecholamines and adrenergic drugs have been recognized by the scientific world. Studies exploring the effect of β_2-AR stimulation upon the production of various inflammatory mediators have deemed β_2-AR agonists as anti-inflammatory agents (see Figure 4.1). Since monocytes and tissue macrophages are a large source of these inflammatory mediators, many investigators have focused on defining the immunomodulatory effects of β_2-AR activity on this particular subset of cells. Indeed, several papers have been published exploring the β_2-AR-mediated modulation of LPS-induced TNF-α, IL-1β, IL-6, IL-10, MIP-1α, NO and superoxide production by monocytes and macrophages. Although many publications have confirmed the anti-inflammatory properties of β_2-AR agonists upon macrophage response, additional research is required to fully characterize these effects. Nevertheless, given the appropriate conditions, the potential to use β_2-AR agonists as anti-inflammatory drugs remains promising.

FIGURE 4.1

Inflammatory Mediator	Effect of β_2-Adrenergic Receptor Stimulation
Pro-Inflammatory Cytokines TNF-α IL-1β IL-6 **Anti-Inflammatory Cytokine** IL-10	↓ Production ↑ Production
Chemokine MIP-1α	↓ Production
Nitric Oxide (NO) iNOS NO	↓ Production
Reactive Oxygen Species Superoxide ($O_2^{\bullet -}$)	↓ Production

FIGURE 4.1 – Summary of Anti-inflammatory Effects of β_2-Adrenergic Receptors upon Inflammatory Mediator Production by Macrophages. Throughout time, several papers have been published demonstrating the anti-inflammatory activities of β_2-AR stimulation upon macrophage inflammatory mediator production. In most cases, β_2-AR stimulation experiments were conducted in conjunction with a known co-stimulatory molecule such as LPS. *Experiments conducted to determine the effect of β_2-AR stimulation upon superoxide production were conducted using PMA or opsonized zymosan as a co-stimulatory source.

