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14. ABSTRACT: The goal for the second year was to identify an optimal delivery vehicle for the injection of Androstenediol (AED) that was non-toxic and allowed an efficient system to ensure bio-availability for the immune regulating hormone. The AED is very soluble in DMSO:ETHANOL but this vehicle when given subcutaneously caused skin ulcerations. A number of other vehicles were tried and finally an injection of sulfobutyl ether β(beta) cyclodextrin, sodium salt containing 10 mg of AED was determined to be a suitable method for administration. During the course of the studies during the year a number of variables were also explored in an effort to optimize the model. Because the delayed healing response following Depomedrol administration was very modest, higher doses of the immuno-suppressant were tried. In addition, we explored using larger wounds plus laparotomies to mimic battlefield scenarios. Although a modulation of white cell populations was observed following AED administration following Depomedrol inhibition showed overall increases in total monocytes and granulocytes and decreased lymphocytes, these changes did not impact overall wound closure. Additional studies returning to the CD-1 mouse model were also pursued to determine if perhaps the rat is resistant to immune modulation but these studies also did not show a significant effect on wound closure. At this point in the project it appears that in order for the AED to reverse inhibited wound closure the model must contain an infectious component. The US Army ISR now has a modified, deep partial thickness, infected burn wound model so that a new strategy will be to employ that model and the mechanistic studies will be pursued here to analyze the white cell populations, cytokines and chemokines in the blood and immuno-staining of the cell populations in the PTFE tubes harvested from the animals and sent here.					
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Introduction:

The overall goal of this project remains to develop an effective clinical strategy employing immune-regulation as a means to reverse altered immune function in stressed combatants and thus reduce infection and restore an optimal wound healing response. Two models are being explored. In the studies being pursued here in the Laboratory of Tissue Repair at the VCU Medical Center immune suppression was achieved using a long acting form of methyl prednisolone called DepoMedrol. Open wounds were then inflicted in either rats (Male Sprague-Dawley) or mice (male CD-1) and then Androstenediol was administered to determine if the impaired healing response would return to normal. Blood was collected for total white cell analyses and their state of activation (CD 11b and CD 45 surface markers). Interleukins such as IL-2, 6, 8 and 10, key inflammatory signals, were also followed. Although some modulations in the white cell counts and state of activation were observed, no significant wound healing response was observed in the absence of an infection.

The major challenge faced this past year was finding an optimal delivery vehicle for the AED and best route of administration to achieve the most significant reversal of the DepoMedrol-inhibited immune response and wound closure. A variety of vehicles were explored and sulfobutyl ether β (beta) cyclodextrin, sodium salt was found to be the best. Therefore continued studies using the deep, partial-thickness, infected burn wound model at the ISR will employ this vehicle (Appendix B).

Another model used in this project is the Walker-Mason infected burn wound model that has been extensively used by the US Army ISR in San Antonio, TX. The problem with that model is that it is very severe and has a high rate of mortality. Recently a better model has been adopted by the ISR where a 2 cm deep partial thickness, infected burn wound is used. This model when it is not infected heals in approximately 14 days whereas when it is infected it heals in about 28 days. When effective agents are employed then the healing response in the infected wound returns

closer to 14 days. Now that the ISR's vivarium is functional again and with this new model in place the research will extensively employ this better model (Appendix B).

The continued research strategy for the coming year will be to focus on the ISR's new burn wound model and samples from these animals will be shipped to the Laboratory of Tissue Repair at the VCU Medical Center for biochemical, cellular, molecular and pathological analyses. This new focus is based upon the apparent need for an infection component in the model to cause stress, altered immune function and thus a delayed wound healing response.

Body:

During the second year, studies were continued to adapt the previous CD-1 mouse model to the Sprague Dawley rat model so the both the Laboratory of Tissue Repair here and the US Army ISR lab in San Antonio would be working on the same animal model. The challenges faced in the first year regarding a bio-compatible vehicle for the delivery of Androstenediol (AED) continued on during the second year. Although AED is very soluble in DMSO:Ethanol it was observed that after repeated subcutaneous injections this vehicle caused an ulceration of the skin and therefore not an acceptable vehicle. Other routes of administration were tried including intraperitoneal and intramuscular and intravenous but they also proved to be non acceptable.

Cremophor, a vehicle that has the ability to dissolve non polar compounds was also explored. However, Cremophor is an opaque liquid and it was impossible to determine if and how much of the AED was indeed in solution. Also when attempts were made to inject the suspension/solution there was a deposit of the AED left at the hub of the needle. Because of these problems, Cremophor was dropped as a likely candidate for AED delivery.

Beta-cyclodextrins are another class of drug vehicles that are widely accepted and are actually used in food products. They are an oligosaccharide in a "cage-like" configuration that can render insoluble materials in a fine suspension that can be

injected. Therefore the bulk of the research during the second year was pursued with the oligosaccharide Beta-cyclodextrins. In addition a better formulation of the cyclodextrins was found to be sulfobutyl ether β (beta) cyclodextrin, sodium salt and therefore will be the vehicle of choice for the continued work.

Other possible variables were also examined such as the use of Hydrocortisone in place of the potent long acting DepoMedrol. The rationale for this study was that Hydrocortisone is a naturally occurring stress response mediator and perhaps would allow for a more appropriate cell signaling response to suppress the immune response. Because Hydrocortisone has a shorter biological half life compared to the synthetic long acting DepoMedrol it was necessary to inject it every 24 hours during the initial phase of the studies. The results of these studies did not show any significant change in the closure of non-infected open wounds.

During this past year a variety of routes for AED administration were also examined. These included subcutaneous, intramuscular, intravenous and intraperitoneal. The conclusion from these studies was that there was no significant difference in the route of administration. However, in the future studies to be engaged with the infected, deep partial-thickness wound model the intraperitoneal route will be the choice for AED administration. This decision is based upon the fact that a greater volume of the AED can be administered by this route plus the peritoneal cavity provides a large, vascularized surface area for the rapid absorption of the drug. In addition the intraperitoneal route avoids first passage through the liver where the AED can be sulfated and metabolized for excretion.

A large number of studies were designed and carried out last year in an effort to optimize the DepoMedrol-inhibited wound healing model. Although the immune response as measured by total white cell profiles and states of activation were modulated following DepoMedrol administration and reverse to some extent by secondary administration of AED, these effects did not translate into any significant alteration of the closure of the non-infected open wound in the rat model. Other

strategies to increase the level of trauma were also explored in an effort to suppress the wound closure response and reversal by AED. These studies explored including a laparotomy and increased wound size and inflicting four wounds instead of 2 wounds. These additional modifications of the basic study model did not significantly alter the overall response. As outlined in brief here, there were many studies pursued this past year and they are far too extensive to include in the Body of this report. Therefore, **Appendix A** contains the entire Masters thesis prepared by F. Jerome Diskin where all of the studies are described in detail.

Therefore, the studies will now progress to the next phase where a deep, partial-thickness, infected burn wound model will be employed. This model more closely mimics the actual battlefield situation where the combatant is stressed, wounded and infection is a major factor that delays the healing response. The US Army ISR protocol entitled “Effectiveness of Silver Based Wound Dressings to Inhibit Infection in Rat (*Rattus norvegicus*) Contact Burn Wounds” is in **Appendix B**. Silver based dressings will be used as positive controls and lines 159 to 178 on page 7 of the protocol outline the AED and AET administration strategies.

Key Research Accomplishments:

- Sulfobutyl ether β (beta) cyclodextrin, sodium salt was found to be the optimal vehicle for the injection of Androstenediol.
- In the absence of an infectious component in the DepoM edrol-modulated wound healing model, very little significant enhancement of the wound healing response can be achieved by Androstenediol.
- Continued studies will focus on the new deep, partial thickness burn wound model being employed by the US Army ISR in San Antonio, TX.

Reportable Outcomes:

“Strategies to Employ Androstenediol to Reverse Steroid Inhibited Healing in a Rat Model of Trauma” a Thesis submitted by F. Jerome Diskin in partial fulfillment of the

requirements for a Masters degree in Biochemistry and Molecular Biology at VCU Medical Center, Richmond, VA. Mr. Diskin paid his own tuition and fees as required by the graduate program and did not receive any compensation from the funded research program. His thesis is in the Appendix A to this report. NOTE: Publication of this thesis has been delayed for 5 years in case the Army needs to approve its release and publication.

Conclusions:

Extensive studies were accomplished this past year in order to define an optimal vehicle for Androstenediol administration. After analyzing a number of potential vehicle options it was decided that Sulfobutyl ether β (beta) cyclodextrin, sodium salt was found to be the optimal vehicle for the injection of Androstenediol. In addition various routes of administration were also explored and included: subcutaneous, intramuscular, intravenous and intraperitoneal routes. It was concluded that the intraperitoneal route would be best suited for future studies because it provides a large vascularized surface area for rapid drug absorption, avoids first passage through the liver where the AED can be sulfated and metabolized for excretion and also accommodates a larger volume for drug administration.

It was concluded from the immuno-compromised studies where either the long acting DepoMedrol was used or the shorter acting, natural Hydrocortisone was used, that secondary administration of Androstenediol was not able to enhance open, non-infected wound closure. This is in spite of the fact that DepoMedrol pretreatment altered the total white cell profiles and state of activation that was reversed to some extent by secondary administration of AED. Therefore it was concluded that the studies need to move to the next phase where a deep, partial-thickness, infected burn wound will be the model of choice.

References:

Not applicable because no new literature citations were needed for the report.

Appendices:

A. "Strategies to Employ Androstenediol to Reverse Steroid Inhibited Healing in a Rat Model of Trauma," a Thesis prepared by F. Jerome Diskin in partial fulfillment for the requirements for a Masters degree in Biochemistry and Molecular Biology at VCU Medical Center .

B. "Effectiveness of Silver Based Wound Dressings to Inhibit Infection in Rat (*Rattus norvegicus*) Contact Burn Wounds" This is the animal protocol for the deep, partial thickness burn wound model being employed by the US Army ISR in San Antonio, TX and will be used for the continued studies of Androstenediol.

Appendix A

Strategies to Employ Androstenediol to Reverse Steroid Inhibited Healing in a Rat Model of Trauma

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

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Abstract

Much of our current understanding regarding trauma, mechanisms of healing, and treatment strategies have evolved as a result of injuries suffered during armed conflict. While our understanding of these processes has advanced during and since these conflicts, treatment methods of traumatic wound healing have failed to progress significantly in the last forty years. The overall objective of this research was to test the hypothesis that the immune regulating hormone Androstenediol (AED) modulates the host's immune system to promote wound healing under conditions where it has been inhibited by stress and infection. In an effort to characterize the effects of Androstenediol on healing following trauma, this research focused on strategies to evaluate which levels of trauma, immunosuppressive agents, and Androstenediol are required to reverse inhibition of healing. Sprague-Dawley rats were assessed for their response to trauma and intervention through assessment of white blood cell levels, cytokine and chemokine expression, and quantification of wound closure. While these studies did provide some trends reflecting modulation of cell counts and protein expression following AED treatment in immune-suppressed animals, measurement of wound closure failed to reveal a significant response.

Introduction

The immune system uses a variety of mechanisms to communicate and coordinate a synchronized response to a range of both perceived and real threats to the health of the host. These threats include foreign viruses, bacteria, parasites,

oncogenic host cells, autoimmune disease and damage from trauma. The mechanisms by which the innate and adaptive immune responses are coordinated to respond to these threats occur through different pathways, including direct cell-to-cell interactions and secretion of signaling proteins. These signaling cascades most frequently result in the activation of formerly naïve cells, recruitment of phagocytic and cytotoxic cells, and as a result, resolution of the threat, and if applicable, initiation of the healing process. Both external and internal influences can stimulate, inhibit, disable, or misdirect this coordination of the immune system thus resulting in a furtherance of the infectious or traumatic process and further inhibition of resolution and healing.

In clinical medicine, one of the most common mechanisms of inhibiting the immune system and healing process are through the use of glucocorticoids. In addition to those glucocorticoids given for the treatment of a broad variety of human ailments, the human body is capable of producing the same potent immunosuppressants. It is well known that the human body is driven to produce glucocorticoids in excess in response to stressors. As with many if not all of the systems in human physiology, there exists a balance. Studies have shown that men and women, who are known to express different sex steroids at difference ages, experience an increase or counter to their own endogenous steroids in response to infection and or trauma. Science has sought for some time to exploit these differences by studying the mechanisms of sex steroids and their ability to create an environment that supports the resolution of infection or trauma while enabling the healing process. Androstenediol, the immediate derivative of the powerful sex

steroid DHEA, has been examined extensively for its ability to modulate the immune system. It has been characterized for having effects in resistance to damage from ionizing radiation, bacterial and viral infection, and following hemorrhage and trauma [1-3]. In studies of mice receiving traumatic injury, androstenediol was shown to reverse the inhibition brought about by the potent glucocorticoid methylprednisolone, and to return healing to a timeline similar to mice receiving no immunomodulatory inhibition (Figure 1). Similar studies have shown that the close derivative of androstenediol, androstenetriol, can improve survival in several animal based shock-trauma models [4, 5]. It may then be hypothesized that given both the healing effects of androstenediol in mice, and the bioactive immunomodulatory effects of androstenetriol in rats, that androstenediol may exhibit a similar immunomodulatory effect on steroid inhibited wound healing in rats.

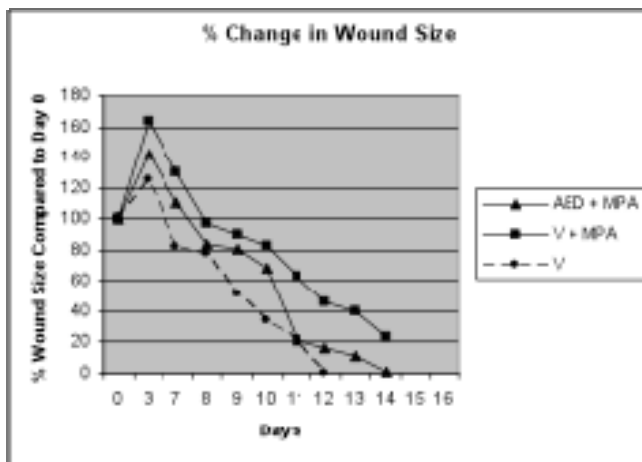


Figure 1: Illustration of the changes in rate of wound healing in murine model. From [6].

Purpose

Of the many complications of battlefield injuries, stress is perhaps the most poorly understood mechanism contributing to the morbidity and mortality of those suffering traumatic injury. As previously described, a number of biologically relevant metabolites of DHEA have been demonstrated to provide significant immunomodulation to the benefit of the test subjects. Currently, the US Army is seeking to demonstrate the effects of improved wound healing in a model simulating full-thickness wounds in male Sprague Dawley rats. While this lab has previously demonstrated the effects of improved wound healing via androstenediol in mice, other labs have shown that administration of androstenediol following trauma/hemorrhage reduces the activity of pro-apoptotic mediators [7]. This reduction in pre-apoptotic mediators may have a role in improved healing following trauma. Our efforts here seek to illustrate this role in the rat model and to characterize the nature of the immunomodulatory effects through analysis of cytokines, chemokines, and gross cellular profile as well as wound image analysis. Specifically, the following experiments represent the pilot studies conducted in order to determine the experimental parameters for the larger research project.

Clinical Significance

According to data collected by the World Health Organization [8], injury deaths worldwide are expected to continue to rise past the recorded 2000 levels of more than 5 million. Composed of deaths from a variety of mechanisms, including

traffic injuries, falls, war, and acts of civilian violence, the American College of Surgeons Committee on Trauma uses a trimodal division of injuries to describe their individual time-course [9]. The first two phases of this trimodal distribution govern events immediately following an injury to hours later and offer little opportunity for intervention beyond the well-proven methods of pre-hospital care and traditional medicine. These patients are either beyond saving due to the severity and immediate nature of their injuries, or are going to benefit from those trained specifically to treat trauma patients within the “Golden Hour”. It is however, the patients who fall within this third phase that persist for weeks to months that allow the opportunity to modulate the immune system to the benefit of the patient. Frequently, these patients succumb days to weeks later due to an avalanche of circumstances, often involving an either over-active or ineffective immune system. While medicine has made strident progress in preventing further illness from external sources, our tools for manipulating the immune system to our advantage remain limited. The careful balance between an overzealous and an underperforming immune system can mean the choice between life and death. Thus, the opportunity to modulate one of the most pervasive and powerful systems in human physiology during a time of compromised health may provide the tools to drastically improve outcomes following traumatic injury.

Mechanisms of Healing

The last generation of science has brought about the characterization of many important physiological processes. Perhaps few are more important than those leading to an understanding of the mechanisms behind the healing of human

tissue and the processes that can interfere with normal healing. Normal healing has been characterized as “acute”; described as a timely, structured, and efficient manner of healing resulting in the closest approximation of undamaged tissue endowed with the functions and appearance present prior to injury. This close approximation of the former state does not come about through haphazard coincidence, but through carefully coordinated steps regulated by specific cells and their signaling mechanisms. The four phases which overlap to produce an acute healing process have been described as: hemostasis, inflammation, proliferation, and remodeling [10].

The hemostatic phase is focused on the restoration of a closed circulatory system following trauma or injury. As is commonly known, this process is primarily mediated by thrombocytes and is initiated by thrombocyte contact with elements of the extracellular matrix. In conjunction with the induction of the clotting cascade, thrombocytes also release immunomodulatory cytokines including TGF- β and platelet-derived growth factor (PDGF). The clotting cascade results in the cleavage of fibrinogen into fibrin and aggregation of the clotting agents.

Following the cessation of hemorrhage now damaged tissue presents the signals necessary to recruit the messengers of inflammation and initiate the inflammatory cascade. Circulating neutrophils are attracted to the site of injury courtesy the ligands displayed on endothelial cells proximal to the site of injury and by the cytokines expressed by thrombocytes present at the site of injury. Within twenty-four hours, neutrophils and macrophages begin their initial tasks of clearing invasive species as well as debris. T and B cells then join the process, and all

contribute to the inflammatory state. The arrival and subsequent activation of all of these inflammatory mediators lay the foundation for a complex physiological response engineered to clear the wound of foreign material and damaged tissue, thus setting the stage for proper scarring, angiogenesis, epithelization, and finally, remodeling. Naturally, as is evident in many wound-healing pathologies, the careful equilibrium between the destruction of damaged tissue and foreign material, and construction of new tissue can be shifted in favor of a chronic wound with unresolved healing characteristics or an “overhealed” wound characterized by excessive production of new tissue. Frequently, these misguided healing pathways are result of improper cellular signaling, including those signaling mechanisms involving cytokines and chemokines expressed in the inflammatory phase of healing.

The chemical signaling that draws the cells of the inflammatory response is also responsible for initiating the fibroblast migration that is hallmark of the proliferative phase. Fibroblasts are rarely found in normal tissue; growth factors and cytokines such as Fibroblast Growth Factor (FGF), PDGF, and TGF- β are responsible for drawing in and activating otherwise naïve fibroblasts [11]. The recruited fibroblasts are responsible for deposition of the most abundant protein in the animal kingdom, collagen. The deposition of collagen into the wound site is followed by a complex pathway of modification and cross-linking resulting in the formation of a scar matrix. In order for the newly deposited tissue to survive, cells will require the oxygen and nutrients that can only be delivered by the vasculature. Again, the same factors that drive recruitment and proliferation of fibroblasts work

in conjunction with low oxygen tension to induce the recruitment and differentiation of endothelial cells and their precursors.

The new combination of tissues, collagen, extracellular matrix, and vasculature now assumes the conformation of “granulation” tissue and waits only for the epithelization and the remodeling phase before assuming up to eighty percent of the strength of native tissue. In order to cover the site of injury, epithelial cells from the margins of the wound begin to migrate toward the center. Through the signaling of growth factors and cytokines, the existing marginal cells dissolve their bonds with the underlying dermis and migrate until meeting epithelial cells from the opposite side. This contact completes the migrational phase and induces the cells to begin proliferating into the differential layers characteristic of the epidermis. During this final phase of healing, the newly deposited matrix is reorganized into a stronger medium, new capillaries merge into larger vascular structures, and metabolic activity finally drops, resulting the familiar scar tissue associated with a healed injury. The final strength of the wound site is largely a result of the re-orientation and cross-linking of collagen fibers that were initially deposited into the wound site. Additionally, the more elastic Type-III collagen initially deposited by fibroblasts is replaced over time by the more rigorous and widespread Type-I collagen. The final remodeling of the epithelial and dermal layers may take several months, or longer under pathological, sub-nutritional, or stressful conditions, and result in a healed wound with up to eighty percent of the strength of the original tissue.

Th-1 vs. Th-2 Responses in Healing

As the immune system has long been held to have a role in the healing process, medicine has also sought to relate some of the known mechanisms in the immune system to their effect on healing. One of the moderately well known immune mechanisms thought to have implications on healing has been the balance between the several species of T helper cells. CD4⁺ T helper cells (Th cells) arise from the same common lymphoid progenitor as the more aggressive CD8⁺ cytotoxic “killer” T-cells [12]. However, their effectiveness comes not from “killing” or simply initiating apoptosis directly in infected cells, although this activity has been demonstrated in vitro, but in several less direct activities that have more widely distributed effects. Namely, CD4⁺ Th cell direct other system to take on characteristics unfavorable toward invading or pathogenic species by either activating phagocytic or antibody producing cells. This therefore provides the difference in distinguishing between CD4⁺Th-1 and CD4⁺Th-2 cells. Specifically, Th-1 cells are associated with the activation of macrophages and dendritic cells (CMI or cell-mediated immunity) as well as some opsonizing antibody classes produced by B-cells, mostly IgG. Conversely, Th-2 cells are associated almost exclusively with the production of antibodies by B-cells, most notable the M, A, and E classes. As some pathogens have evolved to exploit antigen presenting cells such as macrophages and dendritic cells, these variations in characteristics have dramatic implications in the ability to counter disease. While some of the functions of these T-helper cells are dependent on cell-to-cell contact, many of their mechanisms are reliant on cytokines and chemokines being transmitted to target cells. Th-1 cells have been associated

with release of such cytokines as IL-2, IL-3, IFN- γ , TNF- β , TNF- α , and GM-CSF. Of these, IFN- γ , TNF- β , and TNF- α are known to activate otherwise naïve macrophages and IL-2 serves as a *positive* regulator of T-cells, in opposition of the effects of glucocorticoids such as cortisone [13]. Conversely, Th-2 cells are more closely associated with the release of IL-3, IL-4, IL-5, and IL-10, which are more directly related to increased activity among B-cells and their subsequent release of antibodies.

Interestingly, the cytokines released by the individual Th species mentioned here have inhibitory effects on the other, further swinging the pendulum in favor of its own response at the expense of the other. As illustrated before, the inflammatory response mediates the recruitment of a wide variety of cell types expressing an even more varied collection of cytokines, chemokines, and other signaling mediators. This inflammatory response may play a direct role in defining the species of Th cells present at the site of injury. Unlike most cells that reach final differentiation following proliferation, T-helper cells circulate in a naïve state, waiting for the appropriate stimuli to drive them into a Th-1, Th-2, or more exotic lineage. *In vitro studies have suggested that the signaling milieu present at the site of injury and inflammation appears to favor the Th-1 lineage* [14]. The signals involved in driving Th-1 and Th-2 lineages are poorly understood. However, some research has indicated that select cytokines will favor one type of T-helper cell over another. Specifically, IL-12 and IFN- γ are thought to promote a Th-1 helper cell lineage, while IL-4 and IL-6 are expressed in Th-2 differentiation. Naturally, the cytokines thought to promote the expression of one species have also been observed to inhibit the

differentiation and proliferation of the other lineage. The signals thought to be responsible for the differentiation onto the Th-1 lineage at the site of inflammation may be secreted by a number of sources. In addition, signals such as CCL3, CCL4, and CCL5 have been shown to be released by antigen presenting cells such as dendritic cells or macrophages as well as endothelial cells. While these signals do not themselves drive a Th-1 response, they do induce the release of IL-12 from dendritic cells, which has been shown to drive naïve T cells into Th-1 differentiation. While this illustration does present data to implicate Th-1 cells alone in injury and inflammation, it should be noted that as with most responses in biology, there exists a balance between the Th-1 and Th-2 responses that should eventually resolve to the equilibrium seen in normal physiology.

Role of Endogenous Steroids in Healing

Stress has long been tied to numerous changes in physiology in animal models as well as human subjects, including graduate students. These physiological changes manifest in many ways, including changes in body mass, immune cell profiles, as well as changes in the timeline and efficacy of healing. Many mechanisms have been documented as contributing to these manifestations of stress induction, though perhaps none more centrally understood as the role of endogenous steroids. While the moniker can be applied to sex steroids and mineralocorticoids, which are both thought to contribute, the primary actors in the stress response are the glucocorticoids. So named due to their origin in the adrenal cortex and their regulation of blood glucose, the glucocorticoids cortisol, cortisone, and corticosterone have diverse effects in physiology. The major player in humans

however, cortisol, has been identified as having the most widely distributed and powerful effects, particularly on wound healing. Due to the complex nature of glucocorticoids and their receptors, wide variation of effects, and contributing factors, little is known about the effects of cortisol on wound healing. What is known about cortisol is that it does not work alone; indeed studies have shown that it is the interaction with immune cytokines such as IL-1 β , IL-1 α , IL-6, IL-8 and TNF- α that coordinate the effects of stress and cortisol on healing, most likely at the site of injury [15].

When considering the induction of glucocorticoids by cytokines, early reports suggested that the interaction between cytokines and the hypothalamo-pituitary-axis (Figure 2) took place in the brain via defects in the blood brain barrier, more recent papers have suggested that the signaling is provided at local sites of inflammation or injury which are then transmitted to the brain via afferent sensory nerves [16]. Following stimulation in the brain, the classical and well-demonstrated human Adrenocortico System then proceeds via synthesis of the forty-one amino acid corticotrophin releasing factor (CRF) in the hypothalamus. CRF stimulates the production of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH then drives the production of cortisol and cortisone in the zona fasciculate of the adrenal cortex. This extremely powerful modulator of multiple physiologic systems also exhibits powerful responses to feedback inhibition, primarily by increased levels of cortisone and ACTH receptors in the brain. Globally, this activation of the hypothalamo-pituitary-axis with subsequent synthesis of cortisone is seen as itself a feedback mechanism in response to a pro-

inflammatory state [16], and typically favors a Th-2 environment at the expense of Th-1 cells. Other factors responsible for driving the increases on cortisol as well as other mediators are widely varied and include social, environmental, and nutritional effectors [15, 17, 18]. These stress-to-healing relationships typically correlate an increase in endogenous cortisol levels with changes in healing efficacy and timeline. Further research has also demonstrated that removal of the organs responsible for the secretion of cortisol and its derivatives returns the healing process to a near-normal process .

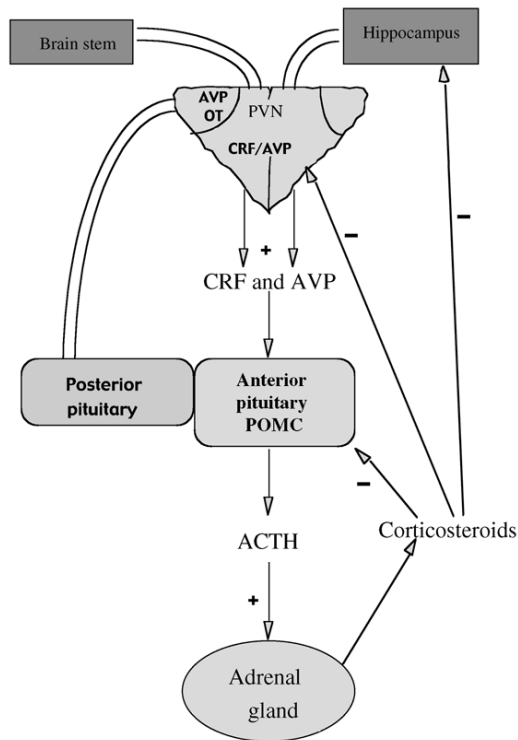


Figure 2: Hypothalamo-pituitary axis

While the activities of the glucocorticoids are closely related between the sexes, the very nature of the sexual dimorphism between males and females provides for comparisons of the effects of sex steroids on response to infection as well as healing.

Male and female study subjects in both human and animal studies have been shown to react differently to different forms of trauma, including frank trauma, burn injuries, as well as trauma/hemorrhage experiments. These studies have drawn relationships between the types of sex steroids expressed in a model and the propensity to recover from or even survive traumatic injury. Indeed, some studies have gone so far as to demonstrate that male subjects are more likely to survive burn injuries while female subjects are better equipped to recover from trauma/hemorrhage injuries [19].

While the relationship between sex steroids and healing may seem to be tenuous at best, studies have shown that in addition to the receptors found in reproductive tissues, mediators of the immune system are also known to have receptors for sex steroids. T-cells, monocytes, macrophages as well as others are known to be modulated by sex steroids. These same cells are also known to play an active role in response to healing following trauma and other insults. Complicating the issue however, is the fact that many of these mediators of the immune system respond in an inverse manner to different sex steroids. Work by Bird et al. demonstrated that estradiol and testosterone promote opposite effects in neutrophil invasion into tissues, following a similar relationship in calcium influx. As the effects of the sex steroids can be shown to have effects on the migration of immunomodulatory cells, this is not the only known effect on healing. The effects of oral contraceptives on coagulation and inflammation are well known within research and medicine. Conversely, research studies have also demonstrated that estradiol antagonists can promote a less coagulable state [20]. As estradiol has been

shown to have a direct impact on the activity of the clotting cascade, it may be hypothesized that this effect may eventually result in a more effective foundation for healing by providing the signals required for the establishment of a clot and eventually a fibrin lattice. However, the effects of the sex steroids with regard to the immune system and healing are not limited to the aforementioned roles.

