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Cytokine and Chemokine Profiles in a Rat Model of Hemorrhagic Shock after Immuno-Modulation by Androstenetriol

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CYTOKINE AND CHEMOKINE PROFILES IN A RAT MODEL OF HEMORRHAGIC SHOCK AFTER IMMUNO-MODULATION BY ANDROSTENETRIOL

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University/Medical College of Virginia.

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SIRS	Systemic Inflammatory Response Syndrome
MODS	Multiple Organ Dysfunction Syndrome
CARS	Compensatory Anti-Inflammatory Response Syndrome
IL-1	Interleukin-1
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor-α
α	Alpha
β	Beta
IL-2	Interleukin-2
IL-8	Interleukin-8
IL-6	Interleukin-6
INF-γ	Interferon-Gamma
NO	Nitric Oxide
NF-κB	Nuclear Factor-κB
HIV	Human Immunodeficiency Virus
ICAM-1	Intercellular Adhesion Molecule
ELAM	Endothelial Leukocyte Adhesion Molecules
NK	Natural Killer
IL-4	Interleukin-4
IL-10	Interleukin-10
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
MCP-1	Monocyte Chemoattractant Protein-1
GRO/CINC	Growth-Related Oncogenes/Cytokine-Induced Neutrophil Chemoattractant
T cells	T Lymphocytes
T_C	Cytotoxic Killer T Cells
T_H	Helper T Cells
B cells	B Lymphocytes
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription
JAK/STAT	Janus Kinase-Signal Transfer Transducer Activator of Transcription
MAPKs	Mitogen-Activated Protein Kinases
HPA	Hypothalamic-Pituitary-Adrenal Axis
AET	Androstenetriol

DHEA	Dehydroepiandrosterone
AED	Androstenediol
PPAR	Peroxisome Proliferator
SA-PE	Streptavidin-Phycoerythrin
VEH	Vehicle
MAP	Mean Arterial Pressure
ELISA	Enzyme-Linked Immunosorbent Assay
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization
AV	Average or Mean
SD	Standard Deviation
R & D	Research and Development

Abstract

CYTOKINE AND CHEMOKINE PROFILES IN A RAT MODEL OF
HEMORRHAGIC SHOCK AFTER IMMUNO-MODULATION BY
ANDROSTENETRIOL

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

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Further understanding of the cellular and molecular mechanisms involved in traumatic injury, and how they are modulated during drug interaction, can facilitate novel treatment strategies for future trauma patients. We hypothesize that the pharmacological agent, Androstenetriol (AET), up regulates host immune response by modulating the continued expression of mediators, including cytokines. In a double-blinded experiment, rats were hemorrhaged, driven by volume or pressure conditions, then resuscitated with fluids and packed red blood cells following a subcutaneous injection of either vehicle or drug. Blood was collected at various time points and cytokine levels were determined by analyses with both multiplex and conventional ELISA assays. Both MCP-1 and GRO/KC increase in surviving animals; trauma increases IL-1α levels in rat plasma,

whereas hemorrhage decreases IL-1α over time; IL-6 plasma levels measured 6 hours after hemorrhage may correlate with mortality; AET may act by mechanisms to modify specific TH1 cytokines (INF-γ) to promote survival.

Literature Review

I. Introduction

1. Purpose

With increasing warfare on both the home front and internationally, a critical need exists to develop innovative life-saving treatment techniques for patients suffering from severe hemorrhage and acute traumatic shock. Both hemorrhage and acute traumatic shock remain the leading cause of combat death in today's society. Studies show 68% of military deaths are the result of hemorrhage and at least 20% of soldiers who die in combat are potentially salvageable [1],[2]. Compared to treatment options for civilians suffering from massive bleeding, the care of hemorrhage patients on the battlefield presents a very distinctive challenge. Tissue injury with hemorrhage, or acute traumatic shock, affects the function of both tissues and organs, accompanied by an intricate amount of adaptive host responses [3]. Because life-threatening hemorrhage affects every organ system in the human body, medical professionals have an obligation to understand the complexity involved in a patient's response to acute traumatic shock. This advanced knowledge will facilitate the development of improved diagnostic and therapeutic strategies. Ideally, these innovative and life-saving treatments will improve the survival rates for combat patients suffering from a massive hemorrhage and acute stress.

1

2. Shock and SIRS

The combined insult of trauma and hemorrhage triggers several protective mechanisms that may have adaptive value to preserve oxygenation. These mechanisms may eventually yield responses that depress the body's immune system and cause systemic inflammation. Shock is defined as inadequate organ perfusion even after adequate fluid resuscitation, which often presents itself as persistent hypotension or the need for vasoactive drugs to increase blood pressure [4]. Victims of septic shock experience fever, falling blood pressure, myocardial suppression, dehydration, acute renal failure and then respiratory arrest. A patient who has been severely injured and has survived initial resuscitation may develop the features of the systemic inflammatory response syndrome (SIRS) and may then deteriorate with features of multiple organ failure that is often fatal. Researchers characterize SIRS as the abnormal generalized inflammatory reaction in organs remote from the initial insult [5]. The fundamental biological mechanisms that underpin SIRS include ischaemia/reperfusion injury, activation of the leukocyte and macrophage systems, and the deleterious effect of numerous mediators of inflammation, especially the cytokines. Sepsis is defined as a clinical syndrome of deleterious systemic response with bacterial infection. When the process is due to an infection, the terms sepsis and SIRS are synonymous [5].

Researchers have demonstrated that the mortality of patients with septicemia remains elevated even with advances in treatment using antibacterial agents [6]. According to reports in 2001, approximately 400,000 patients suffer from septicemia each year in the United States and ultimately 25% of those patients die of septic shock

and multiple organ failure [6]. More recent data, February 2004, reports that mortality from sepsis has increased by 90% over the last twenty years [7]. Furthermore, this epidemic is associated with a hospital cost of nearly 17 billion dollars a year [7].

3. Multiple Organ Failure

Multiple organ failure occurs when two or more organ systems fail. From the progression of SIRS to multiple organ failure a patient may not present with complete organ failure, but individual organs may function abnormally [8]. As a result, multiple organ dysfunction syndrome (MODS) was termed to define the detection of altered organ function in an acutely ill patient where homeostasis cannot be maintained [8]. Approximately 10-20% of all deaths following multiple trauma remain related to the manifestation of irreversible multiple organ failure [9].

Initially, the etiology of multiple organ failure was thought to be uncontrolled infection and MODS was characterized as the life-threatening outcome of successful shock resuscitation [4]. Current research, however, concludes that multiple organ failure results when a host's inflammatory or anti-inflammatory response to injury acts excessively [4]. As illustrated [Figure 1], death usually occurs when the host response to injury is either excessive or insufficient [4]. It is important to note that individual patients vary to the extent of multiple organ failure depending on the balance between the specific injuries with their individual response [4].

[FIGURE 1]

Inflammatory Response
Canadian Journal of Anesthesia 48:502-509 (2001)
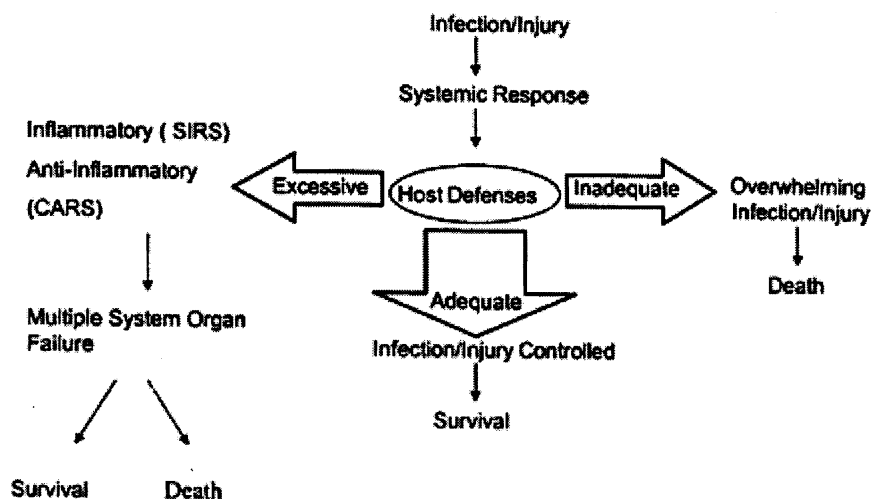
FIGURE 1: Illustrates the balance between an inadequate inflammatory response eventually leading to death and an excessive anti-inflammatory [CARS] or inflammatory response [SIRS] resulting in multiple system organ failure. Survival without multi-system organ failure requires a balanced systemic host response to injury or infection.

Compensatory Anti-Inflammatory Response Syndrome [CARS], HLA-DR on monocytes < 30% and diminished ability of monocytes to produce inflammatory cytokines, such as TNF-α or IL-6 [5].

4. Two-Hit Model

Recent studies have provided the basis for the current "two hit model," a theory to explain how an initial injury, independently of infection, sets the scene for an inflammatory environment conducive to the development of multiple organ failure [10]. The first hit is a result of direct cell trauma, from mechanical or thermal injury, or cell ischaemia, from hypovolaemic shock. This initial injury associated with the first hit primes the body's inflammatory machinery. Humoral systems, including coagulation and fibrinolysis, are activated and cellular sources, including macrophages, lymphocytes, and granulocytes, release potent pro- and anti- inflammatory mediators [9]. The second hit is common in patients suffering from severe polytrauma or burns. More specifically, the second insult (sepsis, additional bleeding, surgery) triggers the release of powerful mediators and unleashes the exaggerated clinical systemic inflammatory response, which leads to multiple organ failure.

The theory suggests that the "second hit" results in autodestructive inflammation, which is the manifestation of severe and sometimes irreversible cell damage induced by mediators, such as cytokines [10]. Inflammation is defined as the activation of leukocytes, the endothelium, the liver, and multiple mediator networks that under normal conditions are held in balance by anti-inflammatory mediators [4]. Following limited injury, the local inflammatory process aims to stimulate the healing process and defend against foreign invading organisms; an appropriate host response to the initial injury remains necessary for survival and recovery [9]. However, with severe trauma and hemorrhagic shock, the inadequate or enhanced activation of the inflammatory cascades,

results in the uncontrolled release of pro-inflammatory cytokines, which leads to cellular

injury and sequential organ dysfunction [9]. Figure 2 illustrates the variety of mediators,

cytokines/chemokines, inflammatory products, and clotting cascades that are active in

propagating the inflammatory response [4].

[FIGURE 2]

Mediators Active in the Inflammatory Response.
Canadian Journal of Anesthesia 48:502-509 (2001)
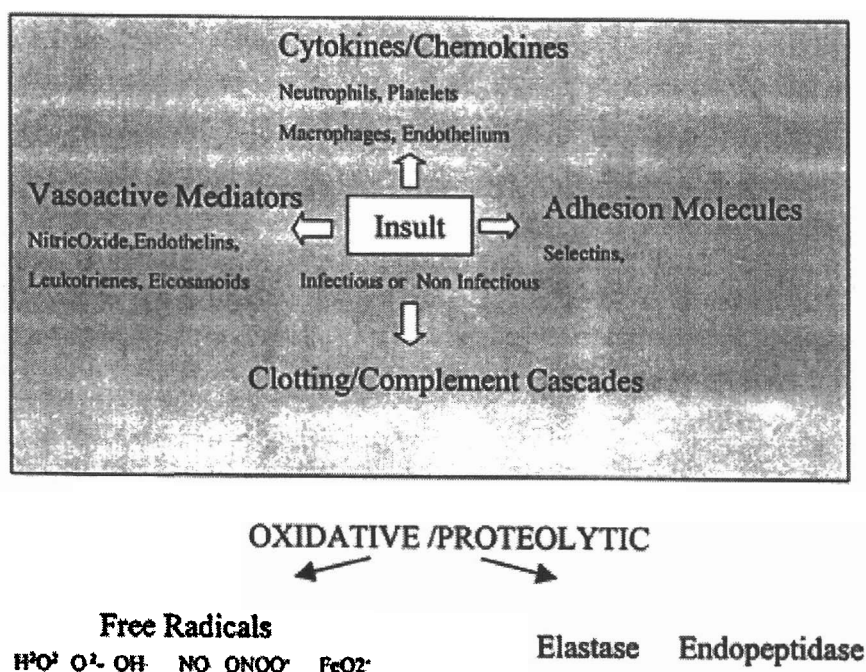
FIGURE 2: Illustrates the inflammatory products that are active in the inflammatory response. Insult activates neutrophils, which promotes a cascade of events. Activated neutrophils recruit other neutrophils with chemokines, they bind to endothelial cells using adhesion molecules, and neutrophils produce pro-inflammatory cytokines to enhance the production of free radicals and proteolytic enzymes (digest proteins) [4]. The inflammatory response must be balanced with the expression of anti-inflammatory cytokines and apoptosis, programmed cell death [4].

5. Overview: Role of Cytokines

Cytokine release is a normal, healthy part of the body's response to insult or infection. Following a harmful insult there is an initial response mediated by the liver, neutrophils, macrophages and the endothelium [4]. During the macrophage response, a variety of inflammatory cytokines, including tumor necrosis factor, interleukin-1, and interleukin-6, are released and these mediators then up regulate receptors on neutrophils and endothelial cells, that in turn stimulate transmigration [4]. As a result of transmigration, other effector molecules (reactive free radical species, endopeptidases) are released by neutrophils, which causes organ damage and further recruitment of activated neutrophils to the injury site [Figure 2] [4]. Researchers have established that septic shock induced by gram-negative bacilli is the result of inflammatory cytokine overproduction, especially tumor necrosis factor (TNF) and interleukin-1 (IL-1), from monocytes and macrophages [6].

Even though current research studies suggest that multiple organ failure is induced through a complex cytokine cascade triggered by pro-inflammatory cytokines, attempts have not been successful in suppressing the actions of these cytokines. Timing and complexity of expression underline the difficulty in therapeutic intervention. The therapeutic challenge in attempting to modulate these pathways is that there are numerous and redundant mediators involved in the progression of multiple organ failure [4]. Cytokines and chemokines often function in multiple complexes where the function of one inflammatory product induces the expression of another in a cascading effect [11]. Furthermore, the expression of these mediators varies over a period of time during the

course of the illness [4]. Adding to the confusion includes the fact that each patient has a highly complex, rigidly regulated network of receptor antagonists and other regulatory agents that continuously modulate the effects of cytokine release [5].

The ability to monitor a patient's inflammatory and immune response following hemorrhagic trauma through cytokine and chemokine measurements remains significant in modern medicine. Animal models reproduce traumatic shock and hemorrhage for the purpose of modulating the cytokine cascade. Therefore, tools to measure the immune/inflammatory response are valuable for animal hemorrhage models. However, modern technology must maintain high sensitivity, specificity, and precision standards in order to produce physiological relevant data.

II.   Cytokines

1. Introduction

Cytokines are important immunoregulators that play a critical role in immune responses, hematopoiesis, inflammation, wound healing, and trauma [11]. In addition, cytokines have recognized systemic effects, that translate into fever, intravascular coagulation, and shock [11]. Generally, the pro-inflammatory cytokines worsen disease states in trauma patients, whereas anti-inflammatory cytokines serve to reduce inflammation and promote healing. The balance between the effects of pro-inflammatory and anti-inflammatory cytokines is thought to determine the disease outcome for patients. Recent studies suggest that susceptibility to disease is genetically determined by the expression of either pro-inflammatory or anti-inflammatory cytokines [12]. Normally,

most cytokines are not detectable in healthy individuals; however, during critical illness many studies identified elevated levels of cytokines [8].

Cytokines form a family of relatively small, secreted proteins that control many aspects of growth and differentiation for specific types of cells. The small proteins have molecular weights ranging from 8 to 40,000 d [12]. Originally, these proteins were described as lymphokines and monokines to indicate their cellular sources [12]. Researchers quickly determined that the term cytokine was the best description for these proteins because practically all nucleated cells are capable of synthesizing cytokines and therefore able to respond to these proteins [12]. Although it is known that these secreted proteins generally contain around 160 amino acids, no amino acid sequence motif or three-dimensional structure exists to categorize cytokines; rather, it is their biological activities that determine their different groups.

2. Pro-Inflammatory Cytokines

Interleukin-1 (IL-1), tumor necrosis factor (TNF), interleukin-2 (IL-2), interleukin-8 (IL-8), interleukin-6 (IL-6), and interferon-gamma (INF-γ) are classified as pro-inflammatory cytokines, which promote inflammation. When these pro-inflammatory cytokines are administered to humans, symptoms such as fever, inflammation, and tissue destruction are observed and in some cases these symptoms manifest into shock and result in death. In addition, these monocyte and lympohocyte proteins (interleukin-1, interleukin-6, and TNF-α) mediate the negative nitrogen balance of injured patients [13]. Reperfusion after ischemia generates high levels of free radicals composed of both reactive oxygen intermediates and nitric oxide (NO). When generated in sufficient

concentrations, free radicals directly injure the myocardium and may even cause cell death [12]. Free radicals activate redox-sensitive transcription factors, including nuclear factor-κB (NF-κB), and trigger the expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and other inflammatory mediators. According to recent research, pro-inflammatory cytokines seem responsible for the wasting symptoms noted in chronic infections [12], such as human immunodeficiency virus (HIV).

A. Interleukin-1

Interleukin-1 is the general name for two distinct proteins, IL-1α and IL-1β. Interleukin-1α and interleukin-1β are biologically equivalent pleiotrophic factors that act locally and also systemically. Locally, this prototypical pro-inflammatory cytokine plays a significant role in the up- and down-regulation of acute inflammation. Systemically, interleukin-1 is a highly toxic compound; minute amounts of IL-1 can induce septic shock. In addition to causing fever, as well as a number of other metabolic changes, interleukin-1 also activates systemic proteolysis in skeletal muscle. Monocytes are the main cellular source of secreted IL-1. Specifically, monocytes predominantly express IL-1β while human keratinocytes express large amounts of IL-1α. Not only is IL-1 responsible for fibroblast and keratinocyte chemotaxis, the pro-inflammatory cytokine also stimulates vascular endothelial cells to express adhesion molecules such as ICAM-1 (intercellular adhesion molecule), E-Selectin, and VCAM-1. Interleukin-1 promotes the adhesion of neutrophils, monocytes, T-cells, and B-cells by enhancing the expression of CAM-1 and ELAM (endothelial leukocyte adhesion molecule). ICAM-1 remains essential for neutrophil recruitment, which results in injury to end organs after endotoxin

exposure [14]. Additionally, IL-1 activates nuclear factor (NF)-κB in various cell populations [14].