FIGURE 4.2

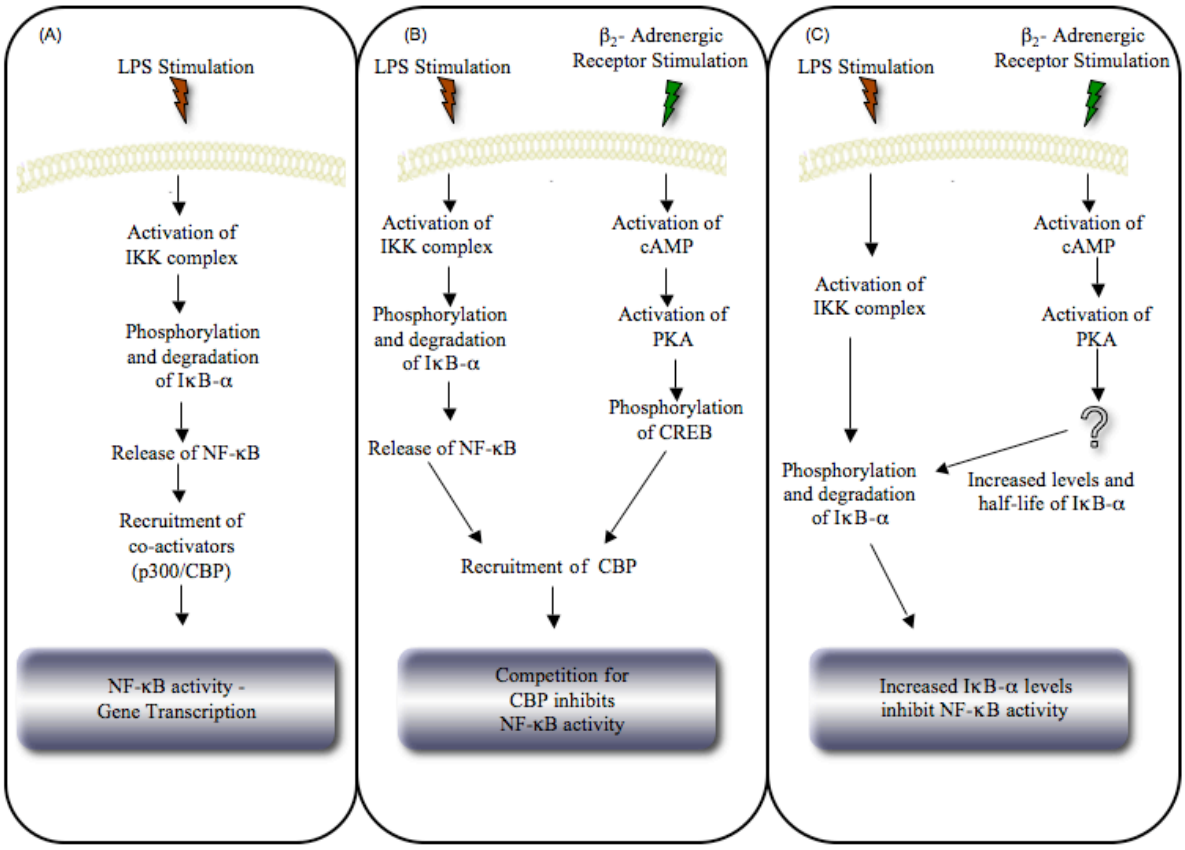

FIGURE 4.2 – Anti-inflammatory modulation of LPS-induced cytokine production by β_2-Adrenergic Receptor Stimulation. (A) LPS activation of macrophages leads to the upregulation of pro-inflammatory cytokines via activation of NF-κB. This occurs following the phosphorylation of IκB-α by the IKK complex. Phosphorylated IκB-α is degraded and NF-κB is free to translocate to the nucleus. Once within the nucleus, NF-κB associates with co-activators such as CBP or p300 and transcription commences. (B) β_2-AR stimulation leads to the activation of PKA. PKA phosphorylates CREB. Phosphorylated CREB recruits CBP, which is also a co-activator of NF-κB. Phosphorylated CREB competes with NF-κB for a limited amount of CBP. The competition for limited quantities of CBP is theorized to result in decreased NF-κB activity and reduced cytokine production. (C) β_2-AR stimulation has been shown to increase cytoplasmic levels and the half life of IκB-α. Since IκB-α is a natural inhibitor of NF-κB activity, β_2-AR-induced increases in IκB-α levels are theorized to result in reduced NF-κB activity and pro-inflammatory cytokine production. The signaling mechanism responsible for the increased levels of IκB-α is undefined but is believed to be PKA-dependent [1, 4, 26, 28].

CHAPTER IV: REFERENCES

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CHAPTER V: PRO-INFLAMMATORY MODULATION OF MACROPHAGE RESPONSE BY β_2-ADRENERGIC RECEPTOR ACTIVITY

Traditionally, activation of β_2-ARs has been reported to possess anti-inflammatory effects upon the immune response [20, 40, 42, 49]. However, a thorough survey of existing literature reveals a small subset of publications describing the pro-inflammatory activities of β_2-ARs located on monocytes and macrophages. These papers have focused on the ability of β_2-ARs to induce the production of several inflammatory mediators including TNF-α, IL-1β, IL-6, and NO [8, 47-49]. Though data from these reports endorse the pro-inflammatory potential of β_2-ARs, this concept is relatively new and has not been fully characterized. The contents of this chapter will provide a comprehensive review of existing literature concerning the pro-inflammatory effects of β_2-AR stimulation on inflammatory mediator production by monocytes and macrophages.

β_2-Adrenergic Receptor Pro-inflammatory Modulation of Cytokine Production.

As described in previous chapters, the immune response is influenced by β_2-AR-mediated immunomodulation. Since monocytes and macrophages express β_2-ARs and are major sources of cytokine production, many researchers have sought to identify the effects of β_2-AR stimulation upon these cells. Accepted dogma dictates that β_2-ARs exert anti-inflammatory effects upon cells of the immune system. Indeed, there is abundant

