REGULATION OF CYTOKINES AND CHEMOKINES DURING THE PROGRESSION OF ACUTE INFLAMMATION IN THE HUMAN WHOLE BLOOD MODEL

by

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Abstract

REGULATION OF CYTOKINES AND CHEMOKINES DURING THE PROGRESSION OF ACUTE INFLAMMATION IN THE HUMAN WHOLE BLOOD MODEL

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Traumatic injuries or infectious challenges activate the innate immune response, initiating inflammation and cytokines and chemokines that are released into the circulation in distinct kinetic patterns. In the clinic, patients often present after the onset of inflammation, however few studies have investigated if the difference in cytokine and chemokine kinetics affects their ability to be regulated by anti-inflammatory reagents. To address this, the whole blood model was used to 1) characterize 24 hour acute inflammation; 2) determine if postponing the addition of the anti-inflammatory glucocorticoid dexamethasone (DEX) until after an inflammatory stimulus had any consequences on its ability to regulate cytokines and chemokines; and 3) determine if the effects of post-stimulus DEX were cell-type specific. The Toll-like Receptor 4 (TLR4) agonist lipopolysaccharide (LPS) or the TLR2 agonist Pam_3-Cysteine-Serine-$Lysine_4$ (Pam) was used to activate the inflammatory response in whole blood. The levels of pro-inflammatory cytokines tumor necrosis factor, interleukin-1 beta, and IL-6 and IL-8 and Growth Related Oncogene alpha (GROα) chemokines were indicators of inflammation.

LPS stimulation of whole blood induced rapid TNF, IL- β and IL-6 protein and messenger Ribonucleic Acid (mRNA) over 24 hours. Pam stimulation caused slower induction of IL-1β and IL-6 protein. Both stimuli induced a continuous increase in IL-8 and GROα protein and mRNA levels. Concomitant addition of LPS or Pam and DEX to whole blood significantly suppressed cytokine and chemokine protein levels compared with either stimulus alone. Six hour DEX significantly suppressed IL-8 and GROα mRNA at 24 hours compared with LPS alone. Pam-induced cytokine and chemokine protein was also suppressed by 6 hour DEX. Additional experiments designed to determine if these effects were cell-type specific indicated that 24 hours after LPS stimulation, isolated neutrophils produced a substantial amount of IL-8 mRNA. Administration of DEX 6 hours after LPS stimulation suppressed IL-8 mRNA levels in neutrophils and monocytes. These data indicate that the administration of anti-inflammatory reagents to a patient presenting in the clinic as little as 6 hours after the onset of inflammation more likely improves inflammation by suppressing the message and thus protein of inflammatory mediators, such as chemokines, which persist beyond 6 hours.

CHAPTER I

INTRODUCTION & BACKGROUND

Introduction

The initiation of inflammation is characterized by the secretion of a myriad of inflammatory mediators, many of which are released into the circulation in distinct kinetic patterns. Relatively little is known about the therapeutic consequences of such distinct kinetic profiles, with virtually no attention given to the possibility that these differences translate into distinct responses to anti-inflammatory therapies. At the molecular level, studies of inflammation have primarily focused on preventing the onset of inflammation, rather than halting progression of the response. As we strive to improve treatment of inflammatory disorders, a more comprehensive understanding of the mechanisms of inflammation and the resulting effects of therapies will be imperative.

Inflammation

Inflammation is the response of the immune system to tissue injury or infection. Invading pathogens as well as harmful internal environments such as ischemia or autoimmune diseases result in the leukocyte secretion of such inflammatory mediators as cytokines and chemokines (1). Components shed from the outer membrane of foreign pathogens bind to receptors on leukocytes and activate the inflammatory response (2). Similarly, during ischemia (or loss of blood flow) cells from the affected organ or tissue secrete cytokines in response the nearby necrotic cells, thereby perpetuating the inflammatory response (3). Characterized by redness, heat, pain, and

swelling, inflammation is a hallmark of a plethora of pathophysiologic processes including cardiovascular disease, meningitis, and allergic asthma (1). Controlled inflammation is an advantageous and necessary process which functions to protect the body against infection and injury. Ideally, inflammation resolves with inconsequential damage to the host (4, 5). However, the loss of control of inflammation can result in chronic disorders such as rheumatoid arthritis, inflammatory bowel disease, and septic shock and can ultimately lead to irreversible damage to tissues and organs and, even death.

Acute Inflammation

Inflammation is frequently divided into two categories: acute and chronic. Acute inflammation is temporary, lasting on the order of minutes to days, whereas chronic inflammation persists over longer periods of time. Although both are characterized by secretion of inflammatory mediators, a hallmark of acute inflammation is the significant increase in acute phase serum proteins such as LPS binding protein (LBP), C reactive protein (CRP), and serum amyloid A (6). Additionally, acute inflammation can be local, confined to a specific area or it can be systemic; in either case, it generally persists only for as long as the stimulus is present. Upon injury or infection, cytokines are secreted by resident cells, leading to the rapid and short-lived constriction of blood vessels. This is immediately followed by vasodilation, which results in an increased blood flow and pooling of leaked fluids in the area of injury. These processes cause the redness and warmth (*rubor* and *calor*) observed in acute inflammation. Edema manifests as swelling (*tumor*) when the increased permeability of the microvasculature allows for the leakage of plasma proteins and other fluids into the injured tissue. Additionally, resident cells

secrete chemokines to rapidly coordinate the recruitment of neutrophils to the site of injury. Here, neutrophils move into the injured tissue and remove the invading pathogen by phagocytosis as well as release of their bacteriocidal granules. As homeostasis is restored, anti-inflammatory mediators such as IL-4 and lipoxins promote resolution by inhibiting the migration of neutrophils as well as promoting the migration of monocytes to the site of injury (reviewed in (5, 7)). This influx of monocytes helps to resolve inflammation by phagocytizing remaining neutrophils.

Chronic Inflammation

Chronic inflammation develops when acute inflammation fails to resolve. This can be caused by persistent pathogen presence, the inability of phagocytic cells to degrade foreign substances, or autoimmune responses (5). The effects of chronic inflammation are devastating and include irreversible tissue damage, as well as the formation of ectopic lymphoid organs arising from the accumulation of lymphoid cells (8). As previously stated, inflammation can be induced by either exogenous or endogenous stimuli. Chronic inflammation is most often the result of persistent endogenous stimuli, including such factors as advanced glycation end products (AGE) and low-density lipoproteins (LDL) (9). The persistent stimuli are continuously detected by phagocytic immune cells such as macrophages and neutrophils, resulting in the subsequent activation of a persistent inflammatory cascade (9). AGE, for example, are ineffectively cleared from tissues, leading to their accumulation in the vasculature (10). This, in turn, results in sustained interaction with the receptor for AGE (RAGE) and sustained inflammation (11, 12). Further, upon oxidation by vascular cells, LDL triggers

an NFκB-mediated proinflammatory response in the arteries, causing in the upregulation of chemokines, cytokines, and other inflammatory markers (13, 14).

Atherosclerosis is a chronic inflammatory disorder of the arteries and the leading cause of death and illness in developed countries (15). Both AGE and oxidized LDL are hallmarks of atherosclerosis. In response to a high cholesterol diet, for example, circulating leukocytes have been shown to bind to endothelial cells lining the inner most layer of the arteries, or the intima (16). LDL oxidation induces leukocyte adhesion molecule expression on the luminal layer of the intima, resulting in monocyte adherence to the endothelial cells (17, 18). The upregulation of MCP-1 by the activated endothelial cells induces the migration of monocytes through the cell layer into the arterial intima where they morphologically transform into macrophages (19, 20). These macrophages express receptors for AGE and other lipoproteins, and as a consequence of the ligand-receptor interactions, they induce reactive oxygen species formation and secrete pro-inflammatory mediators, enabling the inflammatory response (21). They also secrete matrix metalloproteinases, which degrade the extracellular matrix and promote aggregation of macrophages causing plaque formation. When a plaque ruptures, coagulation and wound healing is activated, leading to a narrowing of the artery and ultimately an infarct, or tissue necrosis due to the sudden lack of blood supply (21).

Many diseases are characterized by persistent cytokine expression; however Forrester *et al.* poignantly noted that while cytokines play a similar role in many of these diseases, the pathogenesis of these diseases is widely variable, making it difficult to design effective therapies to combat inflammatory disorders (22, 23). Thus, it is important that further experimentation be undertaken to elucidate the mechanisms which

govern the multi-faceted phenomenon that is inflammation. One way in which to study the mechanisms of inflammation in a model system is to use inflammatory stimuli as well as anti-inflammatory reagents, which allow manipulation and in-depth examination of the process. The studies presented here were performed using heparinized human whole blood as a model of acute inflammation with emphasis on studying the regulation of cytokines

Cytokines

Cytokines are small, water-soluble, signaling proteins and glycoproteins secreted by hematopoietic and non- hematopoietic cells (24). During innate and adaptive immunity, they potentiate cellular communication via autocrine and paracrine mechanisms. In response to injury/infection, cytokines are secreted by inflammatory cells over several hours to days, and in some instances even weeks (1, 2).

It is important that investigation of such a complex phenomenon as inflammation includes careful and focused attention on its fundamental mediators. Since inflammation is a well-orchestrated and tightly regulated process, it was important to first evaluate a broad scope of mediators, and then narrow thefocus on those which were specifically altered by treatment with anti-inflammatory reagents . To ensure that the whole blood model was comprehensive, a diverse selection of mediators including pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines was investigated (Table 1.1).

Pro-inflammatory cytokines

TNF, IL-1β, and IL-6 are classic pro-inflammatory cytokines and have been shown to be rapidly induced within minutes to hours after inflammatory insult, followed by rapid clearance, *in vivo* and *in vitro* (25).

Tumor necrosis factor

Tumor necrosis factor (TNF) was originally identified in 1975 by Lloyd Old as a macrophage-derived factor that could "necrotize" tumors in mice (26). It is a classic pro-inflammatory cytokine secreted by a number of cells including macrophages/monocytes, mast cells, and tumor cells (27, 28). TNF is induced in response to a variety of stimuli including, but not limited to, bacterial endotoxins, oxygen radicals, and viruses. In response to LPS (lipopolysaccharide) stimulation, PBMCs (peripheral blood mononuclear cells) obtained from whole blood rapidly secrete a significant amount within the first three to four hours (29, 30). In addition to being produced in response to direct stimulation, TNF production can also induce various other cytokines including IL-1β and IL-8 (31, 32).

Interleukin 6

Although widely studied throughout the 1980s, IL-6 was not identified as such until long after having first been cloned in 1980 by Weissenbach *et al.* (33). While nearly all nucleated cells have been reported to express IL-6, it is most often produced by monocytes/macrophages in response to endotoxin stimulation (34, 35). During the acute-phase response, IL-6 can induce B cell proliferation as well as regulate hepatic production of acute phase proteins (36, 37).

Interleukin 1β

Interleukin 1β is a member of the IL-1 family of cytokines which consists of IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra), all of which are encoded by separate genes (38). The IL-1 genes are induced by a wide variety of stimuli including LPS, viruses, and TNF (39). Like IL-6 and TNF, IL-1 is most prominently produced in

monocytes/macrophages (29). Upon endotoxin stimulation, for example, IL-1β is rapidly induced in whole blood (40). Although IL-1α and IL-1β are both induced upon stimulation of the inflammatory response, IL-1α remains in the cytosol, whereas IL-1β is processed and cleaved into its active form by IL-1β converting enzyme (ICE) (41, 42). Additionally, direct stimulation with IL-1β can activate the transcription of specific inflammatory genes such as TNF and IL-8 (34). Finally, once bound to its receptor, IL-1 transmits a downstream signaling cascade similar to that observed with TLR binding (43).

Interleukin 6 receptor

The IL-6 (receptor IL-6R) is present on monocytes, hepatocytes and lymphocytes (44). It can be cleaved from any of these cell types and exist in a soluble form (45). The IL-6 protein can bind to either the cell-associated or the soluble IL-6R (45, 46). In the presence of a 130 kDa transmemberane glycoprotein, IL-6R displays a high affinity for the IL-6 protein (46, 47). Upon binding, the IL-6R/IL-6 complex activate factors including Janus kinases (Jaks) which ultimately lead to the activation of STAT-3 (Signal Transducer and Activation for Transcription), also known as APRF (acute-phase response factor) (47).

Anti-inflammatory cytokines

IL-1 receptors

Two IL-1 receptors, termed IL-1RI and IL-1RII, exist. The type I IL-lR is an 80-kDa glycoprotein and the type II a 60- to 65-kDa molecule (48, 49). Whereas IL-1RI is found predominantly on T cells and fibroblasts, IL-1RII is expressed on activated T cells, B cells, monocytes, and neutrophils (48). IL-1RI is essentially pro-inflammatory, as it

activates a downstream signaling cascade in response to IL-1α and IL-1β (50). The type II receptor, in contrast, acts as a decoy receptor and inhibits inflammatory signaling. Specifically, it has been documented to bind IL-1β and prevent its activation of signal transduction (51). IL-1RII occurs as both a membrane-bound and soluble receptor (sIL-1RII) (52). While sIL-1RII is present in the circulation of healthy individuals, it preferentially binds IL-1β and, like its membrane-bound form, does not transmit a signal (50, 52). The sIL-1RII binds the non-processed, pre-IL-1β, preventing its maturation thereby inhibiting signal activation (51). In the present studies, sIL-1RII was examined as part of the panel of anti-inflammatory cytokines so as to ensure that a comprehensive model of inflammation was evaluated.

IL-1 receptor antagonist

The third component of the human IL-1 gene family (previously described) is IL-1 receptor antagonist (IL-1ra). Like IL-1α and IL-1β, IL-1ra binds to the IL-1RI but does not trigger signal transduction (53). In contrast, IL-1ra is a naturally occurring inhibitor which binds to the IL-1RI with nearly irreversible affinity (54). In this way, it functions as an anti-inflammatory cytokine by blocking the binding of IL-1α and IL-1β to the IL-1RI (55). Marketed as Kineret®, recombinant IL-1ra has been used to treat rheumatoid arthritis (56, 57).

Soluble TNF receptors

TNF has two distinct receptors which share sequence homology in their conserved cysteine-rich extracellular domains (58-60). Due to the dissimilarity of their intracellular domains, however, each receptor is thought to have distinct biological functions (61). TNF receptor I (TNFRI) has been described as playing a key role in LPS-

induced septic shock (62). Acting in a pro-inflammatory manner, TNFRI enhances the respiratory burst in neutrophils, increases IL-6 mRNA, and induces NFκB activation (63-65). The function of TNFRII, on the other hand, has been less well defined. It has been shown to play enhance early hematopoiesis (66).

Both receptors can occur in soluble form. Specifically, the *cis*-acting membrane protease TACE (TNF alpha converting enzyme) can cleave immature, membrane-bound TNFR resulting in soluble, biologically active TNFR in the circulation (67). Occurring naturally in healthy individuals, sTNFR are key targets for anti-inflammatory therapies (68, 69). Drugs (such as Enbrel®) which neutralize sTNFRI and sTNFRII have been shown to significantly improve such inflammatory disorders as rheumatoid arthritis by binding TNF protein out of the circulation and preventing downstream signaling (70).

Chemokines

Leukocyte migration dictates the initiation, maintenance, and repair process of inflammation. These processes employ positive/start signals as well as negative/stop signals. Chemokines are start signals which are secreted by resident and circulating immune cells during injury/infection. In response to these signals, adhesion molecules are upregulated and leukocytes are recruited from the circulation. The subsequent sequence of events includes the rolling of leukocytes along the endothelium and culminates with transendothelial cell migration into the injured/infected tissue.

Chemokines are specialized *chemo*tactic cyto*kines* which, upon release into peripheral blood circulation, recruit leukocytes to the sight of injury (71). Chemokines secreted by PMNs and *Mo* at the sight of injury form a concentration gradient towards which additional immune cells migrate. They are characterized by four conserved

cysteine residues near their amino termini which form disulfide bonds. Specifically, CC chemokines have two cysteine residues and induce migration of *Mo* while CXC chemokines have two cysteine residues separated by one amino acid and induce migration of PMNs (71). CXC chemokines can be further classified as Glutamic acid-Leucine-Arginine (ELR)-positive or negative. ELR-positive CXC chemokines contain a Glutamic acid-Leucine-Arginine amino acid sequence immediately before the first cysteine residue in the CXC motif which is critical for activity (72, 73).

Both CC (*Mo*-specific) and CXC (PMN-specific) chemokines were evaluated in the studies subsequently presented in this thesis. Such chemokines included IL-8, Growth Related Oncogene (GRO), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 (MIP-1α) and MIP-1β (Table 1.1). IL-8 and GRO belong to the CXC family of chemokines and thus induce migration of PMNs whereas MCP-1, MIP-1α and MIP-1β are all CC chemokines which induce migration of monocytes and macrophages.

CXC chemokines

Interleukin 8 and Growth related oncogene, are chemoattractants that recruit neutrophils to the site of injury/infection. Both chemokines bind to the CXC receptors found the surface of neutrophils. Secreted by endothelial cells, neutrophils, monocytes and macrophages, augmented and sustained IL-8 is implicated in such disorders as atopic asthma and Crohn's disease (74, 75).

C-C chemokines

MCP-1, MIP-1α and MIP-1β are all CC chemokines which mediate the recruitment of immune cells, namely monocytes/macrophages. MCP-1 has been

implicated in several diseases including rheumatoid arthritis (76), atherosclerosis (77), and inflammatory bowel disease (78). It is induced in response to several stimuli including cytokines, growth factors and the TLR4 agonist LPS (79). Similarly, MIP-1α and MIP-1β induce migration of macrophages during the inflammatory process (80). Produced in macrophages, MIP-1α and MIP-1β can be induced by endotoxin (80) and, like MCP-1, they have been implicated in disorders including inflammatory bowel disease, and rheumatoid arthritis (81). In addition to their traditionally described functions, MCP-1, MIP-1α and MIP-1β have all been shown to be produced continuously during inflammation in a variety of systems (82-85).

Mo and PMNs have been documented to secrete inflammatory cytokines (31), although the extent to which each cell type contributes to the overall inflammatory milieu remains unclear. Several published reports, including those from our own laboratory, have demonstrated that *Mo* secrete both cytokines and chemokines, while PMNs primarily secrete chemokines such as IL-8. Xing *et al.* demonstrated that freshly isolated PBMC strongly induced TNF, IL-1β, and IL-6 following LPS stimulation, while PMNs contributed less than 1.5% of those cytokines (86). In subsequently published experiments, Xing *et al.* further showed that in a mixed cell environment PMNs contributed negligible amounts of TNF, IL-1β, and IL-6 in response to LPS stimulation, but synthesized a significant amount of IL-8 mRNA (87). Additionally, kinetic studies have shown distinct profiles for TNF, IL-1β, and IL-6 compared to IL-8. Our laboratory, as well as others, has demonstrated that following LPS stimulation, TNF, IL-1β, and IL-6 are rapidly induced compared to IL-8 which exhibits a two-phase pattern of production (31, 32).

It has yet to be determined if the distinct cytokine/chemokine kinetic patterns consequently alters the efficacy of anti-inflammatory therapies. As a result, we used the human whole blood model as a physiologically relevant system in which to investigate the effect of post-stimulus DEX treatment on the progression of acute inflammation.

Toll-like Receptors

The function of the innate immune response depends heavily on the body's ability to rapidly detect and defend against a broad range of foreign pathogens. Toll-like receptors are key pattern recognition receptors (PRRs) which bind to conserved molecular structures, found on a variety of pathogens, called pathogen-associated molecular patterns (PAMPS) (88, 89). TLRs are evolutionarily conserved transmembrane receptors with a leucine-rich extracellular domain that is involved in ligand recognition (43, 90). Further, its intracellular Toll/IL-1 receptor-like (TIR) domain transmits the intracellular signal in response to ligand binding (43). LPS is a potent activator of TLR4 and responds a Gram-negative pathogen, while several Gram-positive pathogens signal via TLR2. Therefore, both TLR4 and TLR2 agonists were used to induce inflammation in whole blood.