As already mentioned, cytokines function in multiple complexes. In the immune response, interleukin-1 stimulates T-helper cells that are induced to secrete interleukin-2 and to express IL-2 receptors. Together, IL-1 acts directly on B-cells, promoting their proliferation and the synthesis of immunoglobulins. This interaction functions as one of the priming factors that make B-cells responsive to interleukin-5. In the cytokine cascade, interleukin-1 is needed for the efficient production of IFN-γ and IL-1 also induces capillary endothelial cells to secrete chemokines (MCP-1). The synthesis of IL-1 can be induced by other cytokines (TNF-α, INF-γ) and also by bacterial endotoxins, viruses, mitogens, and antigens. In the inflammatory response, interleukin-1 is capable of inhibiting or promoting its own synthesis, depending on conditions and cell types; the synthesis of interleukin-1 is controlled by a complex feedback loop.

B. Interleukin-6

In the blood, circulating IL-6 levels are slightly elevated during a woman's menstrual cycle, moderately raised in patients with certain cancers (melanoma), and large IL-6 elevations are present following surgery. Previous research has suggested that interleukin-6 serves as both a marker and a mediator for the severity of sepsis [15]. The controversy however questions whether or not IL-6 in sepsis serves as a marker of inflammation, an inducer of altered physiology, or a mediator of organ insult in multi-system organ failure. Remick, et. al suggests that this pro-inflammatory cytokine functions as an important and sensitive indicator of systemic inflammation; plasma levels

of interleukin-6 correlate with mortality in septic patients [15]. According to Remick, studies have shown improved survival statistics in bacterium-derived sepsis models with antibody inhibition of IL-6 [15]. Furthermore, Remick states that a correct antibody dose to interleukin-6 will improve survival in patients diagnosed with sepsis [15]. Current literature has also demonstrated that IL-6 mRNA and protein is produced in the lungs, liver, and intestinal tracts of rats subjected to resuscitated hemorrhagic shock [16]. Interestingly, both the ischemic and reperfusion phases of resuscitated hemorrhagic shock are required for the production of this particular cytokine [16].

Interleukin-6 plays a significant role in several aspects of the inflammatory response, including temperature regulation and metabolic activity. IL-6 is easily detectable in multiple tissues and the circulation of resuscitated hemorrhagic shock individuals [17]. Many different cell types produce this pro-inflammatory cytokine; however, the main sources in vivo are stimulated monocytes, fibroblasts, and endothelial cells. Systemically, interleukin-6 contributes to the initial response after trauma. IL-6 is known to change neutrophil deformability by inducing an accelerated release of immature neutrophils from the bone marrow with higher F-actin content and decreased deformability. Locally, IL-6 increases neutrophil sequestration in the lung. Interleukin-6, a cytokine with numerous biological activities, helps control the induction of the acute-phase response [15]. In addition, interleukin-6 mediates the immunoglobulin class switching. Finally, interleukin-6 stimulates the synthesis of a number of hepatic proteins called acute phase reactants by the liver and induces fibroblast proliferation. In the

cytokine cascade, IL-6 is essential for the regulation of the inflammatory response to the inflammatory stimulus tumor necrosis factor.

Interestingly, a net effect of IL-6 exists in the host inflammatory response. Although IL-6 is mainly regarded as a pro-inflammatory cytokine, literature states that the final effect of IL-6 on the inflammatory response is a result of two countereffective mechanisms [18]. A paracrine response stimulates the inflammatory response, while a endocrine effect diminishes inflammation [18]. However, IL-6 is widely regarded as detrimental because the up-regulation of interleukin-6 correlates with mortality and multiple organ failure in numerous clinical studies [17].

C. Tumor Necrosis Factor-Alpha

In animal models of sepsis, there is a direct correlation between a significant increase in TNF-α synthesis and the development of shock and multiple organ failure. Originally sepsis was believed to result directly from the invading bacteria, but research later recognized that host system proteins, such as TNF-α, induced sepsis. The pleiotropic inflammatory cytokine, TNF-α, is released very early after shock and has a number of cellular functions. Quiescent cells produce this 17.5 kDalton, 157 amino acid protein in extremely small quantities, whereas TNF-α is a major secreted factor in activated cells. Tumor necrosis factor alpha suppresses adipocyte triacylglycerol synthesis, prevents uptake of circulating triacylglycerol by inhibiting lipoprotein lipase, stimulates lipolysis, inhibits release of insulin, and promotes insulin resistance. Recently, Irshad H. Chaudry published findings which suggest that female hormones, such as estrogen, play a crucial role in stabilizing immune responses after trauma-hemorrhage by suppressing the

amplification of TNF-α [19].   This inhibition was shown to prevent the increased lethality from subsequent sepsis [19].   From his results, Chaudry proposes that female sex hormones may be useful in preventing trauma-induced immunodepression through the modulation of TNF-α [19].   It should be noted that efforts to measure TNF-α in the clinical setting remain unsuccessful due to the highly variable and inconsistent occurrence of TNF-α in the blood stream [9].   Johannes Frank noted in 2002 that a correlation between plasma TNF-α concentrations and tissue injury, multiple organ failure, or outcome in trauma patients does not exist [9].

This acute phase protein, considered a proximal mediator of the inflammatory cascade, initiates and regulates the synthesis of several other cytokines and chemokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. Both IL-1 and TNF are inducers of endothelial adhesion molecules [12]. These molecules are necessary for the adhesion of leukocytes to the endothelial surface prior to emigration into the tissues. Whether provoked by an infection, trauma, ischemia, or immune-activated T cells, research indicates that interleukin-1 and tumor necrosis factor start the cascade of inflammatory mediators by targeting the endothelium [12].

D. Interleukin-2

Another pro-inflammatory cytokine, interleukin-2 (IL-2), exerts a key role in the immune system. Specifically, IL-2 is necessary for the proliferation and functioning of T cells by influencing T-cell differentiation and supporting the growth of cytotoxic T cells. In addition, interleukin-2 has an essential role in the induction of lymphokine-activated killer cells. Systemically, interleukin-2 induces activated cells to enter a preapoptotic

phase, increases the levels of production for pro-inflammatory cytokines, enhances the cytotoxicity of natural killer (NK) cells, and increases fibroblast infiltration and metabolism. NK cells compose a small fraction (~2%) of the lymphocytes circulating in the blood that are neither T cells nor B cells. They are identified as natural killer cells because these lymphocytes are already specialized to kill certain types of target cells.

E. Interferon-Gamma

Specific cytokines influence the development of many cell types. Interferon-gamma is a prime example of the pleiotropic nature of cytokines. Secreted interferons, such as INF-α, INF-β, INF-γ, act on neighboring cells to stimulate enzymes that render cells more resistant to virus infection. Specifically, interferons are cytokines that instruct cells to produce proteins, which makes the cells less susceptible to viruses. INF- [Gamma] not only possesses antiviral activity, but this cytokine also activates the pathway that leads to cytotoxic T cells [12]. INF-γ is classified as a pro-inflammatory cytokine because it induces nitric oxide (NO) and supplements TNF activity. In particular, INF-γ activates alveolar macrophages to produce a variety of substances including reactive oxygen and nitrogen species that are involved in growth inhibition and killing of mycobacteria.

3. Anti-Inflammatory Cytokines

Anti-inflammatory cytokines are grouped according to their ability to suppress genes for pro-inflammatory cytokines and the chemokines. In particular, anti-inflammatory cytokines suppress the intensity of the inflammatory mediator cascade [12]. Research indicates that anti-inflammatory cytokines, such as IL-4 and IL-10, suppress the production of IL-1, TNF, chemokines (IL-8), and vascular adhesion molecules [12].

Adhesion molecules aid in the retention of neutrophils during transmigration as these large cells are transiently retained in the microvasculature by purely mechanical factors [4]. The actions of pro-inflammatory cytokines are limited by auto regulatory mechanisms, the anti-inflammatory cytokines; however, the very short half-lives restrict the duration of action [8].

A. Interleukin-4

Although interleukin-4 is essential for the formation of functional antibody-producing B cells and a close relative of interleukin-2, it is also an effective anti-inflammatory agent [12]. IL-4 is a potent, pleiotropic cytokine that directs cellular activation, differentiation, and rescue from apoptosis. In mast cells, interleukin-4 induces the down-regulation of activation receptors and promotes cell death.

B. Interleukin-10

Anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines. Interleukin-10 was first described as a TH2 cytokine that inhibited IFN-γ and GM-CSF cytokine production by TH1 cells in mice. At the present time, IL-10 is known to inhibit the synthesis of a number of cytokines including INF-γ, IL-2, and TNF-β. Specifically, interleukin-10 suppresses IL-2 production following activation and inhibits apoptosis in T cells. For mast cells, interleukin-10 induces histamine release while blocking GM-CSF and TNF-α release. Additionally, interleukin-10 acts as a co-stimulator in the proliferation of mast cells with IL-4 present. IL-10 promotes TNF-α and GM-CSF production in NK cells, potentiates IL-2 induced NK cell proliferation, and facilitates IFN-γ secretion in NK cells primed by IL-18. In neutrophils, interleukin-10 inhibits the

secretion of the chemokines MIP-1α, MIP-1α and IL-8, blocks production of the pro-inflammatory mediators IL-1α and TNF-α, and decreases the ability of neutrophils to produce superoxide.

4. Other Cytokines

Some cytokines induce the formation of important blood cells. Granulocyte/macrophage colony-stimulating factor (GM-CSF) stimulates a specific progenitor cell in the bone marrow to divide several times and then differentiate into granulocytes. Granulocytes are a type of white blood cells that inactivate bacteria and other pathogens. This pro-inflammatory cytokine is secreted by cells of the monocyte/macrophage lineage. GM-CSF also activates and enhances the production and survival of neutrophils, eosinophils, and macrophages that have key roles in the innate immune response.

[FIGURE 3]

Pro-Inflammatory Cytokine Cascade

FIGURE 3: Illustrates the pro-inflammatory cytokine cascade. This diagram reinforces the cellular sources and biological activity for each pro-inflammatory cytokine. Used with permission from Dr. Nathan Menke, Department of Biochemistry, Virginia Commonwealth University/Medical College of Virginia.

[FIGURE 4]

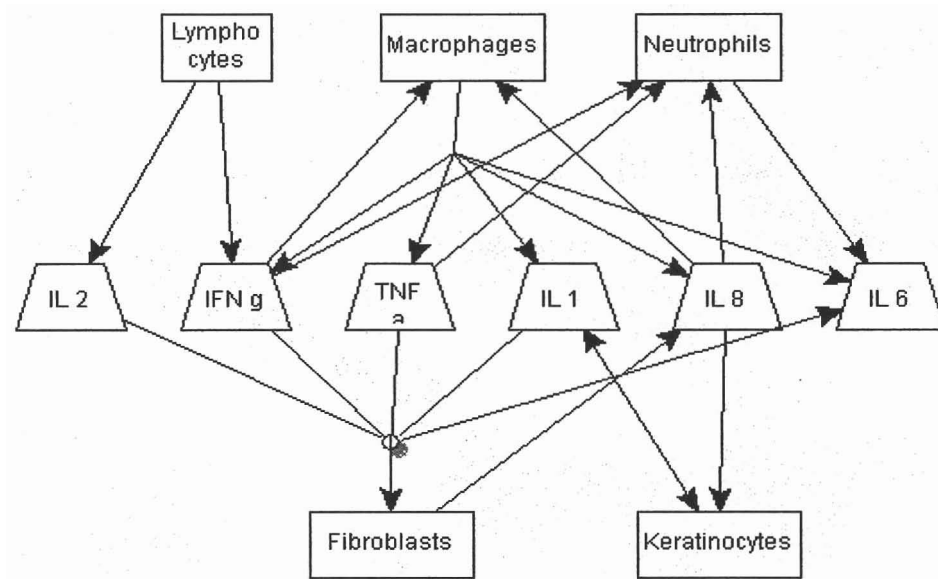Anti-Inflammatory Cytokine Cascade

Anti-inflammatory Cytokines
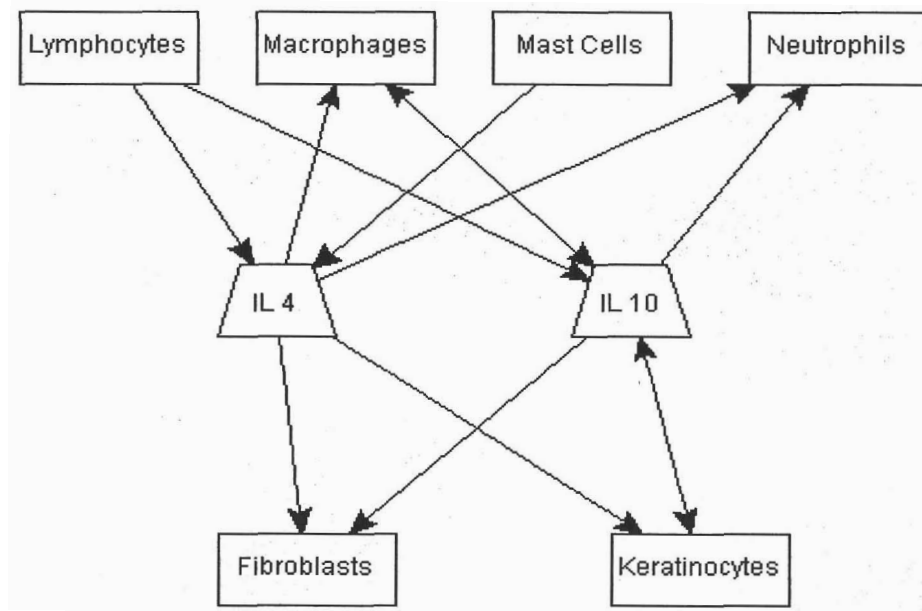
FIGURE 4: Illustrates the anti-inflammatory cytokine cascade. This diagram reinforces the cellular sources and biological activity for interleukin-4 and interleukin-10 as explained in detail above. Used with permission from Dr. Nathan Menke, Department of Biochemistry, Virginia Commonwealth University/Medical College of Virginia.

5. Chemokines

Chemokines are small peptides (8,000 d) that facilitate the passage of leukocytes from circulation into the tissues [12]. More specifically, chemokines are chemotactic cytokines that mediate inflammation and provide directional clues for the movement of leukocytes in development, homeostasis, and inflammation. Chemotactic agents attract adhesion molecules and cytotoxic agents assist these cells in driving the process. The increased secretion of chemokines during inflammation results in the selective recruitment of leukocytes into inflamed tissue. Most alpha [α] chemokines are chemoattractants for neutrophils, whereas beta [β] chemokines generally attract monocytes, lymphocytes, basophils, and eosinophils. Chemokines have been detected during inflammation in most organs, including the skin, lungs, kidneys, GI track, and brain. The main stimuli for chemokine production are early pro-inflammatory cytokines, such as interleukin-1 and TNF-α.

A. Interleukin-8

IL-8, a pro-inflammatory chemotactic and angiogenic C-X-C chemokine, is synthesized by resident and inflammatory cells [20]. Current literature, considers the neutrophil chemoattractant, interleukin-8, as a prime example of the chemokine family. More specifically, IL-8 activates neutrophils to degranulate and cause tissue damage [12]. As a chemokine, IL-8 mediates macrophage and neutrophil chemotaxis, whereas the immunoregulator promotes keratinocyte maturation as a pro-inflammatory cytokine. Johannes Frank reports that IL-8 consistently increases in injured patients during the first 24 hours after injury and IL-8 levels remain elevated for several days following severe

trauma [9]. In the cytokine cascade, pro-inflammatory mediators including, IL-1, TNF-α, INF-γ, and GM-CSF, induce interleukin-8 [20].

B. Monocyte Chemoattractant Protein-1

MCP-1, an example of a prototypical pro-inflammatory chemokine, belongs to the family of chemotactic cytokines known as C-C, or beta-chemokines [21]. Specifically, a highly conserved single gene at chromosome 17q11.2-q21.1 encodes this 76 amino acid basic glycoprotein. A wide variety of cell types including, monocytes, vascular endothelial cells, smooth muscle cells, glomular mesangial cells, osteoblastic cells, and articular chondrocytes, synthesize MCP-1. In addition, MCP-1 is produced in response to pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and INF-γ in the cytokine cascade. During inflammatory responses, MCP-1 is highly chemotatic for monocytes, T lymphocytes, basophils, and NK cells. MCP-1 also regulates adhesion molecule expression and cytokine production in monocytes. Additionally, MCP-1 can induce the proliferation of other effector cells of the immune system. Finally, monocyte chemoattractant protein-1 appears to possess a protective role in ischemia reperfusion injury because MCP-1 has the ability to protect monocytes from hypoxia-mediated apoptosis [17].

C. GRO/KC

Rat GRO/KC (also known as Rat KC or CINC) promotes neutrophil chemotaxis and degranulation. A cytokine-induced neutrophil chemoattractant (GRO/CINC), which belongs to the interleukin (IL)-8 family, acts as a functional chemoattractant for neutrophils in rats. Members of the CXC branch include chemokines that have four

invariant cysteines, where the first two cysteines are separated by one other amino acid (X). Those chemokines containing a glutamic acid–leucine-arginine sequence (ELR) immediately prior to the CXC motif are defined as potent neutrophil chemoattractants. In humans, seven ELR+CXC chemokines have been identified including, interleukin-8 (IL-8) and growth-related oncogenes (GRO)-α, -β, and -γ. Specifically, the GRO proteins chemoattract and activate both neutrophils and basophils. All chemokines in the CXC family play a significant role in the recruitment of neutrophils to tissue in various infectious and inflammatory conditions [22]. KC is defined as a homologue of the human GRO/melanoma growth-stimulatory activity family [23]. Since rats lack an IL-8 homologue, they provide a good model for investigating the roles of other rat ELR+CXC chemokines, including cytokine-induced neutrophil chemoattractant (KC).

6. Cellular Sources

Leukocytes (white blood cells), including neutrophils, macrophages, and lymphocytes, are required in a patient's immune response. Macrophages ingest large particles and cells by phagocytosis. A monocyte is defined as a white blood cell that can ingest dead or damaged cells and provide immunological defenses against many infectious organisms. In particular, circulating monocytes can migrate into tissues, become fixed tissue monocytes, and then develop into macrophages. During shock, the prolonged stimulation of macrophages results in excessive production of cytokines [INF-γ, TNF-α, IL-1, IL-8, IL-6, IL-4, IL-10] and other inflammatory mediators [17]. A neutrophil is a type of white blood cell filled with neutral staining granules and tiny sacs of enzymes that help the neutrophil kill and digest microorganisms it has engulfed by

phagocytosis. Neutrophils form the primary defense against bacterial infection and are produced in the bone marrow. Neutrophils circulate in the bloodstream and then move out of blood vessels into infected tissue in order to attack foreign substances. After neutrophils reach the tissues, they release oxygen metabolites, proteolytic enzymes, and other cytotoxic agents from their granules [14]. Mast cells are important effector cells providing granule and membrane mediators as well as cytokines in inflammatory diseases. Mast cells contain metachromatic granules that store a variety of inflammatory mediators including, histamine and serotonin, proteolytic enzymes that destroy tissue or cleave complement components, and chemotactic factors. Under normal conditions, mast cells are not found in circulation. Mediators released from mast cells are responsible for the four cardinal signs of inflammation: redness, heat, swelling, and pain.