evidence (*in vitro* and *in vivo)* to support the anti-inflammatory properties of β_2-AR stimulation [15, 20, 23, 26, 42]. Though the anti-inflammatory model of β_2-AR activity appears deeply entrenched throughout science, research has demonstrated that the physiological role of the β_2-AR is actually quite diverse [24, 48]. These functional variations often reflect the myriad of conditions under which the β_2-ARs are activated. With this in mind, several investigators have focused on examining the pleotropic immunomodulatory effects of β_2-AR activating agents on numerous cell types under a variety of environmental conditions. For instance, studies have shown that activation of β_2-ARs found on myocytes, pituicytes, adipocytes, cardiac fibroblasts and skeletal muscle cells promoted the upregulation of the pro-inflammatory cytokine, IL-6 [10, 18, 30, 36, 50]. Additionally, Krishnaswamy and colleagues have demonstrated that activation of β_2-ARs on mast cells lead to increased IL-13 mRNA production [7]. Importantly, for the purpose of this thesis, these papers have encouraged additional papers exploring the pro-inflammatory effects of β_2-ARs upon cells of the mononuclear phagocyte system.

Surprisingly, many of the original papers aimed at characterizing the anti-inflammatory actions of β_2-ARs on monocytes and macrophages actually provided evidence to suggest a pro-inflammatory role for these receptors as well. For example, careful examination of the 1992 paper by Severn and colleagues reveals an intriguing, pro-inflammatory caveat within their data. This study characterized the effect of epinephrine and isoproterenol upon TNF-α production by LPS-treated THP-1 monocytes. In most cases, epinephrine/isoproterenol significantly reduced TNF-α production by LPS-treated cells. However, a 24-h pre-treatment with epinephrine actually led to a significant increase in LPS-induced TNF-α production. As described earlier in chapter four, this pro-inflammatory

effect was attributed to reduced cAMP levels and/or epinephrine-induced receptor desensitization. Though these studies suggest the involvement of cAMP, the signaling mechanism responsible for the observed pro-inflammatory phenomenon was not defined by Severn and colleagues [40]. In 1999, Nakamura and colleagues published a paper that also demonstrated the dual immunomodulatory potential of β-AR activation upon macrophages. This set of experiments used LPS-stimulated renal macrophages to study the modulatory effect of β_2-AR agonists upon IL-6 production. In this paper, Nakamura et. al. demonstrate that β_2-AR activation (at varying concentrations) had a "biphasic" effect on IL-6 production. Data from these studies demonstrate that while high concentrations (10^{-6} M) of the β_2-AR agonist, tertbutaline, enhanced LPS-induced IL-6 production, lower concentrations (10^{-8} M) of the agonist significantly reduced IL-6 production by LPS-stimulated macrophages. In an attempt to explain this "biphasic" phenomenon, several potential modulatory mechanisms were explored. Based on data from their studies, Nakamura and colleagues propose that the β_2-AR-mediated downregulation of IL-6 production was secondary to the inhibitory effect of tertbutaline upon TNF-α production. However, no TNF-α inhibitory experiments were conducted to confirm this mechanistic theory. Nakamura et. al. attributed the β_2-AR-mediated upregulation of LPS-induced IL-6 production to increases in cAMP levels. The proposed cAMP/PKA-dependent signaling cascade was supported by the fact that treatment with H-89, a PKA inhibitor, prevented β_2-AR-induced increases in IL-6 production [32, 51]. Although the regulatory mechanisms proposed by Nakamura et. al. are complex and require additional work for clarification, these studies demonstrate that stimulation of β_2-ARs can elicit both pro- and anti-inflammatory outcomes [32]. In conclusion, data from the publications discussed above promote the importance of recognizing how environmental

factors, such as time/duration of treatment and concentration of agonist, can influence the effects of β_2-AR activation upon cytokine production.