Toll-like receptor 4

Toll-like receptor 4 is prominently recognized as the mediator of lipopolysaccharide signaling. As depicted in figure 1.1, in the presence of LPS-binding protein (LBP), LPS is bound by the cell surface PRR cluster of differentiation (CD) 14, which is found on the surface of monocytes (and to a lesser degree) neutrophils. Together, the LBP-LPS-CD14 complex activates a dimerized complex TLR4 by mechanisms not yet fully understood. The activated TLR4 dimer recruits the adaptor

protein Myeloid differentiation primary response gene (88), or MyD88, to its intracellular TIR domain, thereby initiating a downstream, multifaceted signaling cascade. The resulting downstream signaling cascade initiated in response to TLR4 activation ultimately leads to the release and nuclearization of the transcription factor Nuclear Factor κB (NFκB). Normally sequestered in the cytoplasm by its inhibitor, the newly released NFκB translocates into the nucleus where it binds κB sites located in the promoter and the enhancer regions of inflammatory genes(91). This culminates with transcription of TLR-inducible genes which encode a number of inflammatory proteins including the classic pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines (91).

Toll-like receptor 2

Toll-like receptor (TLR2) mediates inflammatory responses to lipopeptides derived from Gram-positive bacteria such as *Mycobacterium* and *Staphylococcus* (92). In 1999 Lien et al. demonstrated that in human PBMC, neutralizing anti-body to TLR2 significantly reduced the amount of TNF protein secreted in response to *Mycobacterium avium* (92). Unlike TLR4, TLR2 does not function as homodimer, rather its activity depends on its heterodimerization together with either TLR1 or TLR6 (93). However, in response to Gram-positive bacteria lipoproteins, TLR2 activates the same signaling cascade as does TLR4 activation (Figure 1.1). Upon binding of Gram-positive bacterial lipopeptides, TLR2 forms a heterodimer with TLR1 or TLR6 on the extracellular surface of immune cells (94). As with TLR4 signaling, MyD88 is recruited to the intracellular domain of the TLR2, leading to the release and nuclear translocation of NFκB. This, in

turn, leads to the transcription of inflammatory genes such as those encoding cytokines and chemokines (93, 94).

Inflammatory stimuli

Lipopolysaccharide

The outer membrane of the double membrane envelope of Gram-negative bacteria contain numerous components which bind TLRs (95). LPS, or lipopolysaccharide, is the main component of the structure of this outer membrane and is composed of an O-antigen side chain, a core oligosaccharide, and Lipid A (96). LPS spans the bacterial envelope: the phospholipids on the inner leaflets are covalently linked to Lipid A, which is found on the outer leaflet (96). Lipid A, is the portion of the pathogen which is recognized by TLRs found on immune cells which upon binding activates the innate inflammatory response. The human immune system is so highly sensitive to endotoxin (from such enteric bacteria as *Eschericia coli*, for example) that low concentrations released into the blood stream can induce severe endotoxic shock, the cause of some 200,000 deaths in the United States, annually (97). Given its potent activation of the immune system, LPS is frequently used as an exogenous stimulatory inducer in both *in vivo* as well as *ex vivo* model systems.

Pam_3 Cysteine-Serine-$Lysine_4$

Lipoproteins from the outer membrane of Gram-positive as well as Gram-negative bacteria have been previously reported to induce lymphocyte proliferation and immunoglobulin secretion (98, 99). Moreover, the activity of the lipoprotein has been narrowed down to its N-terminal region, which contains three glyceryl-cysteine-bound fatty acids (100). Pam_3 Cysteine-Serine-$Lysine_4$ (Pam) is a synthetic lipopeptide first

prepared by Bessler *et al.* in 1983 (101). Pam is comprised of an amino acid sequence which is identical to that of the native lipoprotein, except that it contains three palmitic fatty acid residues (Fig. 1.2).

Similar to triacylated lipopeptides found in Gram-positive bacteria, Pam signals via TLR2/1 heterodimers and has been widely used to study the mechanisms of inflammation. Hauschildt *et al.* reported that Pam induced significant production of TNF, IL-1, and IL-6 in bone marrow-derived murine macrophages (102). As recently as 2007, Pam was shown to dose-dependently increase IL-8 mRNA production by adrenocortical cancer cells similar that induced by lipoteichoic acid (LTA), a natural component of Gram-positive bacteria (103).

In the context of the studies presented here, Pam was a useful tool by which to investigate possible stimulus-specific effects. More importantly, however, Gram-positive bacteria account for more than 50% of all cases of sepsis in the United States (104). Thus, thorough investigation of the mechanisms of inflammation required attention to the role of TLR2-activating agents.

Whole Blood

Since the early 1990s, whole blood has proved to be a convenient model in which to study inflammation and cytokine regulation. It is comprised of red blood cells, white blood cells (or leukocytes), plasma, and plasma proteins. The leukocyte population includes monocytes, neutrophils (PMNs), and lymphocytes.

Lymphocytes

Lymphocytes are divided into three subclasses: T lymphocytes (T cells), B lymphocytes (B cells), and natural killer (NK) cells. While NK cells function to protect

the host against tumors and viruses, T cells and B cells are part of the adaptive immune response and perpetuate cell-mediated and humoral immunity, respectively. The role of lymphocytes was beyond the scope of this thesis, and thus was not investigated.

Monocytes

Monocytes (*Mo*) are mononuclear cells which migrate to sights of injury and/or infection in response to inflammatory signals. They comprise roughly three per cent of circulating whole blood cells and differentiate into macrophages and dendritic cells upon tissue infiltration. *Mo* phagocytize invading pathogens, apoptotic PMNs and their granules. Produced in bone marrow by hematopoietic stem cell precursors, *Mo* have a half life of about thirty-six hours in the peripheral blood stream. Further, they secrete cytokines and chemokines to recruit more immune cells to the site of inflammation.

Neutrophils

Neutrophils are granulated, multinucleated cells which, like *Mo*, are critical in the innate inflammatory response. The most abundant leukocyte in the blood stream, PMNs act within an hour of injury or infection, chemotaxing (or migrating) towards a chemokine gradient and the site of infection (105, 106). They are the first responders in the inflammatory process and are the hallmark of acute inflammation. PMNs mediate inflammation by ingesting foreign pathogens, releasing their germicidal granules, and secreting inflammatory cytokines and chemokines (105).

Other constituents of whole blood

Whole blood also contains glucose, which is an essential source of energy utilized in cellular metabolism. It also contains lipopolysaccharide (LPS) binding protein (LBP), which is necessary for cellular responses to exogenous LPS stimulation (107, 108).

Compared with isolated cell types, whole blood is a more physiologically relevant system in which to study inflammation. Several investigators have shown that it is as effective as, if not better than, isolated peripheral blood mononuclear cells, which can inadvertently become activated during isolation (109, 110). Obtaining whole blood is relatively easy and its manipulation does not require expensive equipment. *In vivo*, systemic inflammation is evidenced by the presence of inflammatory mediators in the peripheral blood; likewise, stimulation of whole blood with pro-inflammatory stimuli results in similar results (25). It has been reported in the literature that LPS, TNF, and IL-1β stimulation of whole blood causes rapid, transient induction of cytokine and chemokine messenger ribonucleic acid (mRNA) levels as well as rapid, transient induction of their corresponding protein levels (25, 111).

Glucocorticoids

Widely used to study the mechanisms of inflammation, glucocorticoids (GC) are lipophilic, low molecular weight steroid hormones which passively diffuse through the plasma membrane to mediate a host of activities including immune suppression, stress control, and overall homeostasis. They mediate their anti-inflammatory effects via protein:protein interactions with transcription factors such as NFκB. In response to an activation signal GC passively diffuses through the cell membrane and binds to the glucocorticoid receptor (GR) located in the cytoplasm (112). The entire GC-GR complex translocates into the nucleus where it can bind directly to transcription factors or to glucocorticoid response elements (GRE) located in the DNA to regulate gene transcription (112). GC can mediate transactivation or transrepression, depending on the moiety to which it binds. In the case of NFκB, for instance, the GC-GR complex can bind

directly to the p65 subunit and prevent its binding to DNA (113, 114). GC can also induce the transcription of the NFκB inhibitor IκB, which leads to the sequestration of NFκB in the cytoplasm (113).

Dexamethasone (DEX) is a potent, synthetic glucocorticoid which has been widely used both to treat inflammation as well as study the mechanisms of inflammation. Bhattacharyya *et al.* showed that macrophages lacking the GR were resistant to DEX reduction of LPS-induced proinflammatory mediators TNFα, IL-6 and COX-2 (cyclooxygenase-2) (115). DEX has also been shown to decrease inflammatory mediator protein and mRNA levels by initiating transcription of the NFκB inhibitor IκB (inhibitor of κB) (113, 114).

Several glucocorticoids of varying potencies exist including hydrocortisone, as well as the synthetic compounds methylprednisolone and dexamethasone (DEX). These steroids work broadly to suppress inflammation and thus are used in the clinical setting to treat a variety of inflammatory events including meningitis, allergic asthma, rheumatoid arthritis.

In a prospective, randomized, double-blind multicenter trial published by de Gans *et al.*, DEX treatment of patients with pneumococcal meningitis reduced the risk of unfavorable events, described as by 10 per cent (116). (A score of 5 on the Glasgow Outcome Scale at eight weeks indicated a favorable outcome and a score of 1 to 4 indicated an unfavorable outcome) (116). Compared with the placebo group, DEX treatment reduced the proportion of patients who died by nearly 50% (116). In a similar study, Michel *et al.* found that daily oral pretreatment with 0.3 mg/kg prednisolone for six days prior to lipopolysaccharide (LPS) inhalation significantly reduced the presence

of the circulating acute phase protein CRP in healthy individuals (117). For the scope of this thesis, the potent anti-inflammatory capacity of glucocorticoids was exploited.

Controversy persists surrounding both dosage and timing of GC administration as a treatment for numerous acute inflammatory disorders (118). Clinical investigations have ranged from high-dose to short-term low-dose GC therapies. In a study conducted by the National Heart, Lung, and Blood Institute ARDS (acute respiratory distress syndrome) Clinical Trials Network, Steinberg *et al.* administered moderate doses of methylprednisolone every six hours for fourteen days to patients suffering from persistent ARDS for at least seven days (119). This moderate dosage was followed by a tapered dose over the remaining seven days. The number of ventilator-free and organ failure-free days and sixty day mortality rates were used as measures of clinical outcome. Although there was a decrease in the number of ventilator-free and organ failure-free days, there was no significant decrease in sixty day mortality rate compared with placebo. Of particular importance was the finding that the timing of GC administration appeared to play a critical role in the efficacy of treatment. Steinberg *et al.* concluded that the risk of death may have been increased by delaying administration of the methylprednisolone for seven days (119). In a similar study conducted by Sprung *et al.* for the Corticus Study Group, low-dose hydrocortisone was administered to septic patients in a similar manner (120). Although low-dose hydrocortisone therapy accelerated the reversal of shock, it did not improve 28-day mortality in any of the experimental groups compared with placebo. These data clearly indicate a gap in current understanding of the role of anti-inflammatory therapies in quelling the inflammatory process.

In contrast, GC have also been shown to have a protective effect when administered after the onset of some inflammatory disorders. Inhaled corticosteroids, for example, are commonly used to treat acute allergic asthma. Characterized by airway obstruction, cytokine and chemokine production, and airway hyperresponsiveness (AHR), asthma is a form of chronic inflammation that is perpetuated by recurring acute inflammatory exacerbations (121). In response to allergen exposure, inflammatory cells such as, macrophages, neutrophils, and eosinophils infiltrate the airway, causing tissue damage and pulmonary dysfunction (121, 122). Ten to fifteen of every one thousand emergency room visits are asthma-related (123), with up to 20% of patients requiring hospital admission (124). Early systemic administration of corticosteroids has significantly reduced the incidence of hospitalization and relapse, and expedited recovery, for severe asthmatics presenting in the clinic with an acute exacerbation (125). Studies utilizing models which mimic the clinical situation have also expanded the understanding of the mechanisms of these effects. Using a novel murine model of asthma, Kim *et al.* demonstrated that dexamethasone inhibits the asthmatic response induced by house dust extract (HDE) containing cockroach allergens (126). Specifically, BALB/c mice immunized and challenged with HDE were treated with 2.5 mg/kg DEX after 24 hours. The investigators reported that 12 and 24 hours after DEX treatment, pulmonary recruitment of inflammatory cells, myeloperoxidase activity in the lung, airways hyperresponsiveness, and total serum IgE levels were significantly reduced compared with PBS (phosphate-buffered saline) treated mice (126). Although few studies have investigated the efficacy and mechanisms of post-stimulus anti-inflammatory

therapies, these studies underscore the importance of experimental models that have clinical relevance.

The conflicting data between the beneficial and detrimental effects of GCs highlight the necessity for a better understanding of the mechanisms of GC actions. Additionally, the movement of medicine toward individual therapy ensures that it will be equally important to gain a more comprehensive understanding of the inflammatory process. To that end, the data subsequently presented in this thesis were undertaken to gain insight into the mechanism by which DEX regulates the progression of acute inflammation in a whole blood model when administered after the inflammatory process has already been initiated. To determine if DEX could effectively regulate the progression of acute inflammation, it was added several hours after an inflammatory stimulus and its effect on cytokines and chemokines was determined. Our aims were threefold: 1) characterize and define our model of acute inflammation; 2) determine if the addition of DEX after the onset of inflammation consequently altered its suppressive capacity; and 3) determine if the effects of DEX were cell-type specific.

Figures and Tables

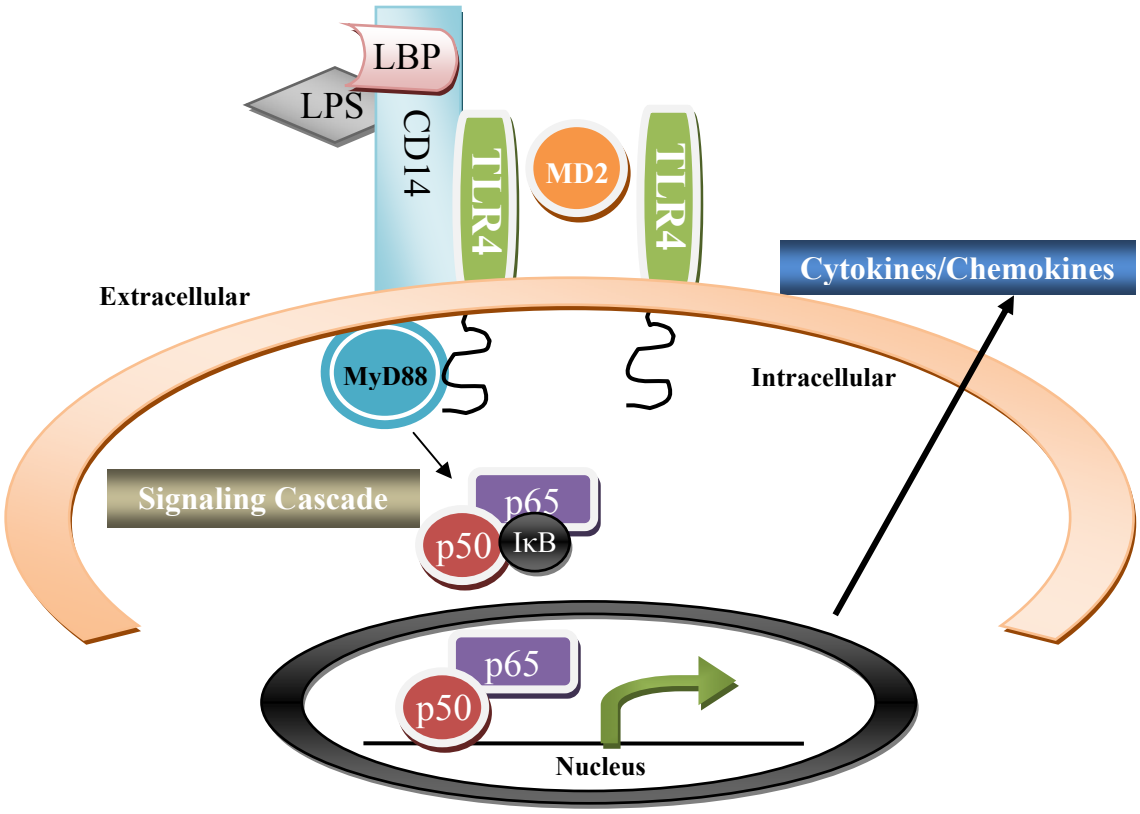

Figure 1.1 Inflammatory cascade. Initiation of inflammation usually begins with binding of PAMPs such as LPS to TLRs or other PRRs. LBP (LPS-binding Protein) and CD14 present LPS to the TLR4 dimer on its extracellular domain. The adaptor protein MyD88 then associates with the intracellular domain, and transmits a downstream signal leading to the release and nuclearization of the transcription factor NFκB. Transcription of inflammatory mediators such as cytokines and chemokines is activated, after which the translated proteins are secreted into the circulation and potentiate the inflammatory response.

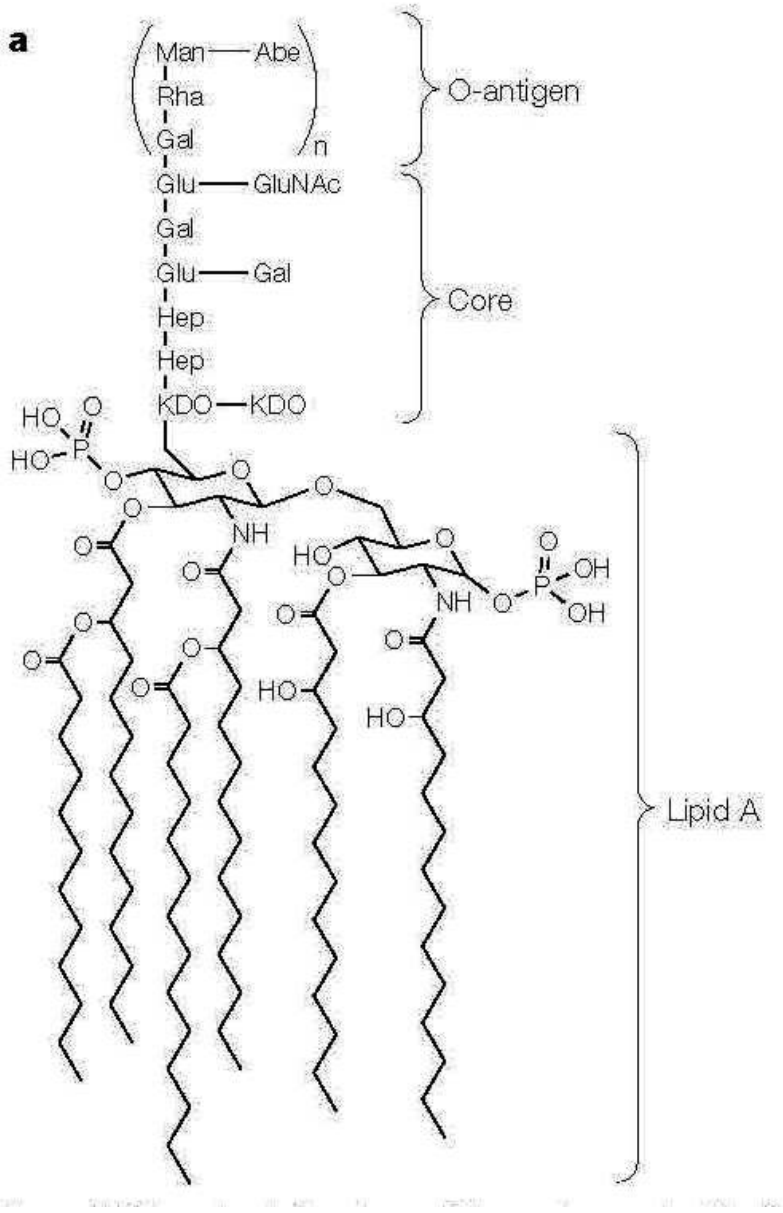

Figure 1.2 Structure of Lipopolysaccharide. LPS is composed of an O-antigen, a core oligosaccharide, and Lipid A (or endotoxin). It signals via TLR4 dimers located on the surface of immune cells. Taken from Miller *et al.* (95).