To further complicate this scenario, the already discussed T-cells are also profoundly affected by the levels of sex steroids. Changes in the immune system, including circulating levels of T-cells, are well known to occur during reproductive years and even more profoundly during the stages of pregnancy. Generally speaking, pregnancy is thought to down-regulate the mechanisms responsible for cell-mediated immunity, while enhancing antibody concentrations and up-regulating humoral immunity [21, 22]. This difference between male and female subjects was further demonstrated in a study in which female mice receiving burns under anesthesia also suffered a 40% decrease in CD4+ T-cells when compared to male animals [23]. Interestingly, while the effects of a burn injury seem to provide a *carte blanche* reduction in the levels of CD4+ T-cells in female animals, Bird states that the trauma-hemorrhage model in male mice drives the balance between the Th-1 and Th-2 CD4+ T-cells into a Th-2 profile, at the expense of the Th-1 function. This effect was ameliorated by the administration of estradiol. This is in contrast to the strictly trauma related model discussed in the Th-1 vs. Th-2 section and several publications supporting the discussion. It should be noted, however, that there are equally compelling data published for opposing perspectives, and the subject awaits resolution.

Much of the research regarding sex steroids and wound healing has centered on the female sex steroids, most likely due to the phased nature of the steroids and the ability to study different levels of endogenous steroids simply by observing the model during different reproductive and stage-of-life time points. Never the less, the effects of the androgens cannot be overlooked. Structurally distinct from estradiol, the androgens are largely thought to have opposing effects when compared to the classical female sex steroids. Work examining the effects of androgens in a rat model demonstrated that dihydrotestosterone provided a wide variety of effects including delaying the migration of epidermal keratinocytes [24]. Human studies on the differences between male and female subjects suffering from trauma are of course subject to happenstance and the existence of a pre-existing study mechanism in place to measure such results. Such studies have sought to quantify the extent of injuries and provide a correlation between the expression of sex steroids among men and women with the appearance of well-known plasma markers for trauma, and finally, to demonstrate a relationship to mortality and morbidity. These studies have largely provided inconclusive data based simply on the diversity of the subjects, their pre-existing health status, injury types, as well as other factors. Perhaps most importantly though, these studies remind that the sex steroids are indeed not fixed, and do respond to trauma and injury as the other steroid classes may. In a study of female subjects following trauma, circulating levels of estradiol were found to have changes in as few as 12 hours following injury [25]. Still further complications of the issues surrounding the effects of sex steroids on trauma and healing are the phased nature of sex steroid expression within the

cycle of menstrual activity as well as the lifetime of a test subject. While there is known modulation of the levels of sex steroids following trauma and/or injury the levels at the time of injury due to life cycle or menstrual cycle may have additional impact on the reaction to injury. Human females of childbearing age, human females post-menopause, as well as men, all have similar levels of circulating estradiol. However, it may be the status of the body and its ability to react to injury with the production of additional steroids that make the difference in responding to insult.

In addition to the complications created by the differences between males in females in the expression of steroids and subsequent effects on healing, there also exist differences in steroid expression between the species considered in these studies. Most of the illustrations in this paper refer to the steroid activity in humans; however, the steroid mechanisms in rodent models are considered to be significantly different. Researchers in the 1950's first documented the predominance of corticosterone expression in rodent plasma, coupled with the almost non-existent expression of cortisol and concluded that rodents fail to express the critical enzyme 17α -hydroxylase [26, 27]. These conclusions have been both supported and refuted in a variety of studies since then. In addition, other authors have postulated that the expression of 17α -hydroxylase may be temporal, increasing and decreasing at different points in life [28]. This however does not change the fact that rat and mouse express primarily corticosterone, while humans are known to primarily express cortisol [29].

Thus the process for healing following trauma or other injury can and is modulated by endogenously produced steroids, including glucocorticoids, sex steroids, and likely mineralocorticoids. These effects vary from general suppression of the immune system, and associated delay and inhibition of the healing process to promotion of certain parts of the immune system and even some enhancement of healing. These effects are dependant on the general health of the subject, age, levels of stress, sexual maturity, and other factors yet to be realized. Generally speaking, the sex steroid estradiol seems to have a beneficial effect on recovery in a trauma/hemorrhage model in both male and female subjects, though the mechanisms are still unknown. Conversely, others have reported that the androgens have a suppressive effect on the immune system, perhaps preventing an overzealous response to disease or traumatic injuries, while *still* others have reported an increase in wound inflammation in response to androgens [30, 31]. In the application of AED (Figure 3), it seems likely that any beneficial effects of the drug would come from its ability to present itself as a stable analog of other sex steroids, perhaps estradiol (Figure 4), which with it bears a strong structural resemblance. The effects perhaps demonstrate this strong structural resemblance in that both estradiol and androstenediol induce in PPAR- γ [32, 33].

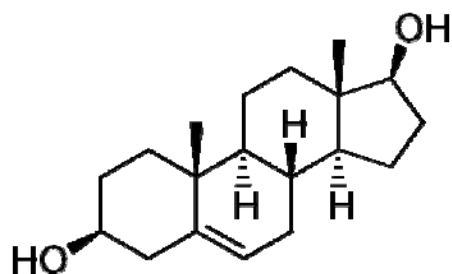


Figure 3: Androst-5-ene-3,17,19-triol

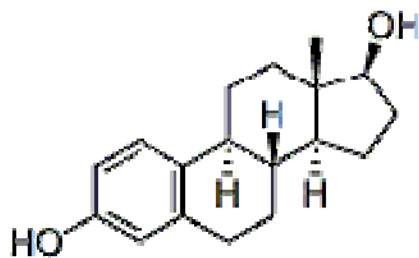


Figure 4: Estradiol

Catabolism of Steroids in Rats

As with other mammals, rats metabolize steroids via the P450 isoenzymes. Previous studies have described the process by which steroids are hydroxylated to less active metabolites and eventually to polar products for excretion in a two-phase process [34]. As there exists a sexual dimorphism in the expression of endogenous steroids, studies have demonstrated that there also exists a sexual dimorphism in the metabolism of both endogenous and exogenous steroids in mammals [35]. As one might expect, the efficiency with which mammals express these isoenzymes and thus metabolize steroids are affected by age, gross health, and in female subjects, by ovulation status and/or pregnancy. Metabolic rates tend to decline with age, and in post-menopausal females.

Other external forces can affect the metabolism of these steroid families as well. The presence of toxins, which receive preferential attention over the steroid families, may delay or inhibit metabolism of the steroids. In addition, there has been a great deal of attention paid to the affect of peroxisome proliferators on the activity of P450 enzymes. These structurally diverse chemical compounds are thought to bind with a variety of nuclear receptors in order to alter DNA transcription and

eventually protein expression patterns. Specifically, these compound/receptor complexes have been shown to alter the expression of P450 enzymes, resulting in alterations to the levels of enzyme expression as well as alterations to the levels of metabolites or precursors produced or altered by the enzymes [36]. This information is important to this research in particular as previous studies have identified an interaction between peroxisome proliferator activated receptors (PPAR's) and sex steroids including androstenediol, estrogen, and testosterone [37-39].

Effects of Methylprednisolone on Healing

As a class, glucocorticoids have been widely associated with suppression of the immune system such that they are routinely employed to curb human diseases such as autoimmune disorders, cancers, as well as injuries suffered as the result of traumatic injury. The side effects of glucocorticoid use are equally as numerous as the diseases they are used to treat, including weight gain, redistribution of body fat, anxiety, as well as sleeplessness and other physiological alterations. In particular, glucocorticoids (both endogenous and exogenous) have been demonstrated to delay and/or alter normal wound healing. The mechanisms behind the activity of exogenous glucocorticoids such as methylprednisolone in the delay in wound healing are still unknown today.

Early efforts by researchers working in traumatic spinal cord injury revealed that trauma resulted in a dramatic increase in the presence of free fatty acid metabolites, particularly arachidonic acid. The presumed sources of this precursor to inflammation were the damaged cell membranes themselves [40]. The results of

this research led the authors to conclude that methylprednisolone was responsible for amelioration of the inflammation cascade through its activity as an antioxidant. Work since then has characterized the mechanism of methylprednisolone as acting through cytoplasmic receptors in order to modify transcription and protein synthesis, thus resulting in inhibiting leukocyte diapedesis to the site of inflammation as well as inhibiting mediators of the inflammatory response and interfering with antibody responses and/or complement. Other authors have sought to demonstrate a relationship between the use of glucocorticoids and changes in the metabolism of L-Arginine through the up-regulated expression of Arginase [11, 41]. As the proper metabolism of Arginine is essential to proper immune response as well as collagen synthesis, the response is predictable. In general, the influence of glucocorticoids has been seen to delay the healing of open wounds, to produce weaker replacement tissue, and to increase the incidence of infection. As with most, if not all systems in physiology in medicine, injury and illness require a balanced immune system. Too much inflammation and healing cannot occur, frequently following by further compromised conditions of health. Conversely, too little inflammation, and the proper influx of mediators are not supported at sites of injury or illness, and foreign agents are allowed to persist. Thus in some instances it is appropriate to support the immune system, while in others, the immune system and inflammation must be countered.

Rat as a Model in Healing

The rat as a model for interpreting the mysteries of science and medicine has been in use for hundreds of years. Today, various rat models are available and are used in the study of trauma, autoimmune disease, cancer, cardiovascular disease, neurological disorders, sexual function and dysfunction, metabolic disease as well as other maladies. The rat genome has been sequenced [42], revealing a homology similar to the other available mammalian sequences, mice and men. Due to the wide availability of numerous knock-out models and vast amounts of physiologic data, the NIH said in 1999 that “in many ways, the rat model is the most appropriate model of human disease” [43]. The NIH report goes forward to state that at the time of publication, there existed over 500,000 scientific publications based on rat models, with approximately 250 strains of genetically modified rats for experimental use. Over ten years later, these statistics have certainly been exceeded with a PubMed search identifying over 700,000 publications associated with the search term “rat”. More specifically to this work, the rat has been used in the research of cutaneous wound healing as far back as 1919 [44]. In the first of a series of two papers completed in 1919, Akaiwa produced experimental data illustrating the changes in healing patterns as a result of the size and depth of the wound induced in the animals [44, 45]. Decades later, work associated with wound healing in the rat model turned to examining the mechanisms behind healing as well as various treatment method to promote healing.

Today, the rat model is recognized as advantageous due to the size, relative inexpensive, availability, and increased metabolic rate of the animal when compared to larger models [46]. At the same time, rats provide for the proper surface area to

induce and evaluate wounds and wound healing in a realistic scale. In addition, the animals are resistant to different physiologic insults and infections and are relatively easy to handle. Despite the problems in comparing “loose skinned” animals such as rats to “tight skinned” animals such as humans, the benefits of shared dermal structures, healing mechanisms, and dermal thermoregulation make for a good, however not perfect, approximation of human physiology, the ultimate goal. The disadvantages of the rat model are mostly based on presence of the subcutaneous panniculus carnosus muscle layer, which contributes to healing by aiding in contraction and collagen formation in a way that is not representative of human physiology [47]. For this reason, the pig is commonly recognized as a superior model, despite its own poor representation of contraction and healing in a humanoid timescale.

Cytokines in Healing vs. Wound Closure

The process of wound healing has been illustrated above as a carefully coordinated process that requires a close synchronization of multiple mediators to facilitate the final return of normal structure and function. This coordination obviously requires a complex mechanism of communication in order to correctly direct the appropriate mediators in a temporal and space sensitive manner (Figure 5). The mediators capable of generating these signaling cascades are products of the cells present at the time of injury as well as those recruited for their various responsibilities, namely those cells of the immune system. There has been a great deal of research done to facilitate a better understanding of the relationship

between these cells, especially their products, and the evolution of the wound in a normal wound healing process.

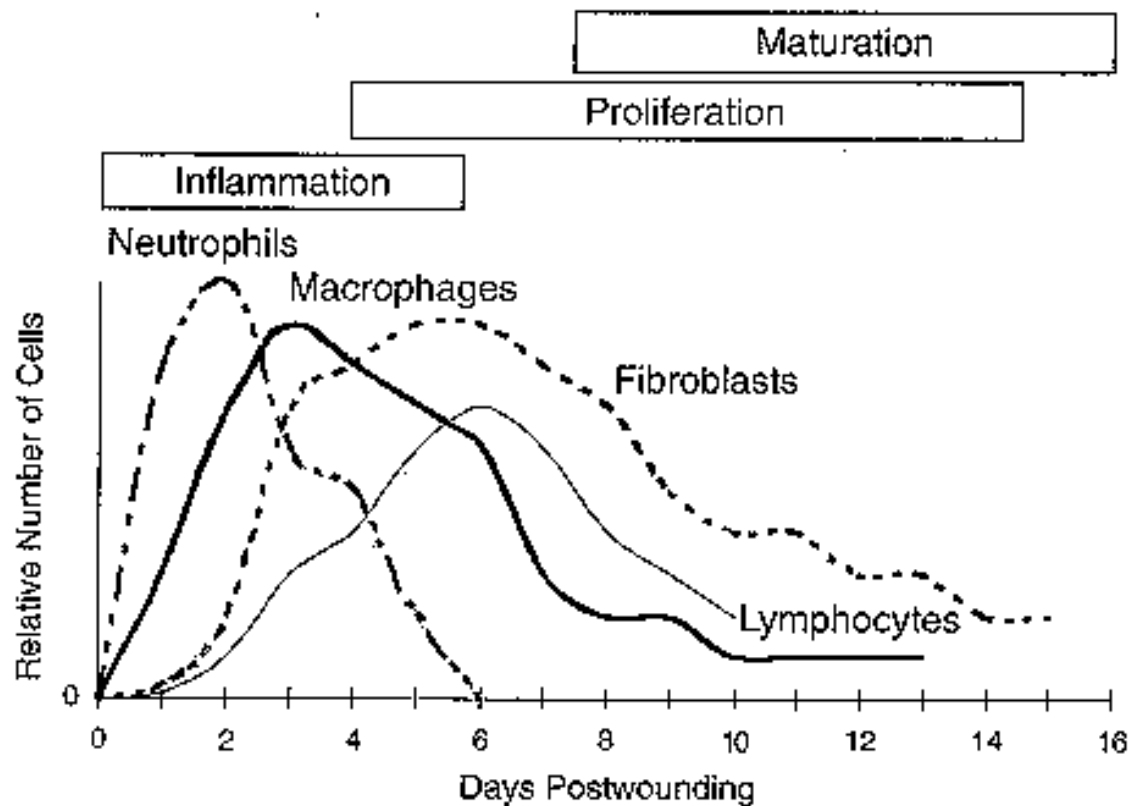


Figure 5: Phase Diagram of Healing Process

The first cells to introduce themselves and their products to the site of injury are those of the readily available innate immune system. Neutrophils are among the first cells present following an injury, and while their primary role is to phagocytize damaged and foreign tissue, the secretion of GM-CSF has also been of recent interest. Perhaps the most central of the innate immune cells recruited to the site of injury are the macrophages. Research in animal models has shown that treatment with anti-macrophage serum, anti-macrophage monoclonal antibodies, and steroids all impair wound debridement and induce fibroplasia, while the introduction of

macrophages directly into the wound site increase wound-breaking strength and increases collagen synthesis [48]. Macrophages are known to secrete several cytokines, including TNF- α , IL-1, IL-6, TGF- α and TGF- β , PDGF, and insulin-like growth factor one (IGF-1). These cytokines elicit different responses among fibroblasts; however, the overwhelming trend is to promote fibroblast migration and proliferation. In contrast, macrophages are also known oxygen radicals and inducible nitric oxide synthase (iNOS), both of which act in an antimicrobial function as well as are damaging to host tissue.

The innate immune system is however, not the only contributor to wound healing. T-cells also migrate to the site of inflammation or injury relatively late in the process. While this timeline suggests that perhaps T-cells are less important in the migration and proliferation of fibroblasts, and thus the wound healing process, research has shown us otherwise. Globally, the removal of the thymus or treatment with antibodies against all T-cells decreases wound breaking strength and collagen content. Specific studies against T-cell subsets have indicated that perhaps regulatory T-cells may play a role in the progression of a normal healing process, while CD8⁺ T-cells may impair normal wound healing. CD4⁺ T-cells also displayed a correlation with increased wound breaking strength, implying that CD4⁺ T-cells may also have an upregulatory role in wound healing [49]. Since histological studies have suggested that the predominant form of CD4⁺ T-cells in the healing wound are the Th-1 type [50], the Th-1 type cytokines are those most likely to upregulate wound healing. The Th-1 cytokines are typified by interleukin-2 (IL-2), but also include IFN- γ , TNF- β , and interleukin-8 (IL-8). While a direct link between these Th-

1 cytokines and fibroblast activity has not been clearly demonstrated, the improvement in wound strength cannot be ignored and more research may be warranted. Indeed, some have suggested that it is through the indirect activity of Th-1 cells on macrophages that wound healing is improved. The relationship between the Th-1 cytokine IFN- γ and the upregulation of macrophage activity thus resulting in increased fibroblast activity has been suggested by some [51]. Despite the obvious importance of fibroblasts in wound healing, the remodeling of the epithelial surface cannot be completed without the assistance of keratinocytes. Keratinocytes are known to express the CXCR2 receptor for IL-8 (and GRO- α) [52]. Thus the secretion of IL-8 by CD4⁺ Th-1 T-cells may also mediate the healing process via keratinocytes. However, as will be discussed, keratinocytes are not the only cells expressing IL-8. Activated fibroblasts also secrete IL-8, perhaps thus promoting the re-epithelization by keratinocytes. Thus, cytokines can have a profound role in affecting the recruitment, activation and secretion of cells both native to and drawn to the site of inflammation and/or injury. These cytokines most likely play a role in the regulation of production of the epidermal tissue, the underlying extra-cellular matrix (ECM), as well as angiogenesis. Removal or abrogation of these cytokines has been demonstrated to alter the wound healing process, as has removal of the cells promoting cytokine production.

Characteristics of Chemokines

CD4⁺ TH-1 Type Chemokines

Introduction to IL-2

Interleukin-2 is and has been one of the most extensively studied and characterized chemokines to date. IL-2 was the first chemokine identified to display the classic characteristics of a hormone signaling complex [53]. Originating from human chromosome 4q28-28, the 153 amino acid precursor for IL-2 is generated from four exons including a 20 amino-acid secretory sequence[54, 55]. Post-translational modifications result in a mature protein of 133 amino acids. Originally identified as a soluble factor contributing to the activation and proliferation of T cells[56], IL-2 was initially identified as TCGF (T-Cell Growth Factor). Through characterized early, initial cell theory suggested that chemokines like IL-2 were less important to cell proliferation than activators such as foreign antigen. It was found later that IL-2 is not only secreted by active T lymphocytes, but is required for activation and cell cycle progression of naïve T cells. In addition, IL-2 has been implicated in activation induced cell death [57]. A 15.5kd protein with variable glycosylation, IL-2 is known to bind to a heterotrimeric receptor including the ever-present common gamma chain.

Biosynthesis of IL-2

As one of the prototypic positive modulators of T-cell activity, IL-2 is unique in that in addition to activating naïve T-cells, it is also produced by the same activated T-cells, creating a positive activation loop[12]. Antigen recognition by the T-cell induces an incomplete synthesis of IL-2, which must be completely enabled by the co-stimulatory signal B7 binding to the T-cell CD28 receptor. This co-stimulation stabilizes IL-2 mRNA and increases its synthesis 20-30 fold. The mechanism by which IL-2 and co-stimulation both allow for increased production of

IL-2 may take place at either, or both, translation or post-translational modification. Translation of the IL-2 gene is regulated by NFAT, with several factors, including TCF-8 contributing to the modulation of transcription. In addition, most IL-2 mRNA produced goes unmodified, and thus is not secreted from the nucleus, indicating yet another mechanism for controlling the levels of IL-2 activity. Biosynthesis of IL-2 has been shown to differ among various age groups, with those over 60 years old showing nearly half of the levels of IL-2 and IL-2R mRNA than in those under 40 years of age [58]. This change was correlated to a similar difference in IL-2 secretion, T-cells displaying IL-2R, as well as IL-2R density of T-cell surfaces. This data may contribute to some of the well-known differences in inflammatory responses between different age groups.

Targets of IL-2

The IL-2 receptor is best characterized in its role in the adaptive immune response as a cell surface receptor expressed almost exclusively on activated T-cells. Perhaps less well understood is the role of the soluble IL-2 receptor in such physiologic mechanisms such as clotting, cell proliferation and healing. In order to better facilitate IL-2 activity, antigen recognition by T-cells promotes synthesis of the alpha chain of the IL-2 receptor, allowing the T-cell to respond to lower levels of IL-2 than with the lower affinity beta/gamma IL-2 receptor. This upregulation of T-cell activity has been linked to a finite quantity of IL-2 binding to receptors, allowing for an “all-or-nothing” response [53, 59]. Receptor expression has been shown to be modulated by other factors such as IL-3, IL-4, IL-5 and IL-6 as well. Work done by Gaya et al. has shown that IL-2 receptor expression can be inhibited in a dose

dependent manner by IL-4 in the first two hours of T-cell incubation following exposure to antigen [60]. IL-2 is known to activate many components of the immune systems such as B-cells, T-cells, monocytes, oligodendroglial cells, and NK-cells. IL-2 activity has also been demonstrated to drive changes in T-cell populations via the Stat-5 and PI3K pathways[61]. In addition to its activity stimulating T-cell expansion, IL-2 has been demonstrated to modulate other surface receptors in various cell lines[61]. Along with its role in the immune system, IL-2 receptors have also been discovered on intestinal epithelial cells [62], and in human proximal tubular endothelial cells [63]. IL-2 has also been applied in some cases to fight various forms of cancer. Given its propensity to activate certain T-cell populations, studies are underway to evaluate the use of IL-2 in battling both solid and blood borne cancers.

Predicted Response of IL-2 to Treatment

As the response to trauma and inflammation at the site of injury has been characterized as a primarily Th-1 type response, it may seem a simple step to conclude that IL-2 levels are most likely elevated during the immune response. However, this is not universally true and the introduction of endogenous steroids with or without the addition of androstenediol should at least further complicate the prediction of response. Human trauma studies tracking cytokine levels in critically ill patients indicate that patients affected only by trauma do not display systemically elevated levels of IL-2, while uninjured patients suffering from infection do present with systemically elevated IL-2 [64]. This may be an example of where cytokines that are expressed locally in response to injury or inflammation

are not expressed systemically, while the response to a potentially widespread threat such as infection elicits a widespread response. However, the widespread distribution of systemic IL-2 is also seen in autoimmune disease, thus explaining the use of IL-2 receptor antagonists and broad-spectrum glucocorticoids in treating autoimmune disease. This of course provides the data for understanding the relationship between methylprednisolone and IL-2. Early work into the relationship between cytokines and glucocorticoids revealed complicated and profound reductions in the levels of IL-2. This reduction was linked to separate mechanisms at differing doses, most reducing IL-2 levels by inhibiting synthesis directly [65]. The last controllable variable in this equation is the administration of androstenediol and the effects on IL-2. Studies evaluating the levels of IL-2 in murine splenocytes indicate a modest increase in production in response to AED [66]. Still other studies demonstrated that a trauma-hemorrhage model followed by cecal ligation and puncture to induce sepsis produced a drop in IL-2 levels that was attenuated by AED [67]. Other sex steroids have differing effects on the production of IL-2 among lymphocytes. Research into the effects of post-menopausal administration of estrogen replacement produced profoundly reduced levels of IL-2 from lymphocytes in culture. While the effects at the site of injury may be different, the combined effects of injury, methylprednisolone, with or without AED are likely to result in little or no effects on systemically measured IL-2.

Introduction to IL-8

Originally purified and characterized in 1987 [68], IL-8 was initially assigned the moniker of monocyte-derived neutrophil chemotactic factor (MDNCF). IL-8 was

considered a tissue-derived factor with similar chemotactic properties as previously characterized factors such as anaphylatoxin C5a, platelet activating factor, or leukotriene B₄. Initial work demonstrated that exposure to IL-8 caused neutrophils to migrate to the extravascular space and caused neutrophil changes in shape, adherence, movement, secretion of enzymes as well as changes in the respiratory burst mechanism [69]. Different then from other known cytokines, IL-8 showed no common homology to such other immune system regulators such as IL-1, TNF, IFN or GM-CSF. IL-8 was shown to evolve from a 99 amino acid precursor including a typical 20 amino acid secretion sequence [70]. Further characterization of the gene and subsequent gene products revealed several forms of IL-8, with the most common form found to be 72 amino acids [71]. Despite the differences in IL-8 from known immunogenic factors such as IL-1 or GM-CSF, IL-8 did indeed show a remarkable amount of similarity to B-thromboglobulin [72], both eventually characterized as CXC type chemokines. Stronger similarities in activity have been demonstrated between IL-8 and a cleavage product of B-thromboglobulin that have been further linked to the relative positions of 4 cysteine residues[73]. Interleukin-8 has been associated with a wide variety of human diseases and has broad implications for targeted therapy. Interleukin-8 has been associated with such human diseases as ovarian cancer [74], breast cancer [75], psoriasis [76], and asthma [77], as well as various autoimmune diseases. One of the most unique characteristics of Interleukin-8 is its physiologic longevity. While most studies of inflammatory cytokines illustrate effectiveness in hours, IL-8 was shown to be

present in immunomodulatory levels at time points greater than ninety-six hours [78, 79].

Biosynthesis of IL-8

A product of human chromosome four, the synthesis of interleukin 8 has been documented in many different cell types. From macrophages [68], to fibroblasts [80], endothelial cells [80, 81], epithelial cells [82], and hepatocytes [83], IL-8 synthesis has been demonstrated in response to a number of different stimuli, including LPS [12], endotoxin, IL-1, implant debris [84] and even hyperosmolarity [79]. Interestingly, while macrophages respond most frequently to stimuli such as LPS, immunogenic agents such as IL-2, IL-6 or interferons fail to produce IL-8 in macrophages [69]. Expression of IL-8 mRNA has been shown to correlate with an increase in NFkB activity [85], raising the possibility that NFkB plays a role in the expression of IL-8. However, still other studies involving suppressed NFkB have suggested that Ap-1, rather than NFkB contribute to synthesis of IL-8 [86]. While these reports seem at odds with each other, they may indeed suggest converging response mechanisms reacting to traumatic vs. infectious stimuli. Response to antigen typically results in a rapid synthesis of interleukin-8, with mRNA detectable within one hour [69]. Once secreted, interleukin-8 is thought to form a dimer, similar to other known chemokines [87]. Given the nature of IL-8 and its recognition as a major mediator in infection, trauma, and human disease it should come as no surprise that methods to inhibit the production and activity of IL-8 have long been sought after. Various agents such as DMSO [78], fluticasone propionate and salmeterol [88], L-arginine [89], and even monoclonal antibodies [90] have been

shown to interfere with IL-8 production, signaling, and/or activation of target cells, thus reducing the inflammatory cascade in many cases.

Targets of IL-8

Complicating our understanding regarding the effects of IL-8 on cell behavior are the receptors known to recognize IL-8. The receptors known as CXCR1, also known as IL-8R α , and CXCR2, also known as IL-8R β , are commonly known to recognize chemokines containing two N-terminal cysteine residues separated by another amino acid, thus designated CXC chemokines. This characteristic sequence is also commonly preceded a glutamic acid-leucine-arginine (ELR) sequence. The many factors matching this profile bind to the CXC receptors with varying affinity and in different combinations, with only two known CXC cytokines binding both CXCR1 and CXCR2 receptors, and the remainder binding only to CXCR2 [91]. The CXC receptors, either one or both, have been shown to be displayed on a number of different cell types, including CD8⁺ T-cells, mast cells, as well as endothelial and epithelial cells. However, the CXC receptors are most commonly recognized for their role in modulating the innate immune system through their expression on neutrophils, monocytes and other myeloid cells. Both of the human CXC receptors map to chromosome two, and lead to the expression of a characteristic 7-transmembrane domain, G-protein modulated receptor, with features similar in structure to the rhodopsin superfamily [92]. The CXC receptors have also been found to be expressed in other primates, chickens, rabbits, cows, mice, and rats, indicating a highly conserved role across nature. The CXCR1 receptor is known to recognize only IL-8 and granulocyte chemotactic protein-2 (CXCL6). IL-8 is also

recognized by CXCR2, which recognizes CXCL6 in addition to the remainder of the other CXC chemokines including several of the GRO chemokines. Both of these receptors seem to act through the classic g-protein coupled signaling cascade, marked by a change in free calcium and granule enzyme release. Much work has been done trying to uncover the seemingly redundant roles of two CXC receptors, with some overlap for ligand and similar, if not identical signaling mechanisms. However, inhibition studies have revealed that CXCR1 seems tied to superoxide anion production and increases in phospholipase-d1, while CXCR2 is related to increases in phospholipase-d2 expression. The downstream results of CXCR2 signaling are less well understood, but the differences in signaling mechanisms may lead to functional differences in the CXC receptors.