Another factor to consider is the co-stimulatory environment under which the β_2-AR is activated. A review of current literature reveals that the immunomodulatory properties of β_2-ARs are almost always studied in conjunction with a known immune activator such as LPS. This co-stimulatory experimental model was designed based on the fact that β_2-AR agonists are often administered to treat complications (blood pressure, irregular cardiac function, etc.) associated with systemic inflammatory conditions such as bacterial endotoxemia and septicemia. However, there are instances when β_2-ARs may be stimulated in the presence of a co-stimulatory molecule other than LPS (i.e. cytokines, microbial by-products, viral components). Recently, Szelenyi and colleagues published a paper exploring the effect of variable co-stimulatory environments upon β-AR inflammatory activity. In this paper, the authors compare the immunomodulatory effect of isoproterenol, a β-AR specific agonist, upon TNF-α, IL-12 and NO production by LPS- or PMA-stimulated monocytes and macrophages. To begin, results from this study confirm previous reports demonstrating that β-AR stimulation decreased inflammatory mediator production by LPS-treated cells. Treatment of PMA-stimulated macrophages with isoproterenol, on the other hand, actually led to increases in TNF-α, IL-12 and NO production. Importantly, these studies were repeated using the β_2-AR-specific agonist, clenbuterol. Results from these experiments were similar to those conducted using isoproterenol suggesting that the β_2-AR subtype may be responsible for these dual immunomodulatory actions.

According to data from Szelenyi's paper, co-stimulatory environments greatly influence the immunomodulatory potential of β-AR activation. This paper also explored the

signaling mechanisms responsible for the co-stimulus-dependent regulation of TNF-α, IL-12 and NO production by β-ARs. Since MAPKs are known to regulate cytokine and NO production, Szelenyi and colleagues explored the effects of β-AR agonists upon LPS- and PMA-induced pERK and p38 activity [9, 16, 19]. Results from these experiments reveal that the dual effects of isoproterenol on LPS- and PMA-treated cells were paralleled by differences in pERK and p38 phosphorylation. More specifically, while treatment with isoproterenol increased MAPK phosphorylation of PMA-stimulated macrophages, β-AR stimulation of LPS-treated cells resulted in reduced levels of MAPK phosphorylation. The data from these studies were in accordance with other publications reporting that MAPK inhibitors can modulate various inflammatory diseases [25, 47]. Notably, a paper by Feng et. al. demonstrates that cAMP elevators were capable of inhibiting LPS-induced IL-12 production via inhibition of the p38 MAPK pathway [16]. Since β-AR stimulation is known to elevate cAMP levels, Szelenyi et. al. theorize that this pathway may be responsible for the isoproterenol-induced reduction in LPS-mediated MAPK phosphorylation. Furthermore, the β-AR-mediated increases in PMA-induced ERK and p38 phosphorylation are believed to be responsible for the increases in TNF-α, IL-12 and NO production in these cells. Taken together, Szelenyi and colleagues propose that MAPKs behave as "molecular switches" that regulate β-AR immunomodulation according to which co-stimulatory molecule is applied. Though more work is required to fully characterize the β-AR signaling mechanisms responsible for these effects, the idea that β-AR agonists may wield dual modulatory effects (depending on the co-stimulatory environment) introduces an additional layer of complexity with regard to their use as therapeutic agents [47].

In addition to being stimulated in combination with a variety of immune activators, it is possible that the activation of β_2-ARs may occur in the absence of additional stimuli. For instance, β_2-AR agonists are often administered to treat patients with asthma. As a result, macrophages in the airways are often exposed to β_2-AR agonists in the absence of LPS and/or other co-stimulatory molecules. Activation of β_2-ARs may also occur by endogenous ligands in the absence of other stimulatory agents. An example of this phenomenon would be the activation of β_2-ARs by circulating catecholamines in patients suffering from chronic stress. Several studies have shown chronic stress increases susceptibility to various illnesses including peptic ulcers, ulcerative colitis, viral infections, asthma, myocardial infarction and depression. It has been proposed that the increased levels of circulating catecholamines associated with chronic stress may be responsible for influencing the patient's immune system and, therefore, their susceptibility to these conditions [14, 34]. Because it is possible that β_2-ARs may be stimulated in the absence of an additional co-stimulatory molecule, it is important to study the effect of β_2-AR agonists alone upon cytokine production.