7. Immune Response

The immune response includes two different subsets of T cells, namely TH1 and TH2. TH1 lymphocytes are characterized by the synthesis and release of IL-2, INF-γ, and IL-12; TH1 lymphocytes drive the inflammatory reaction. Conversely, TH2 cytokines (IL-4, IL-5, IL-10, IL-13) determine an inhibitory immune response. T lymphocytes (T cells) are composed of two groups of cells, the cytotoxic killer T cells (T_C) and the helper T cells (T_H), while B lymphocytes (B cells) produce and secrete antibodies. In response to a viral infection, T_C cells with the proper specificity proliferate to destroy large numbers of virus-infected cells. T_C cells interact with infected host cells through receptors on the T-cell surface. While the infected cell is being destroyed by the killer T cell, T_C and T_H cells bound to the infected cell mature and proliferate, which is

stimulated by interleukins. T_H cells interact with macrophages and secrete cytokines (interleukins) that stimulate T_C, T_H, and B cells to proliferate. Interleukins, which are produced and secreted by a variety of cells, stimulate the proliferation of only those T and B cells with the required interleukin receptors. It should be noted that T and B cells produce interleukin receptors only when they are complexed with an antigen. Therefore, only the immune system cells that have the ability to respond to the antigen proliferate. Populations of activated helper T cells secrete interleukin-2, which in turn stimulates proliferation of neighboring killer T cells and helper T cells having the appropriate interleukin receptors. As a result of this secretion and proliferation series, the number of immune system cells available to recognize and respond to the antigen increases. Another subpopulation of activated helper T cells complexed to macrophages or B lymphocytes secrete interleukin-4. The B cells, stimulated by the interleukins, proliferate and produce soluble antibodies that recognize the antigen.

8. Cytokine Receptors and Signaling Pathways

The structural homology conserved amongst the different cytokines provides significant evidence that all cytokines evolved from a common ancestral protein. Similarly, it has been determined that the various receptors evolved from a single common ancestor as well. All cytokines have a similar tertiary structure, which consists of four long conserved alpha helices folded together in a specific orientation. The structures of cytokine receptors are similar with their extracellular domains constructed of two subdomains, each of which contains seven conserved beta strands folded together in a typical fashion.

FIGURE 5

Pathway of Signal Transduction
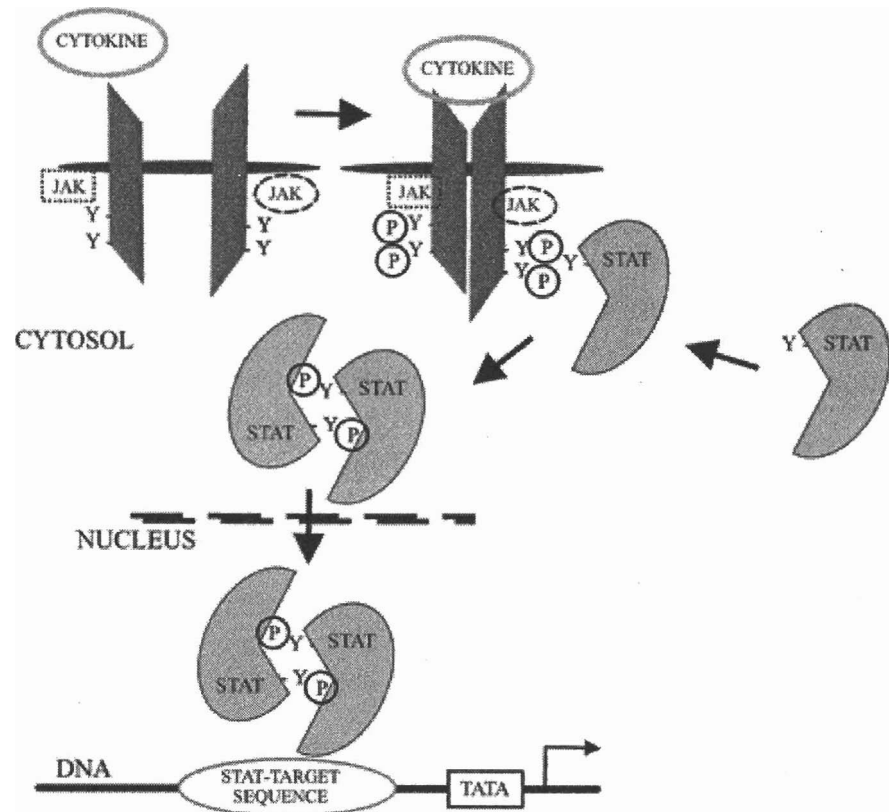
FIGURE 5: Pathway of signal transduction from cytokine binding on a receptor to the nucleus via the JAK/STAT pathway [24].

Cytokines, or local mediators, bind to receptors that activate gene regulatory proteins, which are held in a latent state at the plasma membrane [Figure 5]. After activation, these regulatory proteins move straight to the nucleus, where the transcription of specific genes is stimulated. Activation of transcription factors causes an increase or decrease in the expression of particular target genes. Because cytokine receptors have no intrinsic enzyme activity, they are instead associated with cytoplasmic tyrosine kinases called JAKs, which are activated when a cytokine binds to its receptor. Upon activation, the JAKs phosphorylate and consequently activate cytoplasmic gene regulatory proteins called STATs. These regulatory proteins then migrate to the nucleus, where the STATs stimulate transcription of specific target genes. Different cytokine receptors evoke various cellular responses by activating different regulatory proteins or STATs. The cytokine signal is ultimately turned off by protein phosphatases that remove the phosphate groups from the activated signaling proteins. Tumor necrosis factor (TNF) is an example of a cytokine family that utilizes this JAK/STAT signaling pathway. Interluekin-6 activates the mitogen-activated protein kinases (MAPKs) intracellular signaling cascade [25]. MAPKs have the responsibility of relaying and coordinating the delivery of environmental stress signals to the genome, with ensuing alternation in gene expression [25]. Whether or not a cell responds to a particular cytokine depends on the cell expression of the corresponding receptor. Despite the fact that all cytokine receptors activate similar intracellular signaling pathways, the response of any particular cell to a cytokine signal depends on the cell's collection of transcription factors.

III. Androstenetriol

Both multiple trauma and hemorrhage activate the hypothalamic-pituitary-adrenal axis (HPA) as a defensive mechanism to prevent cell death. It appears that a loss of balance between the hypothalamic-pituitary-adrenal systems is responsible for the deleterious effects observed in the victims of severe hemorrhage and trauma [17]. The hypothalamic pituitary-adrenal axis mediates a cascade of neurohormonal changes whose activation is associated with immuno-suppression. In particular, prolonged activation of the HPA axis shifts a patient's response from immuno-stimulated to immuno-suppressed. As already described in great detail, inadequate or excessive host defenses can be self-destructive, resulting in multiple organ system failure and ultimately death.

Androstenetriol (AET), a physiological metabolite of DHEA, up regulates host immune response, prevents immune suppression and modulates inflammation. The significance of AET came from a discovery that DHEA has the ability to regulate the immune response and protect against lethal bacterial infection and irradiation. DHEA, dehydroepiandrosterone, is a natural steroid hormone produced from cholesterol by the adrenal glands. The testicles, ovaries, and brain also produce DHEA. DHEA is chemically similar to testosterone and estrogen; dehydroepiandrosterone is carried to the tissues and cells where it is easily converted into these sex hormones. DHEA is metabolized along the pathway DHEA $\Rightarrow$ AED $\Rightarrow$ AET. Androstenetriol (AET) is derived from androstenediol (AED) by the addition of a third hydroxyl group at position 7 of ring B. According to recent literature, androstenediol is ten thousand times more

effective than DHEA in preventing infection, and AET is up to one hundred thousand times more potent than DHEA [26].

Traditionally, the immune system is treated as a separate physiological system from the endocrine and nervous systems. However, in 1988 Loria et. al. reported that DHEA could regulate immune responses against lethal virus and bacterial infections [27]. DHEA and particularly, AED and beta AET, have been shown to protect mice from viral, bacterial, and parasitic infections. In a trauma-hemorrhage model, AED improved organ function and altered cytokine production [26]. Literature reports that beta AET up-regulates immunity to increase resistance against lethal infection and lethal radiation [26]. In addition, the beta androstene steroid mediates a rapid recovery of hematopoietic precursor cells after radiation injury.

[FIGURE 6]

AET and HPA Axis Interaction in Traumatic-Hemorrhage
The Japanese Journal of Clinical Pathology 46:6:505-517 (1998)
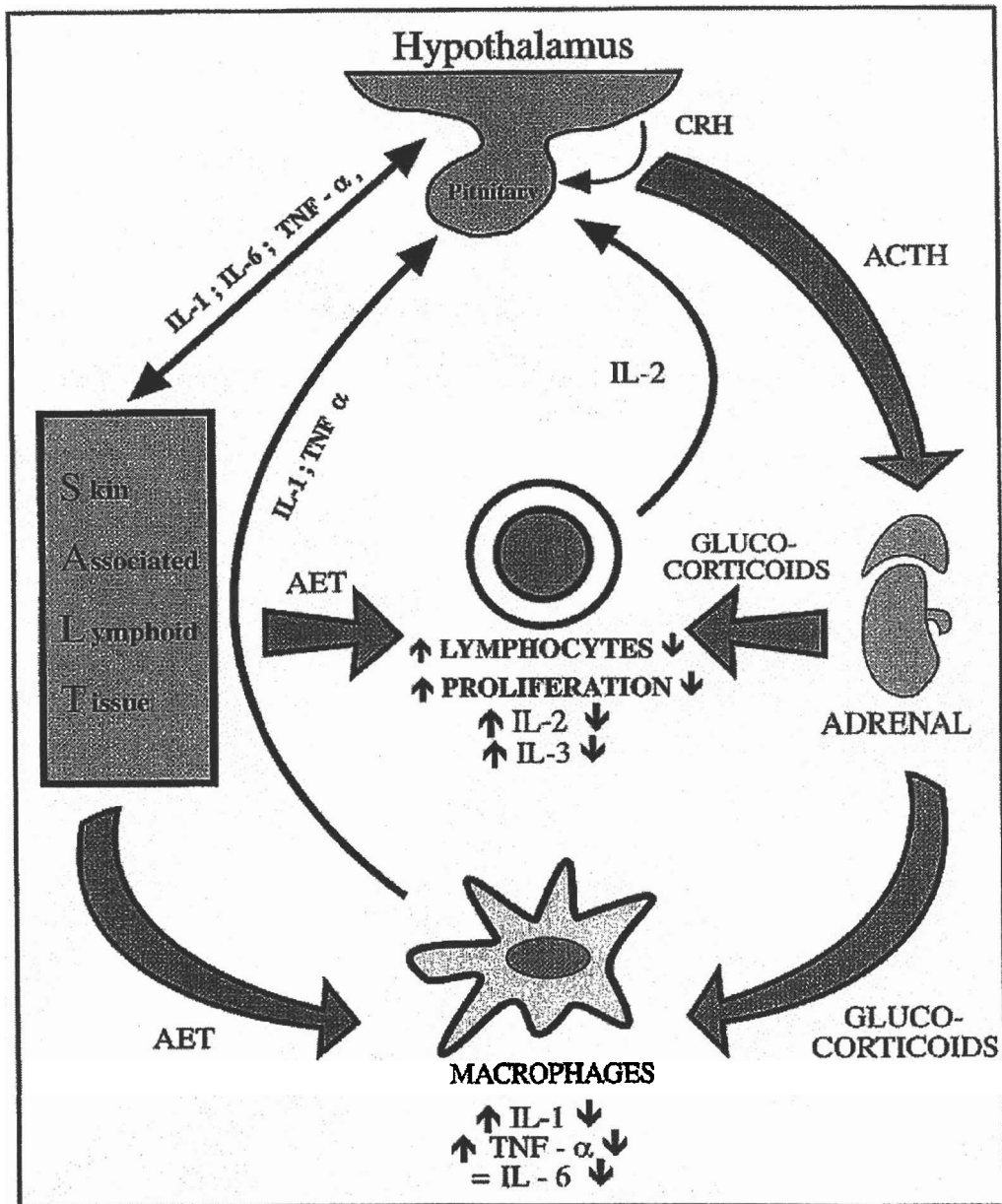
FIGURE 6: Illustrates the possible roles of AET, specifically those that modulate inflammatory cytokines, to counteract the effects of glucocorticoids on the immune system.

AET was reported to counteract the immune suppressive effects of hydrocortisone (cortisol). Therefore, androstenetriol may in part exert its physiological function by neuroimmunological regulatory mechanisms. In the body, these three hormones (DHEA, AED, & AET) balance the widely accepted immunosuppressive action of glucocorticoids, suggesting a possible new immune regulation mechanism. Hydrocortisone classically is used to suppress excessive inflammation in cells treated with an immune challenge. According to published research, DHEA and its metabolites, androstenediol (5-androstene-3 beta-17 beta-diol, AED) and androstenetriol (5-androstene-3 beta-7 beta-17 beta-triol, beta AET), counteract the stress-induced immunosuppressive action of glucocorticoids [15]. Specifically, Dr. Loria concluded in 1997 that DHEA was unable to counteract the immunosuppressive effects of cortisol, while androstenediol counteracted it only at high doses [26]. Conversely, androstenetriol significantly counteracted the immunosuppressive effects of the glucocorticoid on lymphocyte proliferation and cytokine production [15]. In particular, beta androstenetriol increases the levels of TH1 cytokines (IL-2, IL-3, IFN-γ) and counteracts hydrocortisone mediated immune suppression [26]. In rodents, administration of AED during traumatic shock demonstrated a reduction in interleukin-6 circulating levels [17]. Research indicates that in vivo, DHEA, 5AD, and 5AT may have some similar functions; while in cell culture their effects are dramatically different from one another. It is important to emphasize the fact that both androstenediol and androstenetriol have the ability to suppress inflammation, comparable to other glucocorticoid steroid hormones. However,

AED and AET suppress inflammation through mechanisms that do not induce immune suppression.

Corticosteroids are critical in the inflammatory process because of their various roles including the ability to prevent complement activation, inhibit the inducible form of nitric oxide synthesis, and prevent neutrophil adherence induced by endotoxin [17]. In the cytokine pathway, corticosteroids decrease the transcription of TNF and other pro-inflammatory cytokines [17]. On the contrary, corticosteroids increase the transcription of anti-inflammatory cytokines such as IL-1 receptor antagonist [17]. In addition, corticosteroids are known to decrease the release of adhesion molecules [17].

Not only does androstenetriol possess anti-inflammatory properties, the steroid can also potentiate the cellular and humoral immune responses in numerous local and lethal systemic insults. As already mentioned, AET has been shown in recent experiments to improve outcomes from systemic insults such as overwhelming bacterial and viral infections [26]. Through AET regulation, the chronic inflammation associated with both infection and sepsis can be control. Consequently, the morbidity and mortality that normally results from this uncontrolled inflammation becomes reduced or prevented. Current research recognizes that the nuclear receptor factor peroxisome proliferator (PPAR) and prostaglandins have a major role in regulating immune response, inflammation, and wound healing [28]. AET was utilized as the pharmacological agent in this study because it is proposed that AET has the ability to modulate the PPAR beta/delta balance and as result reduce inflammation, which in turn may reduce SIRS, prevent systemic sepsis, and decrease multiple organ failure mortality.

Materials and Methods

I. Sample Preparation

Blood was collected from adult male Sprague-Dawley rats for cytokine analysis. Under sterile condition, the surgeon placed two catheters using PE 50 and PE 90 tubing in the carotid and jugular vein. A ventral midline laparotomy was performed to induce soft tissue injury before the onset of hemorrhage. After recovery, 40% of the rat's total blood volume was hemorrhaged using the carotid catheter over a time period of approximately 15 minutes [Volume Model]. A specific formula was used to calculate the total blood volume for each rat. After 45 minutes from the onset of hemorrhage, resuscitation was initiated with lactated Ringers and packed red blood cells were returned to the rat in intervals over 2.5 hours. At the start of resuscitation, rats were randomized to receive a single subcutaneous injection of AET or vehicle (methylcellulose). Following resuscitation, rats were observed for 4 hours and then returned to the vivarium. Rats were monitored at 24, 48, and 72 hours and any rats surviving to 72 hours were euthanized using Euthasol.

Arterial blood (0.5 mL) was taken from the rat using the carotid catheter at three different time points, baseline, 24 hours, and 72 hours. More specifically, the first blood draw was at the on-set of hemorrhage; therefore baseline is considered pre-hemorrhage /pre-resuscitation. The blood was placed in a heparin coated microcentrifuge tube and

immediately spun at 15,000 rotations per minute for 5 minutes. The supernatant was then extracted using a 100 μL pipette while the pellet was discarded. The supernatant was divided into three labeled plain microcentrifuge tubes and the samples were place in a –20°C freezer. Following final sample preparation for all time points, plasma samples were stored in a –80°C freezer until thawed for cytokine analysis.

A similar procedure was used to collect plasma from rats where blood was removed during a 60-minute hemorrhage while maintaining the mean arterial pressure (MAP) between 35-40 mmHg [Pressure Model]. Specifically, blood was hemorrhaged during the first 15 minutes until the blood pressure was controlled at 35-40 mmHg. This blood pressure was then sustained for the remainder 45 minutes. A third animal model, utilized the pressure-driven hemorrhage procedure, however the subcutaneous injection of either AET or vehicle was delivered 24 hours prior to the start of hemorrhage [Pre-treatment model]. For these two experiments, arterial blood was taken using the carotid artery at four different time points, baseline (post-hemorrhage), 6 hours, 24 hours, and 48 hours. The blood was then treated as described above and the rat plasma was stored in a –80°C freezer until thawed for cytokine analysis.

II.   Cytokine Analysis

1. Rat Cytokine/Chemokine Premixed LINCOplex Kit

The LINCOplex® kit (RCYTO-80K) [St. Charles, Missouri] enabled the simultaneous multi-analyte detection and measurement of fourteen rat cytokine/chemokines. All reagents and components were included in each kit. The overnight assay required at least 5 μL of rat plasma to measure a premixed 14-plex panel.

The rat cytokine/chemokine panel included: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-18, GM-CSF, GRO/KC, INF-γ, MCP-1, and TNF-α. The standard curve for this particular assay ranges between 6.4 and 20,000 pg/ml. Performance characteristics include, sensitivity for plasma is 1-20 pg/ml and accuracy is between 92.8 and 108.6%. Accuracy describes the amount of uncertainty that exists in a measurement with respect to the relevant absolute standard and sensitivity describes the smallest absolute amount of change that can be detected by a measurement. The intra-assay precision is less than 15%, while the inter-assay precision is less than 10%. Furthermore, the cytokine antibodies in this multiplex do not cross react to other analytes in the panel.