Predicted Response of IL-8 to Treatment

The primary role of IL-8 as a chemotactic factor for the recruitment and activation of neutrophils makes it one of the better understood cytokines. Despite their differing roles, the activity of IL-8 expression shares some of the features of the previously discussed IL-2, including high local expression following trauma and inflammation. However, unlike the results we have seen in IL-2, there is research showing ten-fold and five-fold increases in plasma levels of IL-8 among critically ill septic and non-septic patients respectively [93], thus setting the stage for measuring changes after treatment on a widespread scale and providing an indication for the expected response on IL-8 levels to trauma. When exposed to glucocorticoid treatment, IL-8 responds much like other mediators in the immune system, namely by displaying a profound reduction in circulating protein levels. Autoimmune

models such as multiple sclerosis and rheumatoid arthritis as well as non-autoimmune models such as exercise-induced inflammation and clinical patient care studies exhibit both systemic and local increases in IL-8 levels. One of the most common treatments employed in these models as well as in clinical medicine is the administration of glucocorticoids, including methylprednisolone. In these cases, methylprednisolone has been demonstrated to provide profound reductions in the levels of many cytokines, including IL-8 [94, 95]. While the mechanisms behind much of these changes are poorly understood, at least some of these studies have implicated a reduced synthesis of IL-8 by monocytes as a cause. Perhaps due to the high profile of other pro-inflammatory cytokines, there is precious little information with regard to the interaction between androstenediol and interleukin-8. However, since there have been comparisons between AED and estrogen, especially with regard to the possibility of a common signaling mechanism, we are not without guidance. In addition to the commonly known pathologies of IL-8 in inflammation such as exercise-induced inflammation, IL-8 has also been implicated in genetic illnesses such as cystic fibrosis. In both of these circumstances, estradiol has been indicated as a suppressor of IL-8. With regard to inflammation in an exercise-induced model of inflammation, estradiol was demonstrated to reduce synthesis of IL-8 mRNA in skeletal muscle cells. In the latter example, estradiol was shown to reduce the synthesis of IL-8 in a dose dependant manner formerly induced by toll-like receptor agonists [96]. Given these shared effects on the expression of IL-8 from various sources and presumably from differing mechanisms, it seems likely that the effects of *significant* trauma should induce an increase in the expression of

IL-8, which should also be detectable in plasma. However, as both treatment protocols involve at least one suppressor of IL-8, it also seems likely that IL-8 will be reduced, perhaps to undetectable levels, in all but subjects receiving trauma plus vehicle only.

CD4+ TH-2 Type Chemokines

Introduction to IL-6

As yet another of the earlier cytokines, IL-6 had already been identified through multiple experiments and had received monikers such as B-cell stimulatory factor-2, IFN- β 2, 26-kDa protein, hybridoma/plasmacytoma growth factor, and HSF [97]. The primary experiments responsible for the identification of the protein that would eventually become known as IL-6 involved the activation of B-cells in the presence of T-cells. Researchers correctly hypothesized that there must be some soluble mediator responsible for the activation of B-cells in the presence of activated T-cells.

Structurally, IL-6 is a 185 amino acid sequence, composed of a four-helix bundle linked by loop structures with an additional mini helix near the N-terminus [98]. IL-6 plays a number of different roles in physiology, including the T-cell dependant activation of B-cells, the activation of the acute phase response, and changes in bone synthesis. IL-6 is capable of binding both cell membrane receptors as well as a soluble IL-6 receptor. Binding of IL-6 to the membrane bound receptor requires the IL-6 receptor alpha subunit in addition to the ubiquitously expressed glycoprotein gp130. Binding of IL-6 to the heterodimeric receptor complex has been demonstrated to drive signal transduction via both the MAP Kinase and JAK-STAT signaling pathways, resulting in altered transcription and protein synthesis depending on the cell type receiving the stimulus [99].

The soluble IL-6 receptor represents a 50-55kD ligand binding protein that is either a cleavage product or a splicing product of the original 80kD membrane bound receptor subunit. With the ubiquitous expression of gp130 in nearly all cell types, the synthesis of the soluble receptor allows IL-6 to stimulate cell signaling in nearly any cell type. Alternatively, in the absence of the gp130 subunit the soluble receptor subunit may act as an agonist, preventing signaling by IL-6 by sequestering otherwise available IL-6 molecules.

Due to the widespread nature of IL-6 expression both in normal and abnormal physiology, science has produced an antibody designed to interfere with both the membrane bound and soluble versions of the IL-6 receptor. This (tocilizumab) has been approved in Japan, Europe and the US primarily for the treatment of rheumatoid arthritis. In addition, IL-6 has been researched extensively in predicting mortality and morbidity. Today's methods of predicting the clinical outcomes in patients have not changed in decades. Many studies have sought to advance current clinical tools in identifying patients at risk of severe morbidity or mortality following a wide variety of insults. Many of these studies have focused on measuring soluble factors and have sought to identify a relationship between these factors and patient outcome. Needless to say, these studies are complicated by the broad spectrum of patient health histories, mechanisms of illness and/or injury, and treatment pathways. Thus, many of these studies have fallen short of providing conclusive measurements to be correlated with predicting patient outcomes and providing interventions to alter undesired outcomes. Nonetheless, measuring IL-6 has been a target to measure following burns, trauma, hemorrhage, sepsis, or

combinations of these insults. Some studies have drawn impressive relationships between the levels of IL-6 in blood, and mortality, with correlations illustrating that higher levels of IL-6 are indicative of a increased risk of death [100]. I have yet to find evidence of research involving the administration of IL-6 receptor antibodies as a counter to rising IL-6 levels following trauma or other insult correlated with mortality.

Perhaps most interesting, IL-6 synthesis was affected by the administration of estrogen *in vitro* and *in vivo* [101]. In this study, the authors demonstrated that in human bone marrow stromal cells, synthesis of IL-6 was increased 10,000-fold following administration of a mixture of IL-1 and TNF α . The synthesis of IL-6 *and* the expression of IL-6 mRNA were then both reduced in a dose dependant manner following administration of 17 β -estradiol and a combination of IL-1 and TNF α , when compared to cells receiving only the IL-1 and TNF α . With the expression of this powerful immunological mediator altered by sex steroids, or their metabolites, it may not be a long leap to hypothesize that additional sex steroid may be able to also affect the synthesis of IL-6 in a number of different tissues.

Biosynthesis of IL-6

The gene coding for the IL-6 protein consists of five exons and four introns found on human chromosome seven and is characterized as having a structure homologous to G-CSF [102]. Production of IL-6 has been most commonly associated with T-cell mediated activation of B-cells. However, synthesis of IL-6 has also been demonstrated in macrophages, fibroblasts, synovial cells, endothelial cells, glial cells, and keratinocytes [99]. In addition, IL-6 expression has been documented in a

number of different types of cancer cells, including cardiac myxoma, certain bladder cell cancers and certain cervical cancers [102]. Obviously, the synthesis of IL-6 by such a broad selection of cell types illustrates the broad implications of IL-6 and its affects at multiple targets. Similarly, this also means that identifying the relationship between the synthesis by specific cells and the response by specific targets is nearly impossible in an *in vivo* model.

Naturally, the biosynthesis of IL-6 in these many different types of cells is stimulated by many different input ligands. Some of the common factors responsible for stimulating the biosynthesis and release of IL-6 are IL-1, TNF, PDGF, pathogen associated molecular patterns (PAMPs), and LPS [99, 102]. In addition to the most recognized form of IL-6, some authors have shown that IL-6 mRNA can be produced in at least two different mRNA isoforms of IL-6 by alternative splicing. These alternative forms of transcription of the IL-6 gene appeared as fragments with 58 bp deletions and 114 bp deletions of exon 5 and exon 3 respectively [103]. The activity of these alternative-splicing variants is the subject of much speculation. While the structure of the protein remains largely intact, there are specific regions missing that have been related to either the alpha-receptor subunit or the gp130 receptor subunit that bring the binding affinity to these ligands into question. Nevertheless, the existence of alternative isoforms of the IL-6 protein already produced by a wide variety of cells and implicated in a number of normal and abnormal physiological process muddies an already complicated picture.

Targets of IL-6

While, as mentioned, the gp130 subunit of the IL-6 receptor is expressed in many tissue types and aids in the signal transduction of other signaling molecules, the expression of the alpha-receptor subunit is far more exclusive and is the key to any selectivity in IL-6 signaling. Membrane bound IL-6 receptors have been identified in hepatocytes, B-cells, osteoblasts, T-cells, chondrocytes, pulmonary epithelial cells, skeletal muscle, fibroblasts, as well as other cells. Conversely, the soluble IL-6 receptor allows for IL-6 to bind with the possibility of a complete cytokine/receptor complex in any cell also expressing gp130 [104], although it should be noted that some studies have postulated that the soluble IL-6 receptor acts as an antagonist; sequestering IL-6 for breakdown and thus reducing the response elicited by IL-6. Binding of the complete IL-6/receptor complex results in intracellular tyrosine phosphorylation as part of the JAK/STAT signal transduction cascade [97]. The dimer formed by STAT3 then is widely known to translocate to the nucleus and initiate transcription. Naturally, the resulting transcription would be dependant on the cell type and the genetic information exposed for initiation of said transcription.

IL-6 in its role as a pro-inflammatory cytokine also initiates reactions that promote a correction of the pro-inflammatory state. Research manuscripts have indicated that the exposure of microglial cells to IL-6 and TNF- α results in the upregulation of IL-10 mRNA and the increased release of IL-10 by these cells [105]. Alternate studies in monocytes have indicated that IL-10 enhances degradation of IL-6 mRNA, resulting in a decreased translation to IL-6 protein [106]. Overall, more

is known about the final results of IL-6 signaling than the specific cascades producing these results. IL-6 is thought to promote normal physiologic signaling, to be a major mediator of pro-inflammatory reactions, and to promote the uncontrolled growth and proliferation of oncogenic cells.

Predicted Response of IL-6 to Treatment

As IL-6 has been identified and researched extensively as a marker for mortality and morbidity following trauma and/or infection, there is indeed a plethora of information available with regard the effects of some common modulatory factors. Perhaps the most well known characterization of the role of IL-6 in critical injury comes from Remick et al. [100]. In it, the authors convincingly demonstrate the use of IL-6 as a predictor of mortality in mice following a sepsis model. In human studies of trauma patients, IL-6 has been identified as having a positive correlation with severity of injury [93, 107]. Thus IL-6 exists as a common indicator of severe trauma and is detectable in plasma following trauma and or infection. These studies have suggested that there is a threshold value for IL-6 above which mortality is threatened. However, as with the other cytokines discussed thus far, IL-6 is also subject to the widespread and powerful effects of methylprednisolone. Both *in vivo* and *in vitro* models have demonstrated the reduction of IL-6 levels at the site of injury as well as in the systemic circulation following treatment with methylprednisolone [108, 109]. The characterization of effects by Almawi et al. illustrated a total abrogation of mRNA in a dose dependant manner among human peripheral blood mononuclear leukocytes. These observations were made in the presence of fully functional receptors, thus leading

to the conclusion that methylprednisolone acts at the level of transcription, though the possibility of interference at additional sites is a possibility. Conversely, studies in a spinal cord injury model among rats demonstrated a site-specific elevation in IL-6 that was also reduced with administration of methylprednisolone, illustrating both the local and systemic production as well as the reduction of that production by glucocorticoids.

As IL-6 has been considered one of the most important cytokines in predicting outcome following illness or trauma, there is fairly good data with regard to the effects of androstenediol on IL-6. The most convincing studies illustrate a reduction in elevated IL-6 levels following a two-hit model of trauma-hemorrhage and sepsis in rats that also correlates with an increase in survival against subjects not receiving AED treatment [67, 110]. Thus it seems likely that IL-6 levels should be increased with trauma, and also likely that the administration of methylprednisolone, while simulating stress, may reduce IL-6 levels, perhaps even to baseline levels. Furthermore, the administration of AED should further reduce the levels of IL-6 when compared to subjects not receiving AED.

Introduction to IL-10

Initially recognized for its inhibition of T-helper cells, IL-10 has become known as a major element in the modulation of the immune system and the differentiation of naïve immune cells [111]. As more has become known about IL-10, its action has been found to be a direct, massive inhibition of monocytes and macrophages, which results in the subsequent inhibition of Th and NK cells [112]. Specifically, the activity of IL-10 has been shown to inhibit the synthesis of IL-1, IL-2,

IL-3, IL-6, IL-8, IL-12 and IFN- γ in humans [113]. The absence of normal IL-10 levels is found frequently in cases of chronic inflammatory disease and autoimmunity. X-ray crystallographic analysis has characterized the signaling peptide as a non-covalent homodimer of 178 amino acids each and with a structure similar to IFN- γ .

Studies using protein mimics of IL-10 have demonstrated that short peptide sequences of the IL-10 peptide are able to initiate IL-10-like responses, leading to questions as to the relationship between the structure and the function of the intact IL-10 protein. The IL-10 gene is most commonly expressed in T-cells and in monocytes/macrophages. Located in chromosome one in mice and men, transcription of the IL-10 gene results in an mRNA product approximately 2kb long.

The structurally similar human IL-22 has been shown in recent work to bind to a receptor complex consisting in part of the IL-10 receptor. This has also lead to questions regarding the possible cross reactivity of IL-10 and similar proteins with heterogeneous receptor complexes. While probably fortunate for research and unfortunate for human health, mammals are not the only organisms expressing the IL-10 gene in nature. IL-10 gene homologues are expressed in several viruses known to infect humans and other mammals, including Epstein-Barr virus, Equine Herpes virus II, pox virus Orf, and human cytomegalovirus [112]. These IL-10 homologues have been identified as having a structural homology of up to a frighteningly 84%. Needless to say, this implicates IL-10 like proteins and IL-10 receptors in the furtherance of disease in humans and other mammals.

Biosynthesis of IL-10

As previously described, the IL-10 gene is transcribed from an approximately 2kb mRNA and processed into a protein of approximately 178 amino acids.

Translation of the post-translational mRNA produces one half of the IL-10 dimer required for biological activity. The monomer presents as a structure of six alpha helices, contains four cysteine residues, of which only one is conserved among members of the IL-10 family of cytokines, suggesting that disulfide bonds are not important in the conformation of the monomer or dimerization of the superstructure. The monomer is characterized by the V-shaped structure, which is hypothesized to aid in the dimerization of the final active molecule. Research characterizing the massive, rapid release of IL-10 in response to certain stimuli has suggested that there are alternative methods for modulating levels of IL-10 *in vivo*.

Researchers have hypothesized that IL-10 is continuously synthesized at some constitutive level, and that it is post-transcriptional mechanisms that are responsible for controlling overall IL-10 levels. This would allow for a faster modulation of IL-10 levels rather than simple resolution through transcriptional control [112]. The biosynthesis has been characterized as a response or counterbalance to inflammation through synthesis by Th-2 cells in order to downregulate the inflammatory role of Th-1 cells. IL-10 has been shown to serve several functions in this role. Primarily as a stimulator of B-cell activation to increase sensitivity to antigen, IL-10 has also been demonstrated to play roles in suppressing Th-1 response following the induction of anergenic activity as well as perhaps being a constitutive element in balancing inflammation against abnormal

physiology in such autoimmune diseases such as experimental autoimmune encephalomyelitis, IDDM, and rheumatoid arthritis [112].

Targets of IL-10

As already demonstrated, IL-10 has a broad effect on a number different cell types, including T-cell, B-cells, monocytes/macrophages as well as NK cells. The possibility of receptor sites for such a diverse and powerful cytokine must be considered. Especially in view of the fact that although IL-10 is first thought to act as a dimer, but has also been shown to act in protein subunits *and* is expressed by additional organisms. Like other cytokine receptors, the IL-10 receptor is also a dimer, composed of alpha and beta subunits, which constitute the receptor and signaling roles of the IL-10 receptor respectively. In contrast to IL-6, there have been no reports of a soluble receptor for IL-10 *in vivo*, although *in vitro* experiments have been able to synthesize a soluble IL-10 α subunit. Studies have shown that T-cells downregulate expression of one subunit of the IL-10 receptor in response to activation, while macrophages actually upregulate IL-10 receptor expression in response to antigen or other activating stimuli. The expression of the alpha subunit has also been demonstrated to be induced in non-immune system cells, such as fibroblasts, epidermal cells, keratinocytes, and colonic epithelium in response to such stimuli as lipopolysaccharide (LPS), glucocorticoids and other compounds [112].

Predicted Response of IL-10 to Treatment

The role of IL-10 in modulating the immune system following trauma and or infection is generally characterized as a “brake” on the pro-inflammatory mediators of the immune system. Reviewing the human data for IL-10 levels among critically ill patients provides a reminder as to both the balance sought by the immune system *and* the temporal nature of cytokine expression. This conclusion is born from the results of both traumatic and non-traumatic patient studies and their expression of IL-10 during their treatment. While perhaps expecting to see a reduction in the levels of the anti-inflammatory cytokine IL-10 among non-survivors (thus providing an overly pro-inflammatory pathologic state), studies frequently illustrate IL-10 levels at or above levels found among healthy volunteers [64, 114]. The most striking differences in these values were presented when comparing critically ill trauma patients with critically ill patients suffering from non-traumatic sepsis. In comparing the values between survivors and non-survivors within these groups, the authors illustrated little difference in the trauma groups, while the sepsis group displayed a ten-fold difference between survivors and non-survivors with the non-survivors having the higher value. This may either illustrate an overly Immunosuppressed state, thus allowing the pathogen to thrive and ultimately overcome the host, or, it may illustrate a futile effort to apply a brake to a pathologic pro-inflammatory state. Only by evaluating the rest of the patient’s blood values can this determination be made.

This is course also dependent on the temporal value of the cytokine messaging system. The previously discussed IL-10 values among traumatic and

non-traumatic patients were obtained at 48 hours post-admission to the ICU. This of course gives no indication as to the overall temporal pattern of the illness and thus is fairly useless in evaluating the temporal nature of IL-10 in this study. It does however serve as a reminder that the expression of cytokines are temporal in nature and may ultimately be dependent on the expression of other cytokines. With regard to the trauma patients, admission to the ICU following surgery or other interventions to stabilize the patient likely occurs within a fairly close timeline between individuals. With the knowledge that the IL-10 values are the same or similar between survivors and non-survivors of the traumatic injury group, IL-10 may be discounted as a value for predicting mortality. However, the further knowledge that the otherwise healthy group presents with IL-10 values nearly twice that of the entire trauma group indicates an imbalance that may be significant.

When considering the reaction of IL-10 to administration of glucocorticoids such as methylprednisolone, one might expect to see an increase as glucocorticoids are known for their anti-inflammatory properties. This is indeed the case, but the reasons for this are not yet clear. There are indeed examples citing increases in circulating IL-10 following administration of methylprednisolone [115]. However, as glucocorticoids like methylprednisolone are known to downregulate other cytokines, most likely at the level of transcription, the mechanisms in the regulation of IL-10 remains unclear. One group has postulated that the reason for increased circulating IL-10 may be at least partially due to decreased expression of the IL-10 receptor. These authors demonstrated not only that the IL-10 receptor is expressed on CD4+ and CD8+ T-cells, NK cells, monocytes and neutrophils, but also that a

seven day course resulted in a significant reduction in the expression of the IL-10 receptor on these same cells [116]. This of course raises the question as to whether the decrease in receptor expression is due to an active removal of the receptors, or merely the failure to synthesize new receptors once IL-10 has been bound and the receptor/ligand complex has been internalized. Furthermore, this casts into possibility the question as to whether there is not increased IL-10 synthesis following glucocorticoid administration, but merely higher circulating levels due to a lack of available receptors. This would in turn cast further doubt on the possibility of IL-10 to act as an anti-inflammatory cytokine as it cannot bind receptors and thus produce its anti-inflammatory signaling cascade. For the sake of simplicity, we must now merely acknowledge that glucocorticoid administration increases IL-10 levels as part of an already anti-inflammatory process.

Unfortunately, there is a similar poor understanding regarding the effects of androstenediol on IL-10 levels. Work in rats treated with a sepsis model and a sepsis/trauma/hemorrhage (STH) model showed increases in IL-10 in the STH model that were reduced by the administration of IL-10 [110]. These results were demonstrated both in plasma levels of IL-10 and in IL-10 mRNA from liver biopsy. Thus, while trauma in humans produced a profound drop in IL-10 levels following only trauma and no significant difference in the sepsis only group [64], the rat model of sepsis only produced no significant increase while the trauma/hemorrhage and sepsis model produced increased levels that were reduced by IL-10. Therefore, when considering a rat model, we should expect traumatic injury to increase IL-10

levels. Treatment with methylprednisolone may increase IL-10 levels, while further treatment with androstenediol may reduce circulating levels of IL-10.

Materials and Methods

Specific Studies

Based on the previously illustrated hypothesis, background information, and mechanisms, the specific studies to be completed were designed. In order to assess the mechanisms behind the suppression of wound healing by corticosteroids and subsequent restorative features of androstenediol, we sought to conduct preliminary experiments to determine the ratios of wound size, glucocorticoid dose, and androstenediol dose that would provide the best illustration of the data. We thus began with groups of ten male Sprague Dawley rats six to eight weeks old and weighing between two-hundred twenty-five and two-hundred fifty grams. The animals in each group of six or ten were to be divided into animals receiving experimental treatments and those receiving the vehicle control only.

The animals were received into the University Department of Animal Resources and were allowed to acclimate for at least seven days prior to the initiation of the study. On the day before wounding (Day-1), animals were assessed for general health; the animals were then weighed and animals received either an injection of synthetic glucocorticoid or vehicle while under anesthetic using isoflurane in oxygen delivered via anesthetic chamber. Animals were then returned to housing. On the day of wounding (Day 0), animals were again weighed, then anesthetized using isoflurane in oxygen delivered via anesthetic chamber. The animals received prophylactic analgesia (0.1mg/kg buprenorphine, IP), then the dorsum of each animal was shaved and anesthesia depth confirmed by absence of

response to painful stimulus. The wounding site cleansed with iodophore and dried. Two circular full thickness wounds were induced on the shaved dorsum adjacent the spine. Experimental animals received either an injection of androstenediol, or vehicle only. In order to determine the effects of the experimental applications, animals were assessed at time points specific to each group and their expected response. During these periodic assessments, animals were again weighed and photographed for wound image data measurements. On further specific time points, blood was drawn from the tail vein into a syringe pre-loaded with EDTA for use in several assays, including ELISA, Multiplex Analysis, FACS Analysis, and VetScan measurements. During all of these periodic assessments and blood draws, animals were anesthetized using isoflurane in oxygen delivered via anesthetic chamber and assessed for response to painful stimuli. On day 21, the animals were sacrificed by Euthasol injection done under isoflurane anesthesia. Prior to euthanasia and when the animals are under deep anesthesia, blood was drawn from the tail vein or by direct cardiac puncture.

Study Groups

The following study groups represent a selection of approximately 25% of the animal groups studied during the course of this research project. Groups listed in this document were selected to provide a sense of the timeline and strategies employed to generate the desired results within the parameters of the research protocol. While the general approach to each group was similar, changes were made in the dose of glucocorticoids, type of glucocorticoids, dose of healing promoter (AED or AET), size of wound, vehicles and/or injection sites (Table 1).

Depomedrol/AED Group Beginning 248376A

In early July 2009, we were well into the study groups employed to determine the parameters of the larger experiment. The group beginning 248376A was indicative of the previous groups and represented a baseline of the procedures and compounds used throughout the experiment. The animals were received and acclimated as illustrated in the previous sections. On Day-1, each animal was weighed, and received an IM injection of 12mg methylprednisolone. On Day 0, each animal received two 6mm full-thickness wounds, taken by 6mm biopsy punch, on either side of the spine, in the upper dorsal aspect. Control animals then received vehicle, consisting of a mixture of DMSO and ethanol in two subcutaneous injection sites near the wound sites of the animal. Androstenediol (AED) treatment animals received the DMSO/ethanol, which then also contained AED at a dose of .25mg/kg in the same location as the control animals. Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 3, 6, and 9. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Depomedrol/AET Group Beginning 248680

The immediately following group was designated to begin 248680. The animals were received and acclimated as illustrated in the previous sections. Following several weeks of experiments to determine the parameters of the master study using AED, we hypothesized that perhaps the rats would not respond as well to the AED treatment as the mice from the previous experiment might. Thus we decided to try a course of the alternative derivative, *androstenediol*. In addition, we

had found that the combination of DMSO and ethanol were producing epidermal ulcerations at the site of injection. Thus, in this group we also elected to try beta-cyclodextrin as the delivery vehicle. On Day-1, each animal was weighed, and received an IM injection of 12mg methylprednisolone. On Day 0, each animal received two 6mm full-thickness wounds, taken by 6mm biopsy punch, on either side of the spine, in the upper dorsal aspect. Control animals then received vehicle, consisting of beta-cyclodextrin in two subcutaneous injection sites near the wound sites of the animal. *Androstenetriol* (AET) treatment animals received the beta-cyclodextrin, which then also contained AET at a dose of .25mg/kg in the same location as the control animals. Following Day 0, animals then received either a vehicle or AET injection consistent with their group on days 3, 6, and 9. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Depomedrol/AED Group Beginning 252251

Immediately following our foray into the AET trial, we returned to experimenting with AED. However, in this group, we decided to try to increase the administration of AED by injecting at additional sites. In addition, we decided that additional trauma may upregulate the immune response and may thus increase the response of the immune system to AED. The animals were received and acclimated as illustrated in the previous sections. On Day-1, each animal was weighed, and received an IM injection of 12mg methylprednisolone. On Day 0, each animal received two 12mm full-thickness wounds, taken by measurement and hand-cut, on either side of the spine, in the upper dorsal aspect. Control animals then received

vehicle, consisting of beta-cyclodextrin in four subcutaneous injection sites near the wound sites of the animal. Androstenediol (AED) treatment animals received the beta-cyclodextrin, which then also contained AED at a dose of .25mg per injection site in the same location as the control animals (for a total injection of 1mg AED). Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 3 and 9. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Depomedrol/AED Group Beginning 252968

In September of 2009, we were continuing with AED while we also proceeded with modulating other parameters of the experiment. At this time, we felt that additional trauma may further the presentation of the desired results. Thus, the animals were received and acclimated as illustrated in the previous sections. On Day-1, each animal was weighed, and received an IM injection of 12mg methylprednisolone. On Day 0, each animal received two 14mm full-thickness wounds, taken by measurement and hand-cut, on either side of the spine, in the upper dorsal aspect. Control animals then received vehicle, consisting of beta-cyclodextrin in a single intraperitoneal injection site. androstenediol (AED) treatment animals received 3ml of beta-cyclodextrin containing AED at the single intraperitoneal injection site as well. Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 3, 6, 9 and 16. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Depomedrol/AED Group Beginning 254682

In October of 2009, we were concerned about the concentration of AED in the beta-cyclodextrin. In addition, we were unconvinced that the frequency of AED doses were sufficient to provide the biochemical momentum required to facilitate the desired results. Thus in this groups we decided to increase the frequency of injection. The animals were received and acclimated as illustrated in the previous sections. On Day-1, each animal was weighed, and received an IM injection of 12mg methylprednisolone. On Day 0, each animal received two 14mm full-thickness wounds, taken by measurement and hand-cut, on either side of the spine, in the upper dorsal aspect. Control animals then received vehicle, consisting of beta-cyclodextrin in four subcutaneous injection sites near the wound sites of the animal. Androstenediol (AED) treatment animals received 2ml of AED in beta-cyclodextrin in the same locations as the control animals. Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 1 through 9. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Depomedrol/AED Group Beginning 258339

In November of 2009, we continued with our hypothesis that additional trauma would enhance the difference in wound healing between the vehicle and AED groups. In addition, we decided that less inhibition of the immune system might make the results more apparent. The animals were received and acclimated as illustrated in the previous sections. On Day-1, each animal was weighed, and received an IM injection of 8mg methylprednisolone. On Day 0, each animal

received two 14mm full-thickness wounds, taken by measurement and hand-cut, on either side of the spine, in the upper dorsal aspect. In addition, each animal received a laparotomy 4cm in length. Control animals then received vehicle, consisting of beta-cyclodextrin in a tail vein injection. androstenediol (AED) treatment animals received .3ml of AED in beta-cyclodextrin, also in the tail vein. Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 3 through 9. The animals were sampled for blood during the course of the experiment via tail vein and were terminated on Day 21.

Cortisone/AED Group Beginning 261328

January of 2010, we had concerns that the profound immunosuppression initiated by methylprednisolone could not be overcome in this particular animal model. In addition, questions as to the true dose of AED in the cyclodextrin vehicle continued to perplex. Thus we tried an alternative glucocorticoid, cortisone, and returned to the original vehicle DMSO and ethanol. On Day-1, each animal was weighed, and received an IM injection of 48mg/kg cortisone. On Day 0, each animal received two 14mm full-thickness wounds, taken by measurement and hand-cut, on either side of the spine, in the upper dorsal aspect. In addition, each animal received a laparotomy 4cm in length. Control animals then received vehicle, consisting of DMSO/ethanol in two subcutaneous injection sites near the wounds.

Androstenediol (AED) treatment animals received .4ml of AED in DMSO/ethanol, also near the wounding sites. Following Day 0, animals then received either a vehicle or AED injection consistent with their group on days 3, 6, and 9. The animals

were sampled for blood during the course of the experiment via tail vein and were terminated on Day 9.