In 1995, Tomozawa and colleagues published one of the earliest papers exploring the effect of β-AR stimulation upon cytokine production in the absence of a co-stimulus. This study explored the effect of isoproterenol on IL-1β mRNA production by primary rat microglial cells. A Northern blot analysis of microglial IL-1β mRNA production revealed that β-AR stimulation increased IL-1β mRNA levels in a dose-dependent manner. It is important to note that although IL-1β mRNA levels were increased following isoproterenol treatment, Tomozawa failed to explore the effect of β-AR stimulation on IL-1β at the protein level. Additionally, the increases in IL-1β mRNA were paralleled by increases in cAMP levels. Based on this data, Tomozawa et. al. proposed that β-AR-induced increases in IL-1β

mRNA may be mediated by a cAMP-dependent PKA pathway. This theory was supported by the fact that H8, a cAMP-dependent protein kinase inhibitor, blocked isoproterenol-induced upregulation of IL-1β production [49]. However, a recent paper by Tan et. al. presents data that opposes the cAMP/PKA-dependent mechanistic theory put forth by Tomozawa [48, 49].

In their 2006 publication, Tan and colleagues explore the effect of salmeterol, a β_2-AR-specific agonist, upon IL-1β and IL-6 production by RAW 264.7 murine macrophages. Data from these experiments demonstrate that stimulation of β_2-ARs by salmeterol increases IL-1β and IL-6 production by RAW 264.7 macrophages and other cells belonging to the monocytic lineage (J774A.1 macrophages, THP-1 monocytes and BV2 microglia). These β_2-AR-mediated increases were observed at both the mRNA and protein level. Furthermore, exposure of macrophages to ICI 118,551, a β_2-AR-specific antagonist, inhibited salmeterol-induced increases in IL-1β and IL-6. Based on current literature, Tan et. al. initially proposed a signaling mechanism that involved the cAMP-dependent activation of PKA. Once activated, it was theorized that PKA would phosphorylate CREB, which would then bind to CRE sites present in the promoter regions of IL-1β and IL-6 [6, 28, 35, 37]. To determine if β_2-AR stimulation mediates pro-inflammatory cytokine production via the cAMP/PKA/CREB cascade, Tan and colleagues conducted several experiments using various PKA inhibitors (H89, KT5720 and RP-cAMP). Results from this set of experiments demonstrate that treatment with H89, KT5720 and RP-cAMP had no effect upon β_2-AR-mediated increases in IL-1β and IL-6 production. It is important to note that Tan and colleagues also conducted studies using NF-κB inhibitors to demonstrate that NF-κB is not involved in mediating β_2-AR-induced increases in IL-1β and IL-6. This data, which does not

agree with the data from Tomozawa et. al., suggests that pro-inflammatory cytokine production following β_2-AR activation is not mediated via the cAMP/PKA/NF-κB signaling cascade.

Recently, research has indicated that cAMP is capable of activating several molecules (other than PKA) including the guanine nucleotide exchange factor for Rap1 known as EPAC [13]. This prompted Tan and colleagues to explore the involvement of EPAC in mediating the pro-inflammatory effects of salmeterol. Results from these studies indicate that activation of EPAC increases IL-1β and IL-6 production and may contribute to the pro-inflammatory effects of β_2-AR stimulation. Since β_2-AR activity is also known to activate MAPKs via G_s-dependent and G_s-independent mechanisms, Tan et. al. sought to determine if MAPKs are involved in mediating the pro-inflammatory activities of salmeterol [3, 12, 38]. Experiments using ERK 1/2, JNK and p38 MAPK inhibitors demonstrate that inhibition of ERK 1/2 and p38 (but not JNK) MAPKs blocked β_2-AR-induced increases in IL-1β and IL-6 production. Tan and colleagues also show that increased phosphorylation of ERK 1/2 and p38 occurred following salmeterol treatment. Due to the ability of the EPAC/Rap1/B-raf pathway to activate MAPKs, the next step was to determine if this pathway was involved in the β_2-AR-mediated induction of ERK 1/2 and p38 activity [13, 22]. Experiments using a B-raf inhibitor indicate that β_2-AR-induced B-raf activity is required for the increases in IL-1β and IL-6 following exposure to salmeterol. Finally, Tan and colleagues conducted experiments to determine the transcription factors responsible for β_2-AR-induced increases in cytokine production by macrophages. Because stimulation of ERK 1/2 and p38 activates the CREB, activating transcription factor (ATF), CCAAT/enhancer-binding protein beta (C/EBPβ), and/or E-twenty-six (ETS) family of