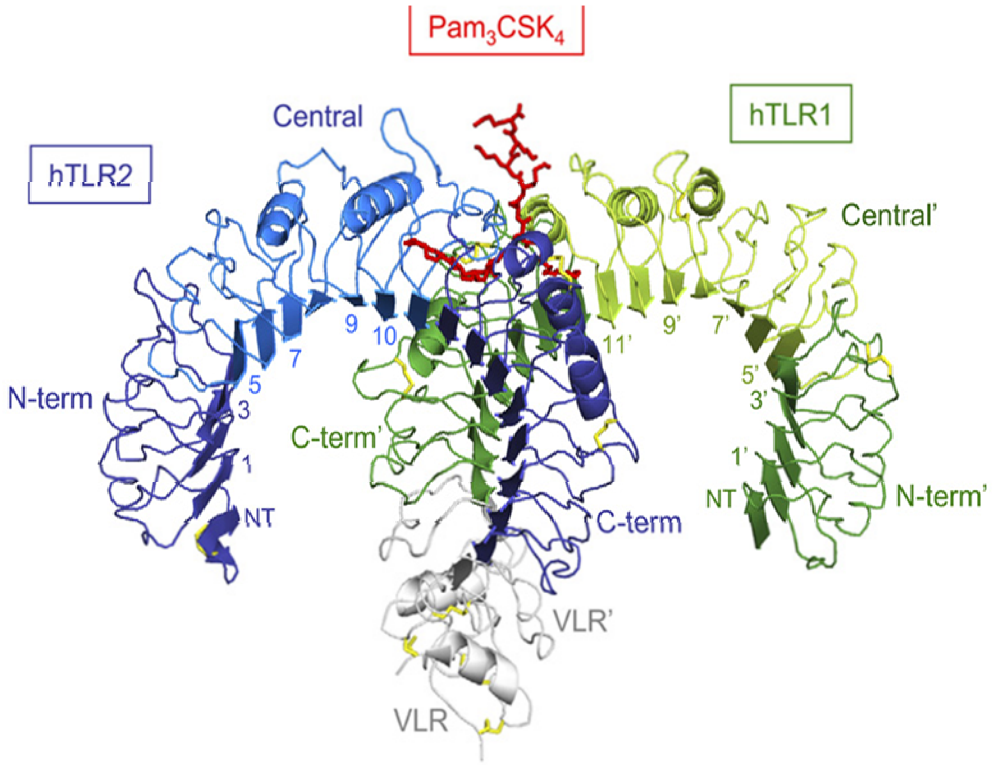

Figure 1.3 Structure of Pam_3 Cysteine-Serine-$Lysine_4$. Pam is a synthetic TLR2 agonist synthesized to mimic the actions of bacterial pathogen lipoproteins (BLPs). It is palmitylated and contains 4 Lysine residues in the COOH domain to mimic the immunomodulatory effects of BLPs (98, 100, 127). Taken from Jin et.al. (128).

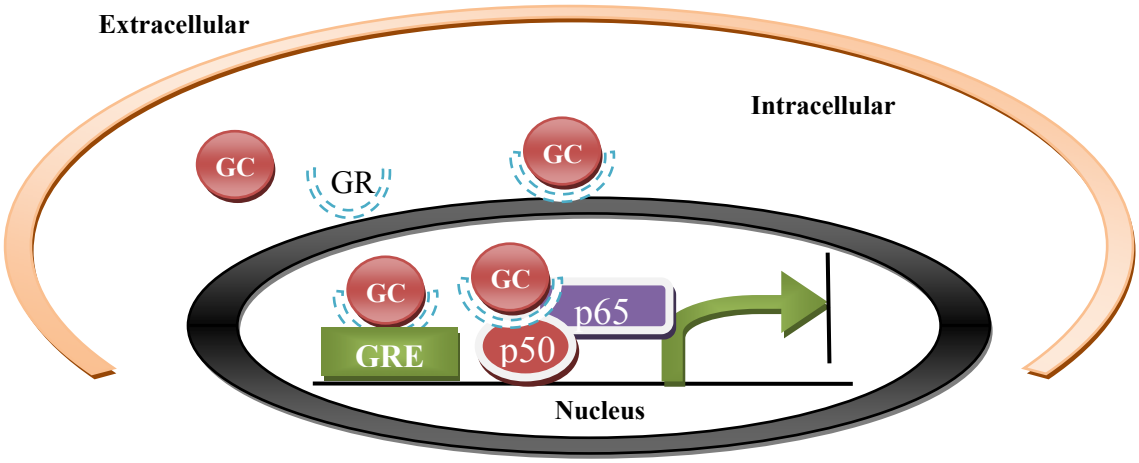

Figure 1.4 Glucocorticoid signaling pathway. GC are present in the plasma. Upon activation, they bind to the nuclear GR and translocate into the nucleus. There, they can bind to GRE on DNA or directly to transcription factors to inhibit gene transcription. GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element.

Table 1.1. Cytokines and chemokines evaluated (34, 81).

Cytokine	Function
Tumor Necrosis Factor alpha (TNFα)	Induces inflammatory cytokines/chemokines
Interleukin 1 beta (IL-1β)	Induces inflammatory cytokines/chemokines
Interleukin 6 (IL-6)	Regulates acute phase proteins
Interleukin 6 receptor (IL-6R)	Binds to IL-6 & activates Jak-STAT pathway
Interleukin 1 receptor antagonist (IL-1ra)	Binds to IL-1RII; no signal transduction
TNF soluble receptor two (TNFsrII)	Binds TNFα from circulation; can transmit signal
Interleukin 1 receptor two (IL-1RII)	Bind IL-1 from circulation; no signal transduction
Interleukin 8 (IL-8; CXCL8)	Recruit neutrophils
Growth Related Oncogene alpha (GROα; CXCL1)	Recruit neutrophils
Macrophage Inflammatory Protein 1 alpha(MIP-1α or CCL3)	Recruit macrophages
Macrophage Inflammatory Protein 1 beta (MIP-1β or CCL4)	Recruit macrophages
Monocyte Chemotactic Protein 1 (MCP-1; CCL2)	Recruit monocytes

Table 1.2. Toll-like receptors

Toll-like receptor	Cellular source	Stimulus
TLR1	Ubiquitous	Downregulated in T cells after PHA exposure
TLR2	PMNs; DCs, and *Mo*	BLPs, PGN, LTA
TLR3	DC & NK cells	Differentiation; decreased upon maturation
TLR4	Numerous including: macrophages, DCs, & ECs	Cytokines & bacterial products
TLR5	*Mo*, immature DCs, ECs, NK, & T cells	Flagellin
TLR6	B cells, Mo, & NK cells	Not induced by cytokines or LPS
TLR7	B cells, plasmacytoid precursor DCs	IL-6; moderately by other cytokines
TLR8	Mo, low in NK & T cells	Gamma interferon, LPS, & moderately by other cytokines
TLR9	Plasmacytoid precursor DCs, B cells macrophages, PMNs, NK cells, & microglia cells	Gamma interferon & LPS
TLR10	B cells & plasmacytoid precursor DCs	Not modulated by cytokines or LPS
TLR11	DCs	Protozoan parasite components

Abbreviations: TLR, Toll-like receptor; PMNs, polymorphonuclear cells; *Mo*, monocytes; NK, natural killer; DC, dendritic cells; EC, endothelial cells; PHA, phytohemagglutanin; BLP, bacterial lipoproteins; PGN, prostaglandin; LTA, lipoteichoiic acid. Table adapted from Janssens et al. (129).

CHAPTER II
CHEMOKINES ARE SELECTIVELY REGULATED DURING THE PROGRESSION OF ACUTE INFLAMMATION

Introduction

Traumatic injuries or infectious challenges activate the innate immune response, initiating acute inflammation which is perpetuated by secreted factors such as cytokines and chemokines. Cytokines and chemokines function in both the innate and adaptive immune responses by perpetuating inflammatory responses via paracrine and autocrine mechanisms (24). Invading pathogens shed their outer membrane components, which, upon binding to cell surface receptors, initiate cytokine and chemokine secretion by inflammatory cells over several hours to days, and in some instances chemokines are produced over several weeks (Fig. 1.1) (130). Published data report that in response to various stimuli, cytokines and chemokines often display distinct protein and mRNA kinetic profiles (31, 32, 131). Cytokines such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), and IL-6 are known to be rapidly induced and cleared while chemokines, such as IL-8 have been shown to have a steady, continuous production over time (25, 32).

Few studies, however, have examined if these distinct profiles result in distinct regulation. In fact, while patients are generally treated after the inflammatory response has begun, much of the investigations of the regulation of inflammation have focused on the inhibition of cytokine and chemokine production following pre-treatment or concomitant treatment with a stimulus and an anti-inflammatory reagent. The present

studies aimed to address this deficit in the knowledge by determining if postponement of anti-inflammatory treatment until after LPS stimulation would consequently alter the ability to regulate cytokines and chemokines. To accomplish this, the potent anti-inflammatory glucocorticoid dexamethasone (DEX) was added to human whole blood either together with or following lipopolysaccharide (LPS) stimulation. Glucocorticoids (GC) are members of the corticosteroid family whose anti-inflammatory properties have been widely exploited both as a clinical therapy and as a tool for understanding the mechanisms of inflammation (132). GC are widely used to treat a number of inflammatory conditions such as rheumatoid arthritis (133), bacterial meningitis (116), and allergic asthma (134). It has been shown to regulate inflammation, at least in part, by suppressing proinflammatory cytokines and chemokines (40, 115).

The whole blood model serves as a more physiologically relevant model than isolated cell types since isolating leukocytes requires washing and centrifugation, both of which are non-physiological processes. In addition, isolation of monocytes often requires depletion of other cell types, where as whole blood allows for stimulation of monocytes while maintaining the natural cellular interactions. De Groote *et al.* illustrated that whole blood had a higher capacity to produce cytokines compared with isolated PBMC (135). Further, Damsgaard *et al.* recently argued that whole blood is a "valid, low-cost method to measure cytokines" (136). They showed that, compared to cytokines produced in PBMCs, cytokines produced in LPS-stimulated whole blood more strongly correlated with cytokines secreted directly from isolated monocytes (136).

The experiments described here were designed to determine if DEX could regulate cytokine and chemokine production after inflammation had been initiated in the

whole blood model. While the whole blood model does not provide the same complexity as *in vivo* studies, the model has been used extensively by numerous investigators to study the regulation of cytokine production (136, 137).

Materials and Methods

Reagents

Heparin sodium derived from porcine intestinal mucosa was obtained from American Pharmaceutical Partners, Inc. (Schaumburg, IL). LPS (*Escherichia coli* serotype O111:B4) and water-soluble DEX were obtained from Sigma-Aldrich (St. Lous, MO; Cat no. D-2915). Capture and biotinylated detection antibodies for ELISA measurement of TNF, IL-1β, IL-6, and IL-8 were purchased from R&D systems (Minneapolis, MN). RPMI-1640 was obtained from Invitrogen Life Technologies (Carlsbad, CA). Oligonucleotide promoters for PCR were purchased from Invitrogen Life Technologies and Integrated DNA Technologies and Integrated DNA Technologies (Coralville, IA). The RNeasy RNA isolation kit was purchased from Qiagen (Valencia, CA) and the One-Step RT-PCR kit was purchased from BioRad (Hercules, CA). The water-soluble tetrazolium (WST) cell counting kit (CCK-8) was purchased from Dojindo Molecular Technologies, Inc (Rockville, MD). The Chromo-LAL reagent was purchased from the Associates of Cape Cod, Inc (Cape Cod, MA).

Blood collection & stimulation

Venous blood was collected from healthy volunteers into heparinzed (10U/ml) syringes. For the kinetics of TNF, IL-1β, IL-6, and IL-8 expression, LPS (to a final concentration of 50 ng/ml) or RPMI 1640 vehicle was added to 1 ml of blood in 1.5 ml tubes, followed by incubation with 5% CO_2 and ambient air at 37^{o}C on a rotating shaker. Blood was spun at 1000 x g for 5 minutes at the time points indicated in the graphs, plasma collected and stored at –20^{o}C for later cytokine analysis. For DEX experiments, dexamethasone was

added to blood at a final concentration of 10^{-6}M (diluted in RPMI-1640) simultaneously with LPS at time zero or six hours after LPS stimulation. These studies have been approved by the Institutional Review Board of Boston University and The University of Michigan.

Blood Gas Analysis

At 0, 6 and 24 hours after LPS, 500 µl of blood were analyzed for glucose and blood gases (pH, pCO2, pO2) using the ABL 800 Flex (Sysmed Lab Inc., Chicago, IL).

Cell viability

Leukocytes were collected after NH_4Cl lysis from four different groups after 24 hours: 1)blood stimulated with vehicle (RPMI-1640), 2) blood stimulated with 50 ng/ml LPS alone, 3) blood stimulated with LPS + 10^{-6}M DEX, or 4) unstimulated blood. Blood was centrifuged at 1000 x g for 5 minutes and the plasma was removed. The packed cell pellet was then added to 14 ml of NH_4Cl buffer (NH_4Cl, $NaHCO_3$, and tetra EDTA) and gently rotated for 5 minutes. The tubes were then spun at 1000 x g for 5 minutes, the supernatant aspirated and 1ml of lysis buffer was used to resuspend the pellet. The mixture was then added to a 1.5 ml centrifuge tube and rotated for 5 minutes. The tube was then spun at 1000 x g and the supernatant aspirated. The pellet was resuspended in 1 ml RPMI. To construct a standard curve to calculate the number of viable cells, unstimulated blood was collected after 24 hours, red cells lysed and the white cells counted using the Beckman Cell and Particle Counter (Beckman Coulter, Inc., Fullerton, CA). For a negative control, cells were heat-killed at 56^{o}C for 30 minutes. 100 µl aliquots of heat-killed, vehicle-, LPS-, or LPS/DEX-stimulated cell suspension were added to 96-well tissue culture plates in duplicate and 10 µl WST solution added to each well and

incubated with 5% CO_2 at 37°C for up to 4 hours. Absorbance was measured at 450 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). WST salt reduced by dehydrogenases from viable cells produced an amount of orange-colored formazan dye which was proportional to the number of living cells.

LPS clearance

Blood was stimulated with 50 ng/ml of LPS, plasma was collected at various time points and LPS levels were determined using the limulus amoebocyte lysates assay. To detect LPS mixed with plasma, LPS was also added directly to plasma and assayed at various time points. To optimally detect the LPS in the assay, the plasma was diluted 1:20 and heated at 95°C for 2 minutes to inactivate the plasma proteins. Serially diluted standards also included 1:20 heat-treated, plasma. Samples or standards were then added to 96-well plates in 100 µl aliquots and 100 µl of Chromo-LAL reagent was added to each sample well. According to the manufacturer's protocol, the delta OD was read at 405 nm every 150 seconds for 2 hours to determine the amount of time required to increase 0.2 absorbance units (reaction time) using the microplate reader. Using the KC4 v3.4 software (Bio-Tek, Winooski, VT), when the log/linear correlation of the reaction time of each standard with its corresponding LPS concentration had a value of ≤0.98, a polynomial curve-fitting model was used to construct a standard curve, and used to calculate the concentration of LPS in each sample.

RNA collection & real-time PCR

Total cellular RNA was purified using the Qiagen RNeasy® kit and 5 µl total RNA was used for reverse transcription and real-time PCR using the BioRad iScript™ One-Step RT-PCR kit with SYBR® Green, according to the manufacturer's protocol. Real-time

PCR was performed on a Biorad iQ5 iCycler in paired reactions. Cytokine gene expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the fold increase relative to vehicle control was calculated using the $2^{-(\Delta\Delta Ct)}$ method (138). Primers (Table 2.1) were generated using Beacon Designer software (Premier Biosoft International, Palo Alto, CA), Primer 3 (139), and OligoPerfect Designer (Invitrogen, Carlsbad, CA).

Cytokine ELISA

Plasma cytokine levels were determined by ELISA according to our previously published methods (140).

Statistics

Statistical analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad, San Diego, CA). Results were expressed as the mean ± SEM. Statistical comparisons were made using a one-way ANOVA followed by the Newman-Keuls multiple comparison post test. For direct comparisons between groups, the student's *t* test was used.

Results

Whole blood

Heparinized human whole blood collected from healthy volunteers was analyzed for glucose and blood gas levels 0, 6, and 24 hours after collection (Fig. 2.1). By 6 hours glucose decreased by 75% (99 to 25 mg/dl) and by 95% (0.5 mg/dl) after 24 hours (Fig. 2.1A). The pH decreased from 7.30 ± 0.02 to 7.20 ± 0.03 by 6 hours, and to 7.00 ± 0.02 by 24 hours (Fig. 2.1B). While the pO_2 remained unaltered (Fig. 2.1C), pCO_2 significantly increased from 50 ± 3 to 74 ± 4 mmHg after 24 hours (Fig. 2.1D).

Given the changes in glucose and blood gas parameters cell viability was examined using the water-soluble tetrazolium reagent (Fig 2.2). Compared with cells heat-killed at 56^{o}C for 30 minutes, significantly more viable cells were detected following whole blood treatment with either vehicle (RPMI), LPS (50 ng/ml), or 10^{-6}M DEX. These results indicate that none of the reagents had any significant cytotoxic effects on cellular viability in the whole blood model.

LPS-induced cytokine and chemokine kinetics

To illustrate TNF, IL-1β and IL-6 kinetics in this model, whole blood was stimulated with the TLR4 agonist, LPS, and cytokine and chemokine protein levels were measured in the plasma. Figure 2.3 A indicates that TNF climaxed early, reaching maximal levels by 3 hours and remaining essentially unchanged for 24 hours. IL-1β and IL-6 displayed nearly identical protein kinetics over 24 hours, both reaching maximum levels within 6 hours of stimulation (Fig 2.3B and C). Cytokine levels remained unchanged after maximal production up to the conclusion of the study at 24 hours. LPS-induced chemokine kinetics, in contrast, yielded different kinetic profiles compared with

the cytokines. LPS stimulation resulted in the continuous increase in IL-8 chemokine protein levels over 24 hours (Fig. 2.3D). IL-8 did not display signs of having reached maximum; it appeared to rise even at 24 hours, consistent with previous reports (25, 32).

Table 2.2 highlights the difference in cytokine and chemokine levels 6 and 24 hours after LPS stimulation. There was no significant difference in TNF, IL-1β, or IL-6 levels between 6 and 24 hours, confirming the impression from the kinetic studies in Figure 2.3 that the levels had plateaued. In contrast, IL-8 levels increased significantly between 6 and 24 hours after LPS stimulation, suggesting continuous production.

Since inflammation is such a well-orchestrated and tightly regulated phenomenon, it was important to evaluate a broad scope of mediators, and then carefully narrow the focus on those which were specifically affected. Thus, LPS-induced anti-inflammatory cytokines were also measured. As depicted in Table 2.3, anti-inflammatory cytokines were not significantly induced following LPS stimulation and were excluded from subsequent experiments.

To document the rapid and transient induction of mRNA, the LPS-stimulated kinetic profile of cytokine and chemokine mRNA in whole blood was investigated. Total RNA was extracted from leukocytes in LPS-stimulated whole blood. Semi-quantitative analysis using real time RT-PCR demonstrated a similar pattern of induction for all three cytokines (Fig 2.4A-C). TNF, IL-1β, and IL-6 mRNA peaked within 2 hours and were nearly undetectable by 6 hours. Similar to protein, chemokine mRNA kinetics were distinct from that of cytokines. LPS-induced IL-8 mRNA gradually increased between 0 and 6 hours, reaching an initial peak 2 hours after stimulation (Fig. 2.4D). By 12 hours,

IL-8 message dropped to near basal levels, after which it rose a second time, continuing to increase at 24 hours.

The next aim was to determine whether progression of acute inflammation characterized by continuous chemokine production was due to continuous LPS signaling. To accomplish this, the kinetics of LPS clearance from whole blood compared with clearance from plasma alone was evaluated. First, LPS was added to whole blood at time 0, followed by Limulus amoebocyte lysate (LAL) measurement after 5 minutes, 6 and 24 hours. Figure 2.5 (solid line) shows that after 6 hours whole blood LPS was significantly lower than at 5 minutes such that it was virtually undetectable. This indicates that LPS was rapidly cleared from whole blood within 6 hours. Additionally, 50 ng/ml of LPS was added directly to plasma. Figure 2.5 (dotted line) shows that LPS levels detected after 6 and 24 hours remained statistically similar to the levels observed 5 minutes after LPS administration to plasma. Thus whole blood clears endotoxin, but human plasma does not.

Delayed DEX fails to suppress TNF, IL-1β, and IL-6

The next set of experiments compared the ability of 10^{-6}M DEX to suppress TNF, IL-1β, and IL-6 protein levels when added to whole blood simultaneously with LPS. Figure 2.6 (panels A-C) shows that concomitant DEX/LPS significantly suppressed TNF, IL-1β, and IL-6 protein levels measured at 24 hours compared with LPS stimulation alone.