Luminex Corporation's xMAP™ Technology involves a patented technology that internally dyes polystyrene microspheres with two spectrally distinct fluorochromes. LINCOplex kits utilize these beads as the foundation for their multiplexed immunoassays. Each signature bead is conjugated to an analyte specific antibody. The rat cytokine/chemokine assay is based on the conventional two-site sandwich method where the mixed beads are incubated with a standard or biological sample in a 96-well plate format. More specifically, each set of beads is coupled with a specific capture antibody. The capture antibody then binds to its specific analyte, followed by the binding of the biotinylated detection antibody. The final result is amplified through incubation with a reporter conjugate, streptavidin-phycoerythrin; the streptavidin-phycoerythrin (SA-PE) binds to the biotinylated reporter and emits a fluorescent signal [Appendix 1: LINCOplex Schematic].

Following incubation, the contents of each microplate well were drawn into a Bio-Plex array reader (171-000005) and precision fluidics aligned the beads in single file through a flow cell where two lasers excite the beads individually. A red classification and a green reporter laser illuminated individual beads to identify each bead's color code and quantify the associated reporter signal.

2. Bio-Rad's Bio-Plex Cytokine Assay

Bio-Rad's Bio-Plex cytokine assay (171-K11070) [Hercules, California] is a multiplex bead-bead assay designed to quantitate multiple cytokines in diverse matrices including rat plasma. One 96-well included coupled beads, detection antibodies, and standards for the detection of IL-1alpha, IL-1beta, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-gamma, and TNF-alpha. Premixed multiplex panels test for the presence of a predetermined set of cytokines in a single sample. The Bio-Plex suspension array system utilizes patented multiplexing technology similar to that of LINCO. The Bio-Plex system uses up to 100 color-coded bead sets, each of which can be conjugated with a different specific reactant and each reactant is specific for a different target molecule. This technology is designed in a capture sandwich immunoassay format. The antibody for the specific cytokine of interest in covalently coupled to color-coded polystyrene beads. The antibody-coupled beads are then allowed to incubate and react with a plasma sample (containing an unknown amount of cytokine) or with a standard solution provided from the company (containing a know amount of cytokine). According to technical support at Bio-Rad, rat standards (recombinant protein) are not always standardized, whereas mouse and human standards for multi-plex assays are obtained from commercial sources. A

Linco representative stated that commercial sources provide a Certificate of Analysis to confirm purity and mass equivalence of products. Bio-Rad also notes that in some cases rat recombinant standards from different companies are better models than theirs in terms of the absolute values.

Unbound protein was removed by a series of washes and a biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads in each well. As a result, a sandwich of antibodies formed around the cytokine and the reaction mixture was detected using streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. As with the LINCOplex, the constituents of each well in the Bio-Plex assay were drawn up into a flow-based Bio-Plex suspension array system and the identity and quantity of each specific reaction was determined using bead color and fluorescence. Fluorescently labeled reported molecules associated with each target protein were used to measure the magnitude of the reaction.

3. Bio-Plex Manager Software

The Bio-Plex Manager software uses a standard curve derived from a recombinant cytokine standard to calculate unknown cytokine concentrations. Bio-Plex system software employs StatLIA 4PL and 5PL curve fitting and provides percentage recover calculations. Data produced by the software were analyzed and imported into Statistical Package for the Social Sciences (SPSS) for statistical analysis. Except for the bead set, default settings were used in the Bio-Plex system software. Any data point in the Bio-Plex System software with a CV% of less than or equal to 50% was included in the final data analysis. Furthermore, any data point above or below the standard curve,

within two standard deviations of the lowest or highest standard, was also included. Triplicate biological samples were used to quantify the cytokine/chemokine amounts. Using the outlier feature in the Bio-Plex system software, one data point in the triplicate was excluded to make the CV% as close to zero as possible. If there was no fluorescence for a particular sample, no outliers were determined and only a duplicate was used in the final analysis. If all samples combined produced the lowest CV% then the entire triplicate data was utilized. Finally, outliers in the standard curve were determined by removing the data point that kept the overall average of the observed/expected * 100 as close to 100% as possible. The range for observed/expected * 100 was any number between or equal to 100 ± 30 (70-130).

4. Titer Zyme® EIA

Enzyme Immunometric Assay Kit (EIA) (Catalog No. 900-086) [Ann Arbor, Michigan]. The TNF-α ELISA Assay employed to measure rat plasma included all materials for the quantitative determination of rat TNF-α in biological fluids. The kit manufactured by Assay Designs utilized a polyclonal antibody to rat TNF-α immobilized on a microtiter plate, which binds the rat TNF-α in the biological sample. Following a short incubation, excess sample was washed away and a monoclonal antibody to rat TNF-α labeled with the enzyme Horseradish peroxidase was added to each well. The antibody binds to the rat TNF-α captured on the plate. Another short incubation was required, the excess labeled antibody was washed out using a wash buffer, and the substrate solution was then added. The substrate reacts with the labeled antibody bound to the rat TNF-α captured on the plate. Finally, a stop solution was added, which turns the blue color in

each well to yellow, and the color generated with the substrate was read at 450 nm. Using standards provided in the kit to draw a standard curve, the optical density reading is directly proportional to the concentration of rat TNF-α in the sample.

Assay Designs' rat MCP-1 TiterZyme® Enzyme Immunometric Assay (EIA) (Catalog No. 900-077) kit is a complete kit for the quantitative determination of rat MCP-1 in biological fluids. The MCP-1 ELISA assay utilizes the same techniques and protocol as described above. The measured optical density is directly proportional to the concentration of rat MCP-1 in either provided standards or biological samples.

Experimental Results

I.   Statistical Analysis

The chemokine and cytokine measurements were analyzed using Independent Sample T-test and Chi-Square statistics; a p value $\leq$ 0.05 was considered significant.

II.   Chemokine and Cytokine Measurements

Comparisons between the enzyme-linked immunosorbent assays (ELISA) and multi-plex plates assessed the precision and accuracy of the multiplex technology and evaluated the sensitivity of each assay.   A uniform comparison was executed using plasma samples from the pressure model to appraise two respectable multi-plex assays employed in the biochemical field.   After numerous experiments utilizing both the LINCOplex and Bio-Plex plates, two specific cytokines were selected for the assessment. We determined that it is important to first screen a full panel of cytokines during pilot studies because not all cytokines are measurable in every assay. The researcher can then choose specific cytokines that give reliable responses to customize future panels for a truncated list.   Utilizing pilot study data, it was determined to customize the LINCOplex kit to include IL-1α, MCP-1, IL-6, IL-10, IL-12p70, INF-δ, IL-18, and GRO-KC. While five cytokines, IL-1β, IL-2, IL-6, IL-10, and TNF-α, were selected for Bio-Rad's Bio-Plex plate.   In particular, interleukin-10 (IL-10) and interleukin-6 (IL-6), produced

reliable data from previous experiments for both plates of interest. As a result, these two cytokines were used for the uniform comparison.

Table 1 demonstrates that many of the baseline (B) values for AET and VEH animals were undetectable when employing Bio-Rad's multi-plex assay to measure IL-6 levels. As noted in the left column [Table 1], only two baseline levels are recorded. The other nine cells are blank because baseline IL-6 levels for these animals were either below the limit of detection or noted non-detectable due to experimental error and/or high %CV. Table 1 illustrates the average or mean (AV) and standard deviation (SD) for each data set. In statistics, a normal distribution does not exist when the standard deviation is greater than the mean. Outlying values can be removed, however, outliers make the data hard to analyze. Without a large sample size, researchers cannot conclude that outliers result from test variation, rather than biological variation.

Table 1 illustrates the variation between two reputable multi-plex assays. The Bio-Plex assay recorded 262.63 pg/mL for plasma sample 64-148759-6 as opposed to 87.82 pg/mL observed by the Lincoplex kit, almost a 3-fold difference for measurable IL-6. For a second plasma sample (65-148760-6), Bio-Rad's assay measured 249.96 pg/mL while 32.15 pg/mL (approximately an 8-fold disparity) was documented using Linco's plate. The cytokine values observed for IL-10 [Table 2] also demonstrated incontinences. Rat 64-148759-B had a cytokine level of 717.93 pg/mL with the Bio-Plex kit and 53.35 pg/mL with Linco. For the biological sample, 53-147134-6, 1162.71 pg/mL was recorded using Bio-Rad's assay, whereas the Lincoplex plate observed the value of 194.81 pg/mL for circulating plasma levels of interleukin-10.

Bio-Rad's Bio-Plex			
Description		**B**	**6 hr**
47-145726-B	AET		
47-145726-6	AET		792.3
64-148759-B	AET	145.52	
64-148759-6	AET		262.63
65-148760-B	AET		
65-148760-6	AET		249.96
67-149126-B	AET		
67-149126-6	AET		145.52
71-149456-B	AET		
71-149456-6	AET		145.52
	AV	**145.52**	**319.19**
	SD		**270.25**

Lincoplex			
Description		**B**	**6 hr**
47-145726-B	AET	6697.34	
47-145726-6	AET		
64-148759-B	AET	38.57	
64-148759-6	AET		87.82
65-148760-B	AET		
65-148760-6	AET		32.15
67-149126-B	AET	163.94	
67-149126-6	AET		135.03
71-149456-B	AET	123.36	
71-149456-6	AET		152.42
	AV	**1755.80**	**101.86**
	SD	**3294.77**	**53.89**

Bio-Rad's Bio-Plex			
66-149125-B	VEH		
66-149125-6	VEH		
74-149767-B	VEH		
74-149767-6	VEH		237.22
52-147136-B	VEH		
52-147136-6	VEH		275.23
53-147134-B	VEH		
53-147134-6	VEH		145.52
54-147135-B	VEH	262.63	
54-147135-6	VEH		959.02
60-148238-B	VEH		
60-148238-6	VEH		249.96
	AV	**262.63**	**373.39**
	SD		**331.02**

Lincoplex			
66-149125-B	VEH		
66-149125-6	VEH		99.76
74-149767-B	VEH	38.57	
74-149767-6	VEH		276.72
52-147136-B	VEH	18.96	
52-147136-6	VEH		430.05
53-147134-B	VEH	1293.03	
53-147134-6	VEH		146.64
54-147135-B	VEH	1395.26	
54-147135-6	VEH		1334
60-148238-B	VEH	111.6	
60-148238-6	VEH		260.03
	AV	**571.48**	**424.53**
	SD	**707.11**	**460.15**

[TABLE 1]

Uniform Comparison: IL-6

TABLE 1: [Pressure Model]
Identical plasma samples were analyzed using both the Bio-Plex and Lincoplex multi-plex technology to measure interleukin-6. AV = average; SD = standard deviation; B = baseline time point; 6 hr = six hour time point; blank cell = value not detectable in assay; concentration values [pg/mL]. Note the inconsistencies between both plates.

Bio-Rad's Bio-Plex			
Description		**B**	**6 hr**
47-145726-B	AET	248.68	
47-145726-6	AET		1813.12
64-148759-B	AET	717.93	
64-148759-6	AET		2855.08
65-148760-B	AET	774.91	
65-148760-6	AET		1583.21
67-149126-B	AET	176.25	
67-149126-6	AET		882.29
71-149456-B	AET	307.92	
71-149456-6	AET		1123.28
	AV	**445.14**	**1651.40**
	SD	**279.68**	**766.50**

Lincoplex			
Description		**B**	**6 hr**
47-145726-B	AET	6096.81	
47-145726-6	AET		
64-148759-B	AET	53.35	
64-148759-6	AET		105.2
65-148760-B	AET	49.16	
65-148760-6	AET		74.88
67-149126-B	AET		
67-149126-6	AET		39.9
71-149456-B	AET	57.58	
71-149456-6	AET		37.87
	AV	**1564.23**	**64.46**
	SD	**3021.73**	**32.03**

66-149125-B	VEH	1189	
66-149125-6	VEH		1686.13
74-149767-B	VEH	231.13	
74-149767-6	VEH		3918.6
52-147136-B	VEH	110.38	
52-147136-6	VEH		6661.94
53-147134-B	VEH	283.79	
53-147134-6	VEH		1162.71
54-147135-B	VEH	525.04	
54-147135-6	VEH		7969.96
60-148238-B	VEH	182.84	
60-148238-6	VEH		3896.72
	AV	**420.36**	**4216.01**
	SD	**402.17**	**2682.84**

66-149125-B	VEH	43.98	
66-149125-6	VEH		102.91
74-149767-B	VEH		
74-149767-6	VEH		324.08
52-147136-B	VEH		
52-147136-6	VEH		382.01
53-147134-B	VEH		
53-147134-6	VEH		194.81
54-147135-B	VEH		
54-147135-6	VEH		821.05
60-148238-B	VEH	57.58	
60-148238-6	VEH		224.77
	AV	**50.78**	**341.61**
	SD	**9.62**	**254.55**

[TABLE 2]

Uniform Comparison: IL-10

TABLE 2: [Pressure Model]
    Identical Plasma samples were analyzed using both the Bio-Plex and Lincoplex multi-plex technology to measure interleukin-10. AV = average; SD = standard deviation; B = baseline time point; 6 hr = six hour time point; blank cell = value not detectable in assay; concentration values [pg/mL]. Note the inconsistencies between both plates.

As noted in the introduction the importance of monitoring chemokine and cytokines levels in response to trauma and hemorrhage may be very important for predicting survival. However, in order to make conclusions regarding the physiological relevance of measured cytokine levels, modern methodologies must first prove to have high sensitivity, specificity, and precision. The variation between these two respectable assays validates concerns regarding the credibility and reliability of rat assays in general and this data questions the legitimacy of cytokine concentrations measured using the multi-plex technology. Therefore, the remaining experimental results presented in this thesis must be regarded as preliminary because of the variation noted when utilizing modern techniques.

A total of 24 animals were used in the volume study. Specifically, 12 rats were randomized in the blinded experiment to receive AET while the other 12 rats were injected with vehicle. Chemokine and cytokine analyses utilized plasma from both AET and vehicle rats that survived to 72 hours. All rats randomized to receive AET survived the hemorrhage and resuscitation, whereas only 9 of 12 rats receiving the vehicle injection survived to 72 hours. Even though the difference in mortality is statistically significant, it should be noted that analysis was performed using a smaller number of vehicle treated animals.

Preliminary results illustrate a trend in the cytokine and chemokine responses following hemorrhagic trauma. In the volume model, those animals surviving hemorrhagic shock and resuscitation had elevated levels of both MCP-1 [Figure 7] and GRO/KC [Figure 9] as compared to baseline chemokine measurements from vehicle

treated animals at the 24-hour time point. As already mentioned, the absolute standardization of both chemokines and cytokines using rat antibodies is problematic. Therefore, an ELISA assay [Figure 8] was used to verify the trend of MCP-1 to validate the chemokine measurements obtained from the Lincoplex kit.

Based on measurements using the Lincoplex kit, analysis indicated that there was an increase of MCP-1 levels in animals surviving hemorrhagic trauma and shock [Figure 7] at 72 hours (VEH 1501.57 pg/mL; AET 1336.00 pg/mL) as compared to baseline MCP-1 levels (105.55 pg/mL). Since drug was not injected prior to taking the pre-hemorrhage/pre-resuscitation plasma sample, the sample size for baseline measurements is 17 (combination of AET + VEH animals). This 13-fold increase of MCP-1 in surviving AET animals is statistically significant with a P value of < 0.0001 (Independent Sample Test). Likewise, there was an increase of MCP-1 in both AET and VEH survivors at 72 hours compared to MCP-1 VEH measurements at 24 hours (156.93 pg/mL). The 8.51 increase between AET 72 and VEH 24 is statistically significant with a P value of <0.0001.

Based on measurements using the MCP-1 TiterZyme® Enzyme Immunometric Assay [Figure 8], levels of MCP-1 are approximately 350 times higher in surviving animals at 72 hours (VEH 94.36 ng/mL; AET 131.13 ng/mL) compared to baseline animals (0.326 ng/mL). The increase of MCP-1 in surviving AET animals is statistically significant with a P value of < 0.001 (Independent Sample Test). The increase of MCP-1 in surviving vehicle animals is statistically significant with a P value of < 0.109. Even though the absolute values of MCP-1 varied greatly between the muli-plex and ELISA

assays, it is possible to conclude that levels of this chemokine are significantly higher in animals surviving hemorrhagic trauma.

[FIGURE 7]

Survival Trend of MCP-1

**Effect of Hemorrhagic-Trauma on Monocyte
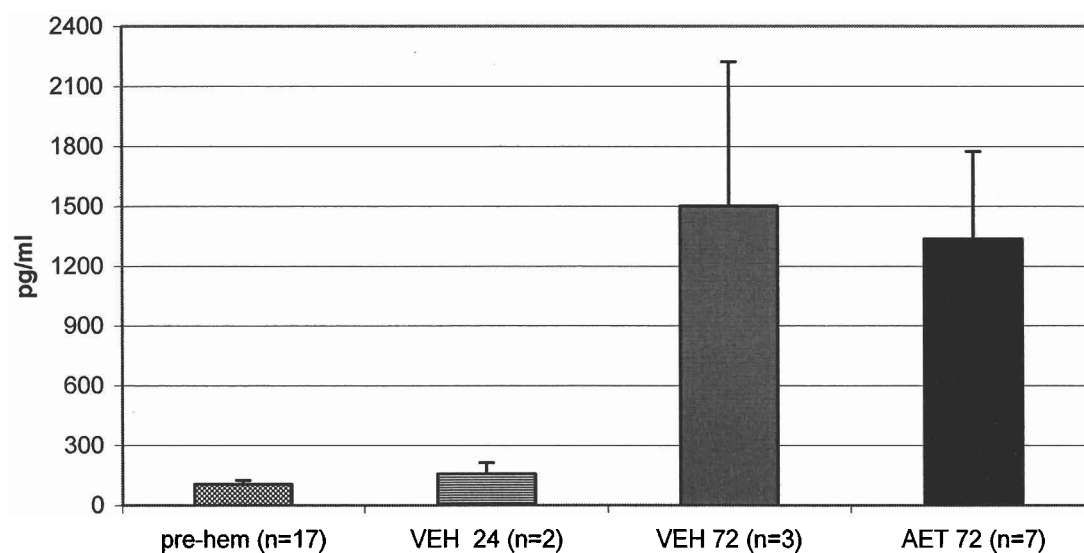Chemoattractant Protein-1 (MCP-1) Plasma Levels**

FIGURE 7: [Volume Model]
     Analysis indicated that there was an increase of MCP-1 levels in animals surviving hemorrhagic trauma and shock at 72 hours as compared to baseline and VEH 24 MCP-1 levels.