transcription factors, Tan et. al. explored the involvement of these molecules in mediating the pro-inflammatory effects of salmeterol. Results from this set of studies demonstrate that ATF-1 and ATF-2 transcription factors are involved in mediating the increases in IL-1β and IL-6 production following β_2-AR stimulation. Taken together, Tan and colleagues have shown that the pro-inflammatory activities of β_2-ARs are not mediated by the cAMP/PKA/NF-κB pathway as initially expected. Instead, these effects are mediated via the ERK 1/2 and p38 signaling pathways (See Figure 5.2).

β_2-Adrenergic Receptor Pro-inflammatory Modulation of Nitric Oxide Production.

As discussed in chapter four, NO is a multi-functional molecule that can act as a vasodilator, neurotransmitter and inflammatory mediator. Years of research have shown that although NO is a vital component of the immune response, excessive NO production during infection can severely damage valuable host tissues [8, 17, 21, 45]. Importantly, many cells produce NO including cells of the monocytic lineage. NO is synthesized from the oxidation of L-arginine by one of three forms of NOS – eNOS (endothelial), nNOS (neuronal) or iNOS (inducible) [17]. Macrophages express the iNOS form of this molecule, which can be induced following exposure to various cytokines, microbes and/or microbial products such as LPS [8, 17]. For years, scientists have recognized the modulatory properties of β_2-AR stimulation with regard to NO production. Though β_2-AR modulation of NO production is generally considered anti-inflammatory in nature, there are several publications that suggest otherwise [8, 44, 46]. Recently, research has shown that macrophages isolated from stressed mice produced higher levels of NO following LPS-stimulation *in vitro* than non-stressed mice. This data suggests that stress is capable of modulating LPS-induced NO production.

More specifically, it has been proposed that catecholamines are responsible for the observed stress-induced modulation of NO production by monocytes and macrophages [8, 41].

In 2003, Chi et. al. published a paper describing the pro-inflammatory effects of β-AR stimulation upon LPS-induced NO production by RAW 264.7 macrophages. Data from these studies demonstrate that both epinephrine and norepinephrine enhanced LPS-induced NO production in a dose-dependent fashion. Studies exploring LPS-induced iNOS expression also showed a dose-dependent increase with respect to epinephrine/ norepinephrine treatment. Furthermore, the use of a β-AR-specific antagonist (propranolol) significantly reduced the enhancing effects of these catecholamines upon LPS-induced iNOS and NO levels. Taken together, this data indicates that β-ARs are responsible for the observed effects. [8]. However, Chi and colleagues did not explore the signaling mechanisms responsible for the pro-inflammatory effects of epinephrine and norepinephrine upon NO production.

Data from the 2005 publication by Lin et. al. also demonstrates the pro-inflammatory activities of β-ARs with regard to NO production. To begin, this study explored the effects of epinephrine/norepinephrine upon LPS-induced NO and iNOS levels. Again, exposure to epinephrine/norepinephrine significantly increased both LPS-induced iNOS expression and NO production by RAW 264.7 murine macrophages. Furthermore, treatment with a β-AR antagonist was capable of blocking these effects. Next, Lin and colleagues advanced their studies by proposing a mechanism responsible for the catecholamine-induced increases in NO production. This theory hinges on the ability of catecholamines to modulate the cellular uptake of L-arginine by LPS-stimulated macrophages. As mentioned earlier, NO is produced by the oxidation of L-arginine by NOS activity. Years of research have demonstrated that