It was then determined if delaying the addition of DEX for six hours would have any consequences on its ability to suppress cytokine protein levels. The results indicate that delaying DEX significantly reduced its anti-inflammatory potency (Figure 2.6A-C).

TNF, IL-1β, and IL-6 protein levels were statistically comparable to their levels following LPS-stimulation in the absence of DEX. These findings suggest that the whole blood model is characterized by early TNF, IL-1β, and IL-6; however these cytokines were not continuously produced and thus were not susceptible to DEX suppression when it was administered after the stimulus. Since the data from Figure 2.4 indicated that TNF, IL-1, and IL-6 mRNA were undetectable after 6 hours, it was not necessary to assess the effects of post-stimulus DEX on their mRNA levels.

Delayed DEX selectively suppresses IL-8

Since LPS-stimulation of whole blood caused different cytokine and chemokine kinetic patterns (Fig. 2.3 and 2.4), the next studies examined the ability of DEX to suppress 24 hour chemokine levels when added 6 hours after LPS stimulation. Whole blood was collected and stimulated as previously described. Figure 2.6 shows that concomitant addition of LPS and DEX to whole blood significantly suppressed IL-8 protein compared with LPS stimulation alone, similar to its ability to suppress TNF, IL-1β and IL-6. In contrast, even when administered 6 hours after LPS stimulation, DEX retained its ability to significantly suppress 24 hour IL-8 protein levels (Fig. 2.6D). Delayed DEX suppressed IL-8 protein by approximately 76% from 14.5±1.8 to 5.3±1.3 ng/ml (Fig 2.6D). Experiments in Figure 2.7 were designed to determine whether the mechanism of suppression was mediated through decreased IL-8 mRNA. Simultaneous DEX and LPS resulted in significant suppression of IL-8 at 24 hours, compared with LPS alone (Fig. 2.7). Delaying the addition of DEX until 6 hours after LPS stimulation did not diminish its suppressive capacity since DEX still significantly reduced IL-8 mRNA by approximately 86% (Fig. 2.7).

Discussion

Inflammation is a rapid, efficient and coordinated response to traumatic or infectious injury, perpetuated in part by the secretion of cytokines and chemokines such as TNF, IL-1β, IL-6, and IL-8. Previous studies have demonstrated that pre or simultaneous treatment with anti-inflammatory reagents, such as DEX, together with a stimulus, such as LPS, prevents the onset of inflammation in part by suppressing proinflammatory mediators (141, 142). However relatively few studies have examined the biological effects of delayed anti-inflammatory treatment. One such study undertaken by Mogensen *et al.* aimed to understand the mechanisms of action of glucocorticoids by examining the effect of DEX prior to, concomitant with, or following N. *meningitides* or S. *pneumonia*-stimulation of peripheral blood mononuclear cells (PBMC) or THP-1 cells. They reported that, similar to our findings, postponing DEX partially suppressed IL-8 induction even when it was added at 7 hours following infection (143). Although these findings were interesting and important, the scope of investigation on the effects of delayed administration of DEX was narrow, focusing only on IL-8 and not including other inflammatory mediators. Further, the experiments were performed in isolated PBMC and THP-1 cells, as opposed to the *ex vivo* model. Thus, to gain further insight into the regulation of inflammation, we examined the effects of 6 hour DEX on LPS-induced cytokines and chemokines in the human whole blood model of inflammation. Here, we provide evidence that delaying the addition of DEX to LPS-stimulated whole blood until 6 hours post stimulation abrogates its suppression of TNF, IL-6, and IL-1β, but delayed addition of DEX still suppresses IL-8.

Blood gas analysis showed minimal changes in pO_2 in unstimulated blood after 24

h, suggesting that gas exchange was taking place. The significant decrease in glucose suggested a possible reduction in cell viability, which was a potential pitfall since glucose is required for cellular respiration and LPS-induced cell signaling events. Normal blood glucose ranges from 80 to 120 mg/dl (150 mg/dl directly after food consumption) (144), whereas ranges below 80 mg/dl are usually considered hypoglycemic. The significant reduction in pH further signaled a potential problem, since the physiologic range of pH of blood is tightly regulated. To address these concerns, the WST (water-tetrazolim salt) assay was performed to determine cell viability 24 hours after stimulation with vehicle, LPS alone, and LPS followed by the addition of DEX. Significantly more viable cells were present in the vehicle, LPS or LPS together with DEX samples compared to heat-killed cells. Further, the data from Figure 4D illustrates ongoing IL-8 production, which indicates that a significant portion of the cells remained viable. In hypoglycemic conditions, cells may compensate by resorting to alternative respiration methods or consumption of other nutrients such as carbohydrates, fats, or proteins as a source of energy (144, 145).

An additional concern was the signaling of LPS in the whole blood model. It could be argued that continuous IL-8 was the result of continuous LPS presence in the system. Also, the anticoagulant heparin has been shown to enhance LPS-induced activation of mononuclear cells in the whole blood model by binding to the LPS-binding protein (146, 147). *In vivo*, LPS is cleared from circulation by binding to Toll-like Receptor 4 (TLR4) expressed on white blood cells (129). Here, we have shown that LPS is rapidly cleared when added directly to whole blood, compared to plasma.

TNF, IL-1β, and IL-6 are classic early and relatively short-lived cytokines while chemokines have been shown to remain for hours to days, and even weeks after stimulation (25, 32, 84). Little data have examined if the distinct kinetic profiles result in differential regulation of the onset of inflammation compared with regulation of the maintenance of inflammation. Our data suggests that in the whole blood model of inflammation a difference does exist. Over 24 hours, LPS stimulation resulted in elevated levels of pro-inflammatory cytokines as well as chemokines after 24 hours. However, when compared with the levels at 6 hours, it was clear that IL-8 was significantly higher. Further comparison of 6 and 24 hour LPS-induced IL-8 mRNA suggested that chemokines were continuously being made even at 24 hours whereas pro-inflammatory cytokine mRNA was undetectable at 24 hours. The most interesting observation was that the continuous IL-8 was susceptible to DEX suppression even when DEX was administered an entire 6 hours after LPS stimulation of whole blood. This was true for both protein and mRNA, supporting our initial hypothesis that chemokines are selectively regulated during the progression of acute inflammation.

Given the lack of investigation of the effects of post-stimulus anti-inflammatory treatments, the present studies utilized the anti-inflammatory properties of glucocorticoids to compare the onset of inflammation with the progression of acute inflammation. One possibility is that DEX works by blocking mediators other than the classical pro-inflammatory cytokines TNF, IL-1β or IL-6. As demonstrated by the data presented here, message for all three cytokines is rapidly induced within 6 hours of stimulation, and thus not present for DEX regulation. However, IL-8, which showed detectable mRNA over 24 hours was receptive to DEX suppression. That the data show

suppression of IL-8 when DEX treatment is postponed until after stimulation suggests that in the clinic anti-inflammatory therapies may work by regulating chemokines, which are persistent, rather than rapidly induced, short-lived pro-inflammatory cytokines.

Figures and Tables

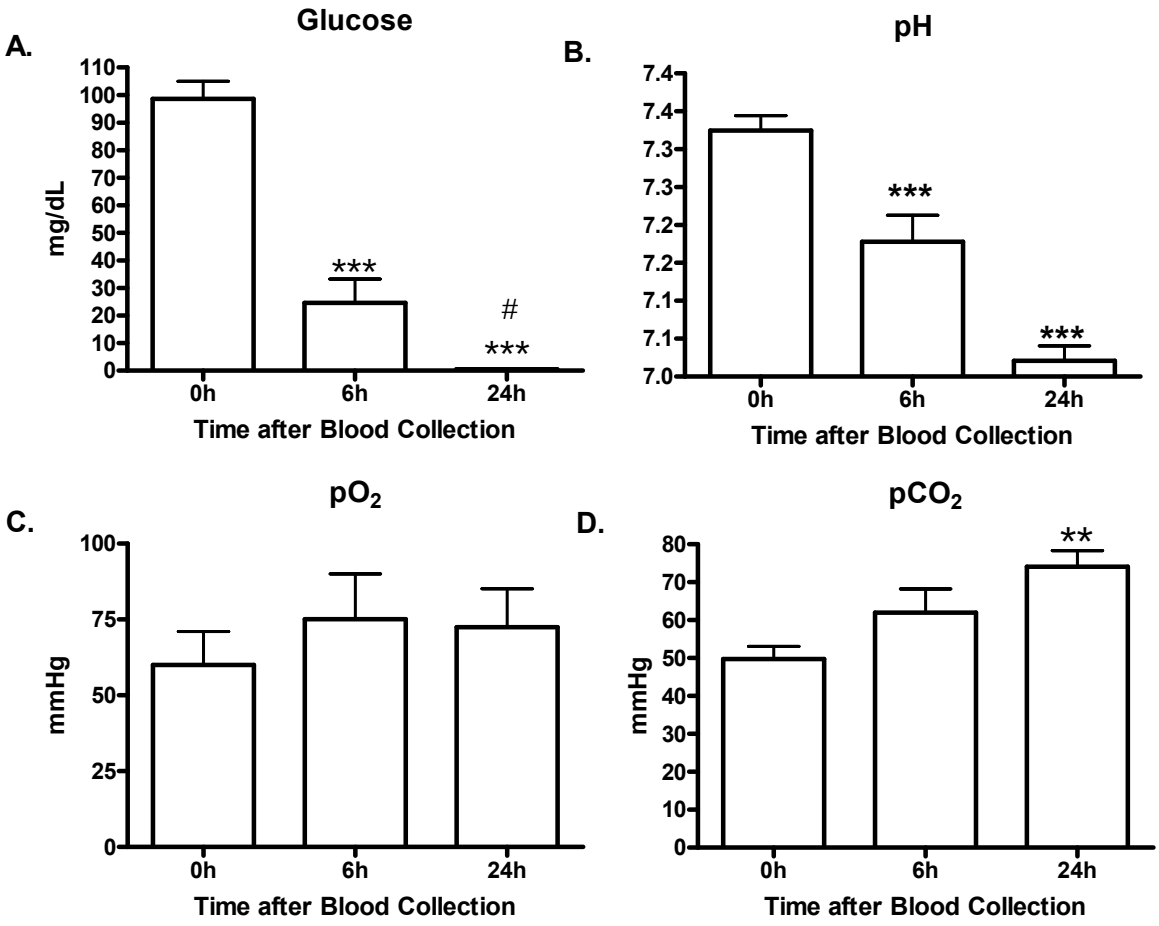

Figure 2.1 Glucose and blood gas analysis. Human whole blood was analyzed for glucose levels (mg/dl) (A), pH (B), and the partial pressure of O_2 (C); and CO_2 (D). Results expressed as mean ± SEM (n=8 donors) compared by ANOVA and Newman-Keuls post test. **p<0.01 and ***p<0.001 compared to 0h; #p<0.05 compared to 6h.

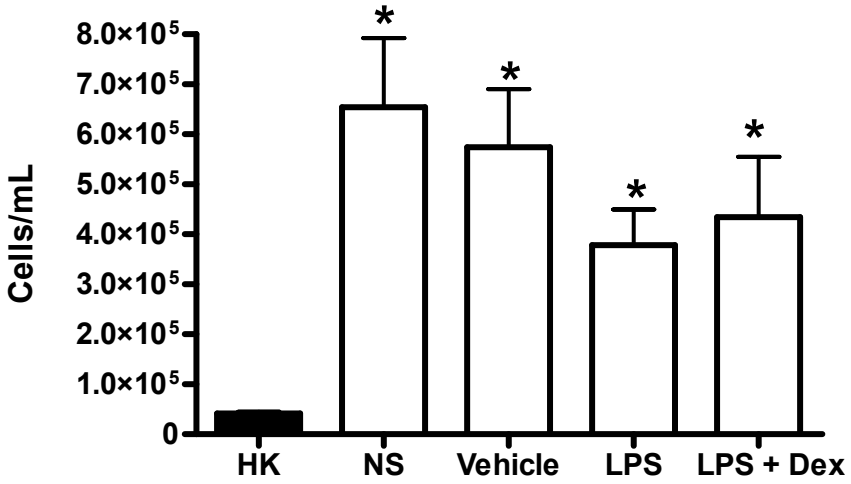

Figure 2.2 Cellular viability. Cellular viability does not change when whole blood is treated with vehicle, LPS, or LPS together with DEX. White blood cells were isolated from 1 mL whole blood 24 hours after stimulation with vehicle (RPMI), 50 ng/ml LPS, or LPS + 10^{-6}M DEX. 10 µl water tetrazolium was added to 100 µl white blood cells and incubated at 37^{o}C for up to 4 h. WST salt reduced by dehydrogenases from viable cells produced an orange-colored formazan dye (measured at 450nm) proportional to the number of living cells. Results expressed as mean ± SEM (n=4-7 donors), *$p<0.05$ vs. HK; compared by One-way ANOVA followed by Newman-Keuls Multiple Comparison post test. HK, heat killed; NS, non-stimulated.

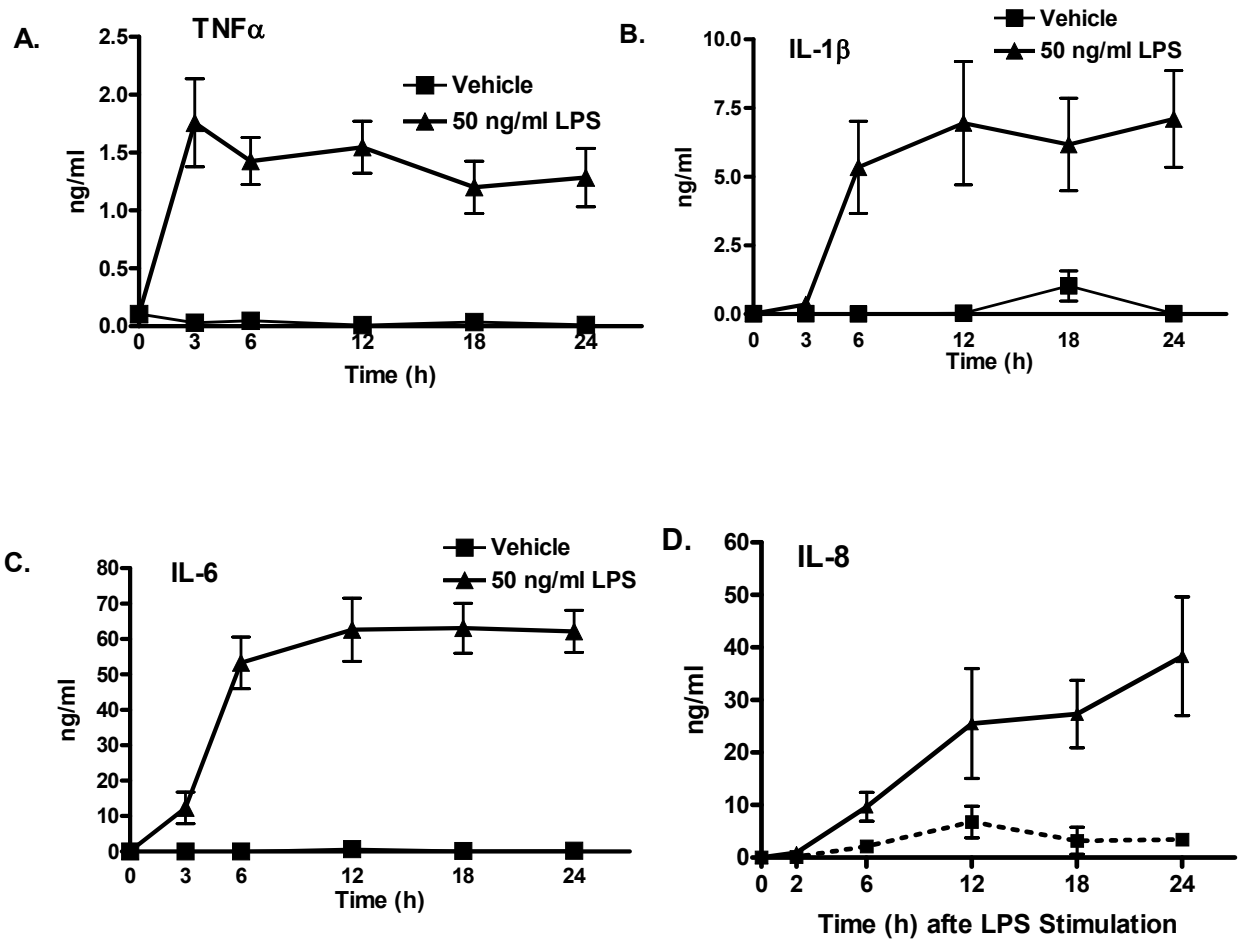

Figure 2.3 LPS-induced cytokine protein kinetic profile. The cytokines TNF, IL-1β and IL-6 plateaud within 6 hours, while chemokine levels continuously increased over 24 hours after LPS stimulation of whole blood. LPS-induction of (A) TNF; (B) IL-1β; (C) IL-6; (D) IL-8 protein in whole blood over 24 hours. Plasma was collected from LPS-stimulated whole blood and cytokine levels determined by ELISA. Results expressed as mean ± SEM (n=4-9 donors).

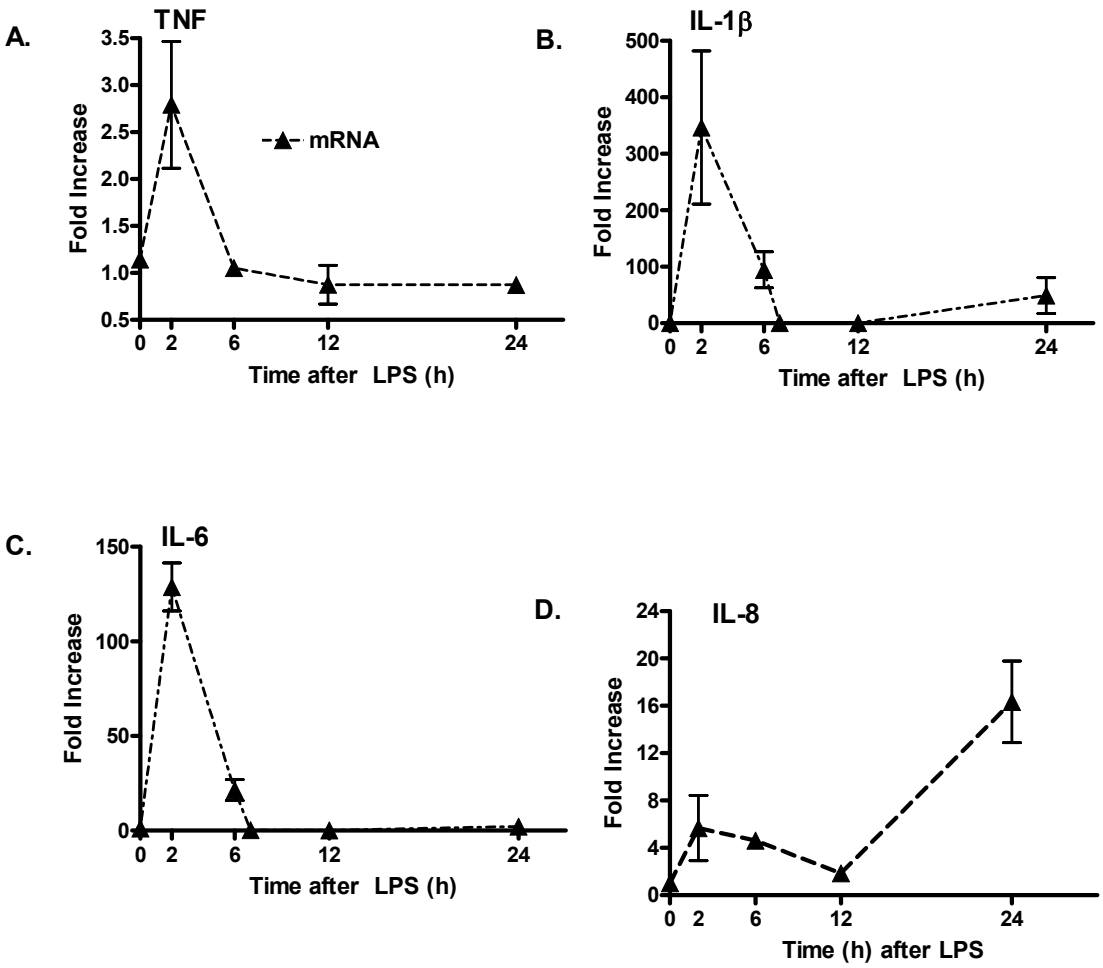

Figure 2.4 LPS-induced mRNA kinetic profile. LPS induction of (A) TNF; (B) IL-1β; (C) IL-6; (D) IL-8 mRNA in whole blood over 24 hours. Total RNA was isolated from leukocytes at each time point and analyzed by semi-quantitative RT-PCR for mRNA levels. Results expressed as mean ± SEM (n=4 donors).