[FIGURE 8]

MCP-1: Survival Trend Validation

**Effect of Hemorrhagic-Trauma on Monocyte
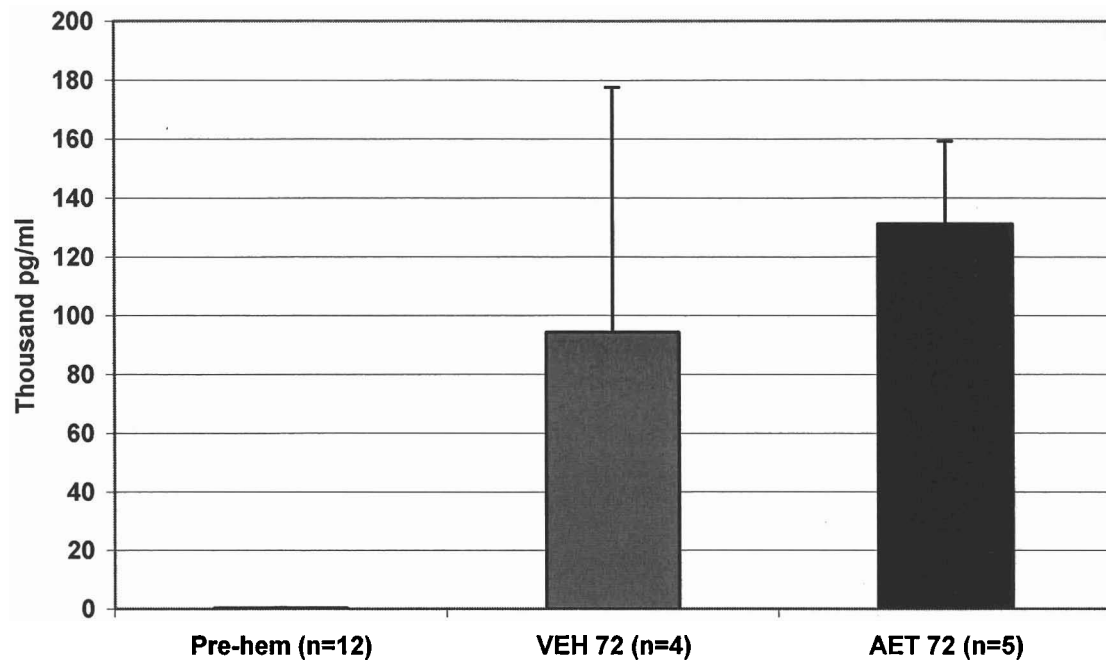Chemoattractant Protein-1 (MCP-1)**

FIGURE 8: [Volume Model]
   Trend confirmation; levels of MCP-1 were elevated in surviving animals at 72 hours compared to baseline measurements.

Based on measurements using the Lincoplex kit, analysis indicated that there was a 2.2 increase of GRO/KC in animals surviving hemorrhagic trauma and shock compared to baseline GRO/KC levels [Figure 9]. Surviving animals had higher levels of this chemokine (365.67 pg/mL) at 72 hours compared to the mean concentration of 164.47 pg/mL for pre-hemorrhaged baseline animals. This 2-fold increase is statistically significant with a P value of < 0.05 (Independent Sample Test). The GRO/KC measurement for survivors was also notably higher than the 120.36 pg/mL observed in vehicle treated animals 24 hours post trauma and hemorrhage. This 3-fold increase is statistically significant with a P value of ≤ 0.03. As noted, the survivor group included both AET and vehicle treated animals; therefore, the elevation of GRO/KC in survivors cannot be attributed to drug treatment.

[FIGURE 9]

GRO/KC and Survival

**Association of Growth-Related Oncogene (GRO/KC)
with Survival**

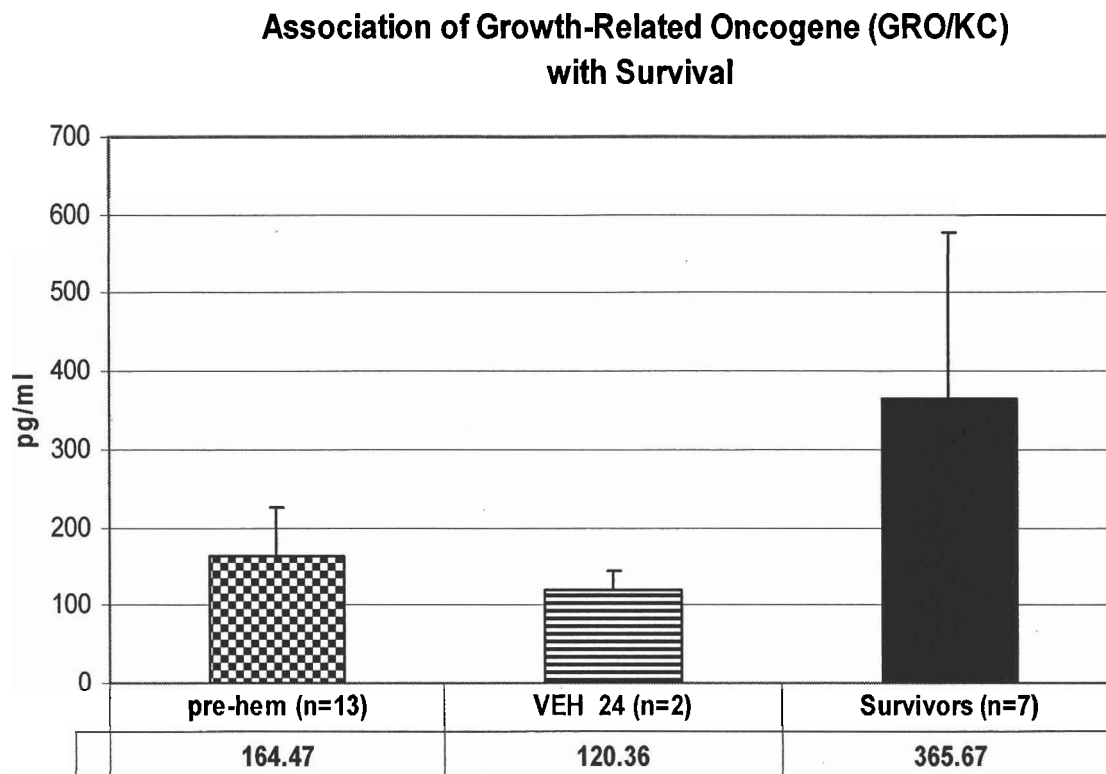|	pre-hem (n=13)	VEH 24 (n=2)	Survivors (n=7)
	164.47	120.36	365.67

FIGURE 9: [Volume Model]

Animals surviving hemorrhage had higher levels of GRO/KC at 72 hours compared to chemokine measurements for both pre-hemorrhage traumatized animals and vehicle treated animals at 24 hours.

IL-1α levels were significantly reduced in untreated animals 24 hours and 72 hours after trauma and hemorrhage as compared to the baseline value for animals in the volume model [Figure 10]. In addition, AET surviving animals had markedly higher levels of IL-1α than the vehicle treated animals surviving 24 hours and 72 hours after injury. Based on measurements using the Lincoplex kit, analysis indicated that there was a 2.5 increase of IL-1α in AET animals surviving hemorrhagic trauma and shock at 72 hours (181.37 pg/mL) as compared to levels of vehicle treated animals at 24 hours (72.11 pg/mL). This increase for AET survivors at 72 hours versus VEH 24 hours is statistically significant with a P value of = 0.017. Likewise, the 3-fold increase of surviving AET animals versus vehicle treated animals at 72 hours (56.99 pg/mL) is statistically significant with a P value of < 0.01. Interestingly, AET treated animals mediated a restoration of IL-1α levels at 72 hours; however the mean concentration for AET survivors was not higher than the mean baseline value reported (246.17 pg/mL). Using an independent sample test, P = 0.212 for AET 72 hours versus pre-hemorrhage levels. Normal levels of IL-1 are below detection of any assay [39]. Therefore, these results may indicate that trauma induces IL-1α because the pre-hemorrhage levels after surgery [placement of three catheters and midline laparotomy] averages 246.17 pg/mL, whereas hemorrhage decreases IL-1α because both the VEH 24 and VEH 72 means after hemorrhage and resuscitation are below pre-hemorrhage levels. The 3-fold decrease between vehicle animals at 24 hours and pre-hemorrhage animals at baseline is significant with a P value < 0.008. The 4.31 decrease of IL-1α between surviving vehicle

animals at 72 hours and baseline (pre-hemorrhage) animals is statistically significant with a P value of < 0.005.

[FIGURE 10]

Effect of AET on IL-1α

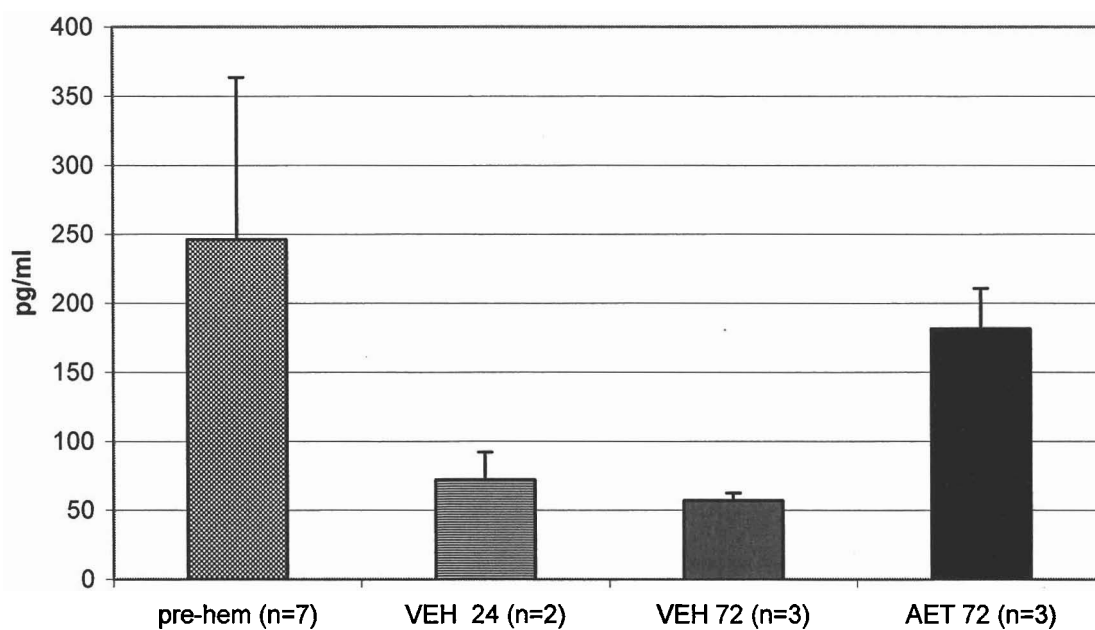**Effect of Androstenetriol on the Pro-Inflammatory Cytokine IL-1 alpha (IL-1 alpha)**

FIGURE 10: [Volume Model]

IL-1α levels were significantly reduced in vehicle animals 24 hours and 72 hours after trauma and hemorrhage as compared to the baseline value. AET treated animals at 72 hours had markedly higher levels of IL-1α than the vehicle treated animals surviving 24 hours and 72 hours.

As demonstrated, by previous results, various rat assays (multi-plex vs. multi-plex and multi-plex vs. ELISA) report different cytokine measurements for the same plasma samples. The validity of modern methodologies remains to be determined; therefore, measurements representing the actual physiological cytokine or chemokine levels present in rat plasma are not reliable at this time. Presently, we elected to base our conclusions on the trends between samples. In the case of MCP-1, the ELISA [Figure 8] was more sensitive than the LINCOplex plate [Figure 7], however ELISA assays are not always more sensitive then the multi-plex technology. With TNFα, preliminary results using Bio-Rad's Bio-Plex Cytokine Assay reported values [Figure 11] ranging from 50 pg/mL to approximately 250 pg/mL. On the contrary, the ELISA assay did not detect any measurable levels for this particular cytokine. Plasma samples diluted in 1:2 ratio were below detectable limits using the TNF-α Titer Zyme® EIA; the manufacturer suggested that a 1:5 dilution was adequate for measuring rat plasma. It is important to note that all assay protocols discourage running "neat samples" because of the matrix effect associated with plasma or serum biological samples.

[FIGURE 11]

TNFα Bio-Plex Measurements

Values of TNF-alpha

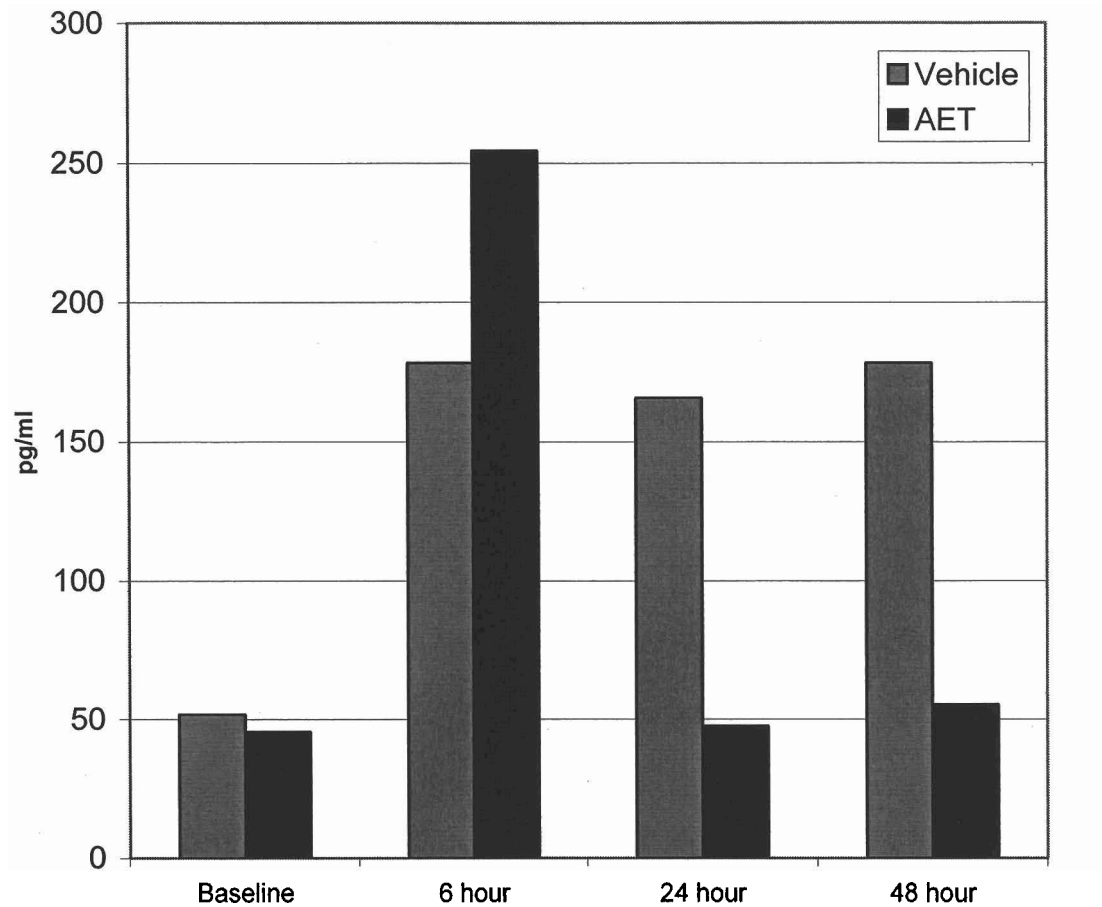
FIGURE 11: [Pre-Tx Model]

Values for TNF-α range from 50 pg/mL to a little over 250 pg/mL. Note these measurements should be regarded as preliminary because of the small sample size for both AET and Vehicle animals.

According to Daniel Remick, several clinical studies have proven that the circulating plasma levels of interleukin-6 (IL-6) predict outcome for septic patients [29]. More specifically, higher levels of IL-6 indicate increased mortality significantly in patients[29]. In his research, Remick concluded that even though multiple different studies have been performed on various cytokines, IL-6 is the only cytokine that has given reproducible results to indicate it is the best predictor of mortality [29]. Mice that died during the first 3 days (n=19) of the experiment had significantly high levels of IL-6 as compared to those mice that lived (n=60). However, Remick notes that there was significant heterogeneity in individual animal response.

[TABLE 3]

IL-6 as a Marker of Prognosis in Trauma

	Alive @ 24 hrs	Dead @ 24 hrs	Total
IL-6 above 400 @ 6 hrs [pg/mL]	2	8	10
IL-6 below 400 @ 6 hrs [pg/mL]	14	4	18
Total	16	12	28

Degrees of freedom: 1
Chi-square = 8.76
p is less than or equal to 0.01.
The distribution is significant.

TABLE 3: [Pressure Model]
Analysis indicated that plasma levels of IL-6 > 400 pg/mL may correlate with mortality in rats.

In accordance with Remick's research, rats were employed from the pressure-driven hemorrhage and resuscitation model to determine whether IL-6 measured 6 hours after surgery could predict survival outcome. It should be noted that Remick tested his hypothesis using a sepsis model in mice, while the 60% cytokine production data was determined using rat plasma from a non-infectious hemorrhage model. Twenty-eight rats were divided into two groups [Table 3], those that survived 24 hours post hemorrhage and resuscitation (n=16) [16/28; 57%] and those that died prior to the 24-hour benchmark (n=12) [12/28; 43%]. Based on measurements using the Lincoplex assay, analysis demonstrated that the survival group had fewer rats (20%) with plasma IL-6 levels > 400 pg/mL than the non-survival group (80%). Consequently, 14 out of the 16 rats (88%) who survived the hemorrhage and resuscitation had plasma levels of IL-6 < 400 pg/mL, whereas only 4 out of the 12 rats (33%) that did not survive past 24 hours had similar measurements. Using a chi-square statistical analysis, the distribution is statistically significant with a P value ≤ 0.01.

Experimental results from the pre-treatment experiment also illustrate trends in the cytokine and chemokine responses following hemorrhagic trauma. A total of 15 animals were used in the pre-treatment study. Specifically, 5 rats were randomized in the blinded experiment to receive AET while the other 10 rats were injected with vehicle. Four rats randomized to receive AET survived the hemorrhage and resuscitation, whereas 7 rats receiving the vehicle injection survived to 48 hours. The AET animal died at 24 hours compared to in the VEH group one animal died at 3 hours, one at 6 hours, and one at 24 hours. Because of the poor difference in surviving data, i.e. 70% in vehicle animals

and 80% in AET animals, conclusions are "risky." Rats were sick with rat respiratory virus (RRV) and we could not complete the experiment.