the cellular uptake of L-arginine is an important regulatory mechanism associated with the production of NO by iNOS [27, 31]. Importantly, data from this paper demonstrates that catecholamine-induced increases in NO production were paralleled by increases in L-arginine transport. Importantly, cellular uptake of L-arginine is mediated by the transmembrane transport system y^+, which is encoded by the cationic amino acid transporter (cat) genes, CAT-1 and CAT-2 [4, 27]. Indeed, members of the CAT isozyme family (CAT-1, CAT-2, CAT-2A and CAT-2B) are required for transmembrane transport of L-arginine. Because studies have shown that CAT-2 and CAT-2B are involved in LPS-induced NO production by macrophages, Lin et. al. theorized that β-AR activity may enhance CAT-2 and CAT-2B expression [33, 39]. Surprisingly, treatment with epinephrine/norepinephrine had no effect upon LPS-induced CAT-2 or CAT-2B expression. Instead, catecholamine treatment increased the expression of the constitutively expressed CAT-1 and CAT-2A isozymes. However, Lin and colleagues did not perform inhibition experiments to confirm the involvement of the molecules in mediating catecholamine-associated increases in LPS-induced NO production. Therefore, more experiments are required to clarify this mechanism. Lin et. al. also theorize about the involvement of NF-κB in mediating catecholamine-induced increases in NO production. This theory is based on the fact that NF-κB is a known transcription factor for a variety of inflammatory molecules including iNOS [5, 27]. Research has also implicated NF-κB activity in mediating the expression of CAT isozymes [2, 11]. Although experiments employing dexamethasone, a NF-κB inhibitor, suggest that NF-κB may be involved in mediating catecholamine-induced increases in NO production by RAW 264.7 macrophages, additional experiments must be done to fully characterize this signaling pathway [27].

Biological Relevance and Future Directions.

Recognition of the dual modulatory activities of β_2-ARs with respect to macrophage function has significantly advanced the understanding of adrenergic immunomodulation. Historically, β_2-AR signaling has been revered as having immunosuppressive effects upon the immune response. The papers discussed in this chapter not only challenge the accepted dogma regarding β_2-AR activity but also introduce potential opportunities for novel therapeutic approaches. It is important to recognize that, under certain conditions, β_2-AR target therapy may actually exacerbate the very conditions they are supposed to improve. A perfect example of this would be the use of β_2-AR agonists as bronchodilating agents to provide acute relief for asthma patients. Theoretically, the anti-inflammatory activity of β_2-ARs would also benefit the asthma-associated inflammation of the airways by inhibiting pro-inflammatory cytokine production by resident cells of the respiratory system, especially macrophages. However, research has shown that extended use of these drugs may actually worsen the condition by prolonging asthmatic activity, increasing airway reactivity and aggravating airway inflammation [1, 29, 43]. In these instances, β_2-ARs may actually be exacerbating the inflammatory response of alveolar macrophages. The paper by Tan et. al. propose that different pathways are responsible for the β_2-AR-mediated bronchodilatory and pro-inflammatory effects. While bronchodilation occurs via the classical β_2-AR-mediated cAMP/PKA/CREB pathway, the pro-inflammatory effects of β_2-AR agonists are mediated by the cAMP/PKA/CREB-independent, ERK 1/2- and p38-dependent signaling cascade. As a result, Tan and colleagues suggest that β_2-AR target therapy can be improved by selectively blocking the pro-inflammatory side effects of β_2-AR activation. This could be done using

MAPK inhibitors or novel β_2-AR drugs that selectively activate the cAMP/PKA/CREB pathway (but not the β_2-AR/MAPK pathway) [48].

Though the subset of papers described in this chapter established a strong foundation to support the pro-inflammatory potential of β_2-AR activity, more research must be done to fully characterize these effects. For instance, several studies are needed to expound upon what is currently known about β_2-AR-mediated increases in TNF-α, IL-1β, IL-6, and NO production by monocytes and macrophages. Furthermore, additional research is required to explore the pro-inflammatory effects of β_2-AR stimulation on the production of other inflammatory mediators such as IL-10, MIP-1α, ROS, etc. Certainly, the signaling mechanisms associated with both the pro- and anti-inflammatory activities of β_2-ARs deserve more attention. By understanding the nature of these mechanisms, scientists are more likely to develop novel, more effective β_2-AR targeted therapies. It would also be interesting to fully define the effects of different co-stimulatory molecules on the dual inflammatory activities of β_2-ARs. These studies would be physiologically relevant since β_2-ARs are known to be stimulated under a variety of conditions during periods of health and disease. If the situations under which β_2-AR activation would result in pro-inflammatory vs. anti-inflammatory activities were clearly defined, clinicians would be better suited to determine whether or not certain β_2-AR therapies should be used.