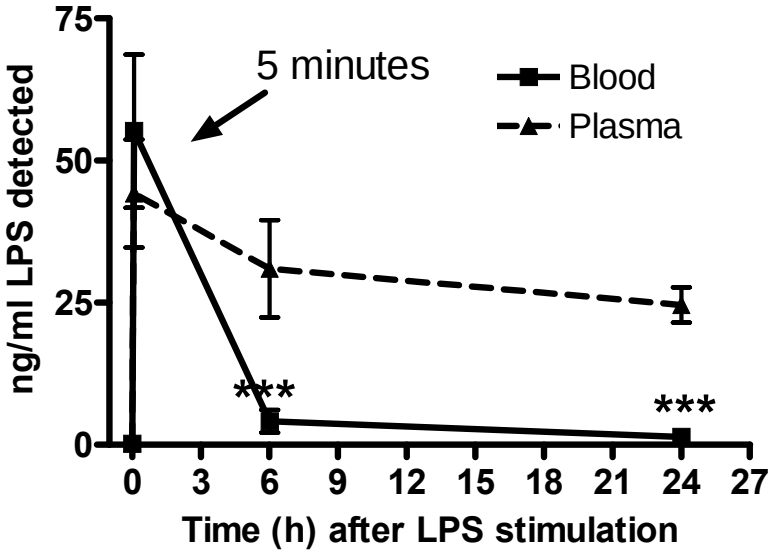

Figure 2.5 LPS clearance in whole blood *ex vivo* model. 50 ng/ml LPS was added to heparinized whole blood (solid line) or heparin anti-coagulated plasma (dashed line) at 0 hours and *LAL* used to measure LPS at subsequent time points. Results expressed as mean ± SEM (n=4 donors) compared by ANOVA and Newman-Keuls Multiple Comparison post test. *$p<0.001$ versus 5 minutes.

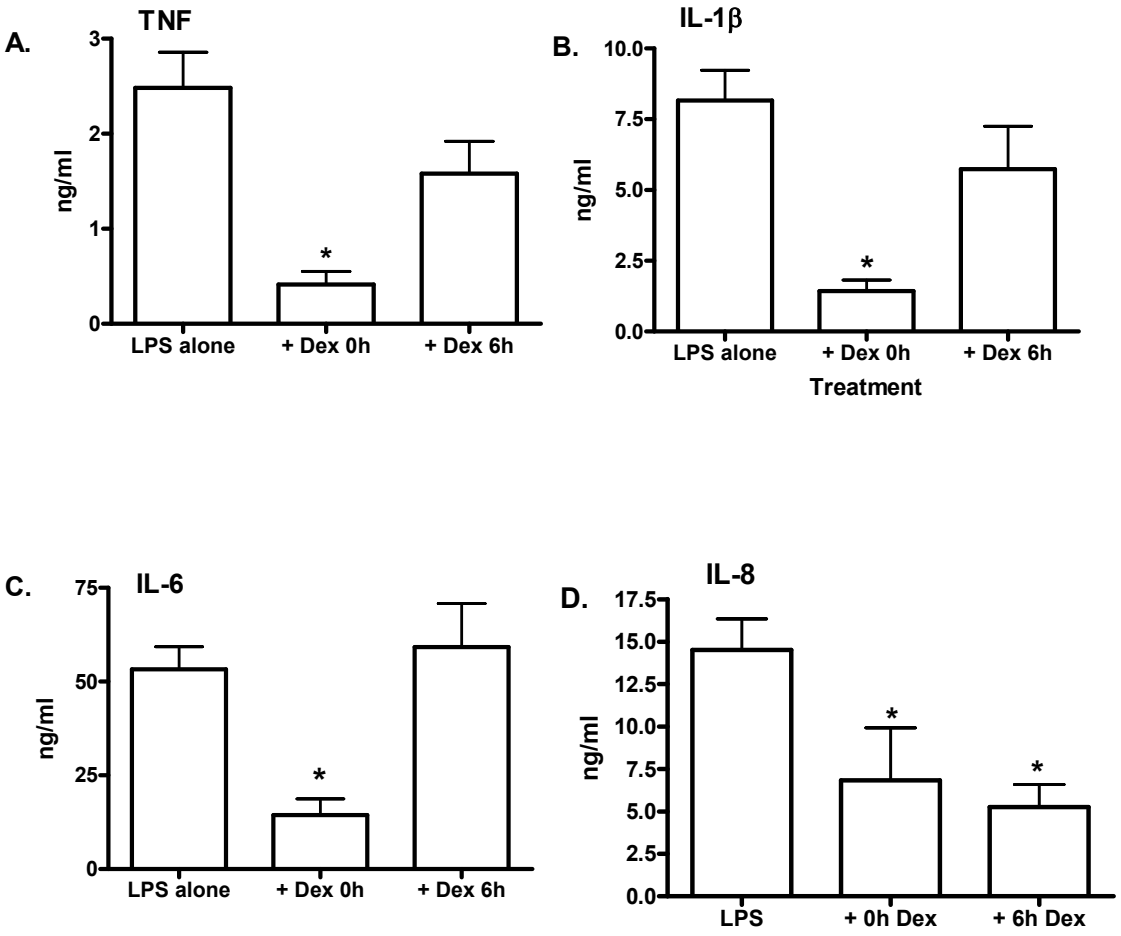

Figure 2.6 Delayed DEX effect on protein. Cytokine and chemokine protein levels following LPS alone, simultaneous LPS/DEX, or LPS + 6 hours delayed addition of DEX treatment of whole blood. TNF (A); IL-1 (B); IL-6 (C); IL-8 (D). Results expressed as mean ± SEM (n=8-13 donors). *$p<0.05$ vs. LPS alone compared by ANOVA and Newman-Keuls post test.

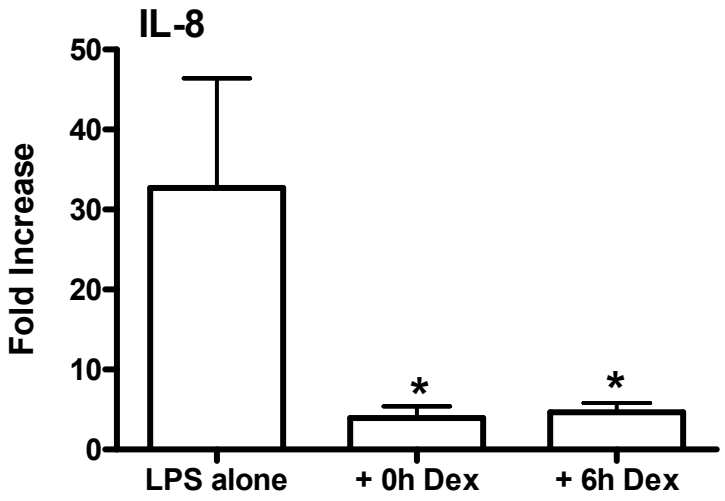

Figure 2.7 Delayed DEX effect on mRNA. Chemokine mRNA levels following LPS alone, simultaneous LPS/DEX, or LPS 6 hours delayed DEX treatment of whole blood. IL-8 (A). Results expressed as mean ± SEM (n=4-8 donors). *$p<0.05$ vs. LPS alone compared by ANOVA and Newman-Keuls post test.

	Sense	Antisense
IL-1β	ATTCTCTTCAGCCAATCTTC	GCACTTCATCTGTTTAGGG
TNF-α	AGCAAGGACAGCAGAGGAC	TGTGGCGTCTGAGGGTTG
IL-6	TCCAGAACAGATTTGAGAGTAGTG	GCATTTGTGGTTGGGTCAGG
IL-8	AGAGACAGCAGAGCACAC	AGTTCTTTAGCACTCCTTGG
GAPDH	TCGACAGTCAGCCGCATCTTCTT	CCAAATCCGTTGACTCCGACCT

Table 2.1 *Oligonucleotide primer sequence sets used for quantitative real-time PCR.*

Cytokine (ng/ml)	6h	24h
TNF	1.4 ± 0.2	1.3 ± 0.3
IL-1β	5.3 ± 1.7	7.1 ± 1.8
IL-6	53.3 ± 7.3	62.2 ± 5.9
IL-8	9.7 ± 2.7	38.3 ± 11.3*

Table 2.2 LPS-induced cytokines and chemokines taken from Figure 3 kinetic graph. The 6 and 24 hour levels of TNF, IL-1β, and IL-6 remain unaltered between 6 and 24 hours. IL-8 increases at 24 hours. Values represent the mean ng/ml of protein ± SEM (n=4-9 donors).

Cytokine (ng/ml)	Vehicle	LPS
IL-1 receptor II	9 ± 1.6	7 ± 0.5
IL-1 receptor antagonist	4.1 ± 1.7	3 ± 1.2
TNF soluble receptor II	2.0 ± 0.4	1.3 ± 0.1

Table 2.3 LPS-induced anti-inflammatory cytokine levels. Plasma was collected from human whole blood stimulated with LPS or vehicle for 24 hours. Values represent the mean ± SEM (n=9 donors). *$p<0.05$ vs. 6h compared by student's *t* test.

CHAPTER III

TLR2-INDUCED CYTOKINES AND CHEMOKINES ARE VARIABLY SUSCEPTIBLE TO DELAYED DEXAMETHASONE TREATMENT IN WHOLE BLOOD

Introduction

Toll-like receptors (TLRs) are cell surface proteins which recognize pathogen-associated molecular patterns (PAMPs) (148, 149). Serving as one of the first lines of defense in the innate immune response, TLRs initiate a signaling cascade which ultimately leads to the production of cytokines, chemokines, and a host of other inflammatory mediators and effectors (150). In the previous chapter, LPS was used to examine the effects of delayed addition of DEX on TLR4 mediated cytokine and chemokine induction. Toll-like receptor 2 is another well-known pattern recognition receptor found on the surface of immune cells (92, 151). Studies in TLR2 and TLR4 deficient mice strongly suggest that while TLR4 mediates LPS signal transduction, TLR2 mediates signaling of microbial components of Gram-positive bacteria such as lipoteichoic acid and peptidoglycan (152, 153).

Invasion with Gram-positive pathogens such as *Streptococcus pneumonia* and *Staphylococcus aureus* activates the TLR2 signaling pathway. Upon binding of Gram-positive bacterial components such as lipoteichoic acid or peptidoglycan, activated TLR2 forms a heterodimer with either TLR1 or TLR6 on the surface of immune cells (94). This is followed by the intracellular recruitment of the adaptor protein MyD88 to the

intracellular domain of TLR2, and ultimately results in the downstream activation of NFκB (91, 154). As described in chapter I, the activation and subsequent nuclear translocation of NFκB leads to the transactivation of inflammatory genes such as cytokines and chemokines (155). Since Gram-positive bacteria account for more than 50% of all cases of sepsis in the United States (104), it was important to determine if postponing DEX treatment until after stimulation with a TLR2 agonist abrogated its suppressive capacity.

To address this, the synthetic TLR2 agonist, Pam_3 Cysteine-Serine-$Lysine_4$ (Pam) was used. As previously mentioned, Pam is a synthetic, palmitoylated lipopeptide comprised of an amino acid sequence homologous to that of the native lipoprotein found in the membrane of Gram-positive bacteria (Fig. 1.2). Widely used to study the mechanisms of inflammation, Pam has been shown to signal via TLR2/1 heterodimers (156, 157). In bone marrow-derived murine macrophages, Pam induced significant production of TNF, IL-1, and IL-6 in (102). Further, it dose-dependently increased IL-8 mRNA production by adrenocortical cancer cells (103). Taken together, these findings imply that thorough investigation of the mechanisms of inflammation requires attention to the role of TLR2-activating agents.

Materials and Methods

Reagents

All reagents were purchased as previously described. $Pam_3CSK_4 \cdot 3HCl$ (product number: ALX-165-066-M002) was purchased from Alexis Biochemicals (Farmingdale, NY).

Blood collection & stimulation

Blood was collected and stimulated as previously described in the "Materials and Methods" section of chapter II. In short, Pam (diluted in RPMI-1640) was added directly to venous blood to a final concentration of 1μg/ml. Plasma was collected at 0, 3, 6, 12, and 24 hours after Pam stimulation and assayed for cytokine and chemokine production as previously described (140).

Statistics

Statistical analyses were performed as described in chapter II. Briefly, results were expressed as the mean ± SEM and statistical comparisons were made using a one-way ANOVA followed by the Newman-Keuls multiple comparison post test. For direct comparisons between groups, the student's *t* test was used.

Results

Pam-induced cytokines

In the previous chapter, the data showed that LPS stimulation of whole blood caused rapid induction of the classic pro-inflammatory cytokines TNF, IL-1β, and IL-6. In the present experiments, the aim was to determine the effects of the TLR2 agonist Pam on cytokine kinetics in whole blood. Pam was added to 1 ml of whole blood at a final concentration of 1 μg/ml and plasma was harvested and assayed for cytokine levels at 0, 2 or 3, 6, 12, and 24 hours. Pam induced a similar TNF kinetic profile as did LPS. TNF secretion was rapid, reaching maximal levels by 3 hours, followed by a rapid decline by 12 hours (Fig. 3.1 A). It remained at basal levels for up to 24 hours. In a separate set of experiments, Pam-induced IL-1β peaked by 12 hours and remained unchanged between 12 and 24 hours (Fig. 3.1 B). IL-6 did not appear to reach maximal levels until 24 hours after Pam stimulation. Figure 3.1 C shows that IL-6 rose steadily between 6 and 24 hours. Further, comparison by paired *t* test indicated that significantly more IL-6 was present at 24 hours than at 12 hours.

Pam-induced chemokines

Data in chapter II showed that LPS stimulation of whole blood induced chemokine profiles that were distinct from those of cytokines. Here, the aim was to determine if Pam stimulation of whole blood would yield similar results. As expected, Pam-induced IL-8 and GROα kinetics were dramatically distinct from TNF and IL-1β. Remarkably, IL-6 kinetics was similar to IL-8 and GROα. Both chemokines were rapidly induced within 6 hours after Pam stimulation (Fig. 3.2 A, B). Unlike TNF and IL-1β, but

similar to IL-6, IL-8 and GROα protein were continuously produced between 0 and 24 hours. They did not display any detectable signs of slowing, even after 24 hours.

Effect of delayed addition of dexamethasone on cytokines

To determine if delaying the addition of an anti-inflammatory therapy effected its regulation of Pam-induced cytokines and chemokines, Pam-stimulated whole blood was treated with 10^{-6}M DEX either simultaneously with or 6 hours after the addition of 1 μg/ml of. Plasma was harvested after 24 hours and cytokine protein measured by matched antibody ELISA, as previously described (140). Since TNF showed little induction following Pam administration (<1ng; Fig. 3.1A), the effect of postponed DEX treatment on TNF was not included in these experiments. As evidenced by the results in Figure 3.3, the effects of post-stimulus DEX treatment were variable amongst IL-1β and IL-6 compared to LPS/DEX treatment. When DEX was added at time 0, IL-6 protein was significantly suppress by nearly 100% (from 104.2±29.3 to 0.6±0.5 ng/ml) similar to the results observed in chapter II when LPS was used as the stimulus (Fig 3.3 A). IL-6 was also susceptible to DEX suppression when it was administered 6 hours after Pam. Delaying DEX for 6 hours significantly suppressed Pam-induced IL-6 to 14.5±5.4 ng/ml (Fig. 3.3A).

Whereas Figure 2.6 showed that adding DEX 6 hours after LPS-stimulated whole blood had no significant effect on IL-1β, Figure 3.3 B shows that 6 hour DEX significantly suppressed Pam-induced IL-1β compared with Pam stimulation alone. Delayed addition of DEX suppressed IL-1β by approximately 60%, from 1.4±0.4 to 0.6±0.07 ng/ml (Fig. 3.3 B). Although simultaneous Pam/DEX apparently suppressed IL-

1β at 24 hours, all of the protein measurements fell below the limit of detection of the ELISA assay, and thus could not be computed in statistical analysis.

Effect of delayed dexamethasone on chemokines

Figure 3.2 suggests that Pam-induction of chemokines was strikingly similar to LPS stimulation. To determine if these similarities translated to delayed DEX regulation, IL-8 and GROα protein were measured 24 hours after simultaneous Pam/DEX treatment and Pam followed by DEX. Compared with Pam stimulation alone, simultaneous DEX suppressed IL-8 by approximately 94% and GROα by approximately 85%. (Fig. 3.4). Similar to LPS, even when the addition of DEX was postponed until 6 hours after Pam induced inflammation, IL-8 and GROα protein were significantly suppressed by approximately 70% and 77%, respectively. Taken together, these data suggest that anti-inflammatory glucocorticoids have the capacity to suppress inflammation when administered after the onset of inflammation, given they (cytokines/chemokines) are being actively synthesized.

Discussion

The present experiments supplement the data observed in chapter II. Although LPS is used almost ubiquitously as an inflammatory stimulant, activation of the TLR2 signaling cascade has proved clinically relevant, as a significant portion of people are present with Gram-positive infections annually (104). Thus, Pam was used as a tool by which to determine if administration of anti-inflammatory reagents after the onset of TLR2-mediated inflammation could still effectively regulate cytokine and chemokine levels. To this end, these data illustrate the effects of Pam stimulation on cytokine and chemokine production in the whole blood system. The effect of DEX was also examined. Initial kinetic experiments revealed that, compared with LPS stimulation, Pam induced similar IL8, GROα, and TNF kinetics, but markedly different IL-6 and IL-1β kinetics (Fig. 3.1 & 3.2). Pam-induced IL-1β did not peak until 12 hours after stimulation. IL-6 was continuously produced over 24 hours following Pam stimulation. TNF, on the other hand, was rapidly induced and appeared to decrease between 3 and 24 hours (Fig. 3.1) similar to LPS stimulation.

Several investigators have demonstrated that the initial TNF (and IL-1β) secreted in response to direct LPS stimulation goes on to further stimulate other cells in a paracrine manner (31, 32). This could be a potential explanation for the apparent decrease in TNF between 3 and 24 hours. On the other hand, all of these data taken together could be the result of slower kinetics induced by Pam. Although Pam stimulation caused a greater increase in IL-6 than did LPS, the rise in both IL-6 and IL-1β were considerably slower than with LPS. Slower kinetics means that adding DEX at later time points could

result in possible suppression, since protein levels were still increasing. As with LPS stimulation, Pam-induced IL-8 and GROα continued to increase between 0 and 24 hours.

In sum, the present studies were performed to enhance the whole blood model of inflammation by including a TLR2 agonist. Since some 50% of all infections are mediated by Gram-positive pathogens, these studies provide insight into the role of post-stimulus GC treatment in regulating cytokines and chemokines. The overall findings were promising, if not conclusive. Although LPS and Pam were both potent induces of cytokines and chemokines in whole blood (notwithstanding TNF), Pam-induced slower kinetics overall. The slower cytokine kinetics resulted in their susceptibility to suppression by delayed DEX treatment. These data suggest that in the clinic, the usefulness of GC as a therapy for a patient who presents with acute inflammation is dependent upon whether or not inflammatory markers remain elevated.