Group	Wound	Steroid	Dose	Mediator	Dose	Vehicle	Sites
248376A	2x6mm	Depo	12mg	AED	24mg	DMSO/ETOH	2xSC
248680	2x6mm	Depo	12mg	AET	24mg	DMSO/ETOH	2xSC
252251	2x12mm	Depo	12mg	AED	16mg	Cyclodextrin	4xSC
252968	2x14mm	Depo	12mg	AED	30mg	Cyclodextrin	1xIP
258339	2x14mm +Lap	Depo	8mg	AED	3mg	Cyclodextrin	1xIV
261328	2x14mm +Lap	Cort.	48mg/kg	AED	32mg	DMSO/ETOH	2xSC

Table 1: Animal Groups and Treatments

Sample Preparation

The analysis of blood samples was critical to the investigation into the mechanisms employed by the drug in question, and second only in importance to the wound sizes during the healing process. Blood samples were taken by tail vein under inhaled anesthesia at various, specified time points. The samples were taken for either use in a single assay, or more commonly, for use in several assays in tandem. In some cases, the samples were divided into groups to undergo individual analysis at later time points, in other cases; a single sample was subject to more than one single assay immediately following harvest. For example, a single blood sample could be assayed by VetScan for total cell counts; since the VetScan technique removes only a small quantity of blood from the sample tube without contaminating the rest, the same sample could then be centrifuged to separate blood plasma from red blood cells. These populations could then be separated via pipette

for further analysis (ELISA and FACS). The sample preparation for each of the specific assays follows.

VetScan

Animals to be sampled were placed under anesthetic by isoflurane in oxygen via anesthetic chamber. Animals were determined to be insensitive to pain and were transferred to a heated pad to maintain body temperature during the procedure. The tail was cleaned with alcohol preparation and blood was accessed in the tail vein via aseptic technique. Samples of at least 150 μ l were collected from the tail vein and deposited in a microtubule pre-loaded with the anticoagulant EDTA. In the instance that the blood sample was to serve both VetScan and ELISA samples, with or without FACS analysis, sample volume was about 600 μ l. Samples were not refrigerated but were kept at room temperature until analysis, usually immediately after the last sample was taken. Sample tubes were loaded into the VetScan machine. The instruments were programmed to read the blood sample according to the species, and to characterize the blood cells. The VetScan HM2 is a self contained, fully automated tool for the complete blood count of cells in whole blood. The VetScan2 measures White Blood Cells and their subpopulations by volumetric impedance. Following dilution of 1:32,000 with two different forms of diluent, electrically neutral blood cells pass through an electrically charged aperture, applying a minimal charge to each cell. Each cell accepts a charge in a manner directly proportional to its size. Diluted blood is then forced through yet another aperture, large enough to accommodate only one cell at a time. Charges are

measured at this aperture and according to the size of the charge, lymphocytes, monocytes, and granulocytes are distinguished from one another.

ELISA

Blood was taken as illustrated above for VetScan. In the instance in which blood was to serve only for ELISA and/or FACS analysis, only 500µl was taken. Microtubes containing whole blood in EDTA were loaded into a centrifuge and centrifuged to separate the intact cells from proteins suspended in the blood plasma. The supernatant from the microtubes was pipetted off above the buffy coat, and was loaded into between one and three fresh, sterile microtubes, depending on the quantity of blood plasma available. The microtubes were labeled with the subject number and date, and were then flash-frozen in liquid nitrogen. Frozen samples were loaded into a -80°C freezer for later use. At the time of assay, samples were removed from the freezer, and placed in ice at room temperature. R&D ELISA assay kits were used for all protein characterization. Kits used were in date and sealed prior to use. Kits were opened and inspected to ensure the stability and quality of the individual reagents. According to the directions provided with the kit, the reagents and assay plate were prepared for introduction of the samples. Samples were processed according to the instructions provided and assayed at the completion of the incubation steps.

Wound Image Data Analysis

In order to accurately characterize the healing process between different treatment groups, our lab sought to employ the use of wound planimetry to provide

an objective, unbiased quantitative measure of the size of the wounds. Animal subjects were photographed using a digital camera at predefined time-points. At the time of photography, the animals were sedated using inhaled anesthetics. Animals were placed on a level, well-lit surface, in a prone position, and were photographed by the digital camera placed at a fixed, predetermined height. Wounds were measured using the Image J software program as available from the National Institutes of Health. Photos were opened in Image J, the scale was set in the program by drawing a line on the scale in the photograph and then setting the photo scale to match the photo. Wounds were then traced by tracing the outline of the open wound. If no open wound was visible due to scabbing, the outline of the scab was traced. Typically, once the scab fell off, there was a center area of open wound, surrounded by intact tissue in the late stages of healing, then surrounded by undisturbed tissue. In all cases where it could be visualized, only the open wound was measured. Wound that had closed completely were not measured, and no tracing measurement was generated. These closed wounds were typically characterized by pink tissue in the outline of the former open wound, surrounded by white, undisturbed tissue. The tracing and area calculations were displayed in a quantitative data summary, which was then overlaid across the bottom region of the photo. Once the wounds were measured and data displayed, a screen shot was then taken and saved into Adobe Acrobat PDF file format for data entry and storage.

Delivery Methods

Vehicles

During the course of these experiments, it became clear that the vehicle used to deliver androstenediol to the animals and their circulation were compromised by complications including damage to the animal model, aggregation of the target drug in vivo, aggregation of the target drug in the delivery instrument, or a simple failure of the vehicle to deliver biologically significant levels of the target drug to the animal model. Below are the profile and rational for selecting the various vehicles.

DMSO and Ethanol

Commonly employed for the delivery of compounds not soluble in polar solvent, DMSO/Ethanol was employed as the first vehicle. We found that the solvent was superior in providing a completely dissolved mixture of AED that delivered completely into tissue. However, despite the documented anti-inflammatory properties of both DMSO and ethanol [78], we found that the solvent induced ulcers in the skin at the site of delivery. This was unexpected as other animal models have employed this vehicle without similar side effects. As the solvent has not only the propensity to modulate the very system we sought to monitor, but also induced additional wounding, we sought out alternate methods of delivering the drugs.

Cremophor EL

Yet another widely popular solvent for the delivery of non-polar compounds has been Cremophor. Popular because it has the ability to solubilize non-polar

compounds in an aqueous solution, we had hopes that this would solve our delivery problems. However, as Cremophor is best known for stabilizing emulsions, we found that we had a compound that would not pass through the needle size necessary to introduce the mixture to the model. Furthermore, we also found that it was extremely difficult to dissolve in the necessary concentration of AED. Also, due to the opaque nature of Cremophor, it was impossible to tell when the target drug had become fully suspended in the vehicle.

Beta-cyclodextrin

Our most recent foray into the search for a suitable delivery vehicle brought to our attention the oligosaccharide beta-cyclodextrins. Beta-cyclodextrins are currently used in pharma, food products, and other chemical applications. Given their unique cage-like structure, it was hypothesized that we could load β -cyclodextrin with molecules of AED, which would then be protected from an aqueous solution and thus protected from precipitation. While there has been some difficulty in determining how much AED exists in the beta-cyclodextrin and how much becomes biologically available, this currently remains our best option in the rat model.

Injection Sites

Due to the various methods approached to deliver androstenediol and our efforts to provide the best route for a biologically active drug, we experimented with several delivery routes. Several of these were mandated by the fact that some vehicles could carry only a small concentration of AED and in order to deliver the

desired amount, we were forced to increase the volume of the total infusion.

Obviously, there are limits to volume that can be introduced under the skin that may not have implications elsewhere. This section details the advantages and disadvantages of each approach in our work.

Intramuscular

Due to the highly vascular nature of muscle tissue, intramuscular injections are commonly applied in both human and veterinary medicine. The sites are generally considered to accept a greater volume than subcutaneous sites, although perhaps less volume than an intraperitoneal injection. In addition, human studies of some compounds have illustrated that intramuscular injections lead to higher peak plasma concentrations, and thus better availability [117]. Conversely, there seem to be fewer complications in the intramuscular site injection than in the intraperitoneal site, though perhaps more risk than that associated with the subcutaneous injection. As several large muscle groups often serve as targets for intramuscular injections, there exists the risk associated with striking or damaging one of the large nerve tracts often associated with the larger muscle groups. In addition, the use of intramuscular injection sites reduces the ability to assess the aggregation of target drug in the animal model, as we saw somewhat frequently in our experiments.

Intravenous

The intravenous delivery of drug presents some of the greatest advantages as well as some of the greatest challenges to our work. The site allows for the delivery

of a significant volume without compromising the health of the model as well as provides the most direct method for guaranteeing the provision of a biologically significant amount of the target drug. In addition, as has been suggested in the use of androstenediol as well as others, this route provides the most direct and diverse exposure for the metabolism that may be required for full biological activity.

Conversely, the technical aspects of securing a patent vein in an anesthetized animal and frequency of drug delivery provide challenges. Inhaled anesthetics are well known to induce vasoconstriction (sometimes intentionally) thus reducing blood flow to the extremities such as a tail vein and making such injection sites difficult to cannulate at best. Also, the repeated introduction of a needle into the rodent tail vein as sometimes required by the volume of drug or by experimental parameters will cause repeated trauma to the extremity, making later venous access more difficult in addition to inducing further trauma and affecting the immune system that is being measured.

Intraperitoneal

Like the other methods, the intraperitoneal injection site has both advantages as well as serious risks. This site presents perhaps the best opportunity to introduce large volumes of drug and vehicle into the animal without risking harm. In addition, if the activity of AED is indeed mediated by activation in the lymphatic system, the intraperitoneal site offers widespread access to the lymph nodes of the abdominal cavity and surrounding structures such as the spleen. Also, the intraperitoneal site also offered us the opportunity to introduce a larger needle gauge, as there are fewer compact structures as in muscle tissue and more room to

work than at a subcutaneous site. This allowed us to mediate some of the vehicle issues that we were challenged with. However, the risks of injection so close to structures prone to induce sepsis or other life-threatening illness in a small animal present their own challenges. The opportunity to lacerate or otherwise damage abdominal organs make this a risky procedure. In addition, the risk of drug aggregation or encapsulation resulting in a massive immune response to the abdominal cavity presents additional risk. While some of these challenges and risks do exist to a smaller extent at other injection sites, mistakes or missteps elsewhere result in death less frequently. However, this is a commonly used technique, and if performed carefully, and with some experience, can be used successfully.

Subcutaneous

The subcutaneous injection site represented for us the most convenient site for introducing drug and vehicle, the opportunity to monitor the external and immediately internal effects of the injection, and to minimize the complications involved with some of the other sites and techniques. However, this convenience came at the price of limiting the size of the bolus that could be introduced beneath the skin, thus limiting the amount of drug that could be delivered at one injection site. In addition, the subcutaneous injection requires a nominal needle size, placing additional pressure on the vehicle system used to apply the compound and the extent to which it is soluble and able to pass through the needle without aggregation. In addition, the location brings into question whether the drug will be encapsulated as was seen with some vehicles, and whether the drug will be offered the proper opportunity for full activation and widespread biological availability.

Results

As was described in the Specific Studies section with regard to the different experimental groups, the following represents the results of the temporal and experimental continuum as we sought to determine the parameters to be used in the larger master study. Thus contained in each subsequent group are different variables such as drugs, dosages, vehicle, injection sites, and/or types and amount of trauma. Included in the data are the wound healing data, the information on changes in cell count, and where available, quantification of specific protein data.

Depomedrol/AED Group Beginning 248376A

The rat group beginning 248376A was measured and given Day-1 treatment on July 6, 2009. During the treatment course, blood samples were taken on days -1, 3, 6, 9, and day 21, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. VetScan data illustrated changes in white blood cells that indicated that the AED group held higher counts, though not always outside the overlap created by standard deviation (Figure 6). While this appears promising, the changes relative to Day-1 data indicate that the total white blood cell counts dropped in both groups, although less in the AED group.

As the predominant white blood cell in these count are the lymphocytes, it should come as no surprise that the lymphocyte counts reflect the same trend (Figure 7). Interestingly, the monocyte and neutrophil counts show data consistent with changes induced by AED, however with opposite trends. Monocytes remain at the same or similar levels as the control groups through Day 9, but begin to separate

thereafter, perhaps reflecting some synergistic effects of both the AED and immune system (Figure 8). When compared to the Day -1 values, the monocyte counts rebound from the initial administration of methylprednisolone (Figure 9). These counts then decline to levels below Day-1 in the vehicle group, while in the AED group they persist at levels at least three times baseline Day -1 levels. While these levels are systemic, they may indicate enhanced healing.

Conversely, the neutrophil counts illustrated by the VetScan granulocyte assay also indicate an initial reduction from baseline levels (Day-1), which then rebound (Figure 10). This rebound is more robust in the AED group and persists at levels higher than the vehicle group throughout the course of the experiment. Despite the perceived benefits of AED on the monocyte counts and the granulocyte counts, and their known beneficial effects on the wound healing process, measurements of wound sizes during the course of the experiment failed to yield any clear benefits.

The trend lines indicate that the greatest difference in wound size exists around time point Day 13 (Figure 11). This may correlate with the early rebound levels in monocyte and granulocyte counts when compared to the vehicle group. However, the replication of the data from earlier experiments that showed complete closure in the AED group while control groups remained open, failed to materialize.

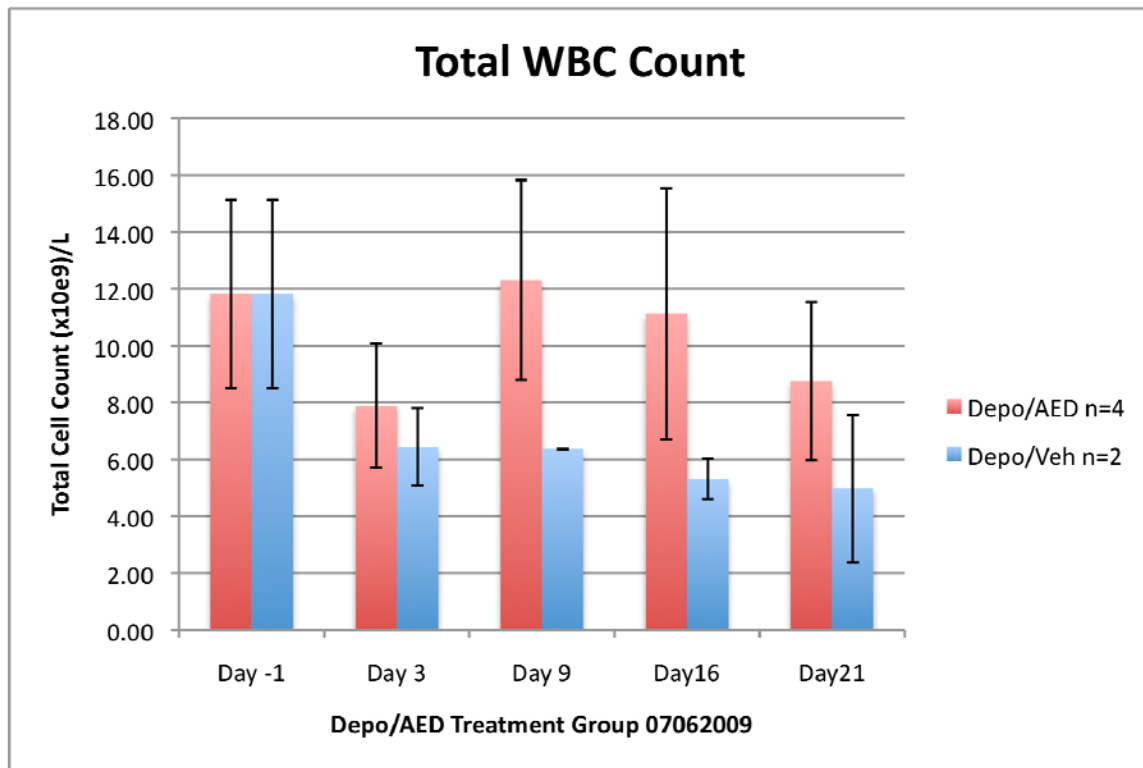


Figure 6: Total White Blood Cell Counts for Rat Group Beginning 248376A

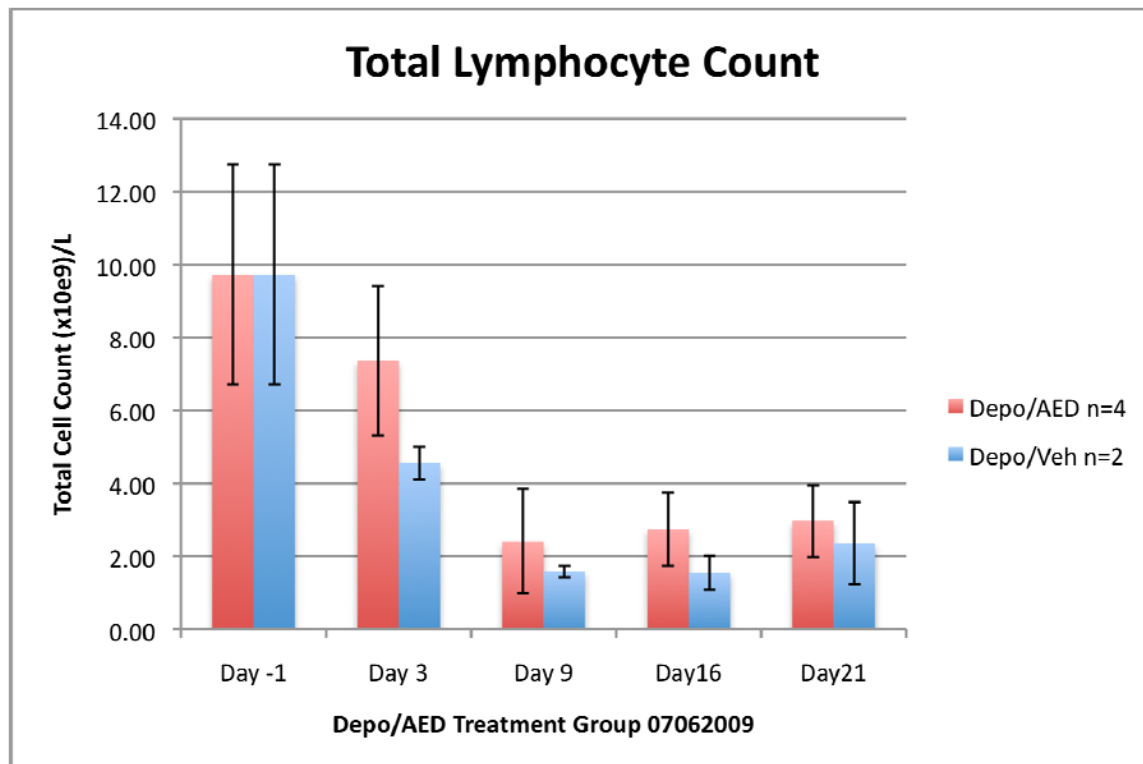


Figure 7: Total Lymphocyte Counts for Rat Group Beginning 248376A

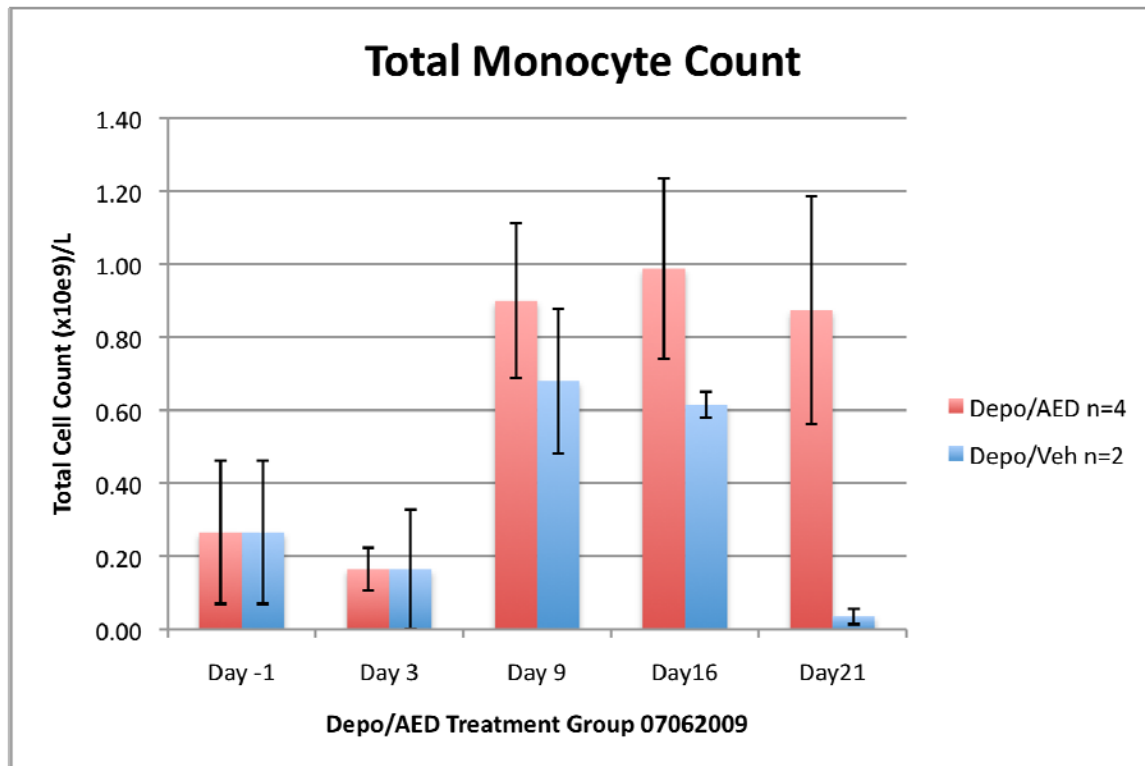


Figure 8: Total Monocyte Counts for Rat Group Beginning 248376A

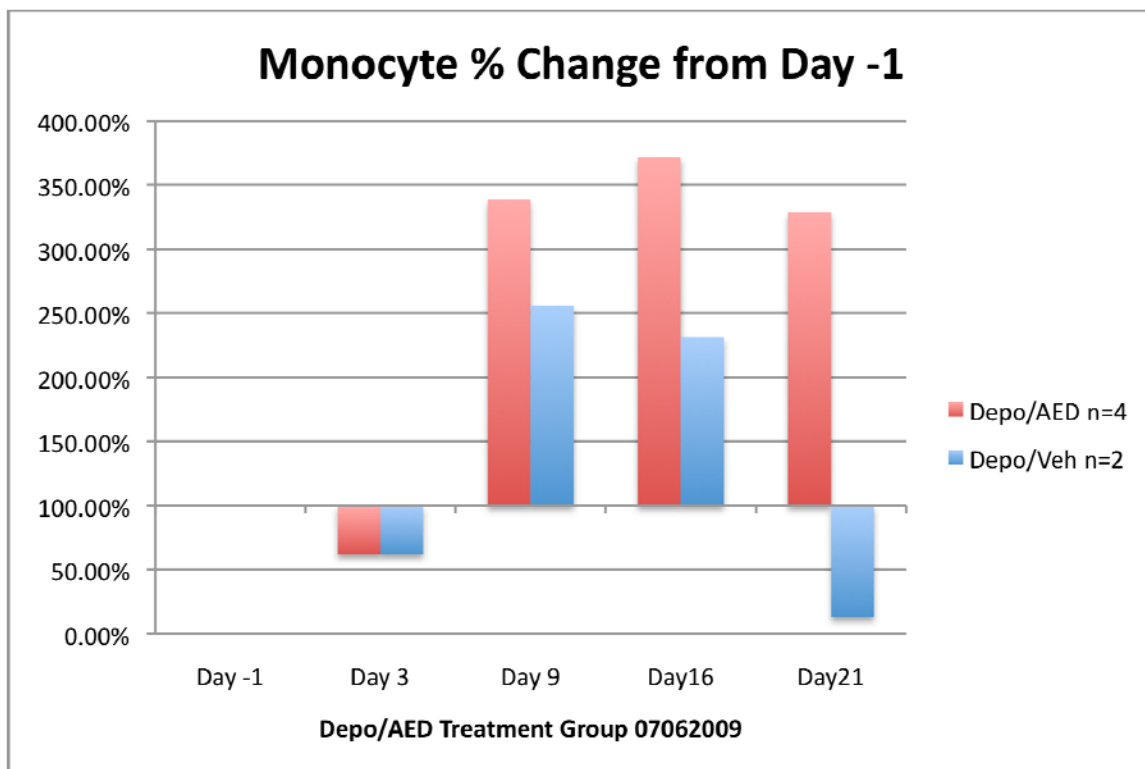


Figure 9: Changes in Monocyte Count from Day-1 Average in Rat Group Beginning 248376A

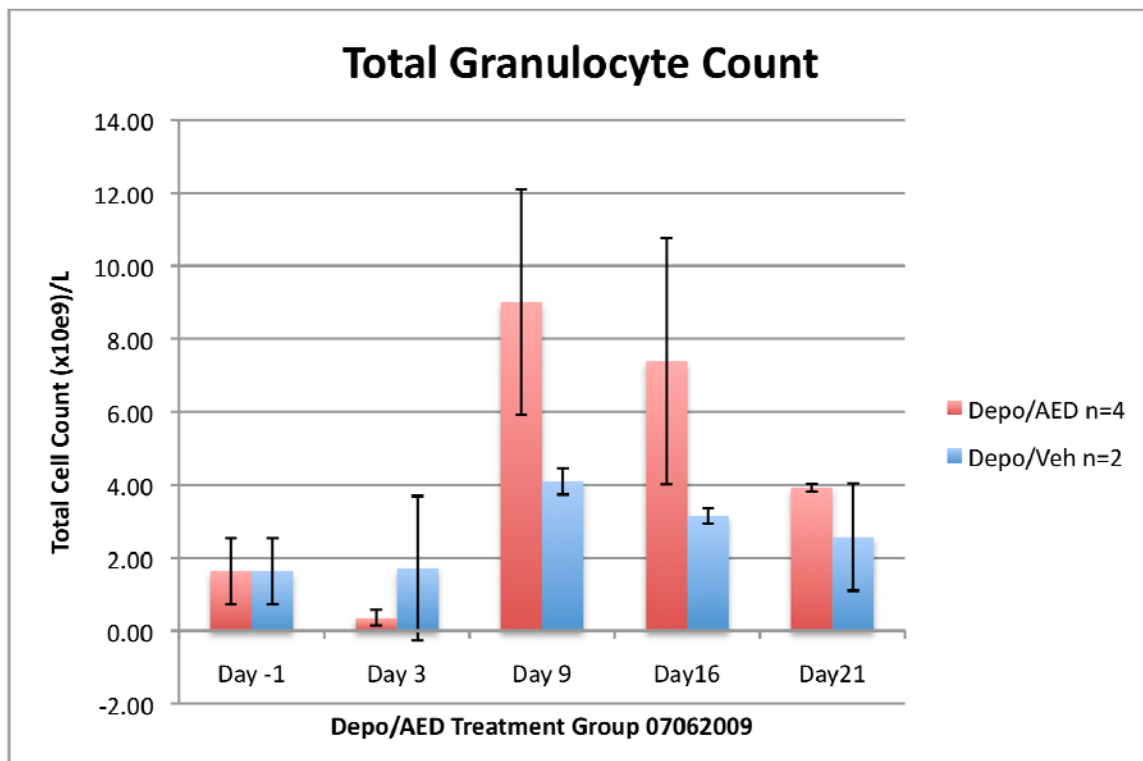


Figure 10: Total Granulocyte Counts for Rat Group Beginning 248376A

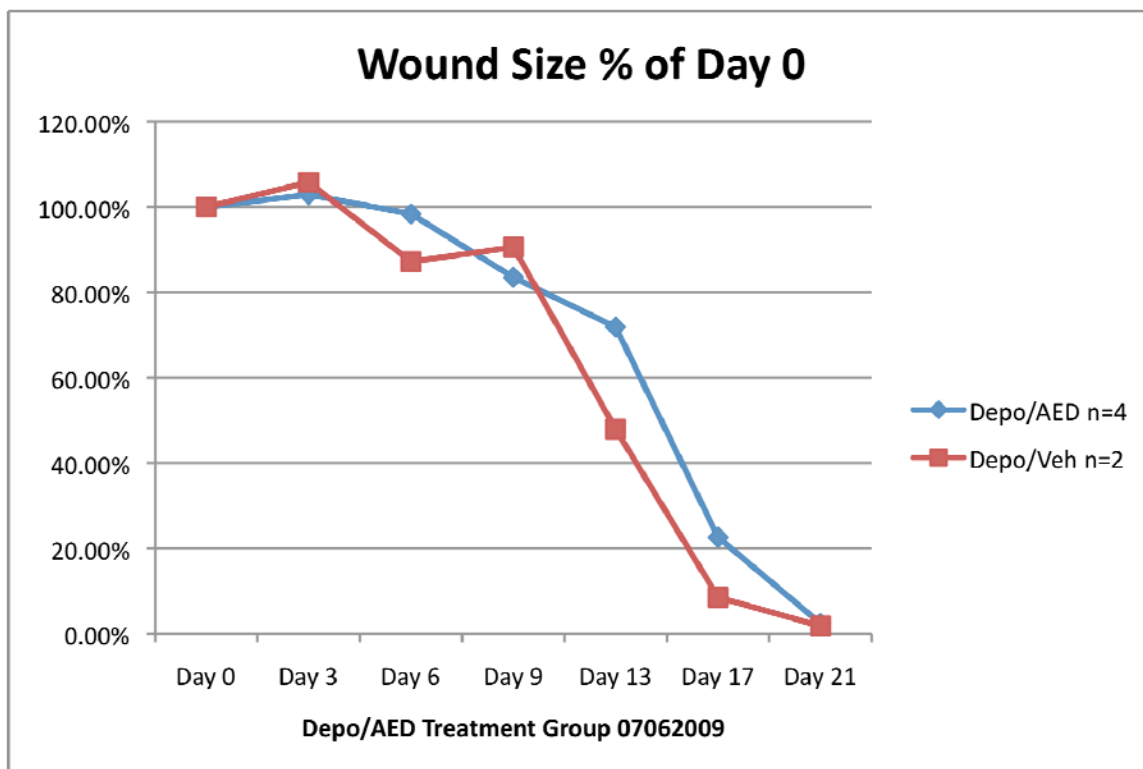


Figure 11: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 248376A

Depomedrol/AET Group Beginning 248680

The rat group beginning 248680 was measured and given Day-1 treatment on July 13, 2009. During the treatment course, blood samples were taken on days -1, 3, 9, and day 16, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. Total White Blood Cell counts in this group were less impressive than with the previous group, with measurements showing both similar total levels and similar trending in the AET group when compared to the vehicle group (Figure 12). This trend was also reflected when comparing the levels against the Day-1 levels, reflecting drops in both groups (Figure 13).