Summary.

Years of research have established that β_2-AR activity influences immune cell function. The stimulation of β_2-ARs present on monocytes and macrophages is generally considered to result in the downregulation of inflammatory mediator production. However, several papers

have recently demonstrated that β_2-AR stimulation may increase the production of pro-inflammatory mediators (see Figure 5.1). A review of these papers suggests that several factors may be involved in dictating the immunomodulatory effects of β_2-AR stimulation upon inflammatory mediator production by monocytes and/or macrophages. Recognizing the dual immunomodulatory capacity of β_2-ARs is essential to understanding the pleiotropic effects of these receptors upon the immune system. Indeed, the dual immunomodulatory properties of β_2-AR activation introduce an additional layer of complexity with regard to receptor function. Furthermore, in order to optimize the therapeutic potential of β_2-AR drugs, it is imperative to recognize both the pro- and anti-inflammatory activities of these receptors.

FIGURE 5.1

Inflammatory Mediator	Effect of β_2-Adrenergic Receptor Stimulation
Pro-Inflammatory Cytokines TNF-α IL-1β IL-6	↑ Production
Nitric Oxide (NO) * iNOS NO	↑ Production

FIGURE 5.1 – Summary of Pro-inflammatory Effects of β_2-Adrenergic Receptors upon Inflammatory Mediator Production by Macrophages. Several papers have been published describing the pro-inflammatory activity associated with β_2-AR stimulation found on cells of the mononuclear phagocytic family. The majority of these studies were done in the absence of a co-stimulatory molecule. *Nitric Oxide experiments were conducted in the presence of LPS as described in the body of this thesis [8, 32, 46, 47, 48, 51].

FIGURE 5.2

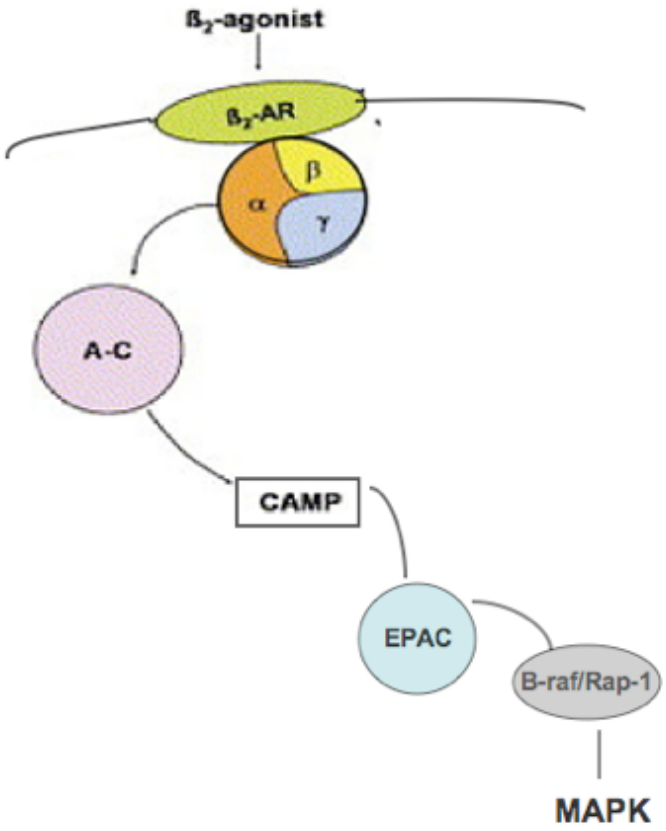

FIGURE 5.2 – Pro-inflammatory Signaling Associated with β_2-Adrenergic Receptor Stimulation of Macrophages. The pro-inflammatory activities of β_2-AR immunomodulation are mediated via a PKA-independent pathway. Following stimulation of the β_2-AR receptor, cAMP activates EPAC, which leads to the activation of B-raf/Rap-1. Ultimately, the ERK 1/2 and p38 MAPK pathways are initiated, leading to the activation of ATF-1 and ATF-2 and the transcription of pro-inflammatory cytokines [24, 48].

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CHAPTER V: REFERENCES

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