Similar to the volume model, pre-treatment data [Figure 12] also indicated that increased MCP-1 chemokine levels promote survival. From this experiment, we cannot prove an AET effect on survival. Based on measurements using the Lincoplex kit, analysis demonstrated that MCP-1 plasma levels increased over time. The elevation of MCP-1 cannot be attributed to drug alone, however the trend demonstrates that surviving animals (those that lived to 48 hours) had increased circulating plasma levels of MCP-1.

Rats were utilized from the pre-treatment hemorrhage and resuscitation model to determine whether or not AET had an effect on INF-γ and survival. Figure 13 demonstrates that for all time points (baseline, 6 hours, 24 hours, 48 hours) INF-γ plasma levels were remarkably elevated in AET animals. After combining the mean INF-γ concentrations from the four different time periods [Figure 14], analysis indicated that the average circulating plasma level for AET animals (7943.88 pg/mL) was notably different that the average circulating plasma level for VEH animals (97.01 pg/mL). The results were statistically significant with a P value of ≤ 0.01.
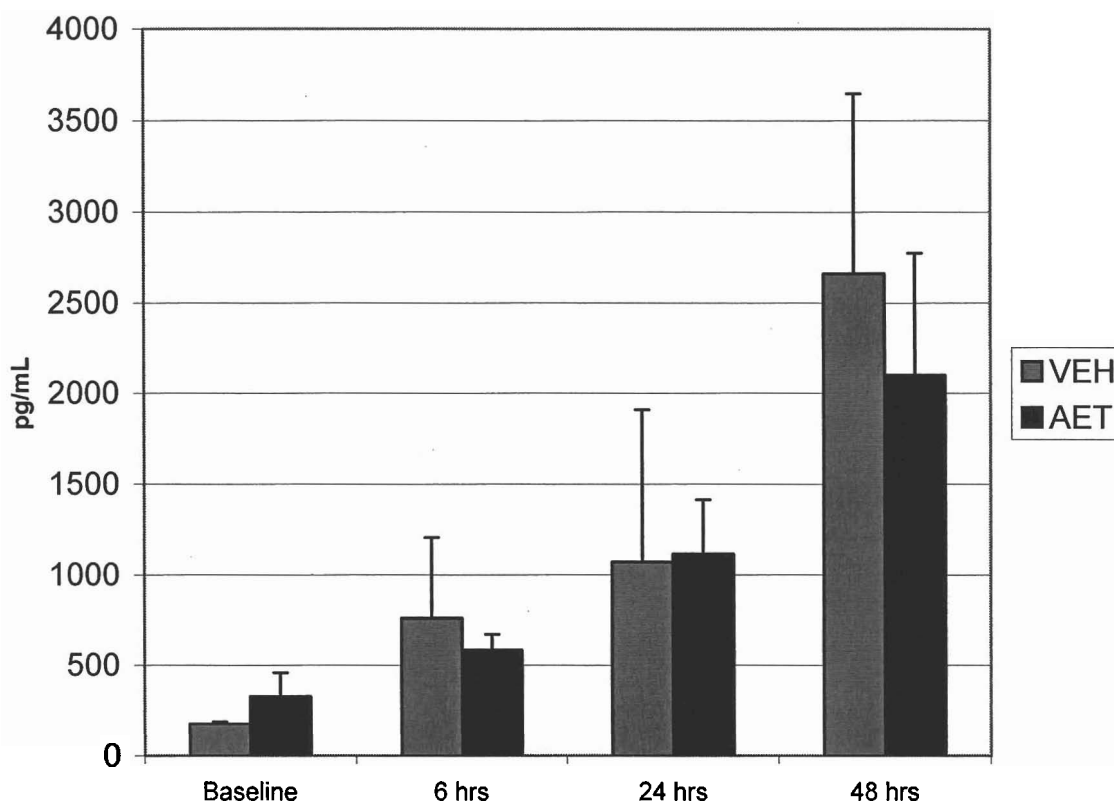
[FIGURE: 12]

MCP-1 Production and Survival

**Effect of Hemorrhagic-Trauma on Monocyte
Chemoattractant Protein-1 (MCP-1)**

FIGURE 12: [Pre-Tx Model]

Analysis indicated that increased MCP-1 levels promote survival, although the elevation of MCP-1 in survivors cannot be attributed to drug alone. VEH sample size: baseline (4), 6 hrs (3), 24 hrs (3), 48 hrs (2). AET sample size: baseline (3), 6 hrs (3), 24 hrs (3), 48 hrs (3).

[FIGURE: 13]

Effect of AET on INF-γ

**Effect of Androstenetriol on the Pro-Inflammatory Cytokine
INF-g (Interferon-gamma)**
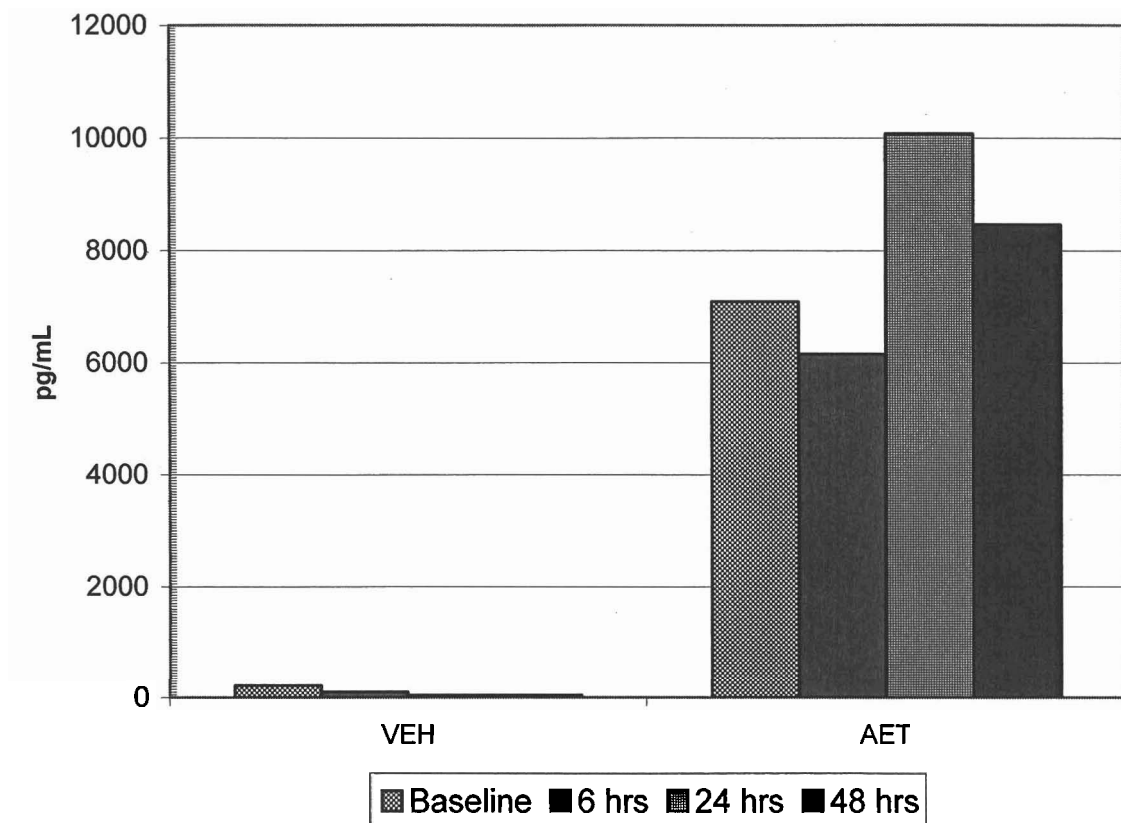
	VEH			AET		
	mean	stdev	n	mean	stdev	n
Baseline	210.72	62.27489	2	7087.0	2420.526	2
6 hrs	94.65	96.2443	2	6154.3	4309.597	2
24 hrs	42.04	15.11794	2	10074.5	9902.415	2
48 hrs	40.65		1	8459.8	6148.258	2

FIGURE 13: [Pre-Tx Model]

Based on measurements using the Lincoplex kit, analysis indicated that AET animals had notably elevated INF-γ plasma levels as compared to VEH animals in the same pre-treatment experiment.

[FIGURE: 14]

Effect of AET on INF-γ
Across All Time-Points

**Effect of Androstenetriol on the Pro-Inflammatory Cytokine
INF-g (Interferon-Gamma)**
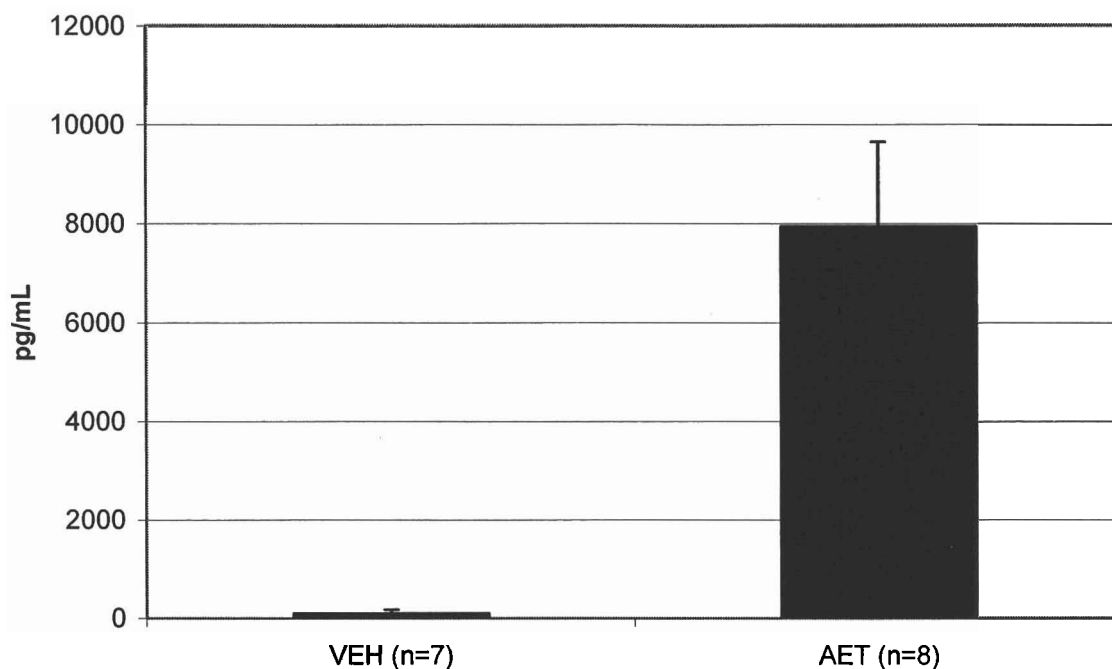

FIGURE 14: [Pre-Tx Model]

Based on measurements using the Lincoplex kit, analysis indicated that the average circulating INF- γ plasma level for AET animals was significantly different than the average circulating INF- γ plasma level for VEH animals across all time-points.

Discussion

I.   Assay Comparison

A uniform comparison [Table 1 & Table 2] was performed using plasma samples from the pressure model to evaluate various assays employed in the biochemical field that determine cytokine concentrations.   After numerous experiments run on both the LINCOplex and Bio-Plex plates, two specific cytokines were selected for the assessment. In particular, interleukin-10 and interleukin-6, had produced reliable data in previous pilot experiments for both plates of interest.   Therefore, these two cytokines were used for the uniform comparison.   In addition, a second comparison was generated using an enzyme-linked immunosorbent assay (ELISA) for MCP-1 [Figure 8] and TNF-α to assess the precision and accuracy of the multiplex technology.

II.   Pilot Study

Prior to the uniform experiment, a pilot study produced results that questioned the validity of modern methodologies. Especially concerning to our research group was the determination that the research and development (R&D) department at Bio-Rad does not differentiate between rat plasma and serum for dilution and measurement purposes. According to the literature, Bio-Plex cytokine assays are multiplex bead assays that quantitate human, mouse, or rat cytokines in diverse matrices, including cell culture supernatants, serum, or plasma. To reduce the matrix effect of the assay, the protocol

requires the researcher to dilute a serum or plasma biological sample using Bio-Rad's Serum Diluent Kit [171-305008].

The matrix effect is defined as a type of interference caused by a constituent of the sample itself. This usually relates to the pH, osmolarity, or composition of the sample. When sample characteristics exceed the limitations tolerated by the assay, a matrix effect will result and sample detection becomes non-linear. Plasma is defined as the clear, yellowish fluid portion of blood in which cells are suspended. Plasma differs from serum in that it contains fibrin and other soluble clotting elements. Plasma is composed of 92% water, 7% protein and 1% minerals. The chief proteins in plasma are: albumin (60%), globulins (alpha-1, alpha-2, beta and gamma globulins) and clotting proteins, especially fibrinogen. These proteins function to maintain the oncotic pressure and transport substances such as lipids, hormones, medications, vitamins, and other nutrients. The proteins are also part of the immune system (immunoglobulins), aid in blood clotting (clotting factors), maintain pH balance, and are enzymes in chemical reactions throughout the body. Serum is defined as the non-cellular portion of blood that remains after the coagulation of whole blood or plasma. Serum is equivalent to plasma without its clotting elements. Our cytokine analyses utilized rat plasma as the biological sample of choice because in vivo plasma is the component of blood that flows throughout the circulatory system. Serum is not physiologically relevant to clinical treatment models because serum is the fluid portion of the blood after it has clotted and the cells have been removed.

Because pilot data using Bio-Rad's Bio-Plex technology did not produce precise and accurate results, we asked technical support to provide us with validation statistics for the rat cytokine assay. Through personal communication with Bio-Rad, it was determined that the person who performed the rat validation has since left the company and according to technical support, they cannot produce validation statistics for rat plasma. Bio-Rad's explanation for this oversight is necessity; marketing data shows minimal use of rat plasma and even less utilization of rat cytokine kits altogether. According to a sales representative, our research team was only the second group to experiment with the rat multiplex kit. The other group evaluated a plate during their demo in which they tested a non-validated kidney perfusate (interstitial fluid) and produced mixed results. After confirmation that Bio-Rad's assay was not validated for plasma, it was suggested by technical support to precipitate out the larger proteins in the plasma sample for the purpose of reducing the matrix effect; therefore, making the substance more resemble serum. A Linco representative, however, concluded that precipitating out larger proteins could remove some of the larger cytokines as well, including those that are bound to carrier proteins (e.g. soluble receptors, etc.).

Another issue that surfaced during our pilot study involved the sensitivity of the rat antibodies as compared to antibodies for human and mouse assays. Bio-Rad concludes that both the rat multi-plex cytokine assays and the traditional rat ELISA assays are notoriously less sensitive than the mouse or human multi-plex assays and ELISAs. The reasoning for this decreased sensitivity results in fact that antibodies used for the rat assays are not of high quality because of the rarity associated with the rat

model; therefore, the rat antibodies are less sensitive in their kinetic profile. Bio-Rad concludes that the antibodies available for rats require more research, which ultimately affects the validity of our results.

III. Evaluating Assays

Plasma samples from the same animals were run on two different multi-plex assays, Bio-Rad's Bio-Plex and Linco's Lincoplex assay. The variation between the two plates caused concern for our research group. For example in Table 1 VEH rat 54-147135-B had a concentration level of 262.63 pg/mL for IL-6 on the Bio-Rad plate as compared to 1395.26 pg/mL measured using the Linco assay. This 5-fold difference in concentration questions the sensitivity associated with both assays. For IL-10 the uniform comparison demonstrated bigger discrepancies in cytokine concentration measurements. Table 2 illustrates that for the AET plasma sample, 47-145726-B, the concentration level of IL-10 using the Bio-Plex assay was 248.68 pg/mL, whereas the Linco plate measured the concentration level of IL-10 as 6,096.81 pg/mL. When comparing measurements from a multi-plex assay to an ELISA, variation also existed. In Figure 7, the mean concentration of MCP-1 for surviving AET animals at 72 hours was 1,336.00 pg/mL in the Linco-plex cytokine assay, whereas the ELISA plate from Assay Designs [Figure 8] produced a mean concentration of 131,131.13 pg/mL for the same group of animals. This 98-fold different for measured MCP-1 is concerning. Through our research we determined that when comparing any antibody driven test to another (ELISA vs. ELISA, ELISA vs. multi-plex, or multi-plex vs. multi-plex), the absolute values calculated by the standard curves from two different assays are not comparable.

The reasoning behind this conclusion comes from experimental data and the determination that each assay uses different recombinant standards and different antibodies.

The standards from one company have different purities and recombinant structures than standards from another company; consequently, the various standards have different binding kinetics to the same antibody. This phenomenon implies that the recombinants between companies are of different genetic construction and produce slightly different products. As a result, the inconsistency between standards influences different curve shapes and different signal intensities because various amounts of the standards bind to the antibodies.

The World Health Organization (WHO) and The National Institute of Standards and Controls set an international calibration method; the WHO assigns an international unit based on a bioassay of the standard, for instance 1000 IU/pg. Most cytokine standards are compared to the WHO bioassay units; however, according to technical support at Bio-Rad not all standards are tested. Additionally, the WHO numbers for different manufacturer standards can differ up to a 1000 fold, while 10 fold is more typical. For example, one assay can calculate 10 pg/ml for one sample and another company can measure 1 pg/ml for the same sample because the standards between manufacturers have different biological or kinetic activity with the antibody. Nevertheless, this does not make one assay more sensitive than the other; the numbers on the curve are relative and importance lies in the trends between samples.

Recommendations for future experiments include purchasing an internal cytokine standard from an independent company and running that standard on the various assays utilized to measure cytokines. Therefore, a known amount of a particular cytokine is analyzed on multiple assays and depending on the observed measurement, the accuracy, precision, and specificity of an assay can be determined. Furthermore, observed measurements for the biological samples can be modified depending on the ratio between the known amount of standard added and the observed measurement for that standard.

Our research suggested that the various modern methodologies utilize different antibodies, which also cause significant differences. Contrary to the standards, the differences between manufacturer antibodies can affect sensitivity. When an antibody cannot bind a significant amount of target at low concentrations due to its inferior binding kinetics, as compared to an antibody with stronger affinity, a loss in detectability at the low-end results. Accordingly, our research determined that, measurements from ELISA plates do excel in some cases when compared to a multi-plex assay; this usually, however, is only limited to one particular target. We concluded that the key for determining sensitivity between assays is to compare the lowest detectable sample on both assays and verify where it falls on the standard curve. If a sample can be detected on one curve and not another, then that particular assay has a sensitivity advantage assuming the results are reproducible.