Figures and Tables

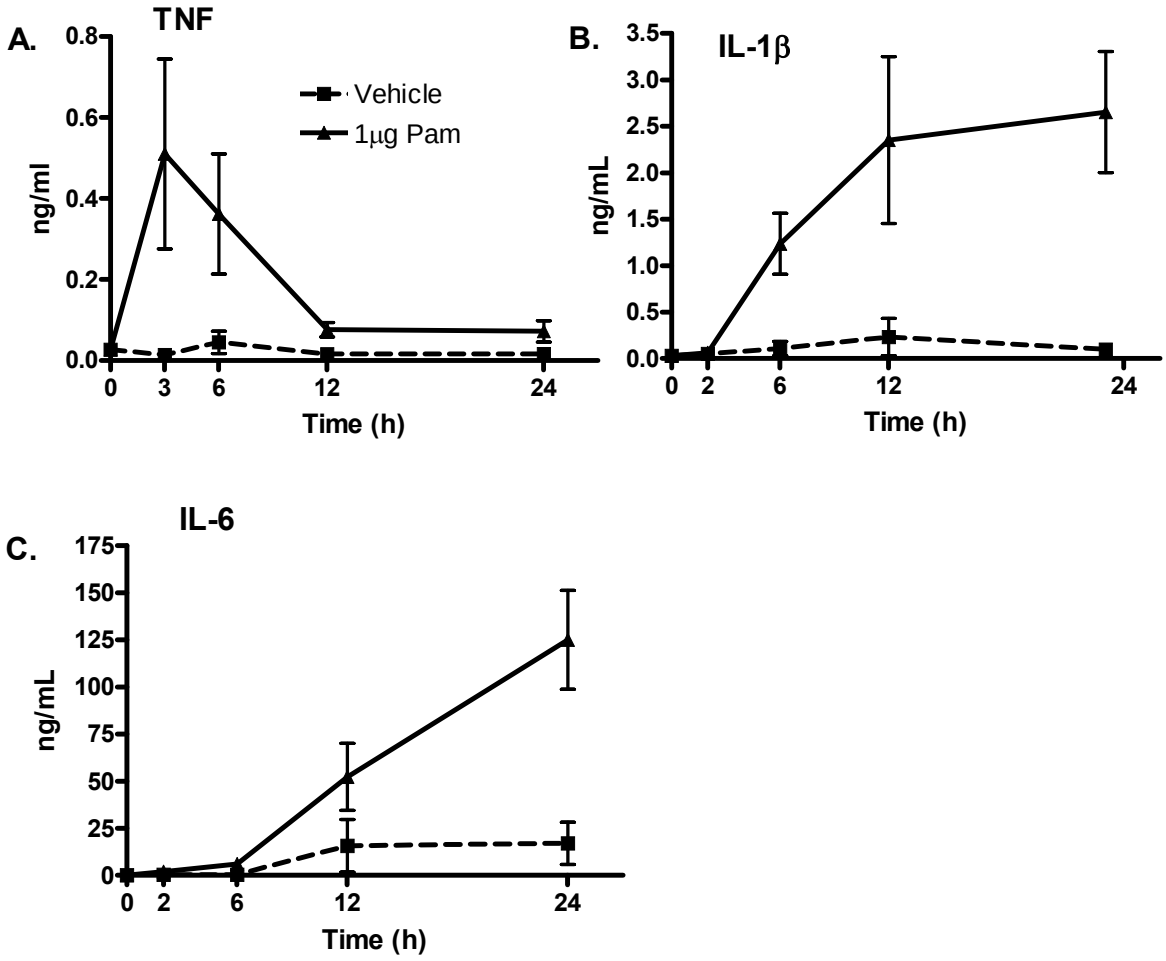

Figure 3.1 Pam-induced cytokine kinetic profiles. Pam induction differed from LPS stimulation.. Pam-induction of (A) TNF; (B) IL-1β; and (C) IL-6 protein in whole blood over 24 hours. Plasma was collected from Pam-stimulated whole blood and cytokine levels determined by ELISA. Results expressed as mean ± SEM (n= 4-8 donors).

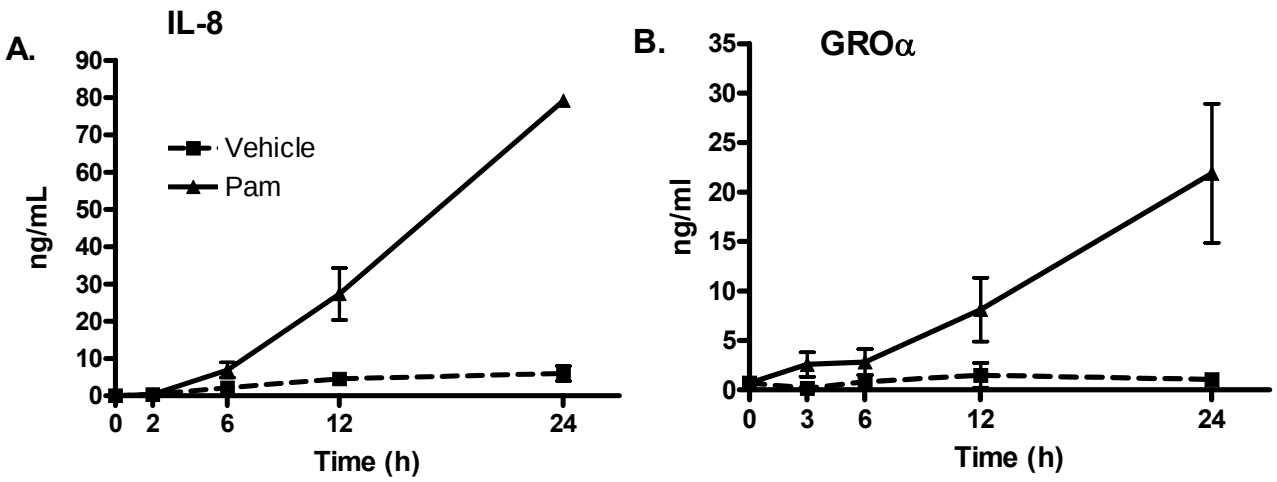

Figure 3.2. Pam-induced chemokine kinetic profiles. Pam induced chemokines showed similar patterns as stimulation with LPS. Pam-induction of (A) IL-8 and (B) GROα protein in whole blood over 24 hours. Plasma was collected from Pam-stimulated whole blood and cytokine levels determined by ELISA. Results expressed as mean ± SEM (n= 4-8 donors).

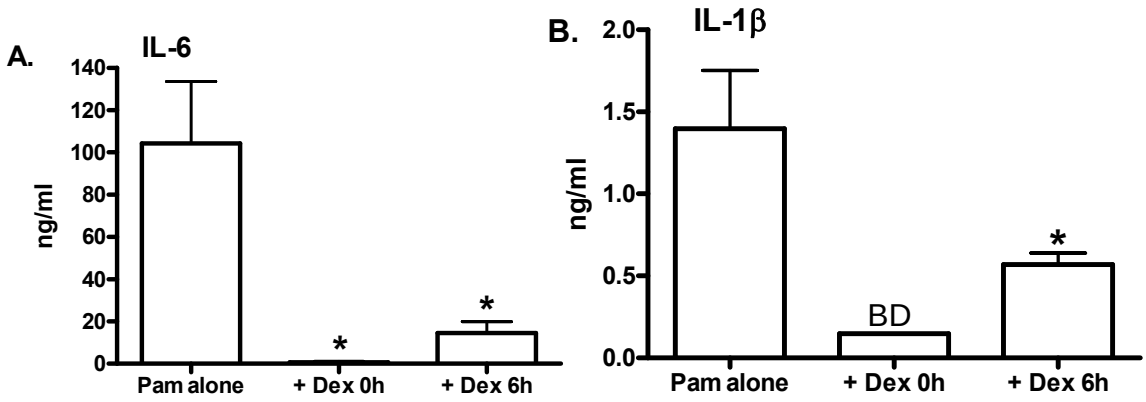

Figure 3.3 DEX effect on cytokines. Delayed DEX suppressed both cytokines. IL-6 (A) and IL-1β (B) levels following Pam alone, simultaneous Pam/DEX, or Pam + 6 hours delayed DEX treatment of whole blood. Results expressed as mean ± SEM (n=4 donors). * $p<0.05$ vs. LPS alone compared by ANOVA and Newman-Keuls post test. BD, below detection.

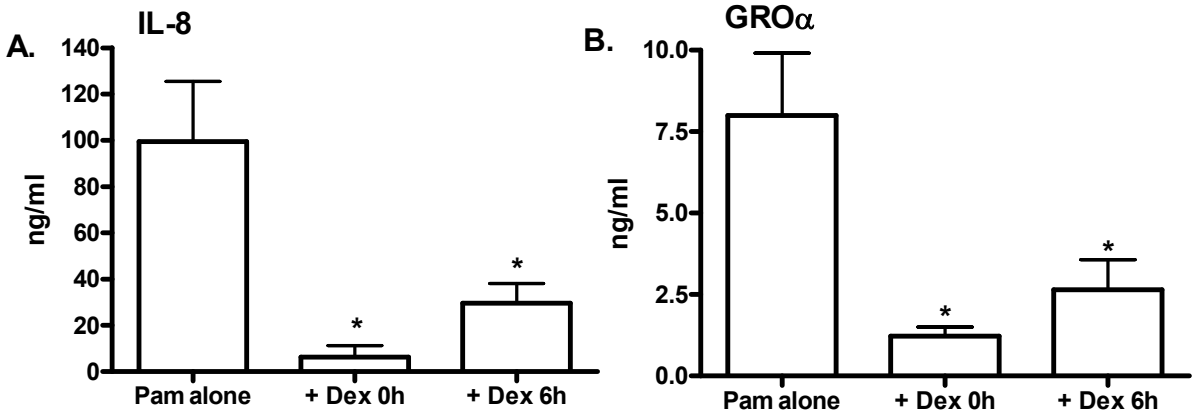

Figure 3.4 DEX effect on chemokines. Chemokine protein levels following Pam alone, simultaneous Pam/DEX, or Pam + 6 hours delayed DEX treatment of whole blood. TNF (A); IL-1 (B); IL-6 (C); IL-8 (D). Results expressed as mean ± SEM (n=4-8 donors). * $p<0.05$ vs. LPS alone compared by ANOVA and Newman-Keuls post test.

Cytokine (ng/ml)	6h	24h
TNF	0.36 ± 0.15	0.07 ± 0.03
IL-1β	1.2 ± 0.33	2.7 ± 0.65
IL-6	6.1 ± 2.3	125.1 ± 26.3*
IL-8	6.9 ± 2.0	79.2 ± 1.4*
GROα	2.8 ± 1.3	21.9 ± 7.0*

Table 3.1 Pam-induced cytokines and chemokines taken from Figures 3.1 and 3.2. Plasma was collected from human whole blood stimulated with Pam or vehicle for 24 hours. Values represent the mean ± SEM (n=4-8 donors). *$p<0.05$ vs. 6h compared by student's *t* test.

CHAPTER IV

DELAYED DEXAMETHASONE SUPPRESSION OF IL-8 IS NEUTROPHIL SPECIFIC IN LPS-STIMULATED WHOLE BLOOD

Introduction

Thus far, experiments have illustrated that TLR2 and TLR4 agonists induce distinct cytokine and chemokine kinetic profiles, with chemokines rising continuously over 24 hours. Further, these distinct profiles resulted in a difference in susceptibility of cytokines and chemokines to delayed dexamethasone regulation, with chemokines being significantly suppressed. In this chapter, the focus shifts to address the source and consequences of the continuous production of chemokines.

Data published by our laboratory as well as others have demonstrated a two-phase production of IL-8 in response to LPS stimulation. In 1991, DeForge *et al.* found that LPS-stimulated whole blood resulted in continuous IL-8 production which was divided into two phases over 24 hours (32). In these studies, IL-8 mRNA proved to be extraordinarily stable over 24 hours; greater even than the β-actin housekeeping gene. Cassatella *et al.* followed in 1993 with studies which showed that PMNs were a significant source of LPS-induced IL-8 (31). In their report, Cassatella *et al.* stimulated freshly isolated PMNs with 1 μg/ml LPS and measured IL-8 secretion over time. They conclude that PMNs secrete IL-8 in response to stimulation with LPS and that this IL-8 is secreted in two phases. Both DeForge and Cassatella *et al.* concluded that the second

phase of IL-8 was not in direct response to LPS stimulation, rather as a result of stimulation from TNF and IL-1β secreted after the initial LPS stimulation (31, 32). Both groups demonstrated that addition of TNF and IL-1β neutralizing antibodies prior to LPS ameliorated the second wave of IL-8 detected in their respective model systems.

In addition to the role of PMNs in IL-8 production, *Mo* are largely thought to be the main contributors of pro-inflammatory cytokines. In response to inflammatory stimuli, TLR expressed on the surface of *Mo* transmit a downstream signal which results in the release and nuclear tanslocation of NFκB (88). The nuclear, activated NFκB binds to NFκB-inducible genes which encode for such cytokines and chemokines as IL-8 (158). As previously noted, Xing *et al.* found that freshly isolated PBMC strongly induced TNF, IL-1β, IL-6 following LPS stimulation, while PMNs contributed less than 1.5% of those cytokines (86). They further showed that in a mixed cell population, PMNs contributed negligible amounts of TNF, IL-1β, and IL-6 in response to LPS stimulation, however they synthesized a significant amount of IL-8 mRNA (87). These studies provided much needed understanding about regulation of LPS-induced IL-8, however it remains unclear the relative contribution of *Mo* and PMN to IL-8 production over time. Further, the role of delayed DEX on the individual cell populations lingers.

In addition to the relative contributions of monocytes and neutrophils, red blood cells may also contribute to the presence, or lack thereof, IL-8 protein detected. Red blood cells are known to serve as a sink for chemokines. They contain an erythrocyte receptor called Duffy Antigen receptor for chemokines (DARC) which has been shown to bind up chemokines from circulation. Controversy persists regarding the function of the sequestered chemokines, with some believing that the DARC simply serves as a sink to

reduce inflammation and the chemokines have no chemoattractant capabilities (159, 160); others have argued that DARC can facilitate the movement of leukocytes across the endothelium (161). In any case, the existence of the DARC made it imperative that red blood cell-associated IL-8 be measured.

In the present studies, PBMCs and *Mo* were isolated from LPS-stimulated whole blood and various assays performed. Frequently, individual cell types are isolated from whole blood, stimulated, and cytokines subsequently measured. These studies were designed to determine the relative contribution of each cell type as well as their susceptibility to DEX regulation. To accomplish this, PBMCs and *Mo* were isolated from whole blood following stimulation. The essential aim was to determine if cellular compensation occurs following LPS stimulation of whole blood, resulting in cell-type specific suppression by post-stimulus DEX treatment.

Materials and Methods

Reagents

The RosetteSep® system (catalog: 15028/15068) was purchased from StemCell Technologies(Vancouver, BC, Canada). NycoPrep™1.077 (Prod. no. 1114550) density gradient media was purchased from AXIS-SHIELD PoC AS (Oslo, Norway).

Blood collection and stimulation, ELISA, RT-PCR

Each of these experiments was performed as previously described in chapter II. Complete blood count was obtained using the Hemavet Multipispecies Hematology Analyzer (Drew Scientific, Dallas, TX).

Monocyte and neutrophil isolation

Monocytes were isolated from whole blood using the RosetteSep® system according to the manufacturer's protocol. Following stimulation with LPS, ethylenediaminetetraacetic acid (EDTA) was added directly to heparinized whole blood at a final concentration of 1mM. Next, 50 µl/ml of RosetteSep® Human Monocyte Enrichment Cocktail was added and mixed well, followed by subsequent incubation at room temperature for 20 minutes. The entire mixture was then gently mixed together with PBS + 2% FBS and 1 mM EDTA and mixed gently. Next, the PBS/blood mixture was gently layered over 2 ml NycoPrep™1.077 density gradient media and centrifuged at 1200 x g for 20 minutes at room temperature with the brake off. The mononuclear layer was then removed and washed with the PBS solution, followed by NH_4Cl lysis as described in chapter II. The cells were then washed a second time and stored at -20^{o}C for later mRNA analysis.

Following monocyte isolation, the remaining density layers were aspirated except for the red blood cell layer. The remaining red blood cell layer was lysed by NH_4Cl lysis as previously described and stored at -20^{o}C for later mRNA analysis.

Statistics

Statistical analyses were performed as described in chapter II. Briefly, results were expressed as the mean ± SEM and statistical comparisons were made using a one-way ANOVA followed by the Newman-Keuls multiple comparison post test. For direct comparisons between groups, the student's *t* test was used.

Results

Effect of a two-hit model

The data in chapters II and III indicate that cytokines and chemokines exhibit markedly different patterns of production in response to either TLR2 or TLR4 stimuli. Since LPS-induced IL-6 reached a plateau by 6 hours compared with IL-8, which continued to increase over 24 hours, it was unclear if the cells in the system were capable of being further stimulated to produce IL-6. To determine if the cells were selectively producing IL-8 at 24 hours, a two-hit model was designed whereby whole blood was stimulated with and initial dose of LPS followed by a subsequent dose of either LPS or Pam. At 24 hours, plasma was harvested and IL-6 measured by ELISA to assess whether the second stimulus could provoke further induction. Figure 4.1 A shows that IL-6 was not further induced when a second dose of LPS was used. However, as depicted in Figure 4.1 B, subsequent stimulation with Pam caused a slight, but significant increase in IL-6 levels at 24 hours.

IL-8 levels in plasma, red, and white blood cells

To verify the cellular composition of the whole blood model, a complete blood count was obtained on unstimulated, heparinized blood at 0 hours. Figure 4.2 shows that while *Mo* comprised roughly 10% of the total cell count, PMNs accounted for more than 75% of the entire cell population. It is important to note that while PMNs are the most abundant cell type in whole blood, *Mo* have nearly 100 fold more CD14 on their surface and thus have a significantly higher capacity to respond to LPS stimulation in the presence of TLR4 and LBP (see chapter I).

The next goal was to determine the location of IL-8 in the various whole blood components. Following LPS stimulation, IL-8 was measured by ELISA in plasma, white blood lysates, and red blood lysates. Figure 4.3 indicates that the majority of IL-8 was located in the plasma (Fig. 4.3 solid bar). Compared with vehicle, plasma IL-8 was significantly increased. Additionally, both the red blood cell and white blood cell lysates had significant amounts of detectable IL-8 compared to vehicle (Fig. 4.3 checkered bar and hatched bar, respectively). However, the cellular components contained significantly less IL-8 compared to plasma alone.

LPS-induced mRNA kinetics: PMNs versus Mo

LPS-stimulated IL-8 mRNA was compared in isolated PMNs and *Mo*. Whole blood was stimulated over 24 hours followed by PMN and *Mo* isolation at 0, 3, 6, 12, and 24 hours. IL-8 mRNA was semi-quantitatively measured in each isolated cell population by RT-PCR. Figure 4.4 shows that over 24 hours, the two populations exhibited different IL-8 kinetics. IL-8 mRNA levels in both PMN and *Mo* increased between 0 and 3 hours, and had declined by 6 hours. By 24 hours, however, IL-8 mRNA levels began to increase again similar to that seen with both LPS-induced IL-8 mRNA and protein (Fig. 2.2 and 2.3). Following stimulation, IL-8 mRNA in PMNs increased roughly 154% from approximately 2.98±0.97 to 4.60±1.60 between 3 and 24 hours. In contrast, *Mo*-associated IL-8 decreased nearly 70% from 1.7±0.5 to 0.5±0.2 during the same time period (Table 4.1). These findings provided a valuable framework from which to design subsequent experiments.

Effects of concomitant DEX on 3h IL-8 mRNA

Measurement of mRNA in Figures 2.4 and 4.4 revealed that IL-8 message rapidly increases between 0 and 3 hours following LPS stimulation. In addition, Figure 4.4 went further in illustrating that the 3 hour peak was the result of IL-8 mRNA production in both PMNs and *Mo*. Given that further data in Figure 2.7 showed that the addition of DEX simultaneously with LPS significantly suppressed IL-8 at 24 hours, the next experiments were designed to verify that concomitant LPS/DEX suppressed IL-8 mRNA at 3 hours as well as to determine if that suppression is cell-type specific. LPS was added together with 10^{-6}M Dex to whole blood and after 3 hours the PMNs and *Mo* were then carefully isolated. The data indicate that IL-8 mRNA is significantly suppressed in both *Mo* and PMN. Concomitant DEX reduced IL-8 mRNA by approximately 85% (from 35±9.4 to 5.3±2.4) compared with LPS stimulation alone (Fig. 4.5 A). Similarly, Figure 4.5 B shows that synchronized LPS and DEX treatment resulted in a nearly 90% reduction in IL-8 mRNA (from 45.7±22.7 to 5.2±1.5) in PMNs. Of note, the overall mRNA induction was nearly 10 times that which was observed in figure 4.4. This was likely the result of heterogeneity amongst donors.