Thus the anticipated responses in lymphocyte counts were also seen. Total lymphocyte count was lowered dramatically (Figure 14), as well as was the counts when compared to Day-1 baseline levels. The trends when comparing the AET animals against the vehicle animals were indistinguishable. Monocytes, however, gave the impression that some difference was perceptible between groups. When comparing absolute monocyte counts, very little difference between groups was apparent, less even when considering the standard deviation between values (Figure 15). However, when examining the values against the Day-1 baseline values, there was a nearly 50% difference in favor of the AET animals, perhaps falsely encouraging given the lack of standard deviation in a measurement of percentage. The granulocyte counts provided no clear direction and indicated conflicting trends in both groups in measuring both total counts and levels against the Day-1 baseline values (Figure 16).

Despite any hope offered by the minor differences illustrated by the major mediators of fibroblast activity (monocytes), wound size data indicated no difference between groups, with day-to-day wound measurements and final closure nearly identical (Figure 17).

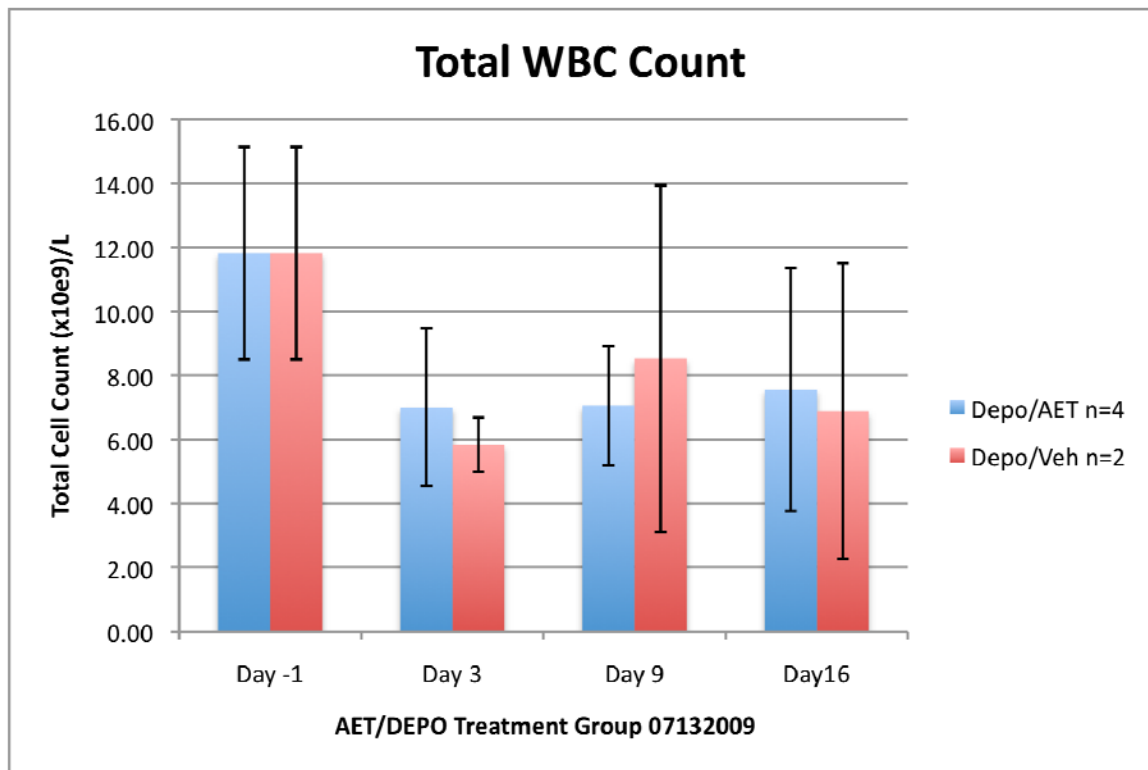


Figure 12: Total White Blood Cell Counts for Rat Group Beginning 248680

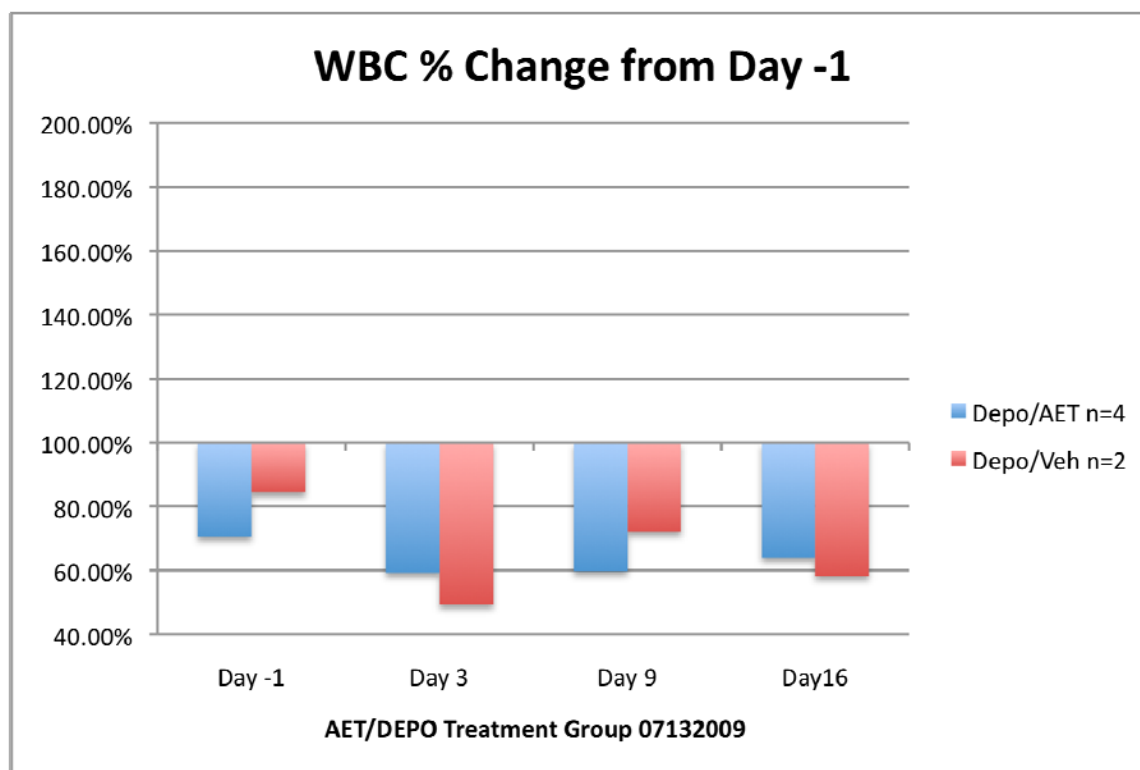


Figure 13: Changes in White Blood Cell Count from Day-1 Average in Rat Group Beginning 248680

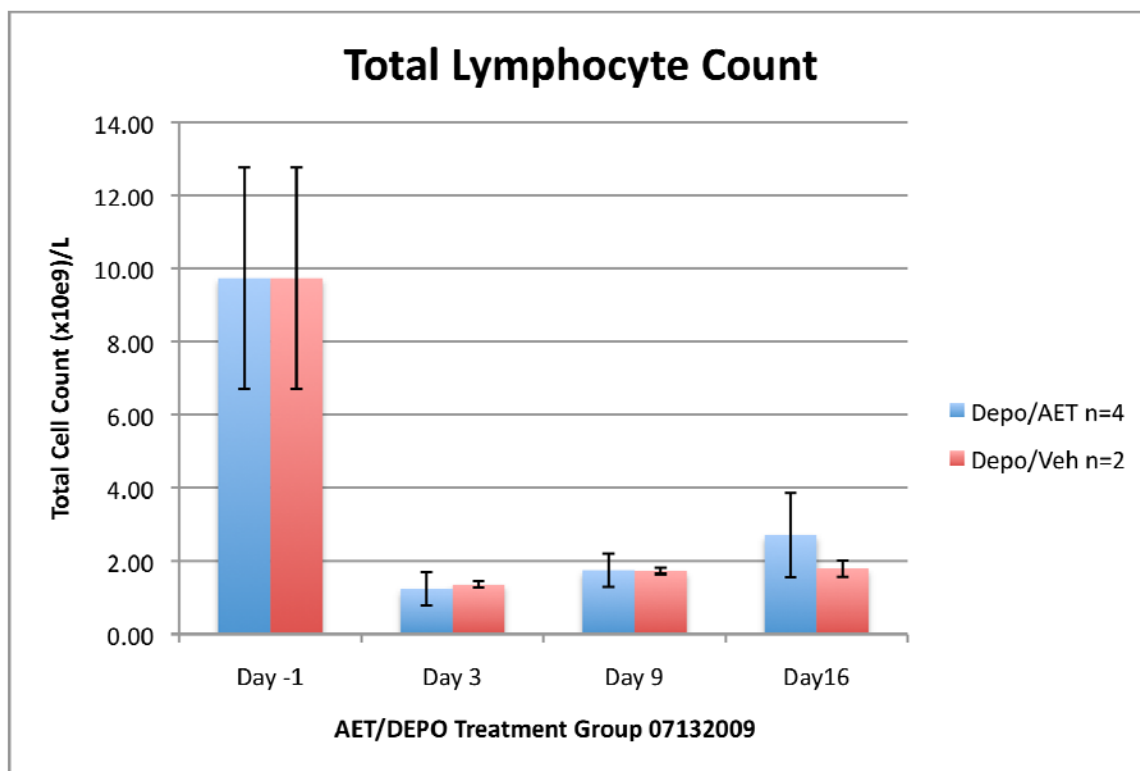


Figure 14: Total Lymphocyte Counts for Rat Group Beginning 248680

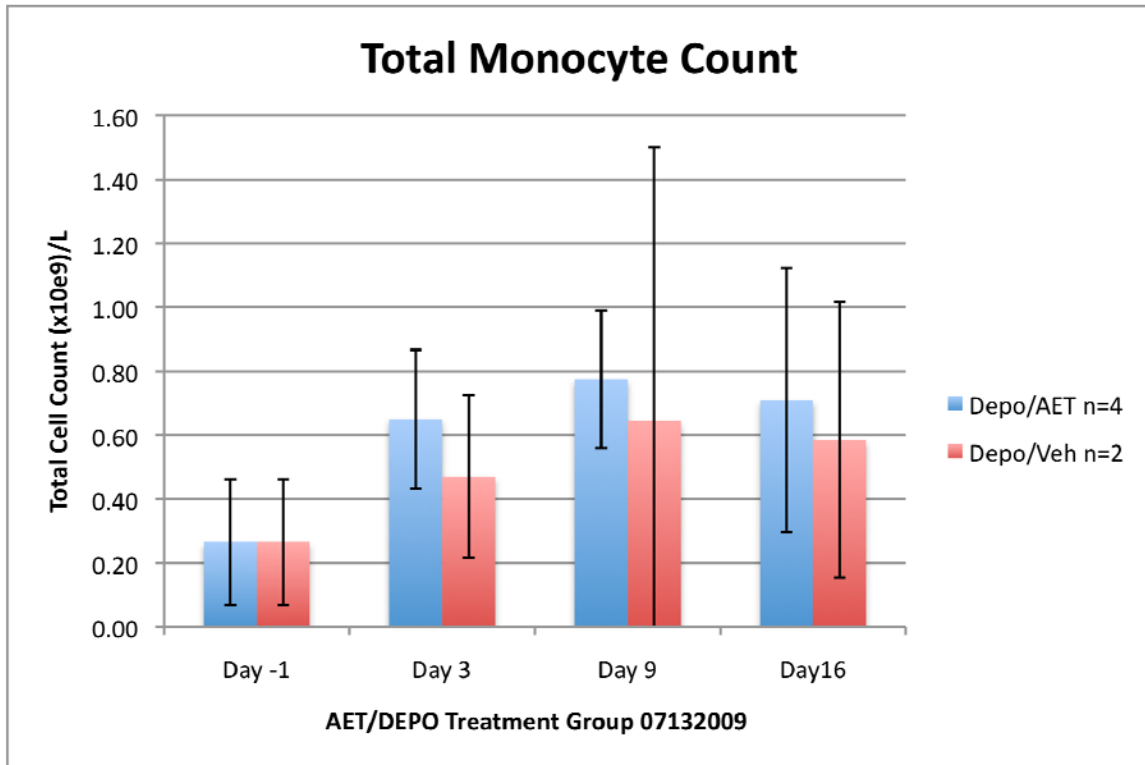


Figure 15: Total Monocyte Counts for Rat Group Beginning 248680

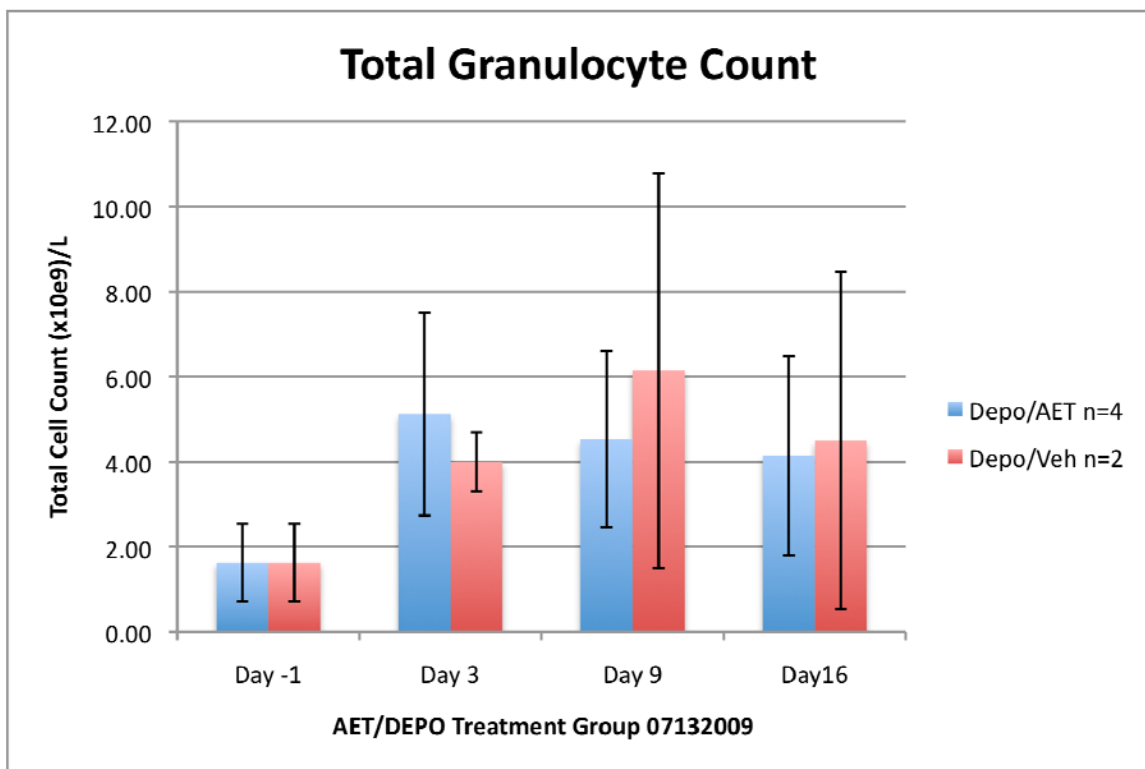


Figure 16: Total Granulocyte Counts for Rat Group Beginning 248680

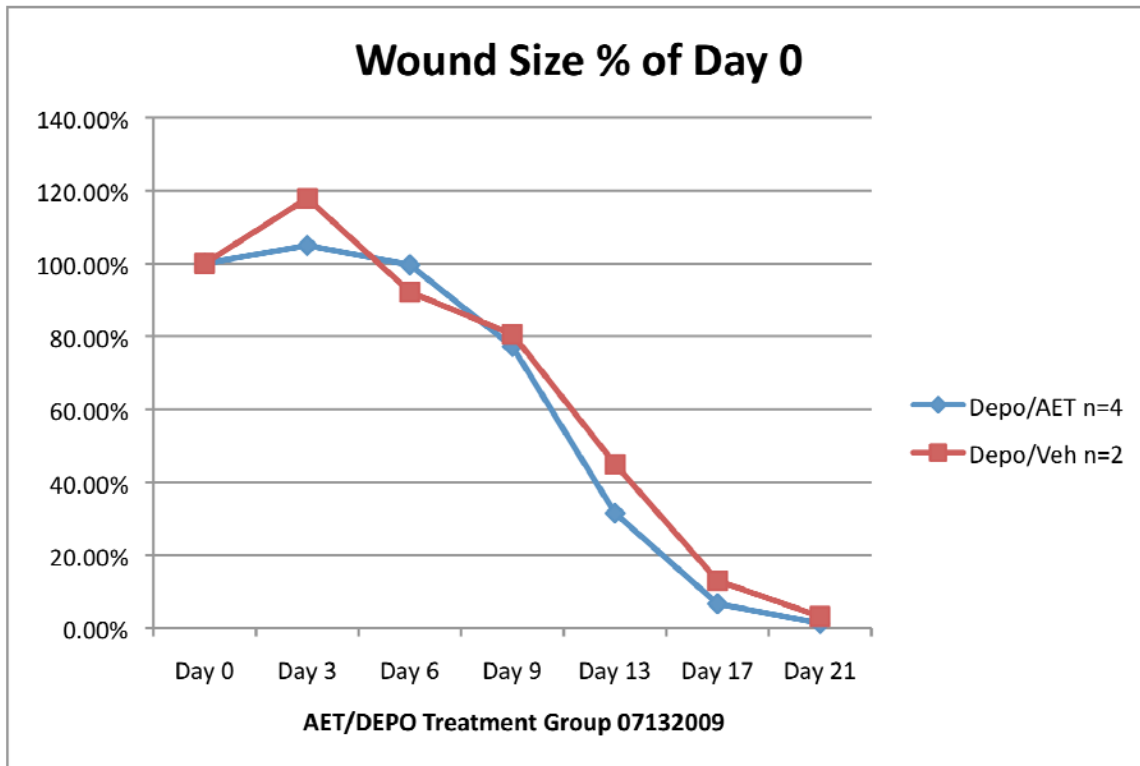


Figure 17: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 248680

Depomedrol/AED Group Beginning 252251

The rat group beginning 252251 was measured and given Day-1 treatment on August 31, 2009. During the treatment course, blood samples were taken on days -1, 3, 9, and day 21, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. The white blood cell count data from this group was extremely variable, with no perceptible trends shown in the absolute cell counting (Figure 18). White blood cell count data referenced to the Day-1 baseline values indicate an immediate drop in counts, with a recovery to near baseline in the *vehicle* group, before a return to nearly identical values (Figure 19).

While usually reflective of the total white blood cell counts, the lymphocyte counts were significantly different, showing none of the modulation that was

indicated in the larger WBC populations. Absolute lymphocyte counts were dropped immediately from Day-1 baseline values, and showed no recovery or difference between groups (Figure 20). Similarly, values referenced back to the baseline values showed no difference between groups (Figure 21). Conversely, the monocyte and granulocyte groups seemed to provide any momentum for the changes seen in the larger WBC population. With regard to the monocytes, Day 9 showed significant increases in systemic counts in both groups (Figure 22). Absolute values showed no significant difference in the values between the AED and vehicle groups, while the percentage values compared to Day-1 baseline give the impression of a difference. Similarly, the granulocyte counts also showed a significant systemic mobilization of granulocytes on Day 9, although no difference was seen between the groups (Figure 23). Values referenced to the Day-1 count indicate an increase in Day 9 among the vehicle animals above those counts in the AED animals, but the deviation among the absolute values seem to discount that conclusion.

In evaluating the wound size data, the effects of the increased monocyte and granulocyte counts seem to be at odds with the better healing of the AED group at Days 3 through 13, especially when considering that the vehicle groups had *slightly* higher values (Figure 24). This of course could have alternative meaning to be discussed later.

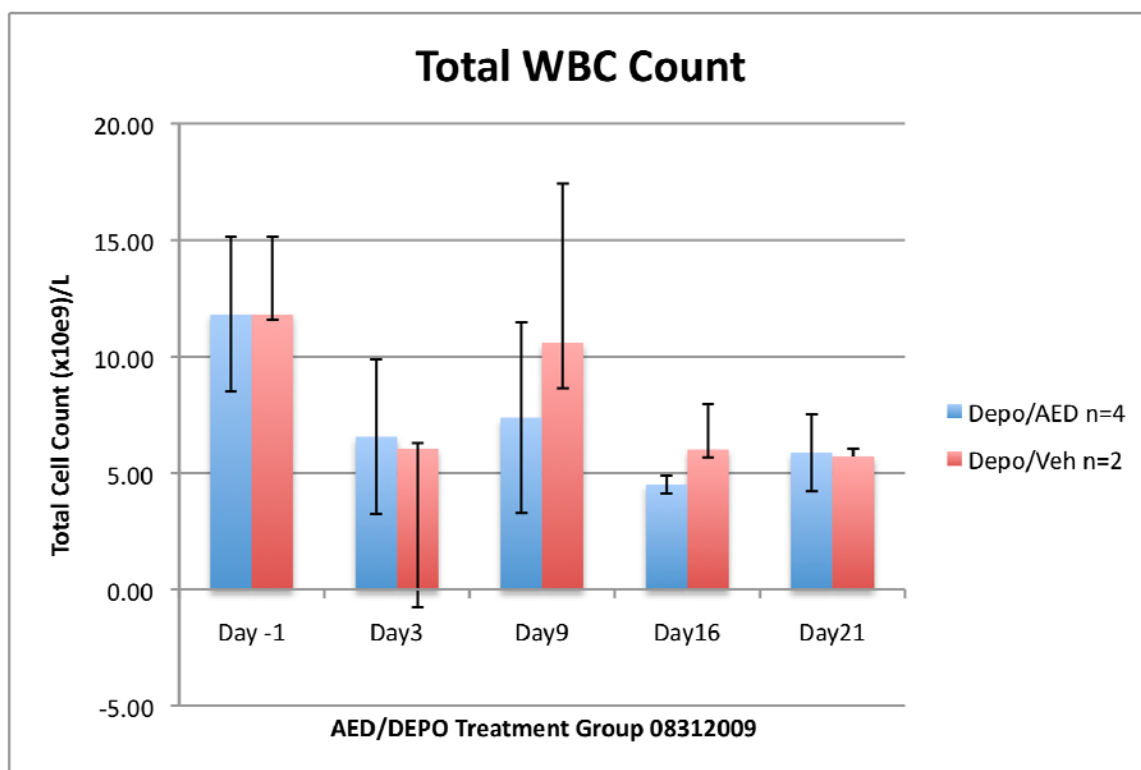


Figure 18: Total White Blood Cell Counts for Rat Group Beginning 252251

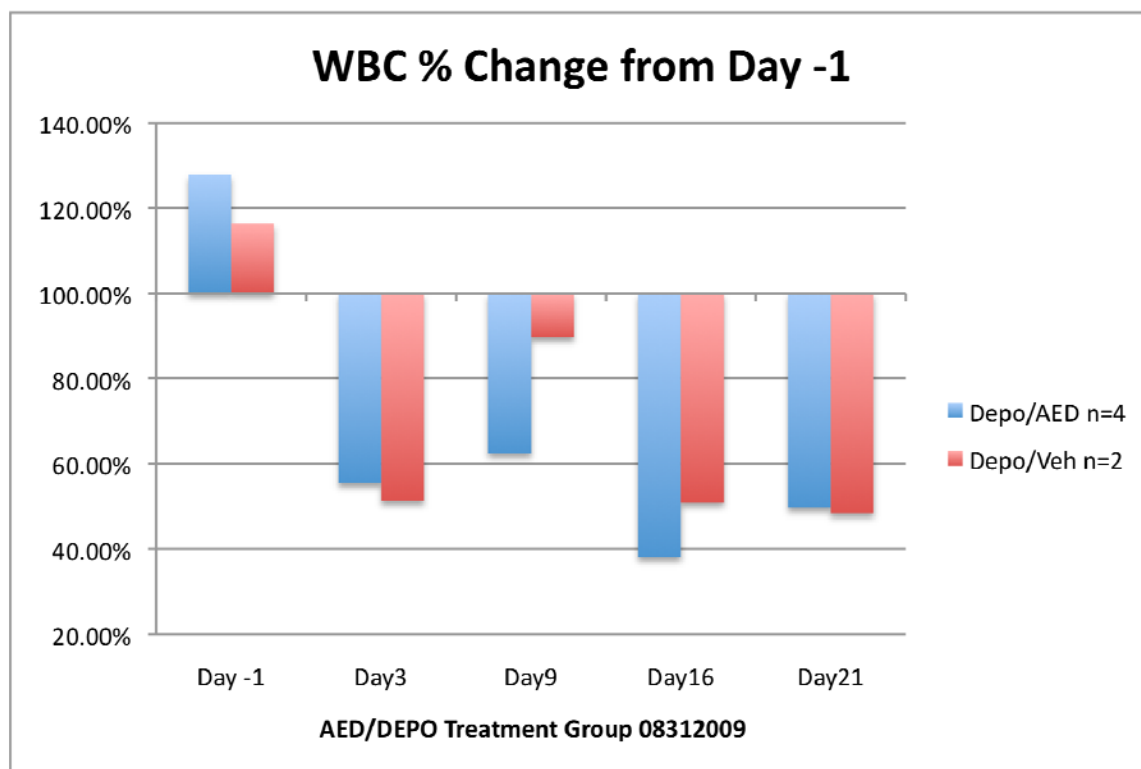


Figure 19: Changes in White Blood Cell Count from Day-1 Average in Rat Group Beginning 252251