Literature states that each antibody pair is made against different epitopes on recombinant antigens. Therefore, even if all the standards match between comparable assays, the results may still vary because of several factors. Inconsistent data results from

inherent components among different assays including, various antibody pairs may not bind to an antigen in its native form with the same affinity, antibody pairs may be binding to a labile site that is easily truncated by serum proteases, and the affinity may be impaired by soluble receptors. Our research also determined that within a single rat mult-plex assay, antibodies are not equally sensitive. We discovered through experimentation that although Bio-Rad's technical support generally guarantees detection for all antibodies to 10-12 pg/mL, most antibodies in the rat Bio-Plex plate are not quality controlled (QC'd) below 50 pg/mL. Because most analyzed cytokine levels fall towards the lower end of the standard curve, we concluded that in the future researchers must first verify QC'd statistics before purchasing a kit.

IV.   MCP-1 Results

Chemokines include low-molecular-weight polypeptides (8 to 16 kDa) that mediate the migration of leukocyte populations toward immune/inflammatory stimuli [30]. Monocyte Chemoattractant Protein-1 (MCP-1) is associated with chronic vascular disorders, such as arteriosclerosis, and congestive heart failure, as well as inflammatory states [31]. According to recent literature, the most potent chemotactic activity of this chemokine involves mononuclear phagocyte activation [31]. Initial studies in a murine model of septic peritonitis demonstrate that the cecal ligation and puncture procedure induces a dramatic increase in MCP-1 production in the peritoneum, followed by an increase in the recruitment of leukocytes [32]. Furthermore, MCP-1 blockade with anti-MCP-1 antiserum significantly decreases the survival rate for rats following surgery and the lack of MCP-1 results in the enhanced recovery of viable bacteria from the

peritoneum [32]. Results from the volume-driven hemorrhage model and pre-treatment hemorrhage model demonstrate that MCP-1 levels increase in surviving animals. MCP-1 is known to counteract hypoxia-mediated apoptosis and attract monocytes. The elevation of MCP-1 in both AET and VEH survival animals [Figure 7 & Figure 12] suggests that the effects of this cytokine may benefit individuals recovering from hemorrhagic trauma and shock. Although the effect of drug cannot be determined at this time, our results indicate that MCP-1 may initiate an inflammatory response to increase survival. As discussed earlier, instead of relying on actual MCP-1 values, researchers are encouraged to relate assays as a relative increase or decrease. The measurements in the volume model using the MCP-1 TiterZyme® Enzyme Immunometric Assay [Figure 8] and Lincoplex Assay follow the same trend, along with the results from the pre-treatment rat hemorrhage model [Figure 12]. All three studies provide data to indicate that the chemokine MCP-1 may play a significant role in the inflammatory survival response after trauma and hemorrhage.

V.   GRO/KC Results

The rat chemokine GRO/KC (also known as Rat KC or CINC) promotes neutrophil chemotaxis and degranulation. Rats lack the IL-8 homologue; current research concludes that rat CINC has a closer sequence homology to GRO than to IL-8, therefore CINC is the rat equivalent of human GRO but not of IL-8 (22). All three isoforms of GRO are CXC chemokines that can signal through the CXCR2 receptor. Recombinant rat GRO/KC is a 7.8 kDa protein consisting of 70 amino acids including the ELR motif common to the CXC chemokine family that bind to CXCR1 or CXCR2. Current

research provides strong evidence that neutrophils have a role in mediating pathology during reperfusion of ischemic tissues [21]. More specifically, CXC chemokines, including interleukin-8 and KC/GROα, guide neutrophils to tissue sites of inflammation [21]. Our results should be regarded as preliminary and future experiments to verify our multi-plex data should include analyses from the growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) ELISA assay [33]. [Assay Designs TiterZyme® Kit (ELISA) cat. #: 900-074]. For the volume-driven hemorrhage model, measurements using the Lincoplex kit [Figure 9] demonstrated that there is a significant increase of GRO/KC in animals surviving hemorrhagic trauma and shock as compared to baseline GRO/KC levels. These measurements indicate that increased levels of GRO/KC may promote survival following trauma and hemorrhage; however, the production of GRO/KC cannot be attributed to drug treatment.

VI.   IL-1α Results

IL-1 is defined as a pleiotropic cytokine thought to participate in the initiation and regulation of the acute-phase response to injury and infection [34]. Infection, sepsis, often results in a significant inflammatory response; a cascade of pro-inflammatory cytokines including IL-1 initiates this response [35]. Increasing evidence suggests that IL-1 has a role in the pathogenesis of Gram-negative septic shock [34]. Interleukin-1 acts on nearby T lymphocytes to stimulate both IL-2 and IL-2 receptor production [8]. Current research demonstrates that a stab injury to the adult mouse brain elicited a prompt and marked increase in levels of transcripts for interleukin-1alpha (IL-1α), considered one of the macrophage cytokines [36]. Specifically, microglia secrete a

number of cytokines, including IL-1α in response to trauma [37]. In other studies, IL-1α induces intestinal inflammation [37]. Literature utilizing rat serum also suggests that stress as determined by serum glucocorticoid levels influences cytokine expression, including IL-1α [38].

Results from the volume-driven hemorrhage model, illustrate that trauma increases IL-1α levels in rat plasma [Figure 10], whereas hemorrhage over time decreases IL-1α cytokine levels especially in vehicle animals. Because normal circulating levels of IL-1 are undetectable in both conventional ELISA and multi-plex assays [39], results from the volume model may indicate that IL-1α initiation occurs during an inflammatory response following both trauma and hemorrhage. More studies are necessary to confirm the role of IL-1α in survival mechanisms. In future experiments, it seems reasonable to utilize SHAM or control rats for the purpose of determining normal circulating IL-1α levels. As already discussed, each assay [multi-plex vs. muli-plex vs. ELISA] analyzes biological plasma samples with different performance characteristics, i.e. accuracy, precision, and specificity. Instead of relying on the literature, future experiments should include SHAM or control rats to determine normal physiological levels in a particular assay.

VII.   IL-6 Results

IL-6 remains as a cytokine with multiple biological functions on a wide variety of cells. Although a variety of cells types produce interleukin-6, monocytes, alveolar macrophages, and Kupffer cells remain the most relevant cell populations to traumatic hemorrhage [25]. Current research proposes that interleukin-6 plays a significant role in

trauma, however it remains undecided whether IL-6 acts as the culprit for producing multiple of organ failure or whether IL-6 is just a valuable biochemical marker for a patient's inflammatory response [25]. According to Dalrymple, interleukin-6 is found in bodily fluids as a result of severe infection, inflammation, burns, and general trauma [40]. Several research groups conclude that plasma levels of IL-6 correlate with severity of organ failure, length of ICU stay, and patient mortality [41]. Carcillo also notes that IL-6 is a marker of tissue injury [41]. In accordance with current literature, our analysis indicated [Table 3] that IL-6 plasma levels measured 6 hours after injury may correlate with mortality in rats. Clinical researchers suggest a trend and correlation exists between IL-6 and mortality; measurements from the pressure-driven hemorrhage model demonstrate this analogous relationship.

Researchers have also demonstrated that IL-6 mRNA and protein were produced in the lungs, liver, and intestinal tracts of rats subjected to resuscitated hemorrhagic shock [16]. Because of the complexities associated with the cytokine cascade, evolving concepts of the septic response give more weight to the importance of local cytokine production, rather than systemic production [16]. In future experiments, it seems reasonable to analyze cytokine production in specific organs as part of the total septic- or hemorrhagic-response picture. Localized cytokine levels may provide better insight into the inflammatory/immune response following hemorrhagic trauma. At this time, since we are not convinced that modern rat methodologies have dependable performance characteristics, cytokine levels derived from localized tissues may provide reliable and reproducible results. Without the matrix effect and low circulating systemic levels

associated with rat plasma, it seems logical to suspect that cytokine measurements from localized tissues will lie closer to the liner portion of the standard curve, thereby resulting in more consistent measurements. In future experiments it remains suggested that researchers concentrate more on localized responses rather than systemic.

VIII. INF-γ Results

In the pre-treatment rat hemorrhage model, analysis indicated that the mean INF-γ circulating plasma level for AET animals was notably higher than the mean INF-γ plasma level for vehicle animals [Figure 19]. For normal serum, INFγ levels are below detection limits [20]. AET up regulates host immune response, prevents immune suppression and modulates inflammation; INF-γ data in the pre-treatment experiment indicate that AET may also act by mechanisms that modify specific TH1 cytokines to promote survival.

IX. TNF-α

Current literature notes that a correlation between plasma TNF-α concentrations and tissue injury, multiple organ failure, or outcome in trauma patients has not been established [9]. In our results, based on measurements using the Bio-Plex kit, values for TNF-α range from 50 to 250 pg/mL [Figure 11]. However, these measurements should be regarded as preliminary because of the small sample size for both AET and VEH animals. For comparison purposes, TNF-α levels in plasma samples from the pressure model were undetectable using both the Bio-Plex assay and the conventional ELISA assay. Because there are concerns regarding TNF-α assays and sensitivity, it seems reasonable in the future to utilize TNF-R1 and TNF-R2 to establish tends. Current

literature states that these two soluble TNF-α receptors demonstrate dependable and consistent results. Researchers have reported that TNF-R1 (55kD) and TNF-R2 (75kD), both part of the physiologic regulation system for TNF-α, were increased after severe trauma or sepsis [9]. Furthermore, elevated TNF-R1 and TNF-R2 correlated with multiple organ failure complications including hypoxia and infection [9].

X.   Conclusion

Trauma and hemorrhagic shock, resulting in multiple organ failure, has transpired into a significant clinical problem in the United States. Despite advances in pre-hospital care and improved diagnostic modalities, trauma and hemorrhage remain a leading cause of morbidity and mortality in the younger population. Additionally, both hemorrhage and acute traumatic shock are associated with the primary cause of combat death for soldiers. Cytokines are powerful mediators and communication molecules capable of regulating various biological functions, including modulation of immune responses and inflammation. In general, cytokines are very labile; therefore, unless a condition exists to stimulate selective expression, cytokines are normally degraded by serum proteases or removed by soluble receptors and hence undetectable. Because normal absolute amounts of cytokines are very low, current research suggests that hemorrhagic trauma induces the excessive production of cytokines to overspill into the systemic circulation, resulting in SIRS and multiple-organ failure [8]. Researchers note that attempts have been made to use cytokine levels to predict outcome in patients presenting with SIRS and multiple-organ failure. However, many studies have identified a wide range of cytokine responses in patients with similar injuries. Because polymorphic sites in cytokine genes have

demonstrated to effect mRNA expression for some cytokines, the differences in cytokine production from one individual to another could be the results of genetic causes [8]. Furthermore, current research has established an association between gender and mortality among trauma patients [42]. Results conclude that sex hormones may be important in traumatic injury outcomes [42]. Our results specifically indicate that both MCP-1 and GRO/KC increase in surviving animals; trauma increases IL-1α levels in rat plasma, whereas hemorrhage decreases IL-1α over time; IL-6 plasma levels measured 6 hours after hemorrhage may correlate with mortality; AET may act by mechanisms to modify specific TH1 cytokines (INF-γ) to promote survival.

Although it is hypothesized that cytokines play a significant role in the pathogenesis of hemorrhagic shock and multiple organ failure, our experimental results demonstrated that numerous pitfalls accompany the measurement of cytokine levels in rat plasma. Consequently, at this time, only preliminary cytokine trends can be determined due to the difficulty associated with interpreting results obtained from both the multi-plex and ELISA assays. Tools to measure the cytokine cascade following hemorrhagic trauma are imperative for discovery into the immune/inflammatory response, however our results suggest that modern rat methodologies lack the sensitivity, specificity, and precision to produce physiological relevant data. The predictive role of inflammatory mediators, including cytokines, with respect to the manifestation of multiple organ failure after severe trauma seems limited. Additional studies are needed to further examine the role of cytokines in trauma and multiple organ failure.

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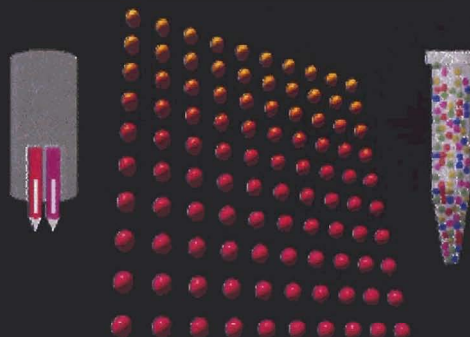
Appendix I:

LINCOplex Technology Schematic
LINCO Research

Color-coded Microspheres
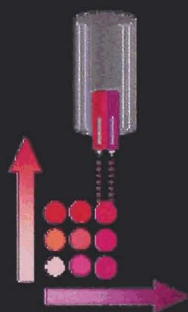
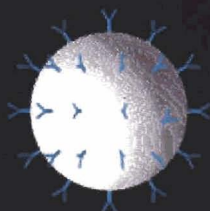Unique microsphere sets are color-coded using a blend of different fluorescent intensities of two dyes.

100 Color-codes = 100 Simultaneous Tests

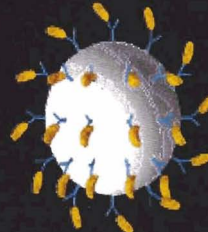Using this method, over 100 distinct microsphere sets can be created.
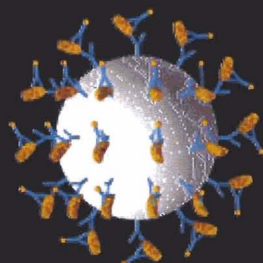
Microspheres as Molecular Carriers

To perform a test, thousands of probes are bound to the microsphere.
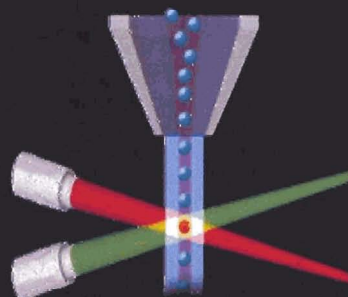
Capturing the Sample Molecule

While suspended in a test sample, the bound probes collect molecules.
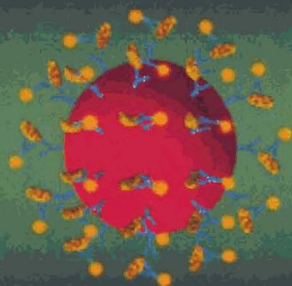
Tagging the Reaction

Fluorescently-labeled Reporter tags bind to the sample molecule.
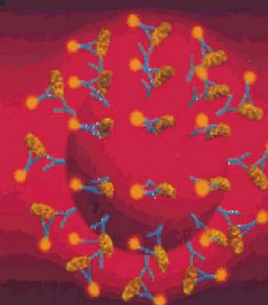

Microspheres in a Fluid Stream

Precision fluidics align the microspheres in single file, and pass them through the lasers one at a time.

One Laser Excites Molecular Tags

Reactions are measured with fluorescent intensity and reported in real time.

Second Laser Excites Microsphere

Fluorescent intensity of the microsphere identifies the reaction.

Appendix II:

Bio-Plex Multiplex Cytokine Assay Protocol

Purpose: To measure the level of cytokines in a sample
☐ IL-1α, ☐ IL-1ß, ☐ IL-2, ☐ IL-4, ☐ IL-6, ☐ IL-10, ☐ GM-CSF, ☐ INF-γ, ☐ TNF-α

Materials:
    ☐ Bio-Plex Rat Serum Diluent Kit [Bio-Rad 171-305008]
    ☐ Bio-Plex Rat Cytokine 9-Plex A Panel [Bio-Rad 171-K11070]
    ☐ Bio-Plex Reagent Kit [Bio-Rad 171-304000]
    ☐ Bulk Scienceware Disposable Pipetting Reservoirs [Fisher 13-712-14]
    ☐ Disposable 96 well Microtiter Plate [Fisher 34-16-02-0-2]
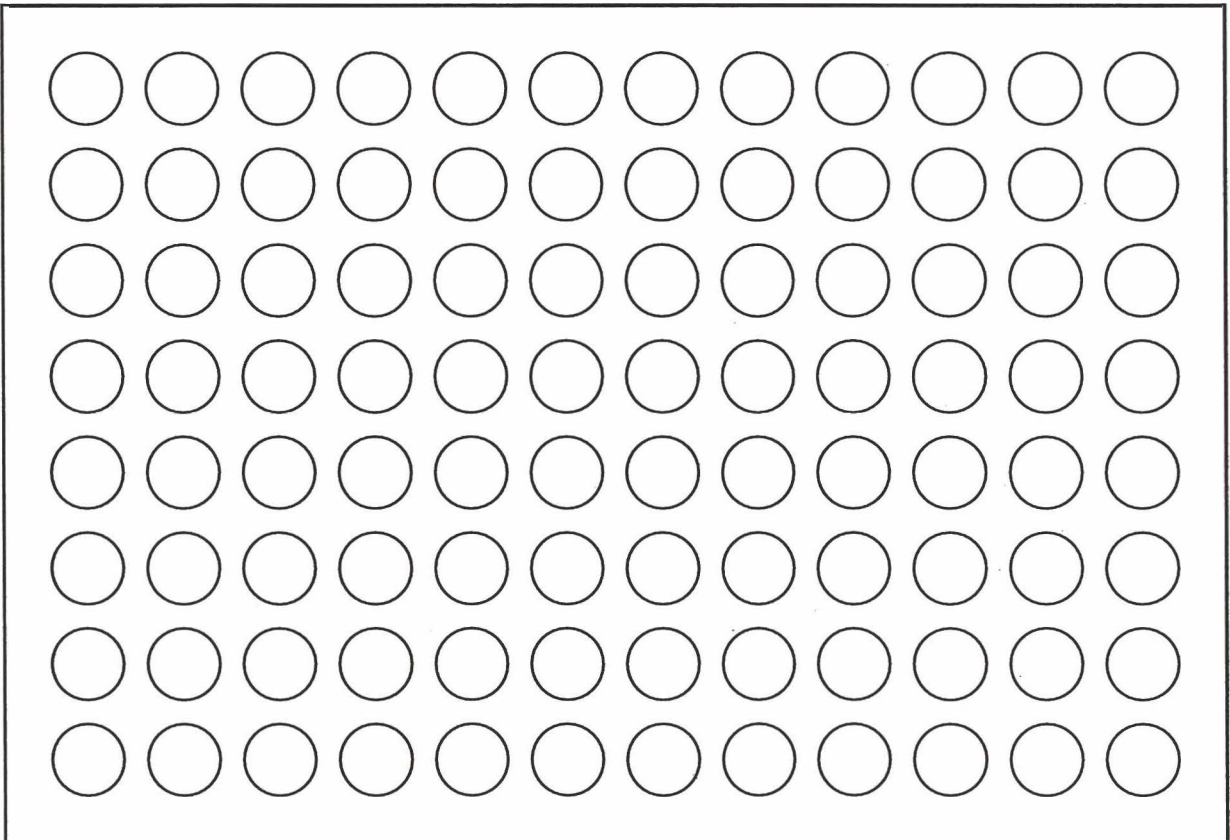
NOTE: DO NOT FORGET TO ADD BLANKS INTO YOUR PLATE SET UP ABOVE. THEY ARE TREATED LIKE SAMPLES ONLY WITH SAMPLE DILUENT AND NO SAMPLE.