Effects of delayed DEX on 24h IL-8 mRNA

Thus far, the data have illustrated that 24 hours after LPS stimulation, mRNA isolated from total white blood cell pellets is significantly diminished by DEX when it is given 6 hours after the onset of inflammation (Fig. 2.7). However, it has yet to be determined if the effects of delayed DEX is cell type specific. To determine if delayed DEX selectively dampens IL-8 mRNA in either PMNs or *Mo* or both, it was added to whole blood 6 hours after LPS treatment. PMNs and *Mo* were then carefully isolated and

mRNA extracted for measurement of IL-8 mRNA. Figure 4.6 A indicates that in *Mo*, IL-8 mRNA at 24 hours was reduced by nearly 90% (from 16.9±2.6 to 1.7±0.5) when DEX was added together with LPS at the time of stimulation. When DEX was administered after 6 hours, it retained its capacity to suppress LPS-induced IL-8 mRNA. In fact, it reduced IL-8 mRNA to 5.8±2.5, approximately 65% (Fig. 4.6 A). Again, similar results were observed with IL-8 mRNA in PMNs. Compared with LPS treatment alone, both simultaneous and delayed DEX diminished IL-8 mRNA (Fig. 4.6 B). PMN-associated IL-8 message was reduced by approximately 85% (from 17.4±8.7 to 2.5±2.1) and 81% (3.2±0.7) when treated together with LPS or 6 hours after LPS, respectively.

Discussion

Data regarding the relative contributions of neutrophils and monocytes to the inflammatory milieu are conflicting. Further, little investigation has addressed the functional and/or regulatory consequences of continuous chemokine production. The studies presented in this chapter were undertaken to determine if the continued increase in IL-8 levels was a result of cellular compensation. Additional experiments were designed to examine if DEX regulation of IL-8 was monocyte or neutrophil-specific.

Experiments were planned to determine if one cell population was responsible for the initial production of IL-8, and a second cell type was responsible for its later production. Given that the broad picture of the whole blood model lends toward the finding that production of cytokines is finite and chemokines continuous, the first experiment in this chapter sought to determine the capacity of the system to be further stimulated. It is well noted that sequential stimulation results in a tolerant phenotype (162, 163). The data from Figure 4.1 indicates that as opposed to inducing tolerance, sequential stimulation can further induce IL-6. Importantly, these data suggest that while whole blood does not continuously produce IL-6 in response to LPS, it has the capacity to do so.

Next, the kinetic profile of IL-8 in both monocytes and neutrophils was obtained. The data suggest that the two cell types have variable IL-8 kinetic profiles. Interestingly, the initial increase in IL-8 apparently resulted from a combined contribution of monocytes and neutrophils (Fig. 4.3). By 24 hours, however, neutrophils appeared to be primarily responsible for the increased IL-8 mRNA detected. DeForge, Cassatella and others have shown that neutralizing antibodies to both TNF and IL-1β ameliorate the

second phase of LPS-induced IL-8. In addition, Kunkel *et al.* illustrated that neutrophils produced a significant amount of IL-8 in direct response to TNF or IL-1β stimulation (164). Taken together, these findings suggest that in the whole blood model of inflammation, neutrophils are the source of the continuous production of IL-8, becoming activated after monocytes in response to LPS-induced TNF and IL-1β.

The next objective was to evaluate the role of DEX in regulating IL-8 levels from the individual cell types. As shown in Figure 4.4, both cell types clearly expressed IL-8 in response to LPS. Consequently, simultaneous or delayed DEX suppressed IL-8 mRNA that was secreted from monocytes and neutrophils at 3 and 24 hours.

Finally, the experiments presented provide an initial framework from which future experiments can evolve. The data suggests that, in the whole blood model of inflammation, both monocytes and neutrophils contribute to the augmented IL-8 levels observed 24 hours after LPS stimulation. DEX significantly suppressed IL-8 mRNA levels in both cell types, implying that despite the cellular source, if mRNA is present, DEX will have an ant-inflammatory target on which to act. The further implication is that, cytokines and chemokines perpetuate inflammation and the effectiveness of GC likely depends on the presence of cytokine/chemokine mRNA rather than protein at the time that a patient presents with an acute inflammatory response. Thus, rapid tests for cytokine/chemokine mRNA, as opposed to protein, would be useful to aid in the treatment of acute inflammation.

Figures and Tables

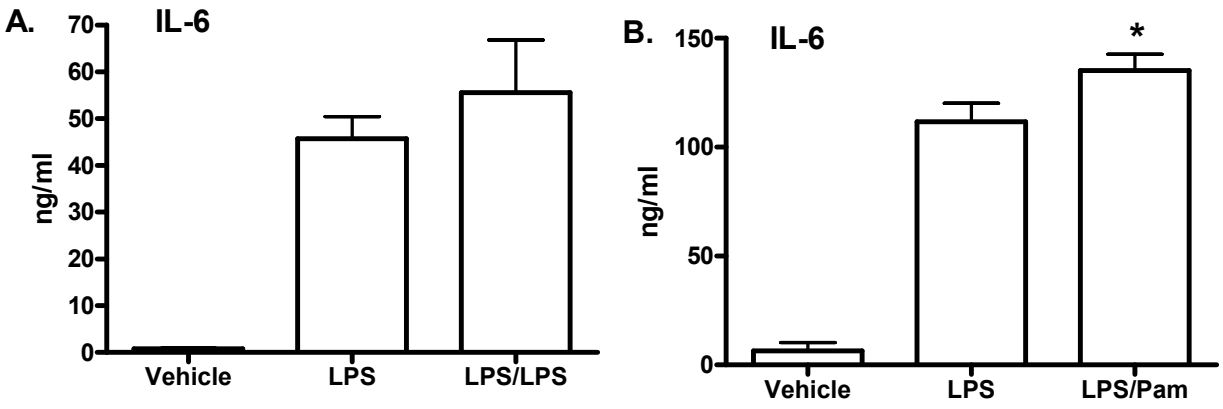

Figure 4.1 Effect of sequential stimulation on IL-6. IL-8 protein levels following sequential stimulation with 50 ng/ml of LPS. Results are mean ± SEM; (n=7-12 donors). *$p<0.05$ vs. LPS alone compared by One-way ANOVA and Newman-Keuls post test.

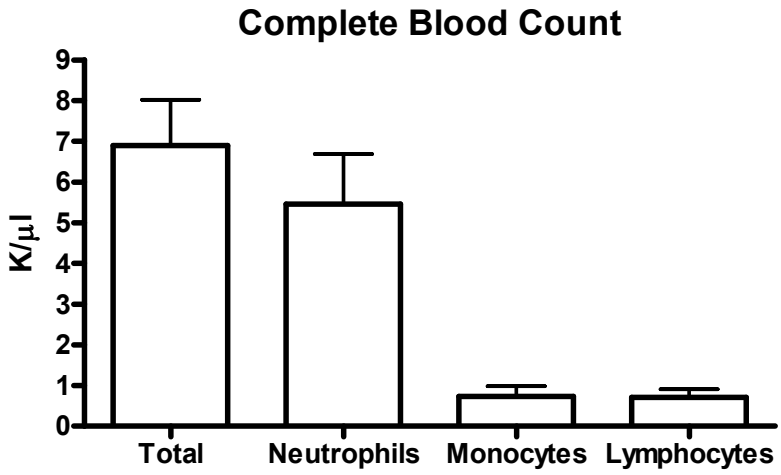

Figure 4.2 Leukocyte composition in unstimulated whole blood. A complete blood count showed a significant portion of the total leukocyte population is comprised of neutrophils. Results are mean ± SEM; (n=7 donors).

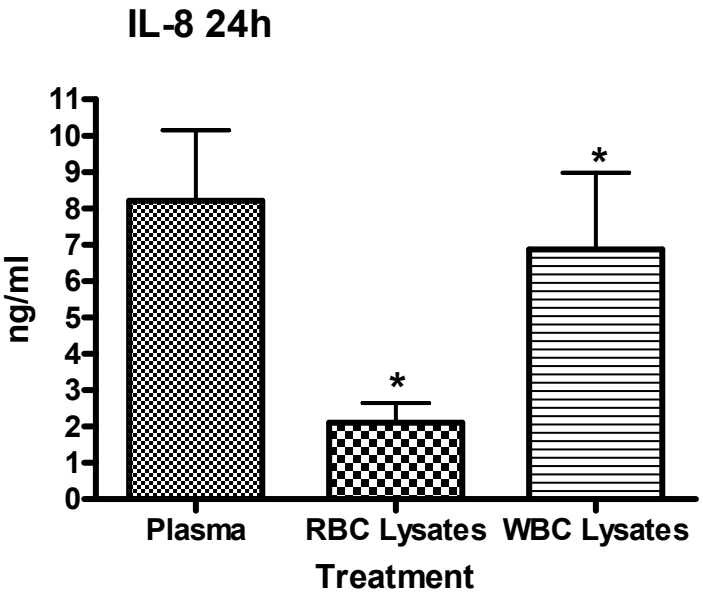

Figure 4.3 IL-8 distribution in whole blood. IL-8 was primarily present in the plasma and the white blood cell lysates obtained from whole blood stimulated with 50 ng/ml LPS for 24 hours. Results are mean ±SEM; (n=7 donors). *$p<0.05$ vs. LPS-stimulated plasma compared by One-way ANOVA and Newman-Keuls post test.

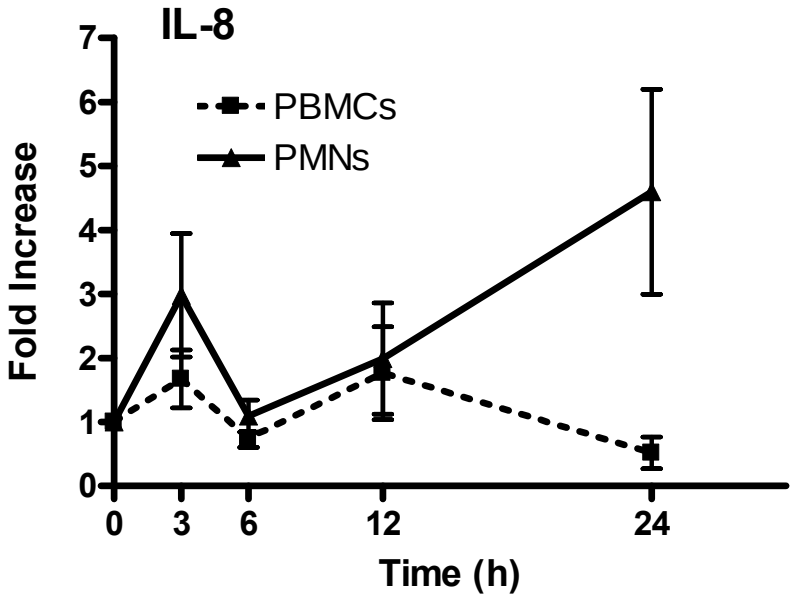

Figure 4.4 Leukocyte-specific IL-8 mRNA kinetics. mRNA from monocytes (PBMCs) and neutrophils (PMNs) was assayed at the observed time points. All data are the mean ± SEM; (n=4 donors).

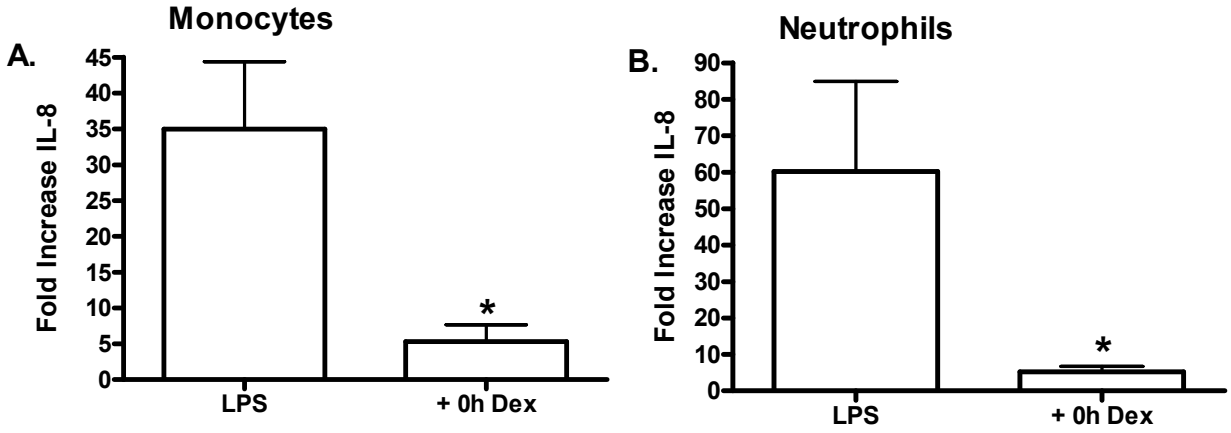

Figure 4.5 Effect of DEX on 3h leukocyte IL-8 mRNA. Concomitant DEX suppressed IL-8 mRNA in monocytes (A) and neutrophils (B) 3h after LPS stimulation. Results are mean ± SEM; (n=4 donors). *$p<0.05$ compared by student's *t* test.

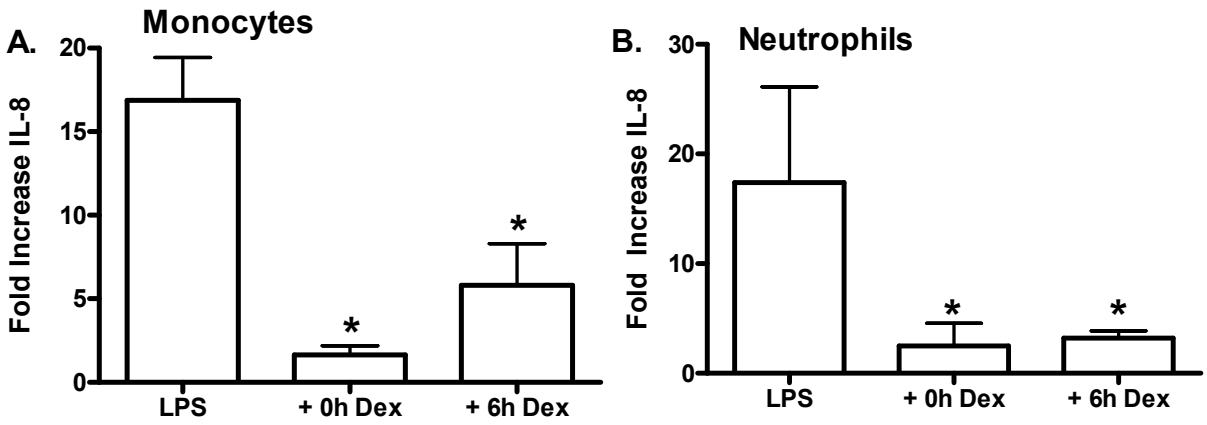

Figure 4.6 Effect of delayed DEX on 24h leukocyte IL-8 mRNA. Addition of DEX either simultaneously with LPS or 6 hours after LPS suppressed IL-8 mRNA in both monocytes (A) and neutrophils (B) 24 hours after LPS stimulation. Results are mean ± SEM; (n=4-6 donors). *$p<0.05$ vs. LPS alone compared by One-way ANOVA and Newman-Keuls post test.

CHAPTER V

CONCLUSION

The overarching hypothesis of this thesis holds that delaying anti-inflammatory therapy until after the onset of inflammation reduces its ability to regulate cytokine levels. Since cytokines represent crucial markers and mediators of inflammation, understanding the various possible mechanisms of their regulation is important. The experiments described in each chapter were designed to address three questions: 1) What is the acute cytokine profile induced in stimulated whole blood? 2) How does the temporal administration of anti-inflammatory drugs affect this profile? and 3) What is the cellular source of the observed effects? Novel findings presented in this dissertation include:

1. IL-8 is selectively suppressed by post-LPS DEX treatment in whole blood
2. Both monocytes and neutrophils are a significant source of IL-8 levels 24 hours after LPS stimulation
3. Dexamethasone suppression of IL-8 is significant both in isolated monocytes and neutrophils

Inflammation is characterized by the secretion of a myriad of pro and anti-inflammatory cytokines and chemokines and is often treated with glucocorticoids. While most studies have evaluated the mechanisms by which anti-inflammatory treatments prevent the onset of inflammation, patients generally present in the clinic after the onset of inflammation. Allergic asthma, for example, is a chronic inflammatory disorder

perpetuated by recurring acute inflammatory exacerbations (121). Previous studies have determined that administration of corticosteroids shortly after the onset of an asthma exacerbation significantly reduced the incidence of hospitalization and relapse, and expedited recovery in the clinic (125). Although few studies have investigated the efficacy and mechanisms of post-stimulus anti-inflammatory therapies, these studies underscore the importance of experimental models that have clinical relevance.

Given the myriad of inflammatory cytokines that characterize the innate immune response, a comprehensive understanding of these cytokines and their interplay would be beneficial. Thus, the first portion of the experiments described here were undertaken to gain insight into the regulation of up to 11 cytokines and chemokines. The data presented in chapters two and three demonstrated that in response to TLR2 and TLR4 agonists, cytokines and chemokines displayed distinct kinetics over 24 hours in the whole blood model of inflammation. While previous studies have shown varying cytokine induction patterns in response to numerous stimuli such as LPS, Pam, lipoteichoic acid, and phytohemagglutanin (PHA) (34, 91), none of these studies evaluated a broad range of mediators at one time.

In response to TLR2 or TLR4 agonists, cytokines and chemokines displayed unique patterns of production, both at the protein and mRNA level. While cytokines displayed finite production, chemokines were continuously produced over the 24 hour time during which they were measured. In addition, the early kinetics of cytokines and chemokines differed. The use of neutralizing antibodies against TNF and IL-1β has been shown to suppress the second wave of LPS-stimulated IL-8 (31, 32). These findings demonstrated that the second phase of IL-8 (and possibly GROα as well as other

chemokines) is a result of secondary stimulation by TNF and IL-1β secreted in direct response to LPS signaling. The present studies demonstrate that neutrophils contribute significantly to the production of IL-8 mRNA 24 hours after LPS stimulation. Both monocytes and neutrophils produced a significant amount of IL-8 that was suppressed by DEX treatment. That cytokines were not affected by post-stimulus DEX suggests that its (DEX) regulatory capacity is dependent upon the presence of mRNA which it can suppress. The significant contribution of neutrophils to the IL-8 levels at 24 hours suggests a potential paracrine-autocrine feedback loop is induced in response to LPS stimulation. In the summary figure (Fig. 5.1), LPS added directly to whole blood directly stimulates monocytes to secrete TNF and IL-1β. In turn, neutrophils are recruited to the site of injury where TNF and/or IL-1β directly stimulate neutrophils. Since neutrophils have been shown to produce IL-8 in response to pro-inflammatory stimulation, it is possible that they secrete other chemokines (158). This, in turn recruits more neutrophils and other leukocytes to the area of injury. The resulting loop likely continues until the presence of the stimulus is removed; i.e. no more TNF or IL-1β is available for signaling (Fig. 5.1).

There are several methods by which to determine if this feedback loop truly exists. Much of the data presented here certainly suggest that is the case. However, future experiments should address a number of different questions. First, it will be important to determine if neutrophils are truly capable of secreting other chemokines. This study must be comprehensive in nature and include measurement of C-C chemokines, which are specific for neutrophils (i.e. GROα) as well as CXC chemokines (such as, MCP-1 and the MIPs) which are specific for monocytes and macrophages (81). Neutrophils have been

reported to stimulate other CXC chemokines including interferon-γ-inducible protein (IP)-10 (165) and the monokine induced by interferon-γ (MIG) (166), as well as C-C chemokines such as MIP-1α (167) and MIP-1β (168), suggesting that neutrophils may be responsible for recruiting other leukocytes, which could also function in the feedback loop. Techniques such as Fluorescence-Activated Cell Sorting or FACS should be employed. It is a sensitive assay with the capability to detect intracellular factors in a single cell. Double staining for cell surface molecules and intracellular cytokines will provide compelling evidence about the true specific nature of what is taking place inside the cell. The studies described here provide the necessary foundation to answer such mechanistic questions about this process. Given the tendency of the immune system toward homeostasis the answer will likely reveal some attempt by the leukocytes in the whole blood system to resolve inflammation.