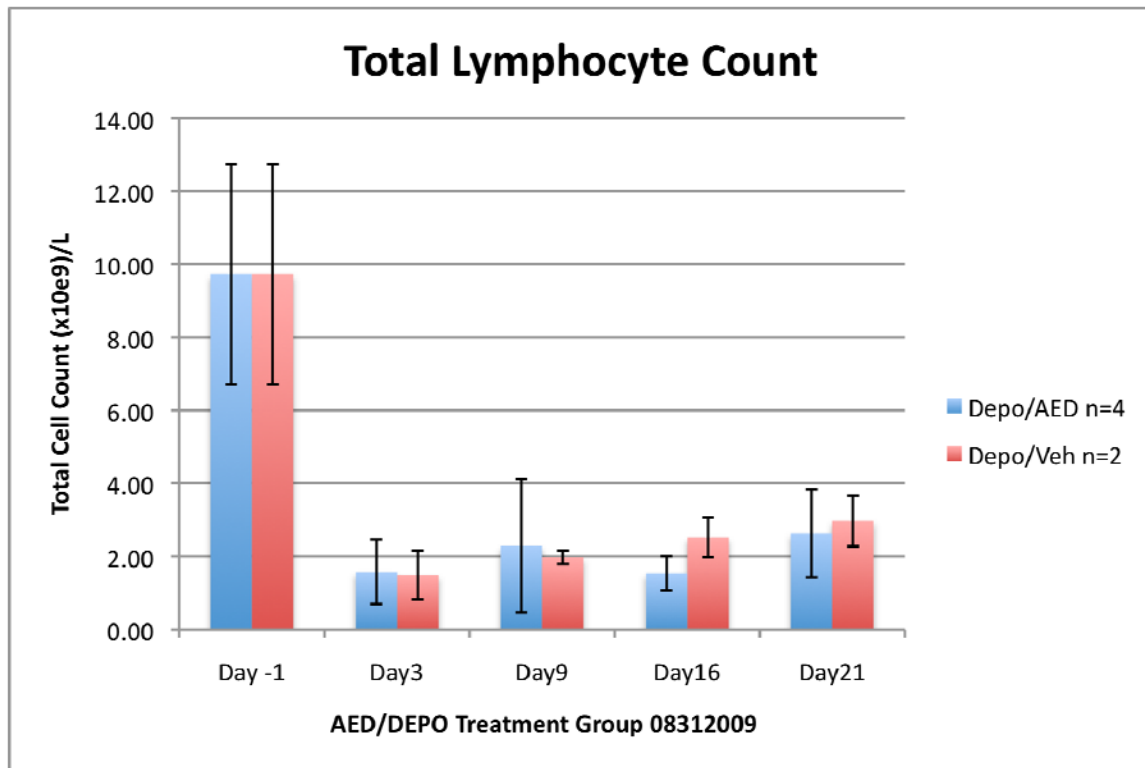


Figure 20: Lymphocyte Counts for Rat Group Beginning 252251

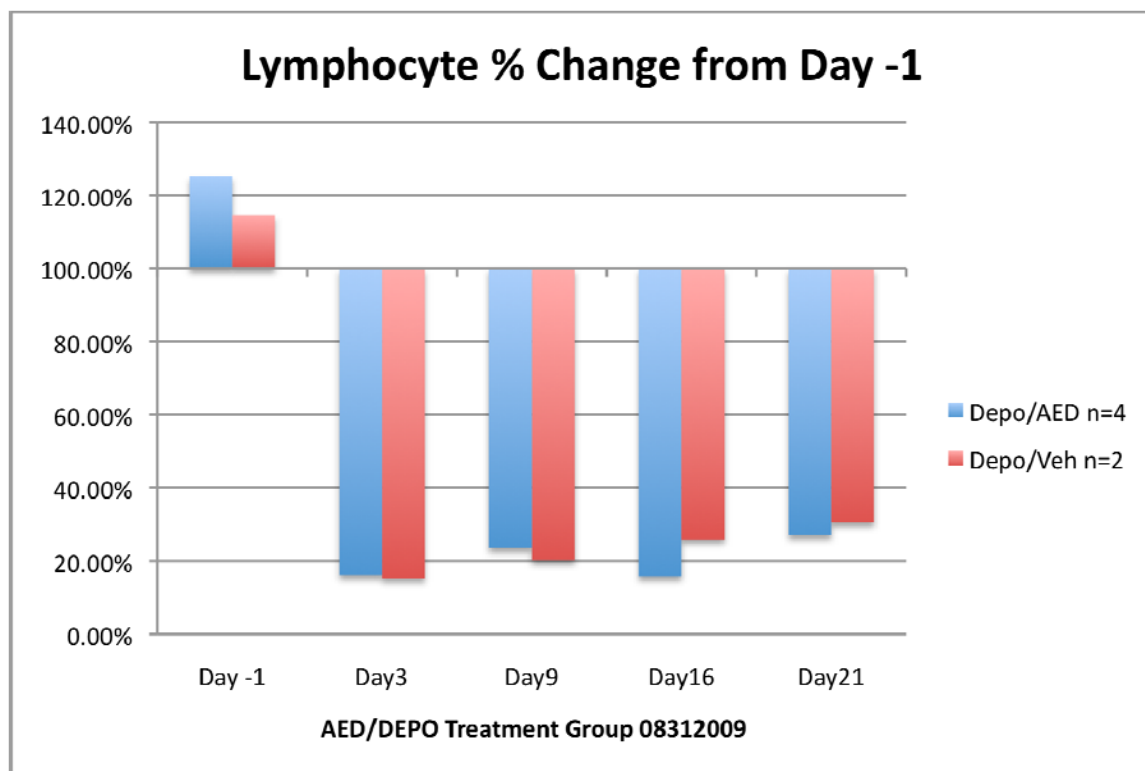


Figure 21: Changes in Lymphocyte Count from Day-1 Average in Rat Group Beginning 252251

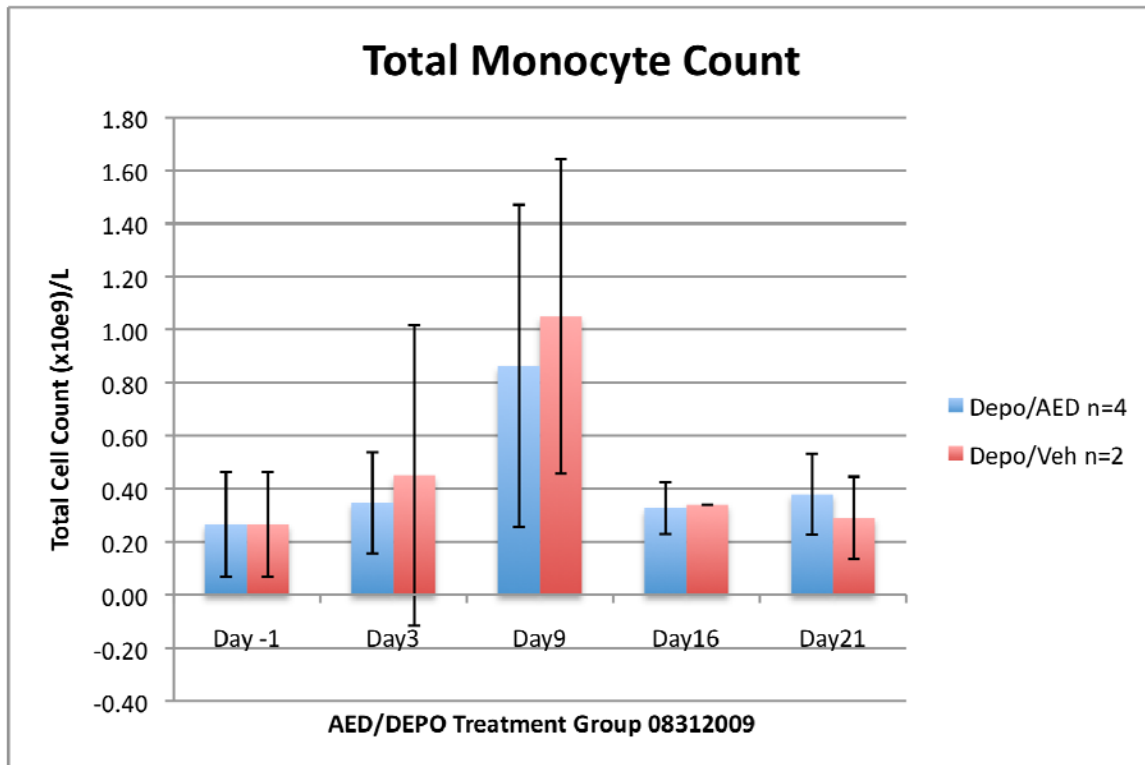


Figure 22: Total Monocyte Counts for Rat Group Beginning 252251

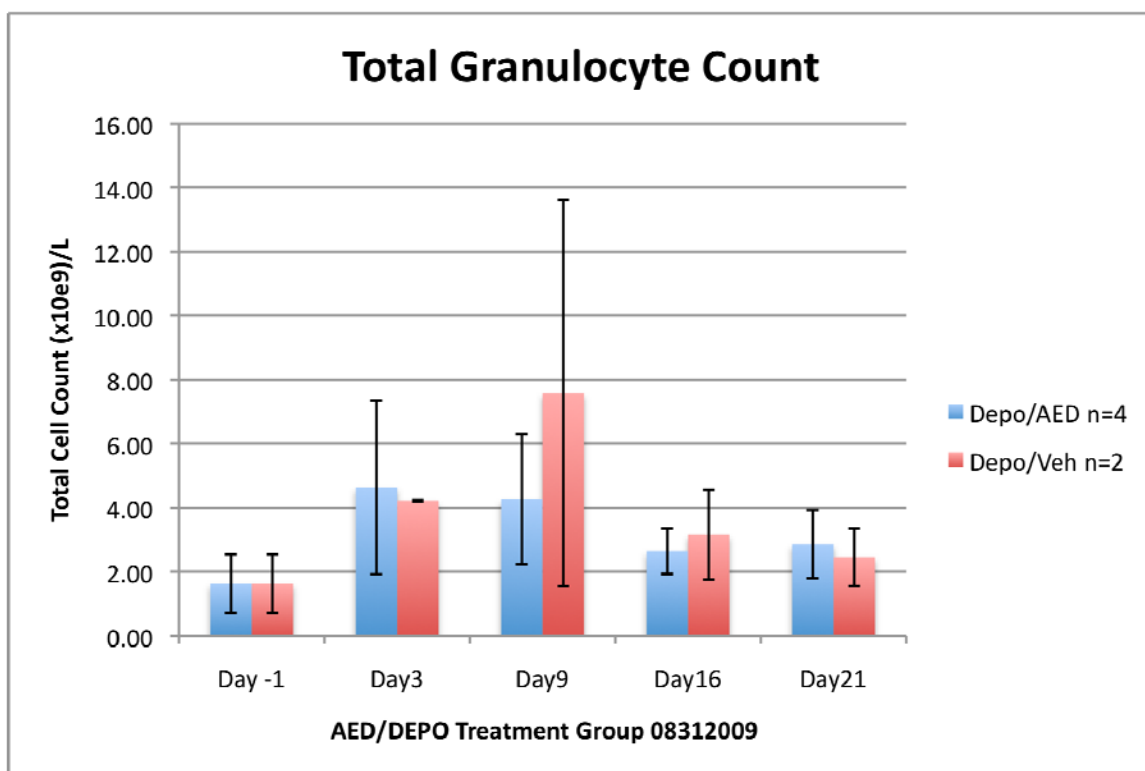


Figure 23: Total Granulocyte Counts for Rat Group Beginning 252251

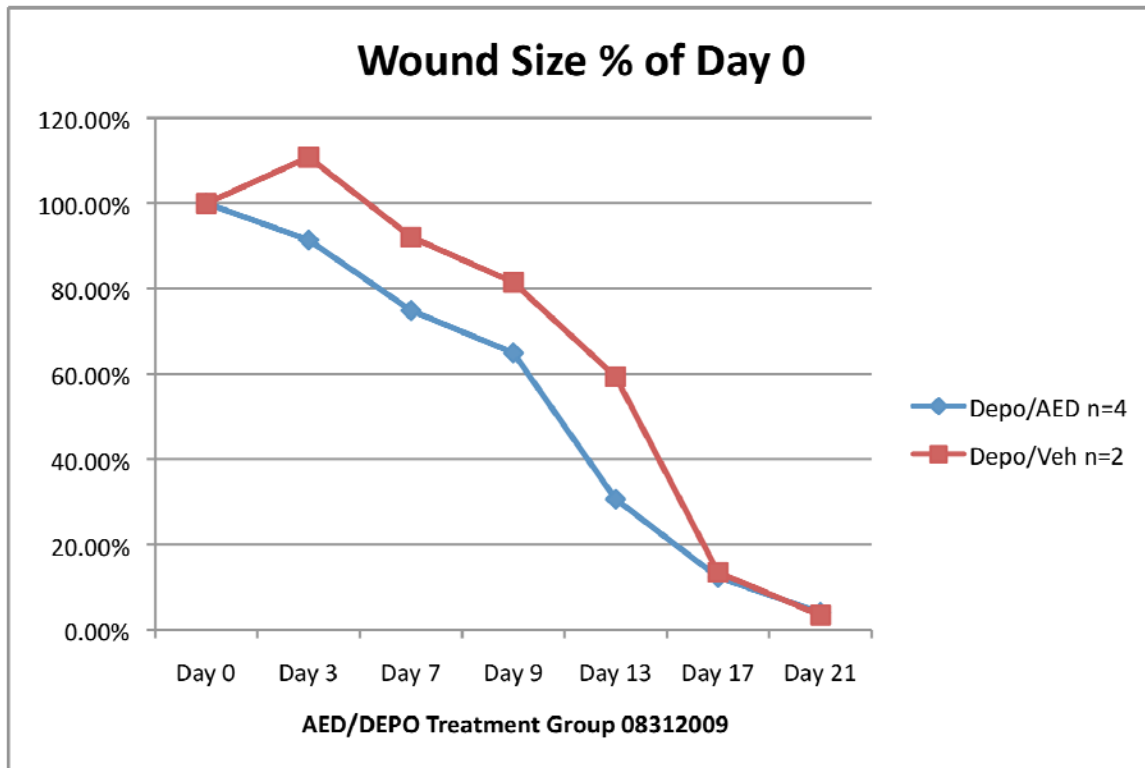


Figure 24: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 252251

Depomedrol/AED Group Beginning 252968

The rat group beginning 252968 was measured and given Day-1 treatment on September 14, 2009. During the treatment course, blood samples were taken on days -1, 3, 9, and day 21, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. White blood cell counts were, again in this group, without a discernable trend nor were they favorable to one group or another. Absolute cell counts indicated a slight advantage to the AED group. However, because of the number of subjects, the standard deviations reduce the meaning of these results (Figure 25). The values referenced to the Day-1 baselines indicate a similar benefit, with the AED group indicating a better recovery from the depression presumably induced by the methylprednisolone (Figure 26).

Lymphocyte counts indicated a curious response in this group. For the first time among these specific animal groups, the lymphocyte count indicated a recovery trend from the initial reduction. This trend was however reflected in both the AED and the vehicle group (Figure 27). The changes in Day-1 values indicate that the group started with slightly higher values than the average Day-1 animal, and that the two groups also trended in a similar fashion (Figure 28). Again, as was seen in the last group, the monocyte and granulocyte trends seem to provide the momentum for changes in the total white blood cell counts. Absolute monocyte values trend in a similar manner to the WBC counts, with values favoring the AED group early in the series, then dropping and eventually matching those of the vehicle group (Figure 29). The values when referenced to the Day-1 counts also show a dramatic difference favoring the AED group, which then rebound to less than baseline values before correcting again (Figure 30). The granulocyte counts also trend in a similar fashion, however, without the drop and subsequent correction from Day 16 to Day 21. The circulating granulocyte values gradually build in both groups, peaking at Day 16, with increases to the AED group, before returning to near baseline values on Day 21 (Figure 31). This trend is repeated in the counts referenced to the baseline Day-1 values, with counts greatly exceeding baseline counts, before returning to near normal (Figure 32). With the exception of Day 16, there seems little difference in the number of circulating granulocytes, of which neutrophils are the clear majority, between the two study groups (Figure 32). In contrast to the last group that indicated a slight advantage to healing in the AED group, this group indicates the opposite (Figure 33).

Despite the perceived advantages illustrated when the values are compared against the Day-1 counts, the standard deviations indicate that most of these values are the same if not statistically insignificant, and thus these trend lines indicating healing rate are most likely the same.

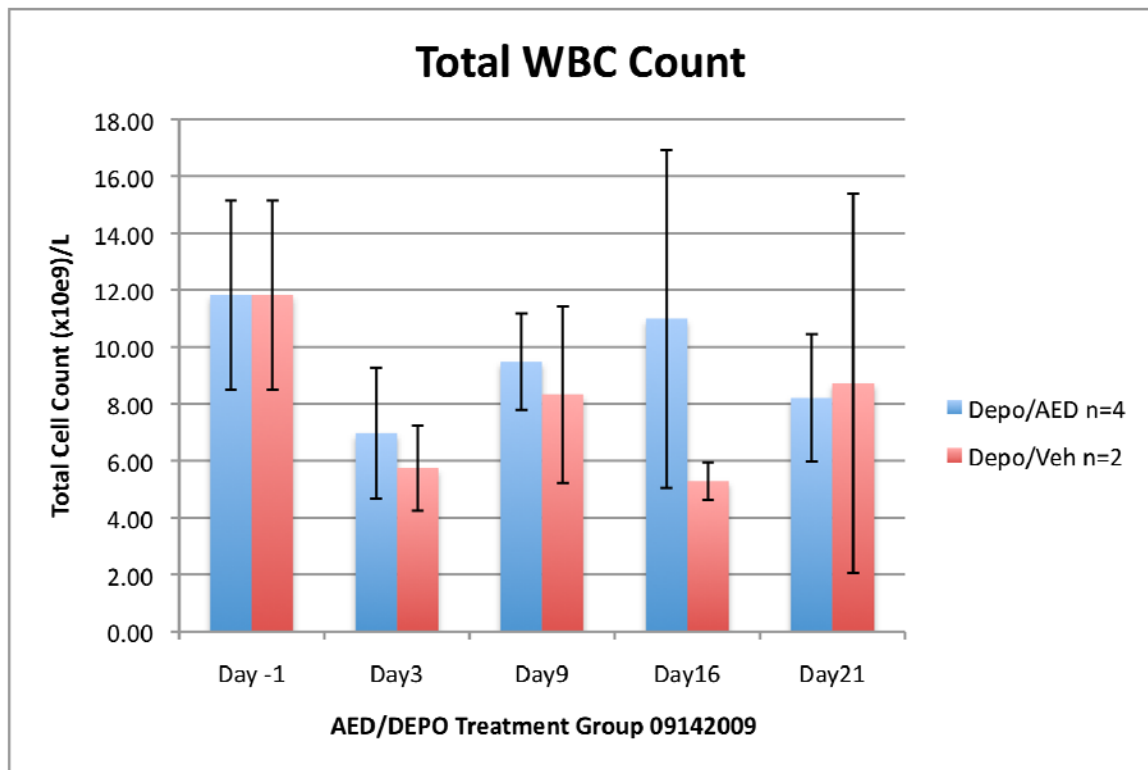


Figure 25: Total White Blood Cell Counts for Rat Group Beginning 252968

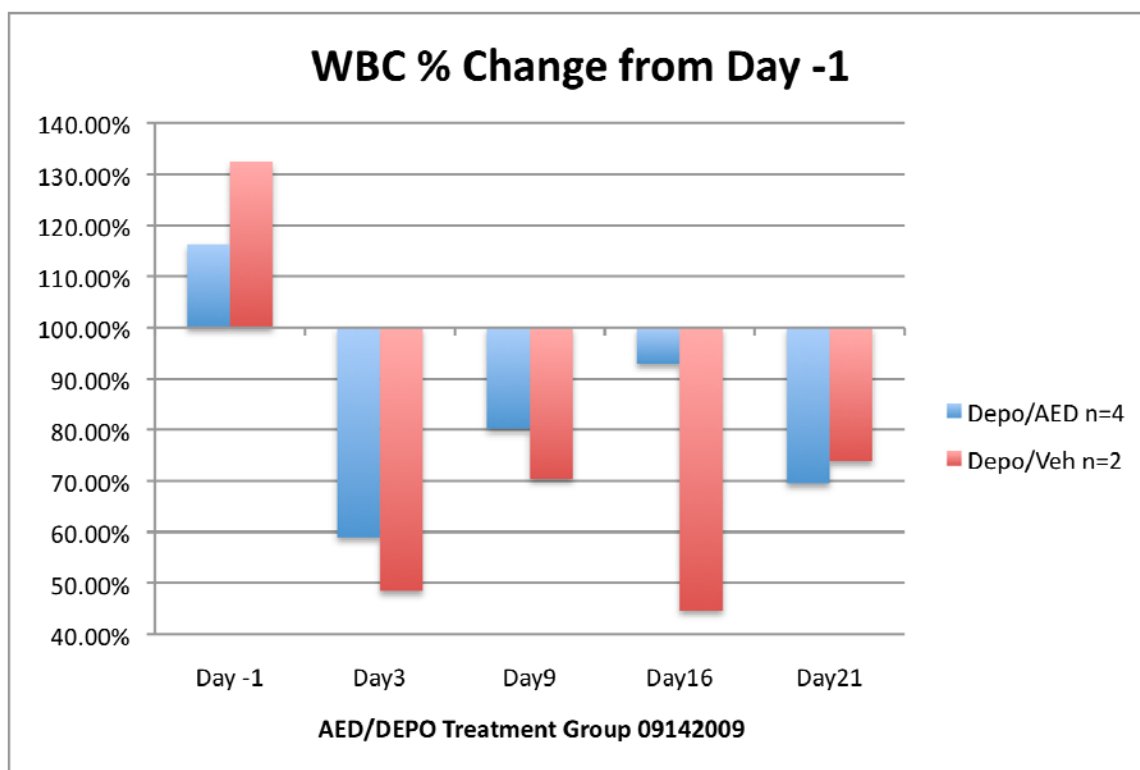


Figure 26: Changes in White Blood Cell Count from Day-1 Average in Rat Group Beginning 252968

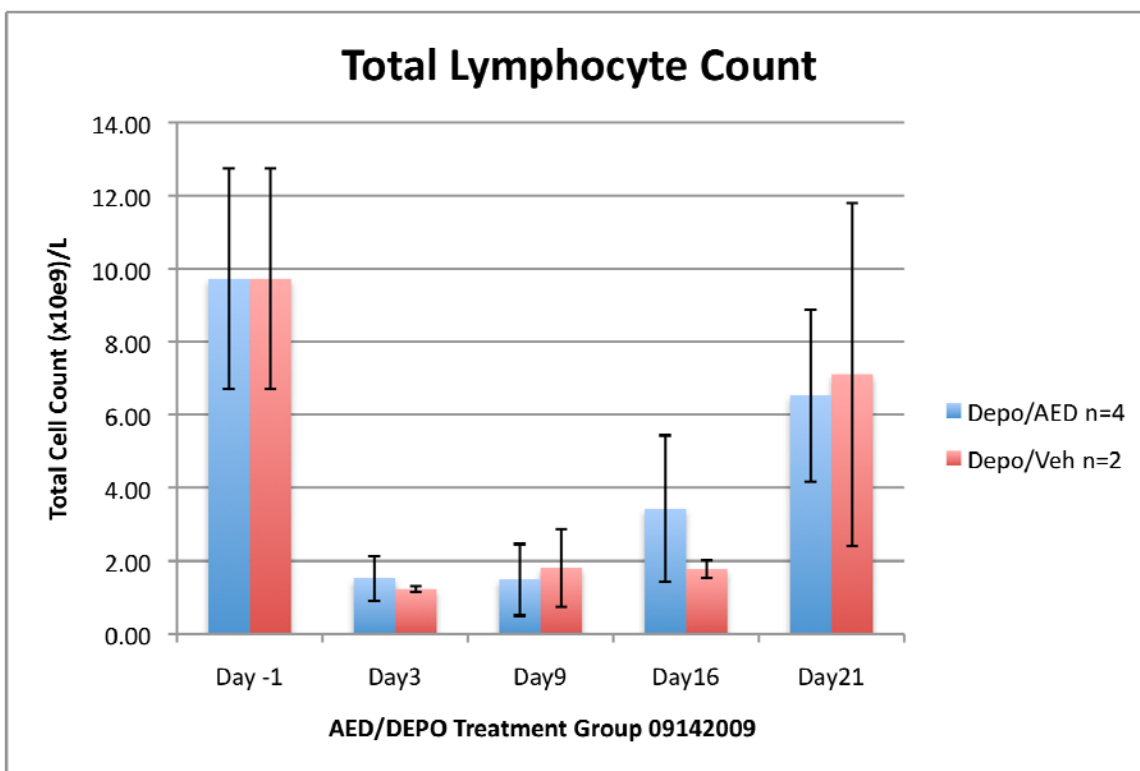


Figure 27: Total Lymphocyte Counts for Rat Group Beginning 252968

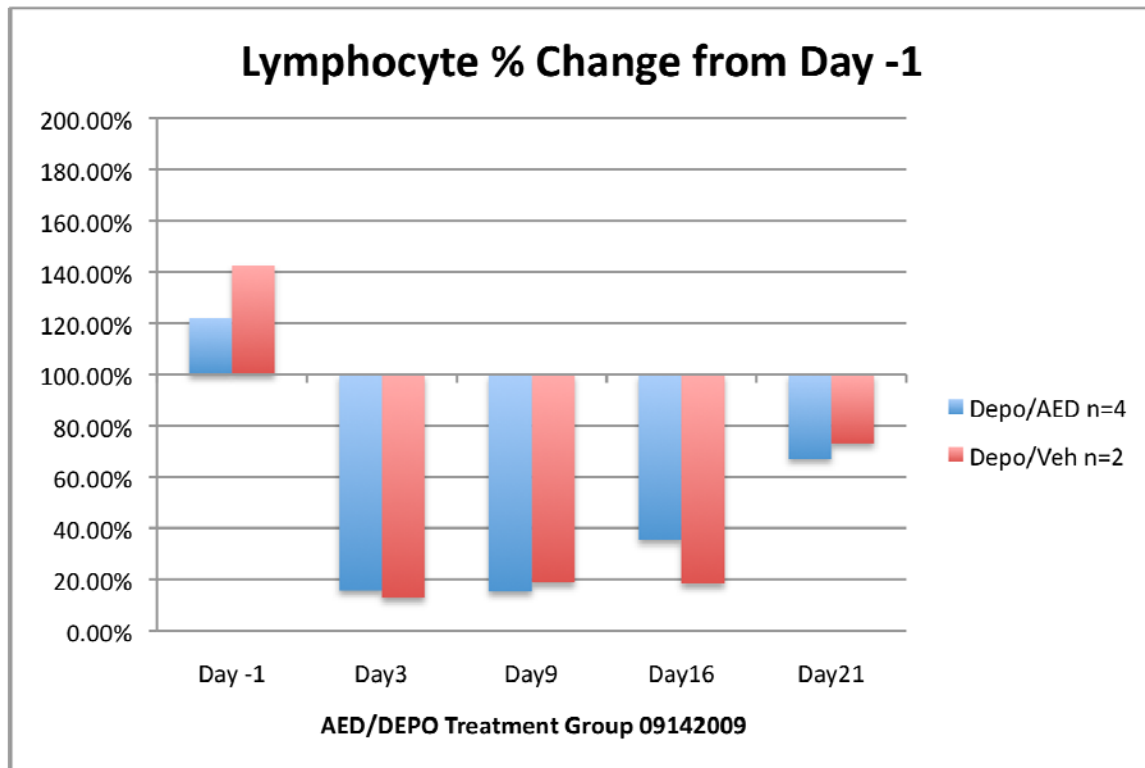


Figure 28: Changes in Lymphocyte Count from Day-1 Average in Rat Group Beginning 252968

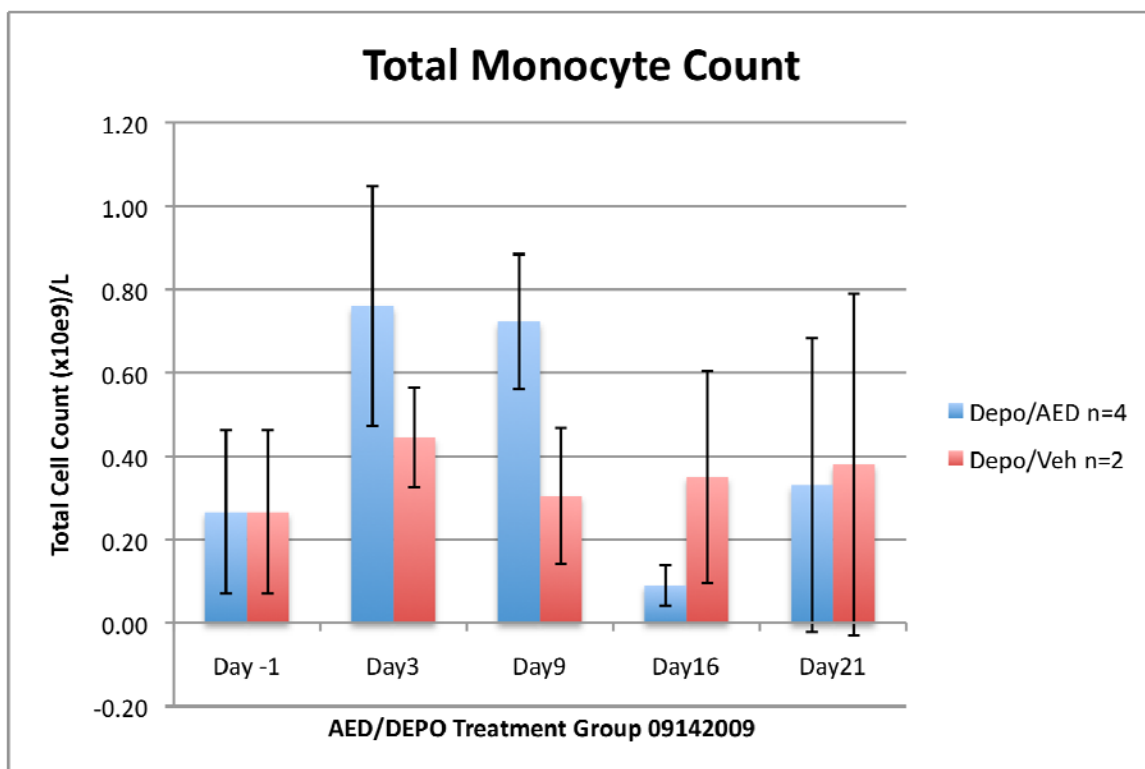


Figure 29: Total Monocyte Counts for Rat Group Beginning 252968

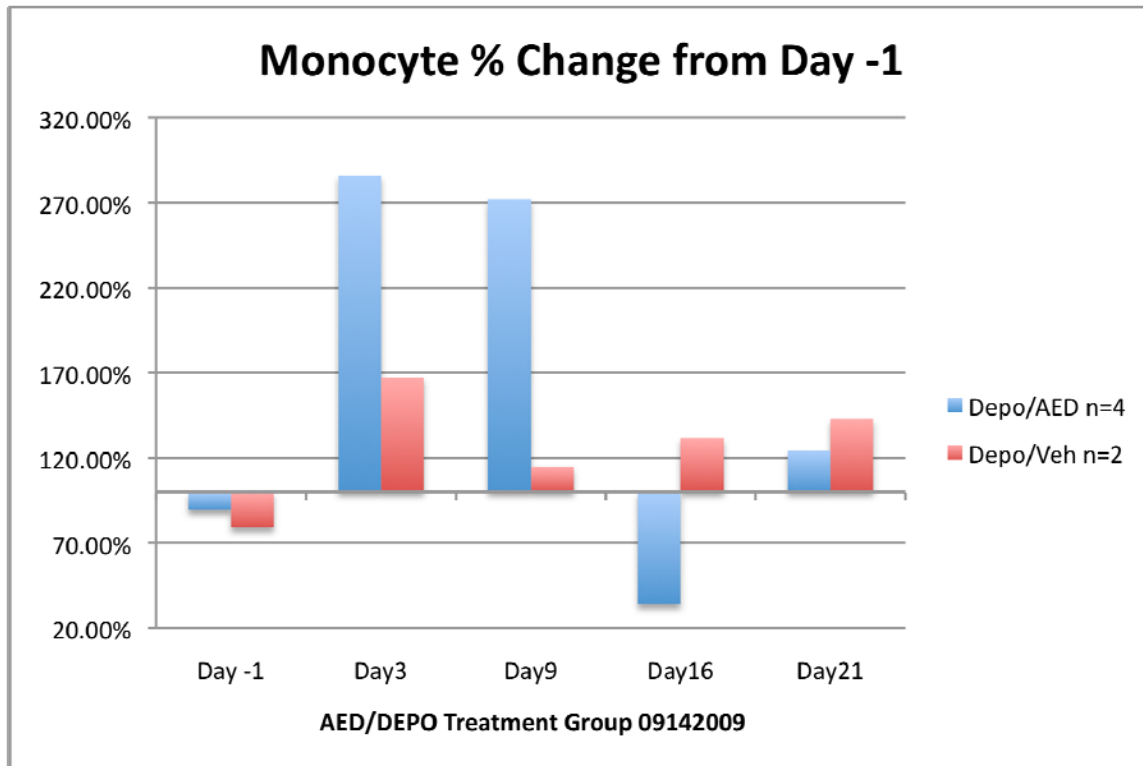


Figure 30: Changes in Monocyte Count from Day-1 Average in Rat Group Beginning 252968