Calculations:

# of required wells __

# of extra wells   __ (2 wells for every 8 required wells)
            __ total number of wells for dilution calculations
☐ *Bead Dilution*

50 μl/well
2 μl of anti-cytokine bead (25x) stock solution/well

__ x 2 μl = __ of anti-cytokine bead (25x) stock solution (assay module)
total wells
__ x 48 μl = __ of **Bio-Plex Assay Buffer A** (cytokine reagent kit)
total wells
__ x 50 μl = __ total volume
total wells

☐ *Detection Antibody Dilution*

Detection antibodies for premixed panels containing **2 to 9 target analytes** are supplied in a **50x** stock solution. Detection antibodies for premixed panels containing **more than 9 target analytes** are supplied in a **25x** stock solution.

    ☐ *2 to 9 targets*
    25 μl/well
    0.5 μl of detection antibody (50x) stock solution/well

____ x 0.5 μl = __of detection Ab (50x) stock solution (assay module)
total wells
____ x 24.5 μl = __ of **Bio-Plex Detection Ab Diluent A** (cytokine reagent kit)
total wells
____ x 25 μl = __ total volume
total wells

OR

□ > *9 targets*
25 μl/well
1 μl of detection antibody (**25x**) stock solution/well

__ x 1 μl = __ of detection Ab (25x) stock solution (assay module)
total wells
__ x 24 μl = __ of **Bio-Plex Detection Ab Diluent A** (cytokine reagent kit)
total wells
__ x 25 μl = __ total volume
total wells

□ *Streptavidin-PE Dilution*

50 μl/well
0.5 μl of streptavidin-PE (100x) stock solution/well

___ x 0.5 μl = __ of streptavidin-PE (100x) stock solution (reagent kit)
total wells
____ x 49.5 μl = __ of **Bio-Plex Assay Buffer A** (cytokine reagent kit)
total wells
____ x 50 μl = __ total volume
total wells

<u>REMINDERS:</u>

- BRING ALL BUFFERS AND DILUENTS TO ROOM TEMP PRIOR TO USE.
- TURN ON THE BIO-PLEX SYSTEM NO EARLIER THAN 2 HR PRIOR TO PLATE READING, PROBABLY AFTER STEP 1O OR 11.
- BRING CALIBRATION BEADS TO ROOM TEMPERATURE 30 MINUTES OR MORE BEFORE THE ASSAY.
- RECONSTITUTE STANDARDS IMMEDIATELY AND ALLOW 30 MINUTE SOLUBILIZATION
- DO NOT FORGET TO ALLOW FOR 2 REPLICATE BLANKS

<u>PREPARE STANDARDS:</u>

(Use a standard curve for tissue culture supernatants ranging from 1.95 pg/ml to 32,000 pg/ml. Use a standard curve for serum, or plasma samples ranging from 0.2 pg/ml to 3,200 pg/ml.)

1.95-32,000 pg/ml Standard Curve

1) Reconstitute lyophilized multiplex cytokine standard to a stock concentration of 500,000 pg/ml.
   (a) Add **50 μl** of sterile distilled water to lyophilized standard.
   (b) Vortex gently for 5 sec.
   (c) Incubate on ice for 30 min.

2) Prepare a serial dilution of the 500,000 pg/ml standard stock solution.

0.2-3,200 pg/ml Standard Curve

1) Reconstitute standard in **500 ul** sterile water (multiplex standard stock is 50,000 pg/ml)
2) Proceed with dilution series.

Remember:
The calculated concentrations of samples will only be accurate, if the standards are diluted in the same media as samples.
   a) Tissue culture supernatants-use the same tissue culture media as that utilized to grow cells.
   b) Serum, or plasma samples-use the STANDARD DILUENT from either the serum, or plasma diluent kit, as appropriate. Warm the diluent bottle at 37°C if there is any precipitate.

12.8 μl

Multiplex standard stock (500,000pg/ml)								
Stock (μl)	12.8	50	50	50	50	50	50	50
Medium (μl)	187.2	150	150	150	150	150	150	150
pg/ml	32000	8000	2000	500	125	31.25	7.8	1.95

PREPARE SAMPLES:

1) <u>Tissue Culture Supernatants</u>-Cytokine levels in 'neat' samples are typically above the range of the standard curve. Samples may therefore need to be diluted before assaying. 50 ul sample is added to each well.
2) <u>Serum/Plasma</u>-Dilute serum/plasma samples 1:4 in serum/plasma SAMPLE DILUENT as required. The sample diluent can be warmed at 37°C to dissolve precipitated material in the bottom of the bottle. 50 ul DILUTED sample is added to each well.
3) For tissue culture supernatants, a volume of 60 ul is appropriate for microtiter plate, since 50 ul will be transferred to the filter plate.
4) In the case of serum, the serum samples should be diluted 1: 4 in serum sample diluent (see serum diluent kit). Place 15 ul serum and 45 ul serum sample diluent in each well (to give 60 ul) of microtiter plate; 50 ul will then be transferred to the filter plate.

PREPARE BEADS:

1) Determine the total number of wells that will be required.
2) Vortex the anti-cytokine bead (25x) stock solution at medium speed for 20 sec.
3) Prepare a 25 fold working dilution of the anti-cytokine bead (25x) stock solution in **Bio-Plex assay buffer A**.
   (a) DOCUMENT ALL VOLUME CALCULATIONS.
   (b) PROTECT THE BEADS FROM LIGHT.
   (c) KEEP ALL BEAD SOLUTIONS ON ICE WHEN NOT IN USE.

PREPARE ASSAY:

Cover all unneeded wells with plastic adhesive plate sealer.

1) Pre-wet the filter plate with **100 μl** of **Bio-Plex assay buffer A.**
2) Vacuum filter.
3) Add Beads.
   i) Vortex the working Bead solution at medium speed for 20 sec and add **50 μl** to each well.
4) Vacuum filter.
   i) BLOT THE BOTTOM OF THE FILTER PLATE AFTER THIS AND EVERY VACUUM FILTRATION
5) Filter wash **2X** with **100 μl** of **Bio-Plex wash buffer A.**
6) Add standards.
   i) Vortex each standard for 5 SEC and immediately add **50 μl** to the appropriate wells.
   ii) Remember to also include the appropriate blanks.
7) Add samples.

      i)   Vortex each sample for 5 SEC and immediately add **50** μl to the appropriate wells.

8) Cover the wells with a fresh plastic adhesive plate sealer, and blot the bottom of the plate.
9) Cover with foil and incubate for **30 min at room temperature** with shaking.
      i)   FIRST SHAKE THE PLATE AT 1,100 RPM FOR 30 SEC.
      ii) REDUCE SPEED TO 300 RPM FOR THE REMAINDER OF THE INCUBATION.

10) **TURN ON THE INSTRUMENT READER AND PLATE PLATFORM AND RUN THROUGH THE START UP PROCEEDURE**

PREPARE DETECTION ANTIBODY:
(DURING PRECEEDING INCUBATION AND 10 MIN PRIOR TO USE)

11) Determine the total number of well that will be required.
12) GENTLY VORTEX the multiplex detection antibody stock solution.

    * Note: The concentration of the detection antibody stock solution will vary as a function of the degree of multiplexing for the premixed panel.
    For **2-9** premixed analytes, the detection antibodies are provided in a **50x** stock solution.
    For **10 or more** premixed analytes, the detection antibodies are provided in a **25x** stock solution.

13) Prepare the appropriate working dilution of the detection antibody stock solution in **Bio-Plex detection antibody diluent A.**
      (a) DOCUMENT ALL VOLUME CALCULATIONS.
      (b) PROTECT THE ANTIBODIES FROM LIGHT.
      (c) KEEP ALL DETECTION ANTIBODY SOLUTIONS ON ICE WHEN NOT IN USE.

14) Remove the plate sealer and filter wash **3X** with **100** μl of **Bio-Plex wash buffer A.**
15) Vortex the detection antibody working dilution gently and add **25** μl to each well.
16) Cover the wells with fresh plastic adhesive plate sealer, and blot the bottom of the plate.
17) Cover with foil and incubate for **30 min at room temperature** with shaking.
      i)   FIRST SHAKE THE PLATE AT 1,100 RPM FOR 30 SEC.
      ii) REDUCE SPEED TO 300 RPM FOR THE REMAINDER OF THE INCUBATION.

PREPARE STREPTAVIDIN-PE:
(DURING PRECEEDING INCUBATION AND 10 MIN PRIOR TO USE)

18) Determine the total number of well that will be required.
19) VIGOROUSLY VORTEX the streptavidin-PE (100x) stock solution.

20) Prepare a 100 fold working dilution of the streptavidin-PE (100x) stock solution in **Bio-Plex assay buffer A.**
      (a) DOCUMENT ALL VOLUME CALCULATIONS.
      (b) PROTECT THE ANTIBODIES FROM LIGHT.
      (c) KEEP ALL DETECTION ANTIBODY SOLUTIONS ON ICE
          WHEN NOT IN USE.

21) Remove the plate sealer and filter wash **3X** with **100 µl** of **Bio-Plex wash buffer A.**
22) Vortex the Streptavidin-PE Working Dilution and add **50 µl** to each well.
23) Cover the wells with fresh plastic adhesive plate sealer, and blot the bottom of the plate.
24) Cover with foil and incubate for **10 min at room temperature** with shaking.
      i)   FIRST SHAKE THE PLATE AT 1,100 RPM FOR 30 SEC.
      ii) REDUCE SPEED TO 300 RPM FOR THE REMAINDER OF THE
          INCUBATION.
25) Remove the plate sealer and filter wash **3X** with **100 µl** of **Bio-Plex wash buffer A.**
26) Resuspend the beads in each well with **125 µl** of **Bio-Plex assay buffer A.**
27) Cover the wells with fresh plastic adhesive plate sealer, and THOROUGHLY BLOT THE BOTTOM OF THE PLATE.
28) SHAKE THE PLATE AT 1,100 RPM FOR 30 SEC.
29) REMOVE THE PLATE SEALER and read the plate.

COUNT 100 BEADS/REGION IN A SAMPLE VOLUME OF 50 UL.

Appendix III:

Lincoplex Protocol

Rat Cytokine/Chemokine Lincoplex Kit Protocol

I. Preparation of Reagents for Immunoassay

 A. Preparation of Antibody-Immobilized Beads
  o Antibody-Immobilized Beads are premixed
  o Sonicate beads for 30 seconds
  o Vortex beads before use

 B. Preparation of rat Cytokine Standard Cocktail
  o Reconstitute the Rat Cytokine Standard Cocktail with 250 uL Deionized Water to give 20,000 pg/ml concentration of standard.
  o Invert vial several times to mix.
  o Vortex the vial for 10 seconds.
  o Allow vial to set for 5-10 minutes and transfer standard to an appropriately labeled polypropylene microfuge tube.

 C. Preparation of Working Standards
  o Label six polypropylene microfuge tubes 5000, 1250, 312.5, 78.13, 19.53, and 4.88 pg/mL.
  o Add 120 uL of Assay Buffer to each of the six tubes.
  o Prepare serial dilutions by adding 40 uL of the 20,000 pg/ml reconstituted standard to the 5000 pg/ml tube.
  o Mix well and transfer 40 uL of the 5000 standard to the 1250 pg/mL tube.
  o Mix well and transfer 40 uL of the 1250 standard to the 312.5 pg/mL tube.
  o Mix well and transfer 40 uL of the 312.5 standard to the 78.13 pg/mL tube.
  o Mix well and transfer 40 uL of the 78.125 standard to the 19.53 pg/mL tube.
  o Mix well and transfer 40 uL of the 19.53 standard to the 4.88 pg/mL tube and mix well.
  o The 0 pg/mL standard (Background) will be Assay Buffer.

Standard Concentration (pg/mL)	Volume of dH2O to Add	Volume of Standard to Add
20,000	250 uL	0

Standard Concentration (pg/mL)	Volume of assay Buffer to Add	Volume of Standard to Add
5000	120 uL	40 uL of 20,000 pg/mL
1250	120 uL	40 uL of 5000 pg/mL
312.5	120 uL	40 uL of 1250 pg/mL
78.13	120 uL	40 uL of 312.5 pg/mL
19.53	120 uL	40 uL of 78.13 pg/mL
4.88	120 uL	40 uL of 19.53 pg/mL

D. Preparation of Controls
  o Reconstitute each Rat Cytokine Control I and Rat Cytokine Control II with 250 uL deionized water.
  o Invert the vial several times to mix and vortex.
  o Allow the vial to set for 5-10 minutes and then transfer to appropriately labeled polypropylene microfuge tubes.

E. Preparation of Plasma Samples
  o Centrifuge samples at 3000xg for five minutes prior to assay set-up.
  o Dilute 1 part of rat plasma with 4 parts of Serum Matrix.
    i. For duplicate samples
    ii. Add 24 uL rat plasma to 96 uL of Serum Matrix
    iii. For triplicate samples
    iv. Add 48 uL rat plasma to 192 uL of Serum Matrix

F. Preparation of Wash Buffer
  o Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution.
  o Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water.

G. Preparation of Serum Matrix
  o Add 1.0 mL of deionized water to the bottle containing the lyophilized Serum Matrix.
  o Add 4.0 mL of Assay Buffer to the bottle containing the lyophilized Serum Matrix.
  o Mix and let stand for at least 10 minutes at room temperature to allow complete reconstitution.
  o Mix well before use.

II. Immunoassay Procedure

** Allow all reagents to warm to room temperature before use in the assay.

1) Diagram the placement of Standards, 0 (Background), 4.88, 18.53, 78.13, 312.5, 1250, 5000, and 20,000 pg/mL, Controls I and II, and samples on Well Map Worksheet in a vertical configuration. It is recommended to run the assay in duplicate.
2) Block the filter plate by pipetting 200 uL of Assay Buffer into each well of the microtiter plate. Seal and mix on a plate shaker for 10 minutes at room temperature.
3) Remove the Assay Buffer by vacuum.
4) Remove any excess Assay Buffer from the bottom of the plate by blotting on paper towels.
5) Add 25 uL of Assay Buffer to the 0 Standard (Background).
6) Add 25 uL of Assay Buffer to the Sample wells.
7) Add 25 uL of each Standard or Control into the appropriate wells.
8) Add 50 uL of serum matrix to the Background, Standards, and Control wells.
9) Centrifuge samples and add 50 uL of 1:5 diluted samples into the appropriate wells.
10) Vortex Bead Bottle and add 25 uL of Mixed beads to each well. During addition of mixed beads, shake bead mix intermittently to avoid bead setting.
11) Seal, cover with aluminum foil, and incubate with agitation on a plate shaker overnight (18-20 hours) at 2-8 °C.

Well Identification	Assay Buffer	Standard/Control/Sample	Serum Matrix	Mixed Beads
0 Standard	25 uL	-	50 uL	25 uL
4.88 Standard	-	25 uL	50 uL	25 uL
19.53 Standard	-	25 uL	50 uL	25 uL
78.13 Standard	-	25 uL	50 uL	25 uL
312.5 Standard	-	25 uL	50 uL	25 uL
1250 Standard	-	25 uL	50 uL	25 uL
5000 Standard	-	25 uL	50 uL	25 uL
20,000 Standard	-	25 uL	50 uL	25 uL
Control I	-	25 uL	50 uL	25 uL
Control II	-	25 uL	50 uL	25 uL
Plasma Sample	25 uL	50 uL (1:5 diluted sample)	-	25 uL
Plasma Sample	25 uL	50 uL (1:5 diluted sample)	-	25 uL

12) Gently remove fluid by vacuum filtration.

13) Wash plate 2 times with 200 uL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash.

14) Remove any excess Wash Buffer from the bottom of the plate using paper towels.

15) Add 25 uL of Detection Antibody Cocktail into each well. (Note: Allow the Detection Antibody to warm to room temperature prior to addition)

16) Seal, cover with aluminum foil, and incubate 2 hours with agitation on a plate shaker at room temperature. **DO NOT VACUUM AFTER INCUBATION.**

17) Add 25 uL Streptavidin-Phycoerythrin to each well containing the 25 uL of Detection Antibody Cocktail.

18) Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature.

19) Gently remove all contents by vacuum.

20) Wash Plate 2 times with 200 uL/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash.

21) Wipe any excess buffer on the bottom of the plate with paper towel.

22) Add 100 uL of Sheath Fluid to all wells.

23) Cover with aluminum foil and resuspend the beads by shaking on a plate shaker for 5 minutes.

24) Run plate on Luminex.

25) Save the data and evaluate the median fluorescence units using appropriate curve-fitting software.

26) A 5 or 4-parameter logistic method is recommended.

27) When calculating final sample concentrations, divide samples by 2 since twice the volume was added and the multiply by 5 since the samples were diluted 1:5.

Appendix IV:

ELISA Assay Performance Characteristics

Performance Characteristics and Sample Recoveries
for TNF-α and MCP-1

Tumor Necrosis Factor-α (TNF- α)	Assay Designs: Catalog No. 900-086
Performance Characteristics	• Sensitivity: 420.7 pg/mL Intra Assay %CV: Low – 9.7 ■ Medium – 9.7 ■ High – 3.9 Inter Assay %CV Low – 9.0 ■ Medium – 3.1 ■ High – 1.0 • Cross Reactivities: ■ Rat TNF-α (100%) ■ **Other rat cytokines (less than or equal 0.1%)**
Sample Recoveries	• Sample ■ Tissue Culture Media 91.6 % Recovery ■ Rat Serum 87.4 % Recovery ■ Recommended Dilution ■ Greater than or equal to 1:5

Monocyte chemoattractant protein-1 (MCP-1)	Assay Designs: Catalog No. 900-077
Performance Characteristics	- Sensitivity: 20.45 pg/mL - Intra Assay %CV: - Low – 5.2 - Medium – 5.9 - High – 4.6 - Inter Assay %CV - Low – 3.5 - Medium – 3.6 - High – 4.5 - Cross Reactivities: - Rat MCP-1 (100%) - Other rat cytokines (less than or equal 0.1%)
Sample Recoveries	- Sample - Tissue Culture Media 99.6 % Recovery Rat Serum 93.6 % Recovery - Recommended Dilution 1:8 - 1:128

Vita

Kristin Elizabeth Paccione was born on July 25, 1981 in Montgomery County, Ohio, and is an American citizen. She graduated from Mills E. Godwin High School, Henrico County, Virginia in 1999. Kristin received her Bachelor of Arts in Health Policy/Bioethics [Echols Scholar Program] from the University of Virginia, Charlottesville, Virginia in 2003. She received her Graduate Certificate in Anatomy & Neurobiology from Virginia Commonwealth University/Medical College of Virginia, Richmond, Virginia in 2004.