Monocytes have significantly more CD14 receptors on their surface than neutrophils. CD14 is a member of the cluster of differentiation (CD) cell surface marker proteins and it functions as a coreceptor for LPS in the presence of LPS binding protein. According to Antal-Szalmas *et al.*, resting monocytes contain significantly more CD14 than neutrophils (169). Additionally, Wright and Dentener *et al.* demonstrated that CD14 plays a critical role in the release of cytokines from monocytes following LPS stimulation (170, 171). Although monocytes produce substantially more cytokines, the data presented here suggest that neutrophils are responsible for a significant portion of IL-8 mRNA production 24 hours after LPS stimulation (Fig. 4.5 and 4.6). Up to this point, the argument has merely focused on whether neutrophils have any contribution to the inflammatory milieu. Specifically, some published reports have argued that neutrophils

are a significant source of proinflammatory cytokines (172), while others contend that despite their minimal capacity to produce pro-inflammatory cytokines, they preferentially produce anti-inflammatory cytokines (87). The studies presented here demonstrate that, despite the fact that significantly fewer CD14 receptors are present on the surface of neutrophils, in response to LPS stimulation of whole blood, they produce a substantial amount of IL-8 (Fig. 4.5 and 4.6).

Another potential future study should explore the molecular mechanisms of continuous chemokine production. These data as well as others provide an explanation of the two phases of IL-8 as well as evidence that the second phase is selectively suppressed. However, much remains unknown about the prolonged stability of the IL-8 mRNA over time. While the currently described whole blood model extends for only 24 hours, other investigators have demonstrated that chemokines can be continuously produced for even longer periods of time. For instance, Yamashiro *et al.* showed that stimulation of neutrophils with supernatant from phytohemagglutinin (PHA)-stimulated monocytes could induced prolonged MCP-1 mRNA expression for up to 72 hours and protein secretion for up to 5 days (83). Their data also showed that IL-8 and MIP-1α mRNA was elevated for at least 48 hours. Additionally, McManus *et al.* showed that in response to LPS, human fetal microglia (the resident macrophages of the central nervous system) induced a steady increase in MCP-1 mRNA over 48 hours (84). While both authors contend that the persistent chemokine mRNA is due to secondary activation by TNF and/or IL-1β, few studies have addressed the molecular mechanism by which this occurs.

Numerous studies have investigated mRNA stability as well as its regulation by certain proteins. Messenger RNA contains adenylate/uridylate-rich elements (AREs) in their 3' untranslated regions, which are conserved signals for rapid mRNA degradation (173, 174). In the absence of stabilizing proteins, AREs confer instability to the mRNA, which is essential for gene regulation (82). It is possible the chemokines contain AREs which are different from those contained in cytokines. This difference could mean that in response to TNF stimulation, chemokine mRNA is susceptible to binding by stabilizing proteins such as HuR or tristetraprolin (TTP). This would lead to prolonged chemokine mRNA over extended periods of time following a single stimulation. Thomas Hamilton's group showed that mRNA produced during an inflammatory response depends on mechanistically distinct AREs (175). If this is true, perhaps TNF induces the transcription of such stabilizing proteins as HuR and TTP which are specific for AREs present in chemokines. Biochemical and molecular analysis might reveal a novel mechanism by which chemokine mRNA is selectively stabilized during an inflammatory response.

As with any large body of work, a few pitfalls befell this dissertation. First, the dependence on volunteers for blood donation proved to be an issue. In the event that the supply of donors is low, the ability to obtain a truly hetereogenous representation of the population is skewed to some degree. One way to combat this issue would be to obtain blood from commercially available facilities (i.e. The Red Cross, etc.). These studies, however, rely specifically on the acute response to inflammation and thus require stimulation within one hour of being drawn. To the contrary, several studies have examined cytokines in blood which has been subjected to freezing and thawing. It would be beneficial to design experiments which recapitulate a selection of the current

experiments in freeze-thawed blood to determine how the results compare. This could save time and money in the long run.

The most obvious way to circumvent the issues which arise from using whole blood as a model would be to transition into a mouse model. Although the administrative process of such a transition could be cumbersome, the mouse model could prove to be invaluable. First, the role of neutrophils and monocytes could be manipulated more freely in a mouse model than in the whole blood model. Specifically, the presence of vital organs and the peritoneum provide very useful tools which can be utilized to evaluate the effect of post-stimulus DEX treatment on such phenomena as real-time chemotaxis, organ injury, and necrosis. Even with these advantages, however, the whole blood model still proved to be a powerful tool in which to study inflammation.

Chapter IV suggested that the mechanism of continuous production appears to occur via the differential cellular regulation of IL-8 mRNA. However, there are several other potential mechanisms to pursue based on the findings in this dissertation. First, the biochemistry of the neutrophil response to LPS-induced TNF and IL-1β should be examined. Is there some factor that allows neutrophils to respond, but prevents monocytes? Do TNF and IL-1β participate in an additional autocrine feedback loop whereby monocyte response to either is attenuated? Given that cytokines and chemokines signal identically, what is the molecular mechanism by which only chemokines are secreted from neutrophils? Here, comparison of the minimal promoter sequences necessary for cytokine and chemokine (perhaps IL-6 and IL-8 to start) can be evaluated. Mutation of each of the promoter sequences might reveal a novel site which is specific to chemokines that allows them to be made by neutrophils. There must exist some

fundamental difference between IL-6 and IL-8 that confers the specific message for why one is made by neutrophils while the other is not.

In sum, this body of work provides insight into the mechanisms by which DEX regulates the progression of acute inflammation. These data indicate that, in order for DEX to have any effect on a progressing inflammatory response, mRNA must be present. When TNF, IL-6, and IL-1β mRNA levels were undetectable, DEX had no suppressive effects on cytokine protein. In contrast, IL-8 mRNA was detectable over 24 hours and thus susceptible to DEX regulation whether it (DEX) was administered together with LPS or 6 hours after LPS stimulation. Although TNF, IL-6 and IL-1β are considered classic pro-inflammatory cytokines responsible for mediating the inflammatory response, they are rapidly induced and subsequently short-lived (25). However, patients who present in the clinic with an acute asthmatic exacerbation, for example, are successfully treated with glucocorticoids (125). Thus, the findings here provide evidence that the mechanism by which early systemic corticosteroid treatment works to regulate the progression of acute inflammation might be via suppression of continuously produced cytokines and/or chemokines. Further experimentation will expound upon these studies by investigating the molecular mechanisms of this regulation.

Figures and Tables

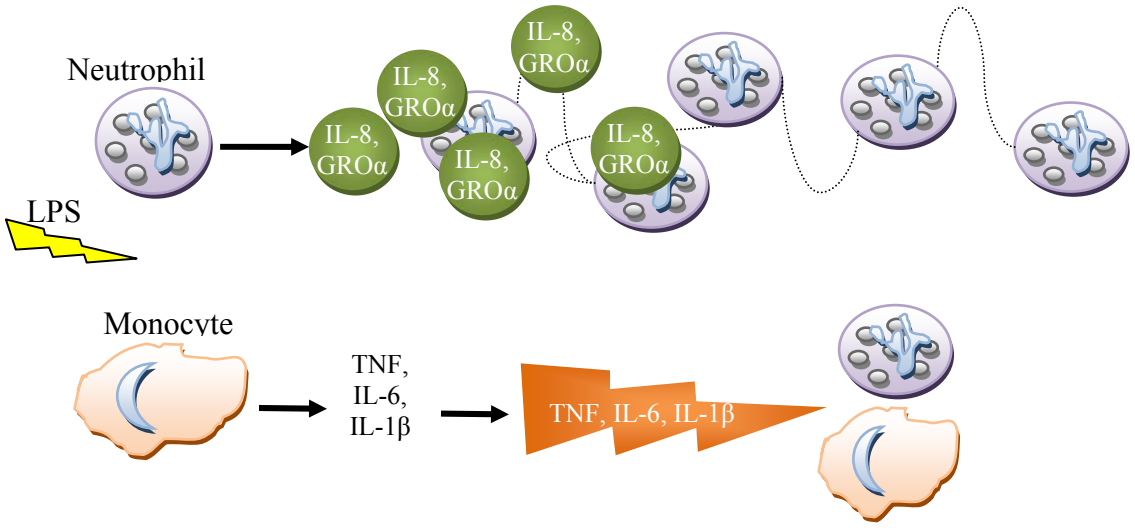

Figure 5.1. Model of LPS-induced paracrine-autocrine response. In response to LPS, monocytes and neutrophils secrete cytokines and chemokines; chemokines recruit neutrophils to the site of injury (paracrine) and cytokines stimulate neutrophils and monocytes to produce more chemokines (paracrine); chemokines are secreted from neutrophils and recruit more neutrophils to the site of injury (autocirine).

Appendix A: Additional chemokine data

LPS-induced chemokines and 6h DEX

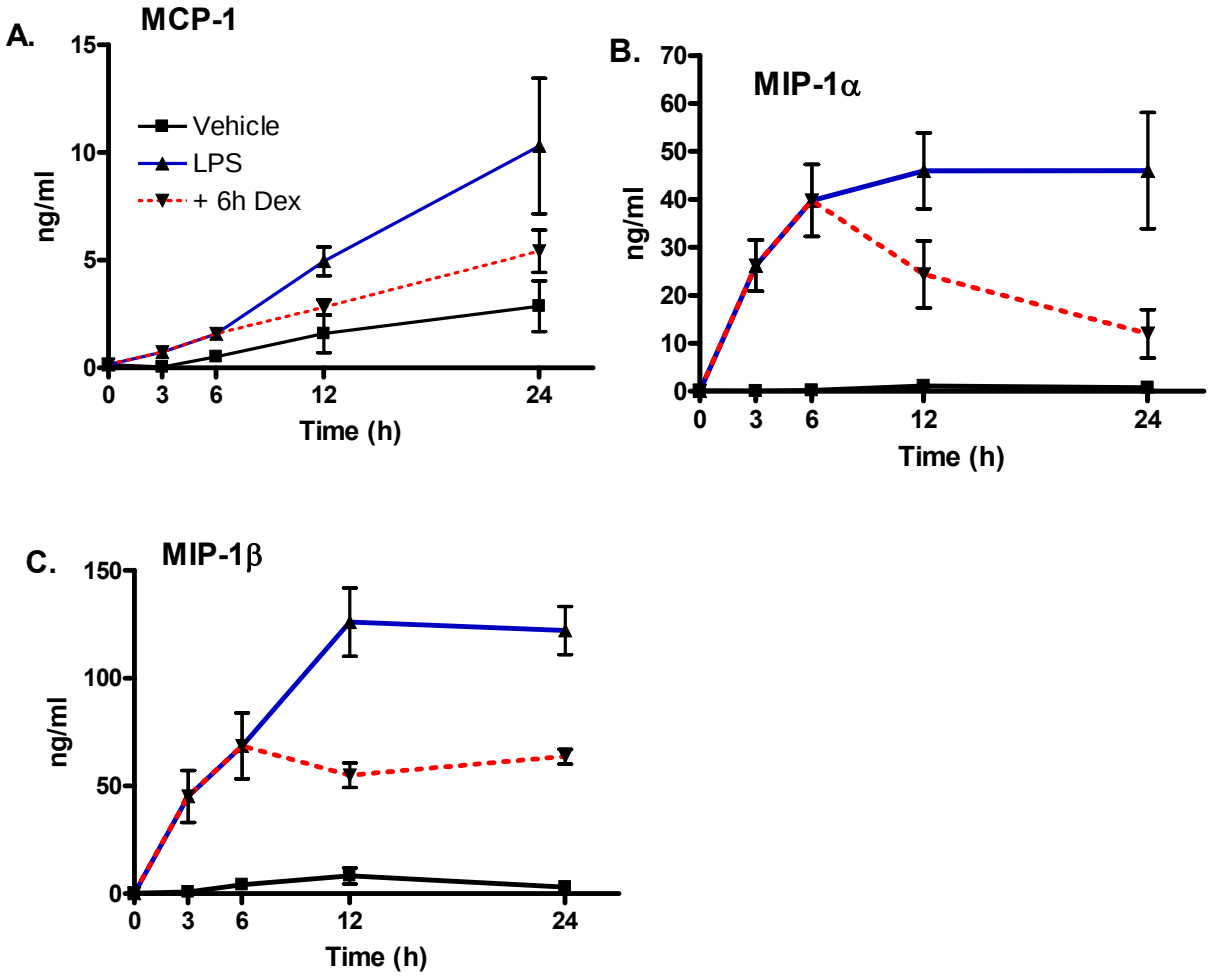

Figure A.1 Effect of delayed DEX on LPS-induced chemokines. LPS stimulated whole blood followed by DEX treatment at 6 hours. DEX consistently suppressed MCP-1 (A); MIP-1α (B); and MIP-1β (C). Results are mean ± SEM; (n=3-12 donors).

Pam-induced chemokines and 6h DEX

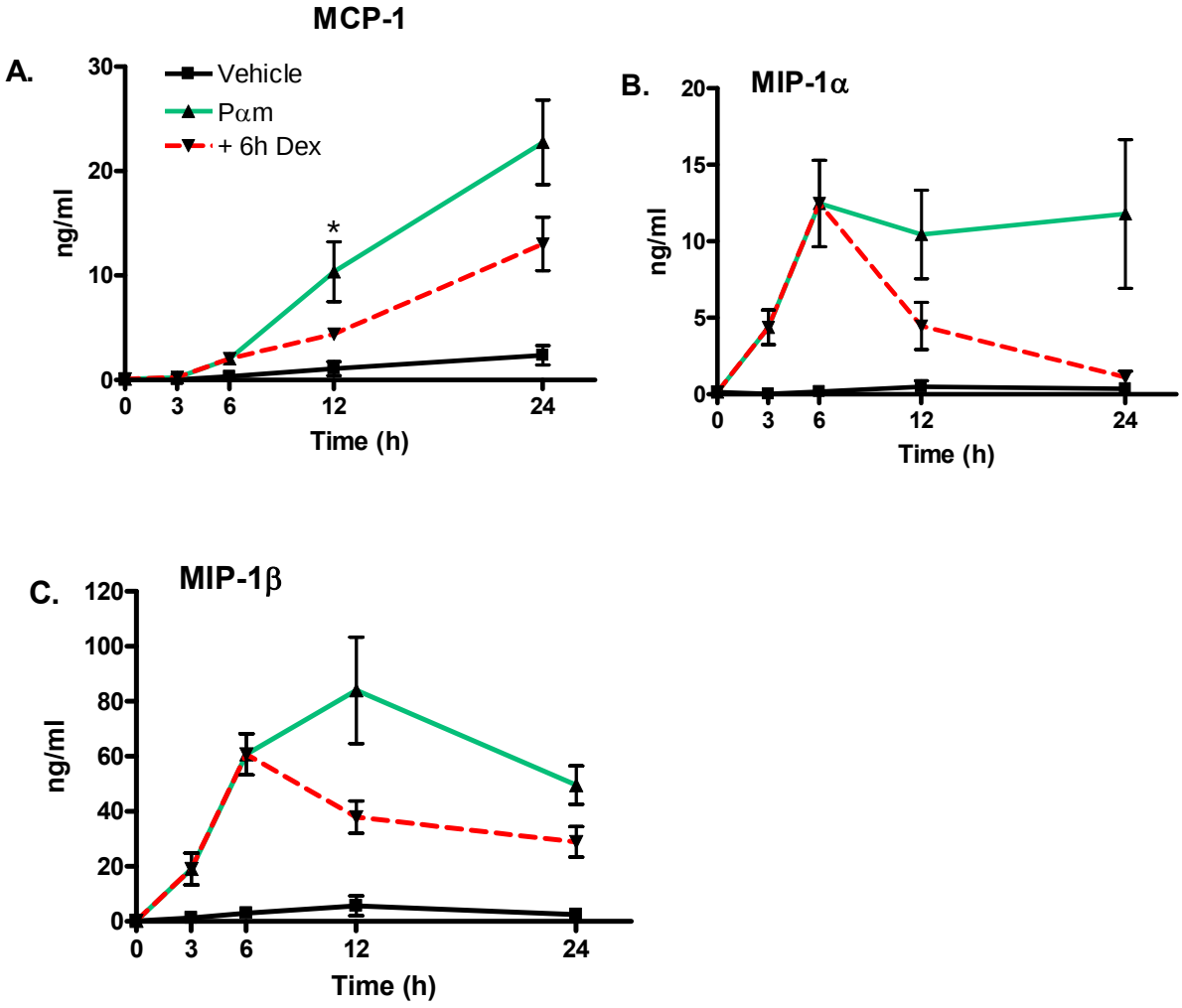

Figure A.2 Effect of delayed DEX on Pam-induced chemokines. Pam stimulated whole blood followed by DEX treatment at 6 hours. Delayed DEX consistently suppressed MCP-1 (A); MIP-1α (B); and MIP-1β (C) over 24 hours. Although these data were consistent, mRNA data was inconsistent and thus these data were included in the appendix rather than the body of the dissertation. Results are mean ± SEM; (n=5-8 donors).

Appendix B: LPS-induced GROα data

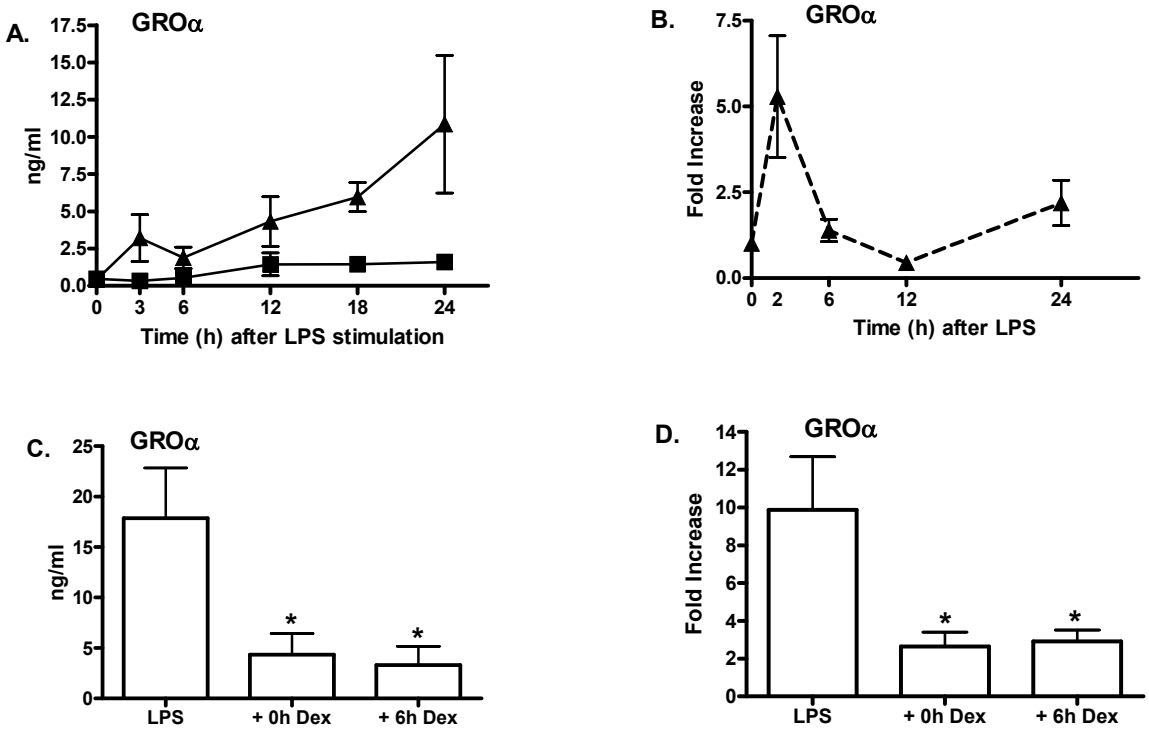

Figure B.1 LPS and DEX effects on GROα. (A) LPS-induced GROα kinetics. 50 ng/ml LPS was added to whole blood and GROα protein measured at the specified times. (B) LPS-induced mRNA kinetics. Total RNA was isolated from LPS-stimulated whole blood at the specified time points and assayed for mRNA by real time PCR. (C) Effects of delayed addition of DEX. DEX was added together or 6h after LPS stimulation and GROα protein measured at 24 hours. Although significant, several of the data observed fell below the limit of detection of the ELISA. (D) Effect of delayed addition of DEX on mRNA. Experiment was similar to (B), but GROα was measured in mRNA. Resultes are mean ± SEM; (n=4-12 donors). *$p<0.05$ vs. LPS alone compared by One-way ANOVA and Newman-Keuls post test.

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