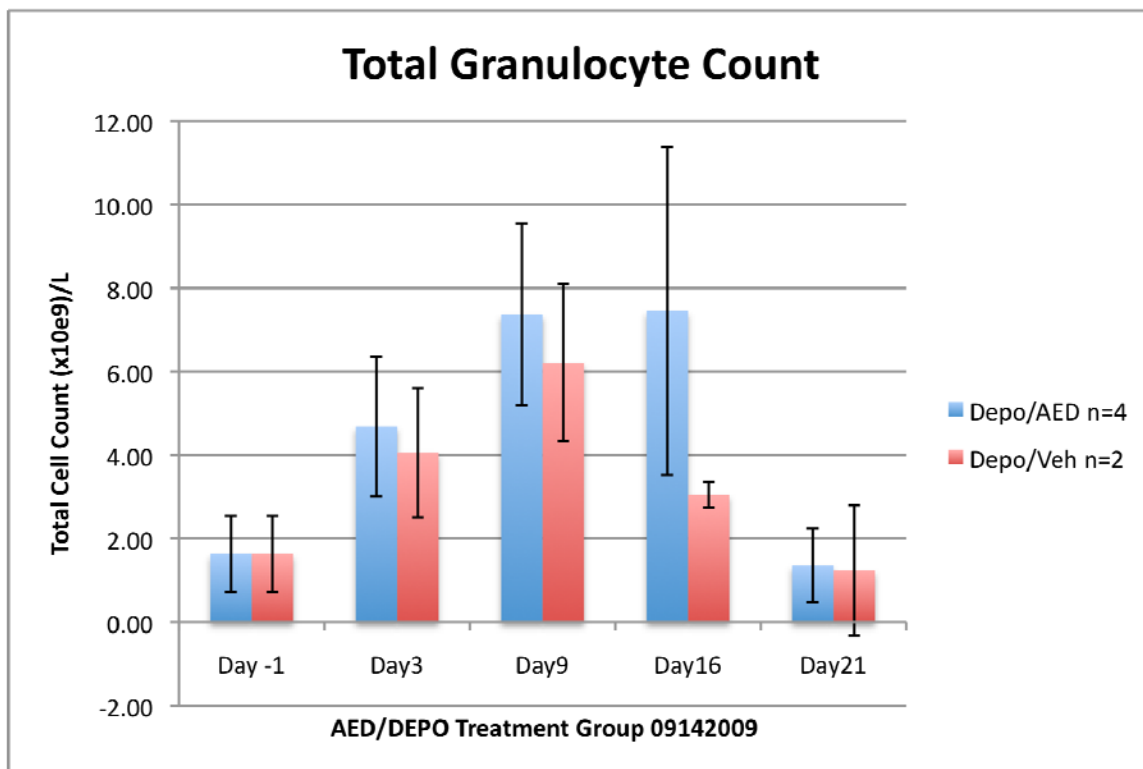


Figure 31: Total Granulocyte Counts for Rat Group Beginning 252968

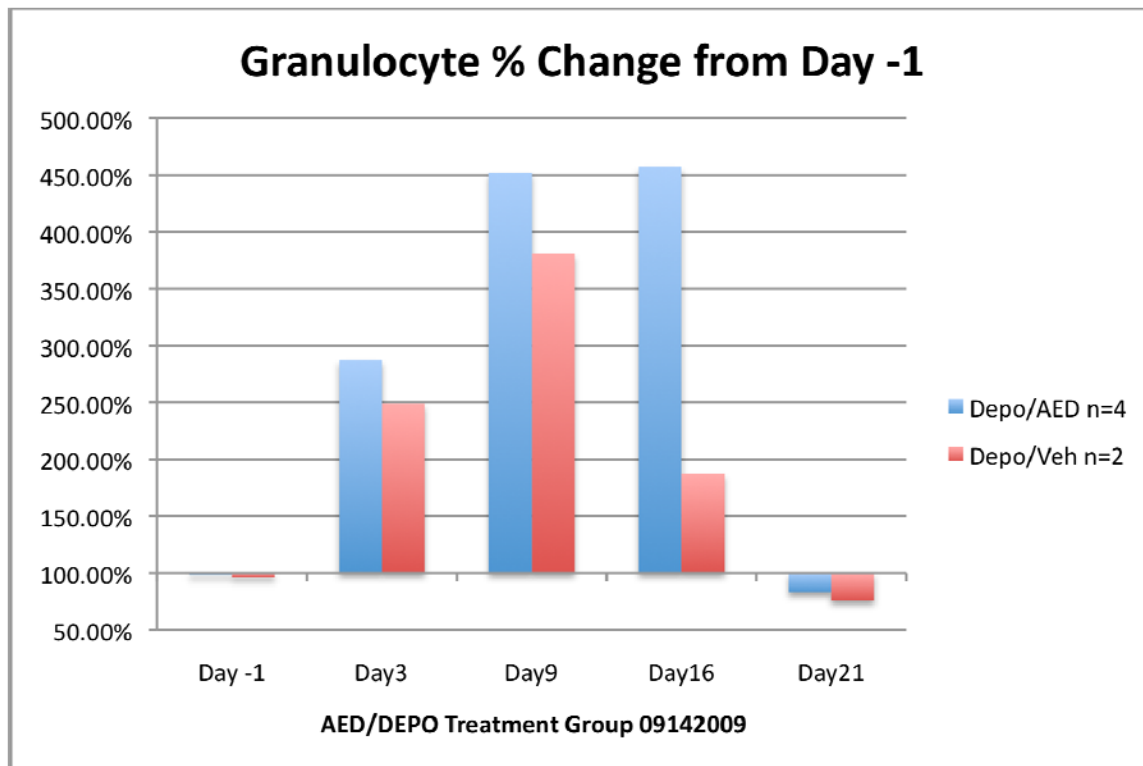


Figure 32: Changes in Granulocyte Count from Day-1 Average in Rat Group Beginning 252968

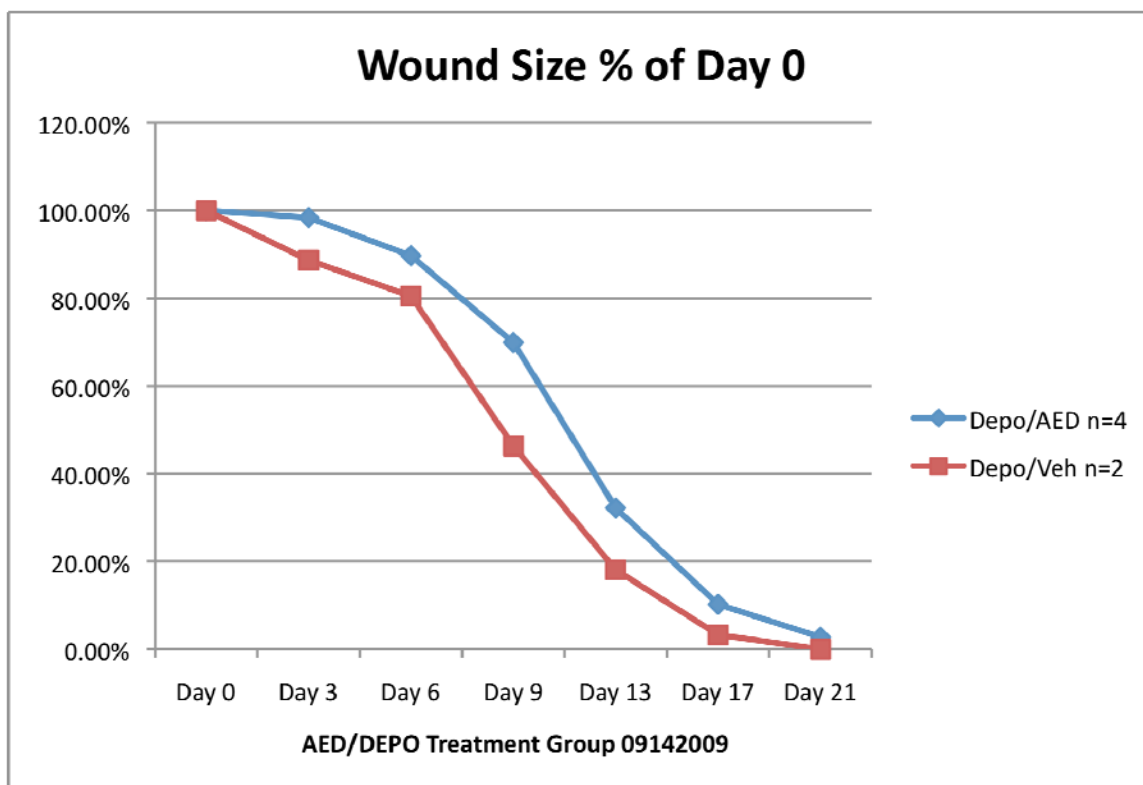


Figure 33: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 252968

Depomedrol/AED Group Beginning 254682

The rat group beginning 254682 was measured and given Day-1 treatment on October 5, 2009. During the treatment course, blood samples were taken on days -1, 3, 9, and day 16, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. As with other groups, the total white blood cell counts dropped and stayed relatively low through the course of the experiment (Figure 34). The pattern in this group shows slight differences indicating minimally higher levels of white blood cells in the AED group. The values for standard deviation indicate that these counts are without merit. When the values are compared to the Day-1 baseline count, there is a similar picture presented, with slightly higher WBC counts in the AED group throughout (Figure 35).

As with the last few groups, the trends in lymphocyte values drop from Day-1 values and fail to recover. There seems no apparent benefit in either group (Figure 36). When compared to the referenced Day-1 counts, the drop is significant to less than 20% of normal values (Figure 37). The monocyte counts paint a different picture, however, especially when compared to the course of AED administration. The monocyte counts in the vehicle group drop following the administration of methylprednisolone and induction of trauma. However, the AED group sustains their monocyte counts and even shows a slight increase from the baseline values. This is particularly interesting as the AED administration continued to Day 9, at which point the AED group monocyte levels drop again. At the same time point (Day 16), the vehicle group's monocyte counts rebound above baseline. As compared to the average Day-1 values, this particular group is already starting at less than 70% of

normal. When the following values are compared as a percentage to the Day-1 baseline values, they indicate a drop in over 80% within the vehicle group, which then gradually recovers to eventually exceed the Day-1 values (Figure 39). In this analysis, however, the levels of the AED monocyte counts seem far less impressive. The AED group indicates a slight drop, small increase, followed by yet another drop. Given the statistical significance of the absolute values, these changes are probably non-existent. However, it is important to note the lack of change as compared to the drop in the vehicle group, especially when compared to the course of AED administration. The granulocyte counts in this group are also particularly fascinating. The AED group displays granulocyte levels that exceed the vehicle group's levels though the final time point, Day 16 (Figure 40). Again due the small animal group, these differences appear to be meaningless in all but one time point. The trend, however, should not be ignored. When considering this same data referenced to the Day-1 average baseline values, this group also starts below "normal" baseline levels. These counts recover significantly from the initial values and gain momentum throughout the experiment. This change is significantly pronounced within the AED group, up to 50% more than within the vehicle group (Figure 41). Thus while the standard deviation within the absolute cell counts cast this data into question, the trend in the group seems to indicate that the AED groups realized increases in both monocyte and granulocyte counts in a manner consistent with the day to day administration of AED. However once again, these changes failed to manifest in any changes wound healing. Both the vehicle and AED groups

proceed on nearly identical courses with the AED group actually further behind the vehicle group in final healing (Figure 42).

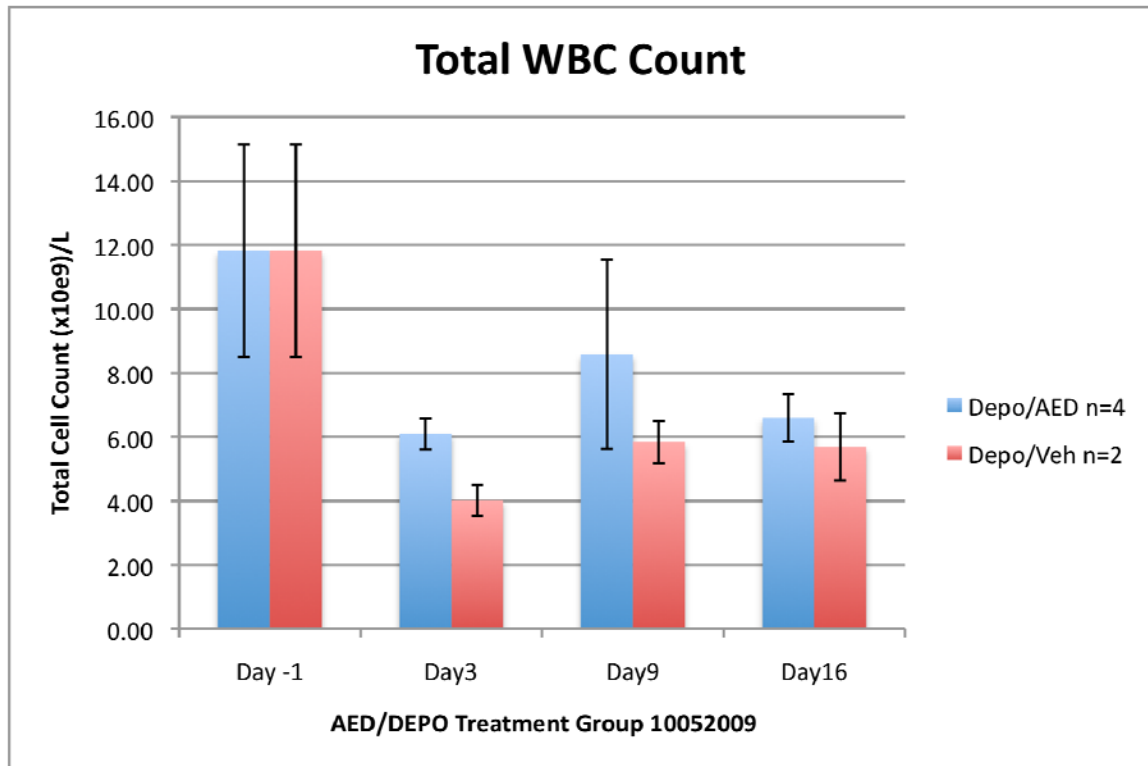


Figure 34: Total White Blood Cell Counts for Rat Group Beginning 254682

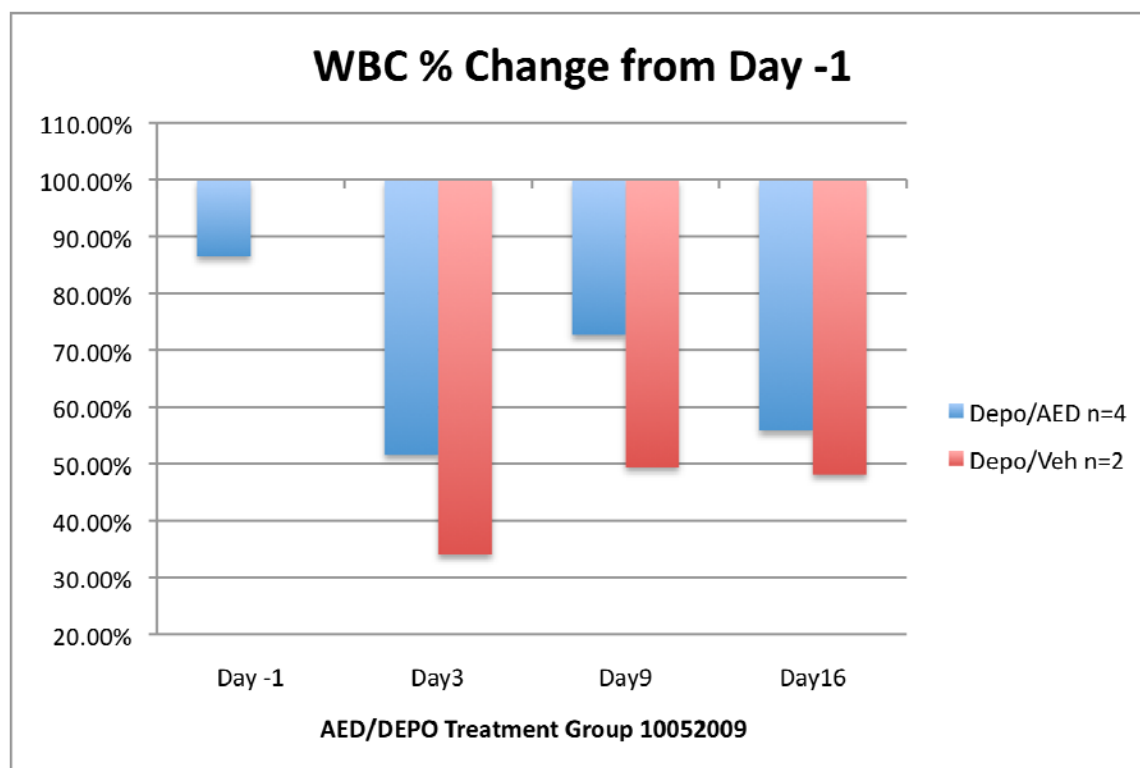


Figure 35: Changes in White Blood Cell Count from Day-1 Average in Rat Group Beginning 254682

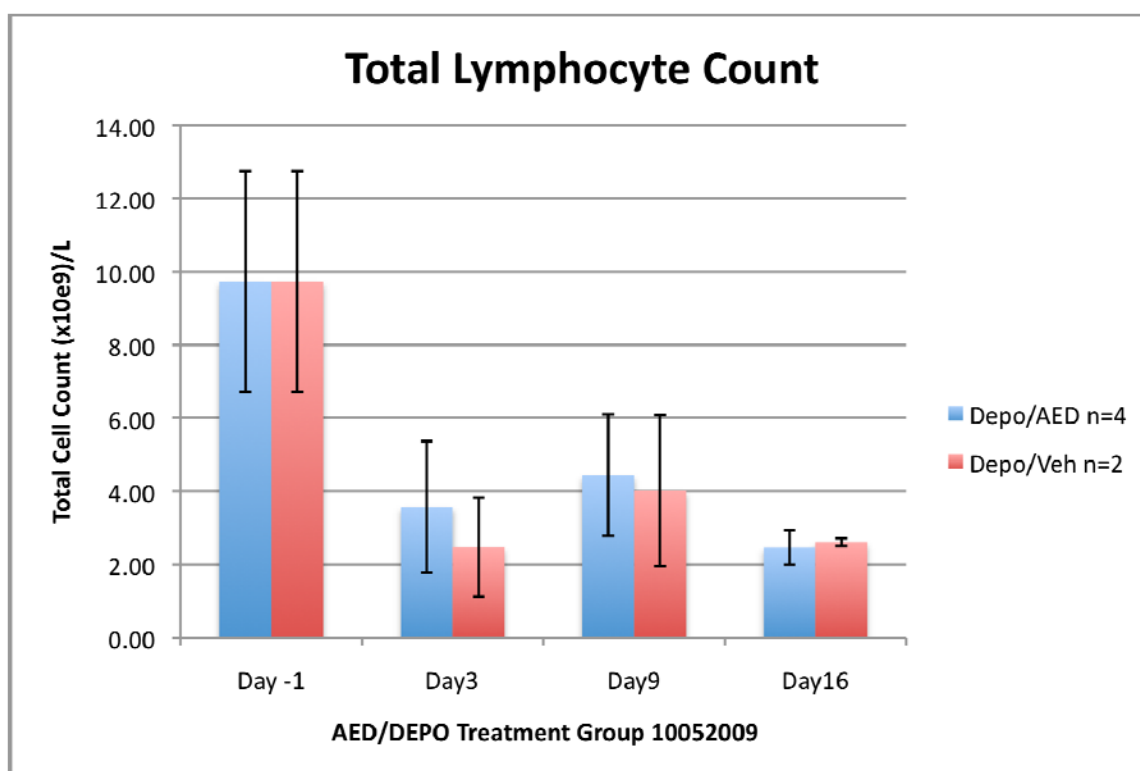


Figure 36: Total Lymphocyte Counts for Rat Group Beginning 254682

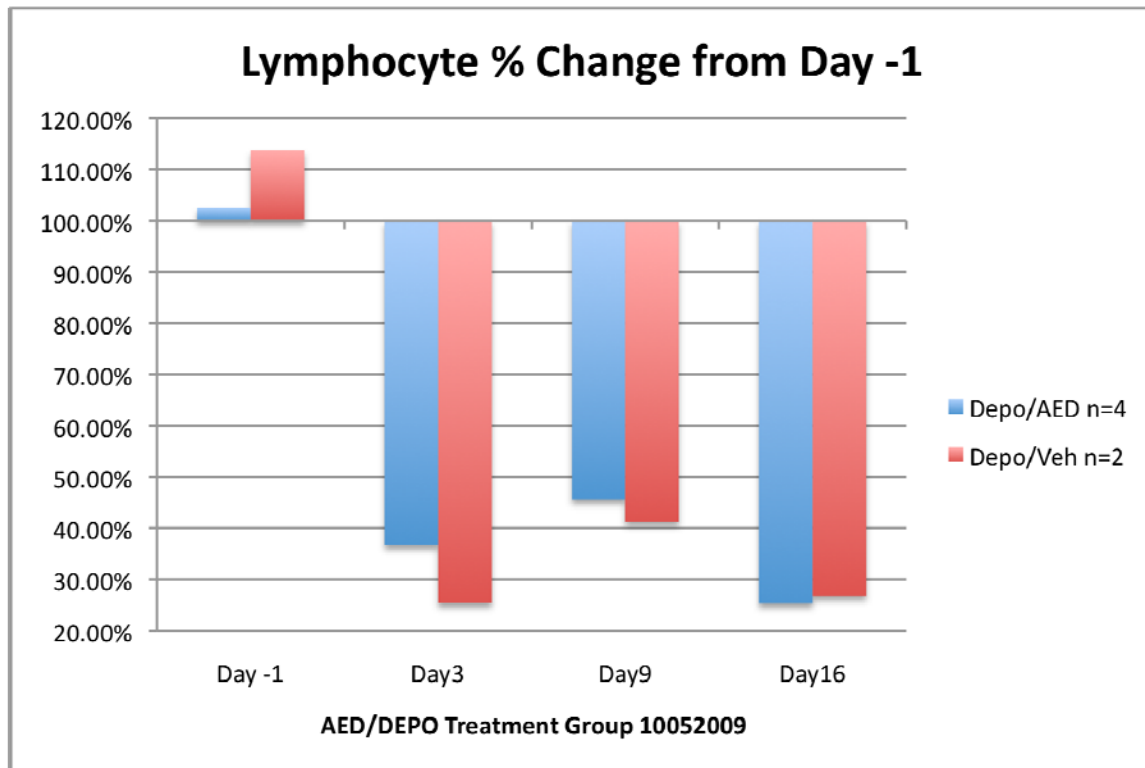


Figure 37: Changes in Lymphocyte Count from Day-1 Average in Rat Group Beginning 254682

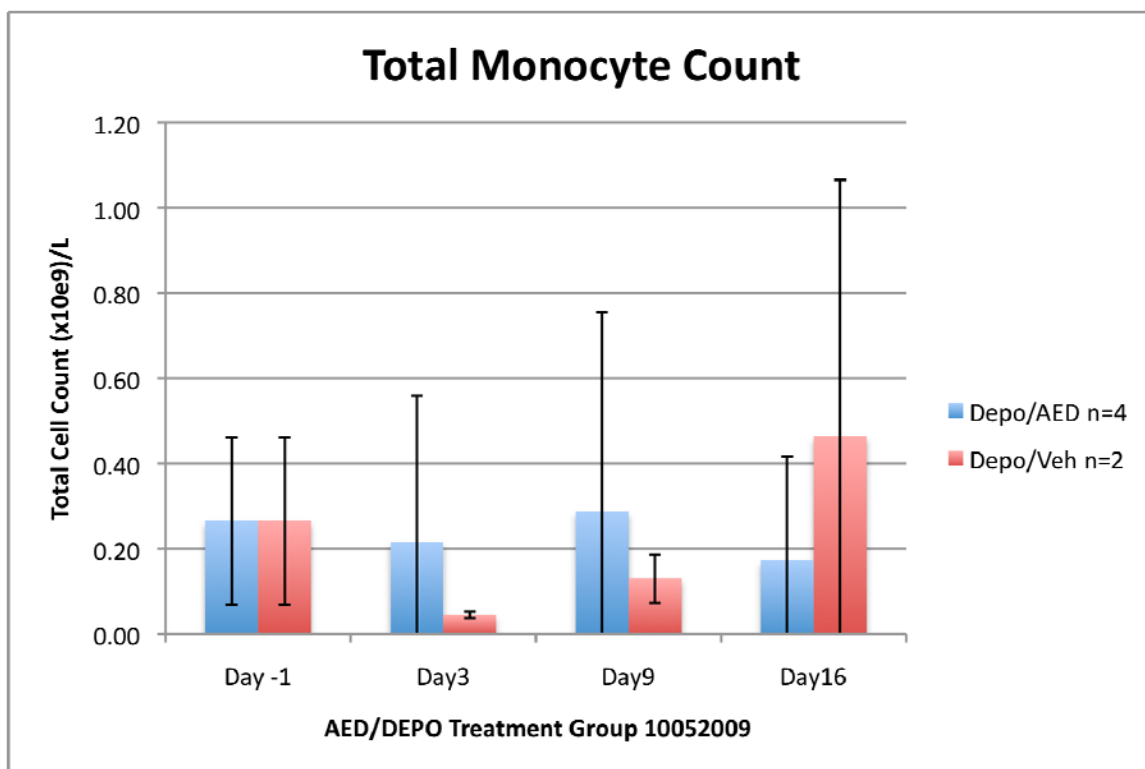


Figure 38: Total Monocyte Counts for Rat Group Beginning 254682

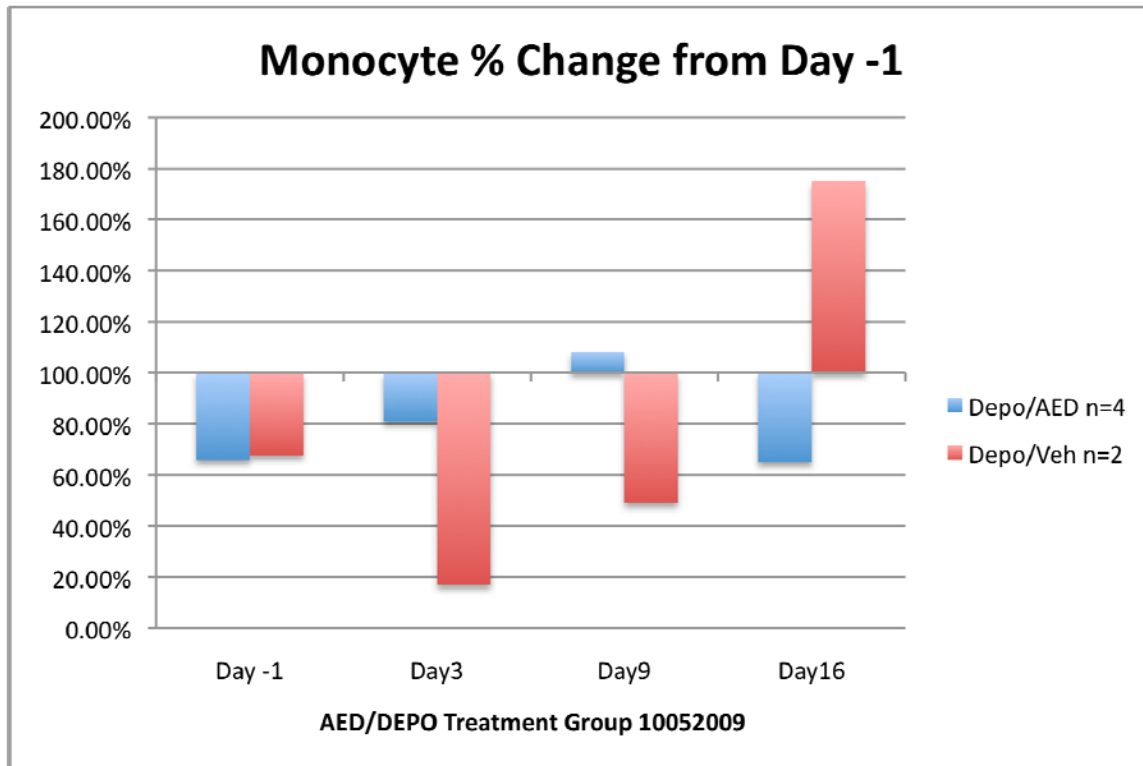


Figure 39: Changes in Monocyte Count from Day-1 Average in Rat Group Beginning 254682

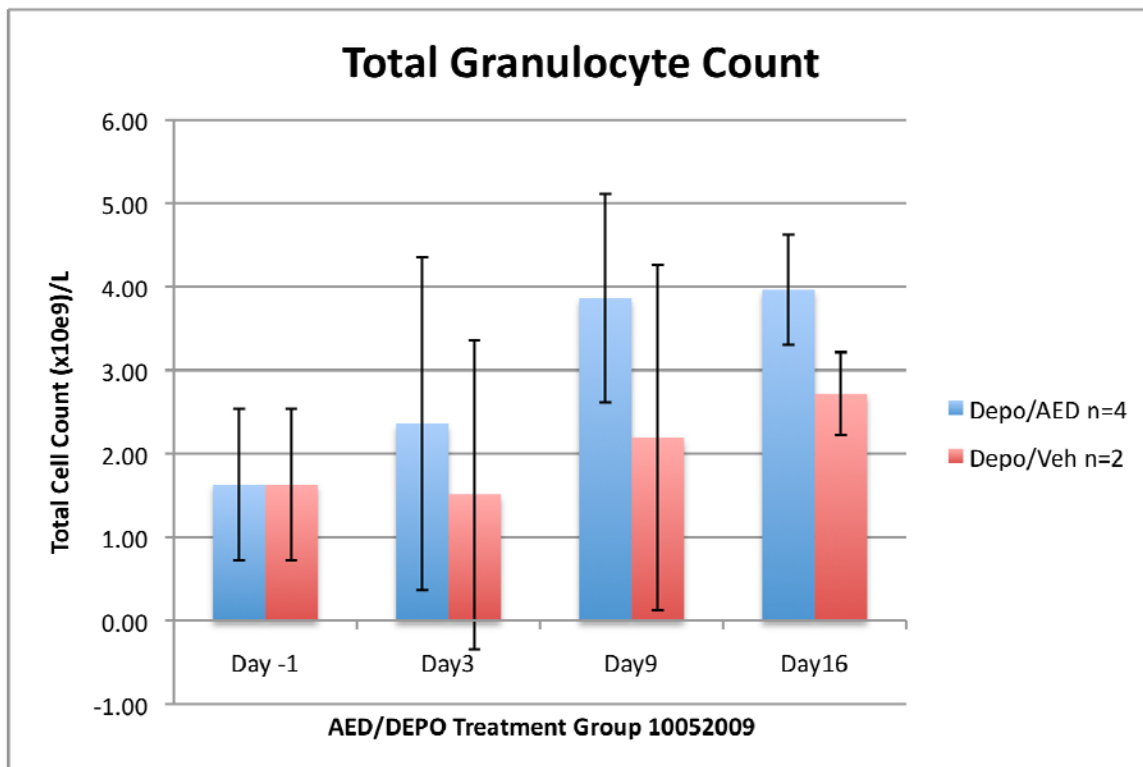


Figure 40: Total Granulocyte Counts for Rat Group Beginning 254682

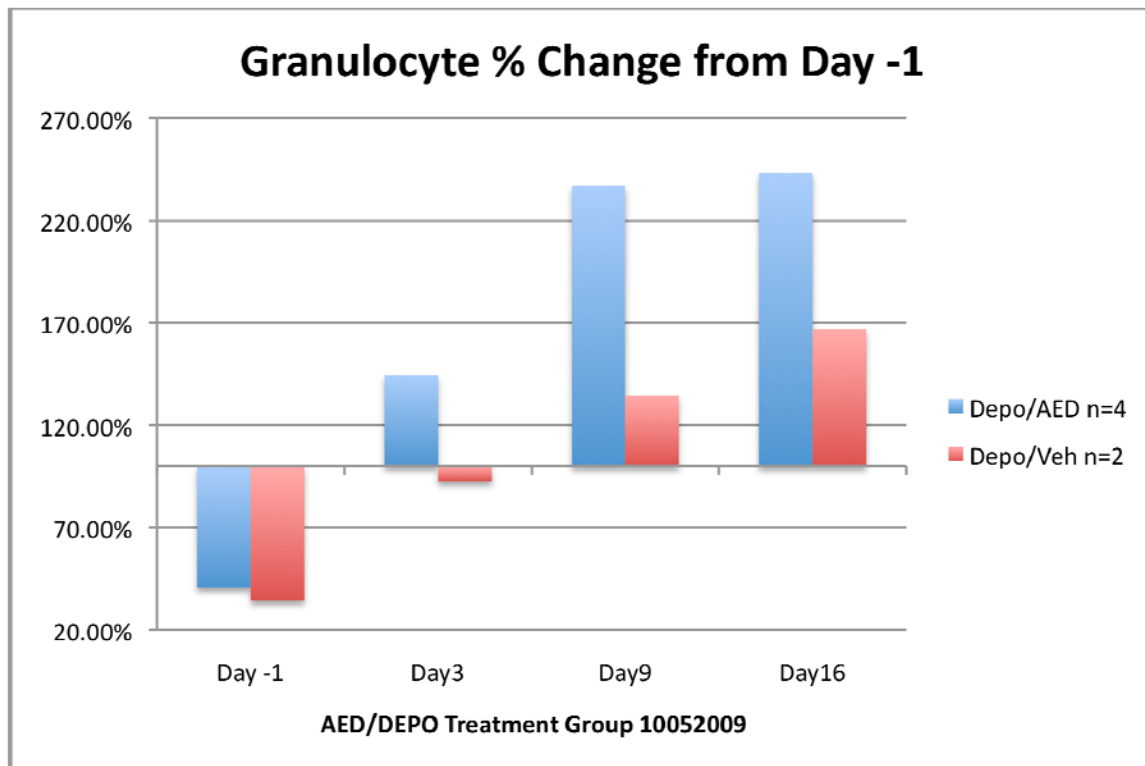


Figure 41: Changes in Granulocyte Count from Day-1 Average in Rat Group Beginning 254682

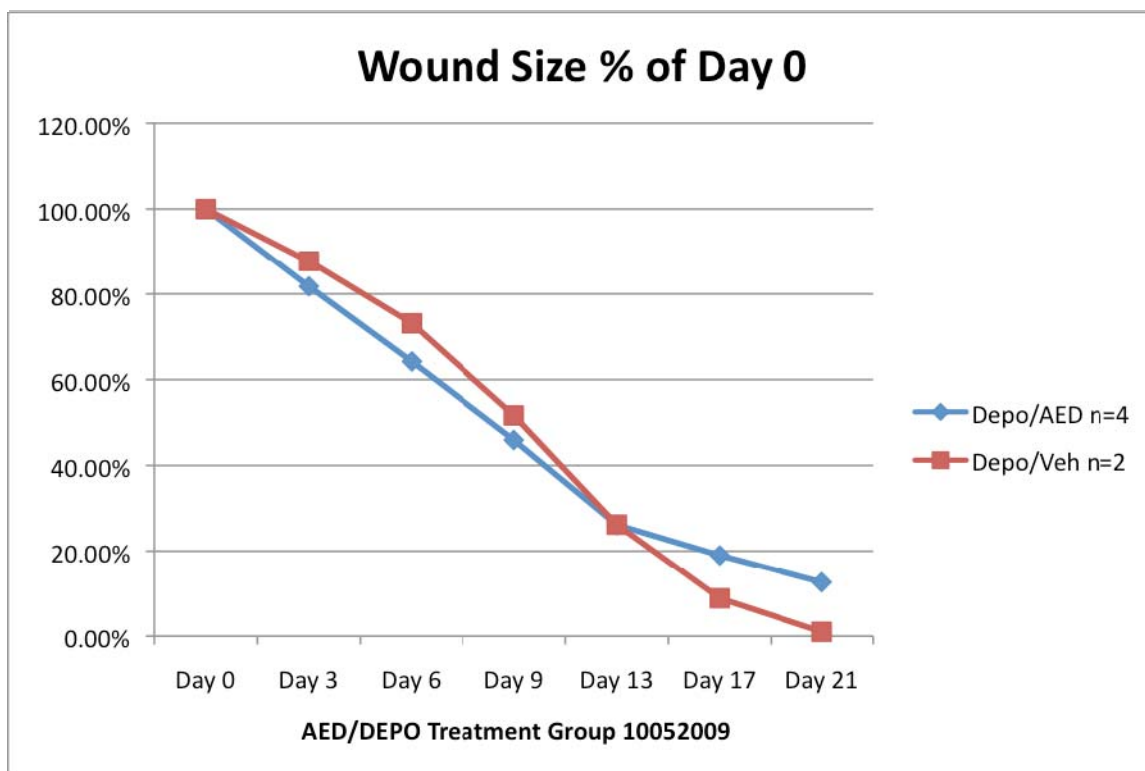


Figure 42: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 254682

Depomedrol/AED Group Beginning 258339

The rat group beginning 258339 was measured and given Day-1 treatment on November 23, 2009. During the treatment course, blood samples were taken on days -1, 3, 16, and day 21, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. As previously illustrated, animals in this group received a lower dose of methylprednisolone as well as the induction of additional trauma in the form of a laparotomy. It was the groups hope that this would represent less immuno-suppression in the face of additional up-regulation of the immune system in response to the additional trauma, thus resulting in a better response to the AED. The following data also presents a unique perspective within this experiment as this group not only included the AED and vehicle subsets, but also a control group (PBS/VEH) as well as a group to distinguish the AED effects from those of the methylprednisolone effects (PBS/AED). In addition, because of the unique nature of this group, it was selected to do a further analysis of the function of the immune system by evaluating some of the protein mediators expressed under various circumstances.

The total white blood cell counts presumably immediately illustrate the effects of the methylprednisolone, even at the lower dose. The total white blood cell counts indicate that both the groups receiving the methylprednisolone drop significantly, with little to no recovery in either the AED or vehicle group (Figure 43). Conversely, the groups not receiving the methylprednisolone appeared to suffer no changes, again with no changes attributable to the AED. Interestingly, all groups presented with significantly lower counts on Day+1, perhaps due to the

effects of the trauma itself. When compared to average Day-1 baseline levels, the Day+1, counts drop well over 80%, with little recovery in the methylprednisolone groups, and recovers beyond baseline levels in the AED and vehicle groups not receiving the immunosuppressive steroid (Figure 44). Interestingly, this same data indicates minor increases in WBC count in the DEPO/AED group above that of the DEPO/VEH group.

Lymphocyte counts for this groups and each individual subset is almost identical to the white blood cell values. Following trauma, all T-cell groups drop to values near normal, with recovery only in the groups *not* receiving the methylprednisolone (Figure 45). Values compared to the average Day-1 counts also follow this trend. Immediately following wounding, all groups display a lymphocyte count below 20% of normal. As with the overall WBC counts, the groups *not* receiving methylprednisolone recover to lymphocyte levels above normal, with the AED/VEH groups doing slightly better. Groups receiving methylprednisolone, however, remain with lymphocyte levels below 40% of normal (Figure 46).

Efforts to characterize the activity and possible activation of T-lymphocytes by analyzing IL-2 levels resulted in unreadable levels. The IL-2 ELISA assay revealed an accurate standard curve down to levels of 31 pg/ml (Figure 47). However, our assay of Day-1 and Day +1 blood samples in group beginning 258339 failed to reveal any detectable IL-2 levels. Rat IL-2 levels are typically less than 15pg/ml, so this result is not completely unexpected. However, we would presume to see some systemic expression of IL-2 with increased T-lymphocyte activity. We also sought to determine some level of T-lymphocyte activity through the

characterization of IL-6 levels. As with the IL-2 ELISA, we found the standard curve for the IL-6 assay to provide for an accurate reading of levels to just less than 62 pg/ml (Figure 48). Again normal levels of IL-6 in rats are found to be less than the lowest detectable level in this assay. While this assay indeed provided some data, it indicates modest levels of IL-6, at time points inconsistent with a response induced by trauma or drugs (Figure 49). Furthermore, without any clear relationship between treatment and group, it seems that the IL-6 levels in these animals did not respond to either drug or physical damage.

Monocyte counts also failed to add any clarity to this group as well. After an initial drop in monocyte levels across all groups, due either to the trauma, methylprednisolone, or both, monocyte levels returned to near normal levels also in all groups (Figure 50). These minor changes did not favor any one group over another, and they did not seem to reflect any identifiable trends (Figure 51). In an effort to characterize any interaction between monocytes and T-lymphocytes in response to any trauma or drug administration, we sought to assay interleukin-10. Unfortunately, circulating IL-10 levels also failed to reveal any useful information. While again we were able to discern a useful standard curve (Figure 52), there were yet again no detectable levels of IL-10 in this group of animals for time points Day-1, Day+1, or Day +3. With normal levels typically below the lowest detectable levels for this assay (32 pg/ml), this result was again predictable for the baseline levels, but disappointing for the later time points.

In this group, it is the granulocyte/neutrophil counts that finally lent some clarity to activity corresponding to treatment or drugs. Raw granulocyte counts

illustrate a response to either drugs, or trauma or both but Day +1, with a recovery of levels shortly thereafter (Figure 53). Perhaps most interestingly, the PBS/AED group indicated the least variation among any of the subgroups surveyed while the DEPO/AED group showed a persistent advance after recovering from the initial insult. Trends are encouraging when evaluated against the Day-1 average baseline values. When evaluating the DEPO/AED group against the DEPO/VEH group, both groups show an increase over the experiment, with the AED group showing higher systemic granulocyte levels (Figure 54). Strangely, PBS/AED levels not only failed to keep pace, they indicated a continuous drop in levels. In an effort to characterize the recruitment of neutrophils, the circulating levels of IL-8 were assayed. The standard curve for interleukin-8 revealed that levels should be detectable as low as between 7 and 15 pg/ml (Figure 55). As was the case with the raw granulocyte counts, the IL-8 assay also provided some interesting trends (Figure 56, Figure 58). While circulating IL-8 levels failed to change significantly in either of the groups treated with methylprednisolone, both the PBS/AED and the PBS/VEH groups expressed interesting increases in IL-8 expression from Day-1 to Day +1. The PBS/AED expressed the most clear-cut change in expression among the groups assayed.

As has been the case with other groups, the androstenediol seemed to provide some minor benefits to healing during the course of the experiment, although it presented no overall benefit with regard to the final wound closure (Figure 58). It should not be unexpected that the subsets of the groups illustrated the most accelerated healing values, at times exceeding that of the

methylprednisolone animals by as much as 40% wound closure. Perhaps most disappointing was that the animals receiving no inhibition and *no treatment* at all, benefited the most, presented as the most closed wounds when measured against the original wound size during the entire course of the experiment.

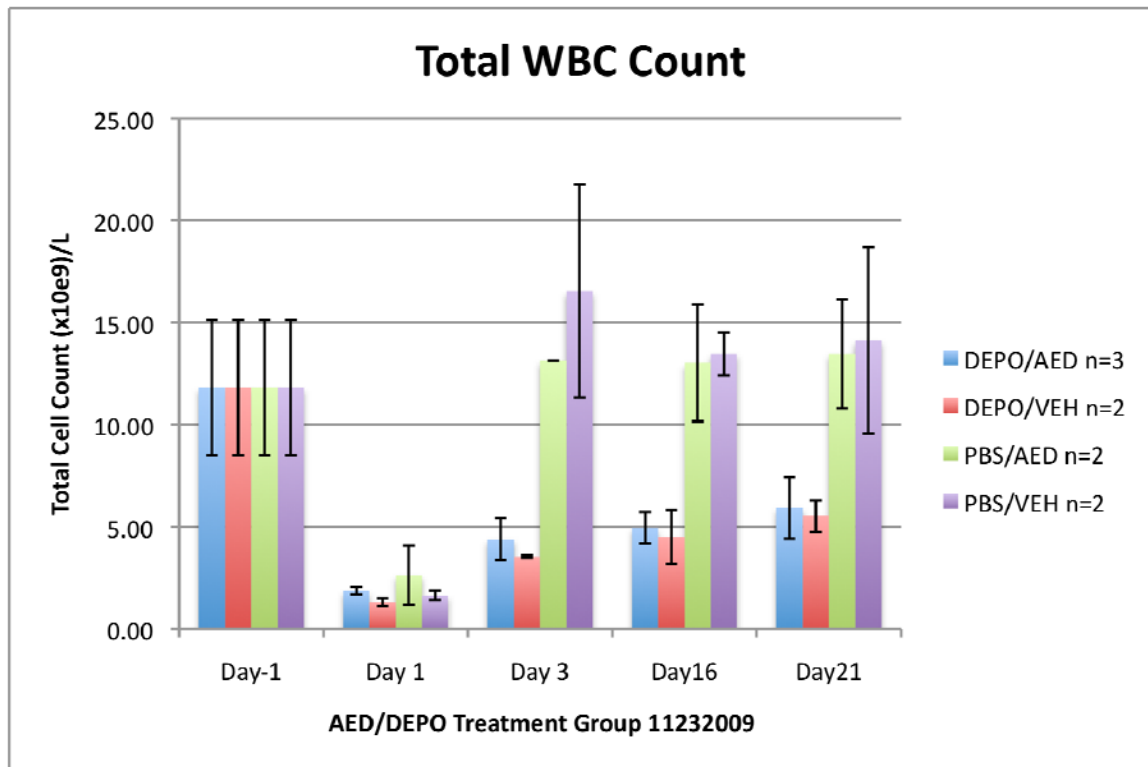


Figure 43: Total White Blood Cell Counts for Rat Group Beginning 258339

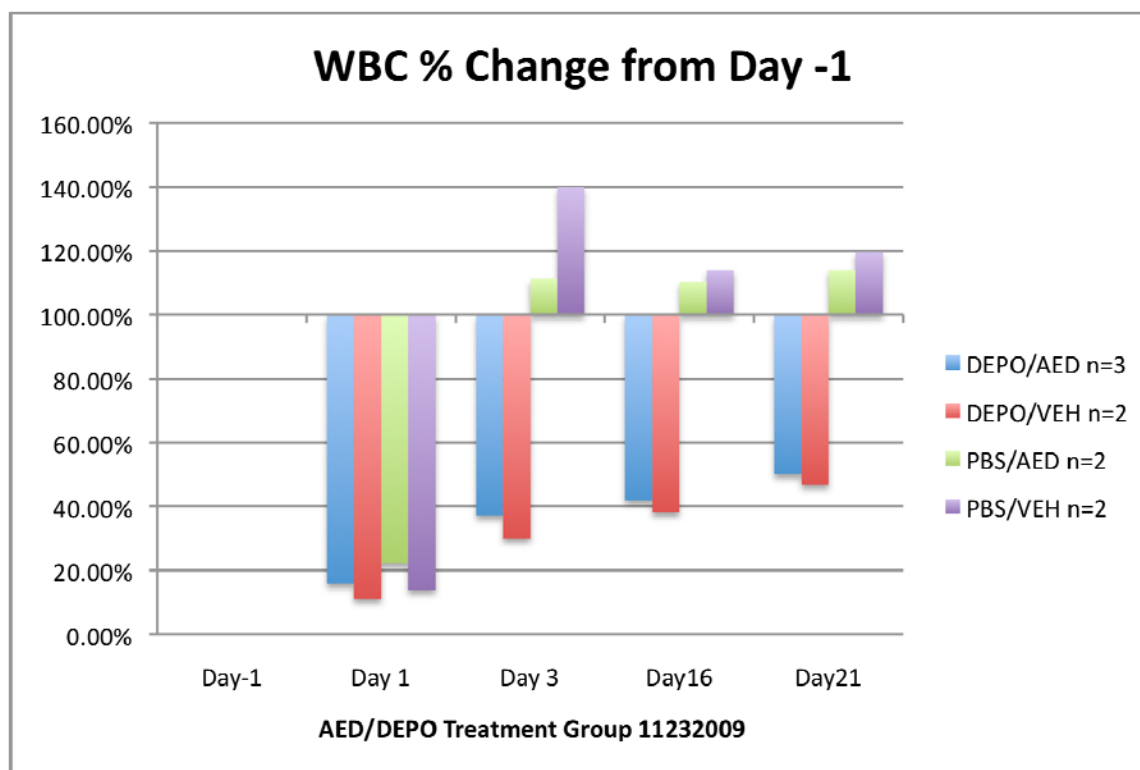


Figure 44: Changes in White Blood Cell Count from Day-1 Average in Rat Group Beginning 258339

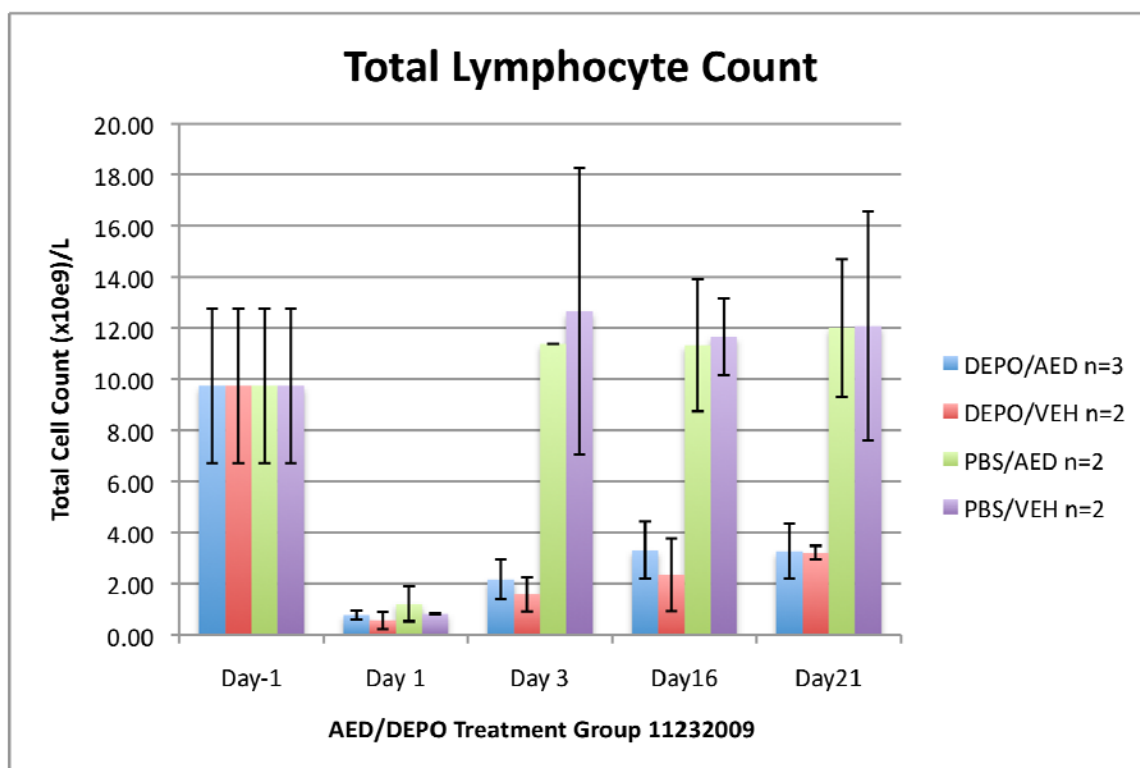


Figure 45: Total Lymphocyte Counts for Rat Group Beginning 258339

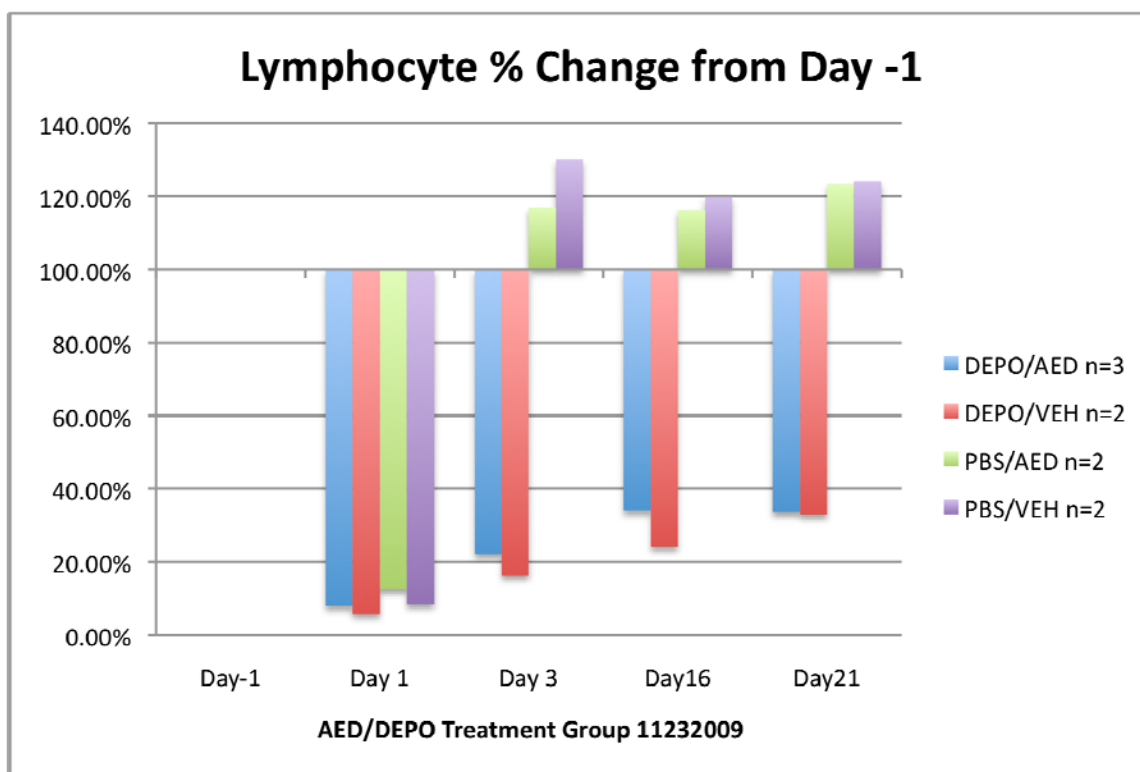


Figure 46: Changes in Lymphocyte Count from Day-1 Average in Rat Group Beginning 258339

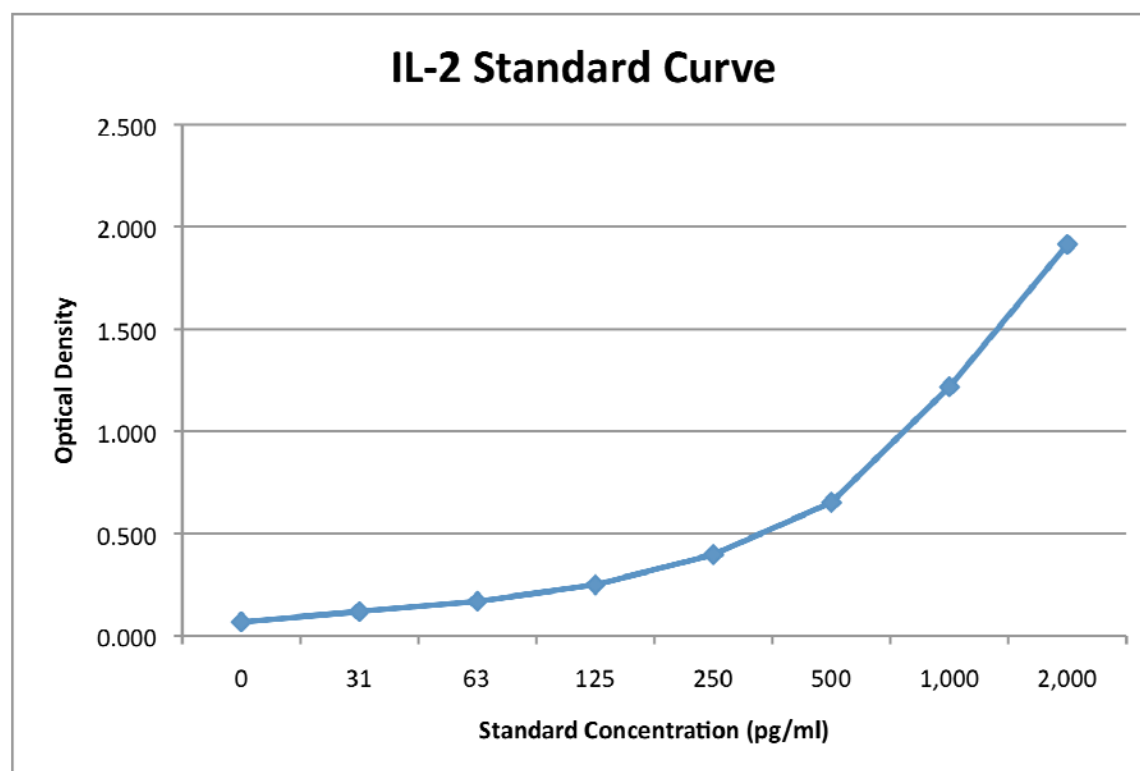


Figure 47: Standard Curve for IL-2 ELISA for Rat Group Beginning 258339

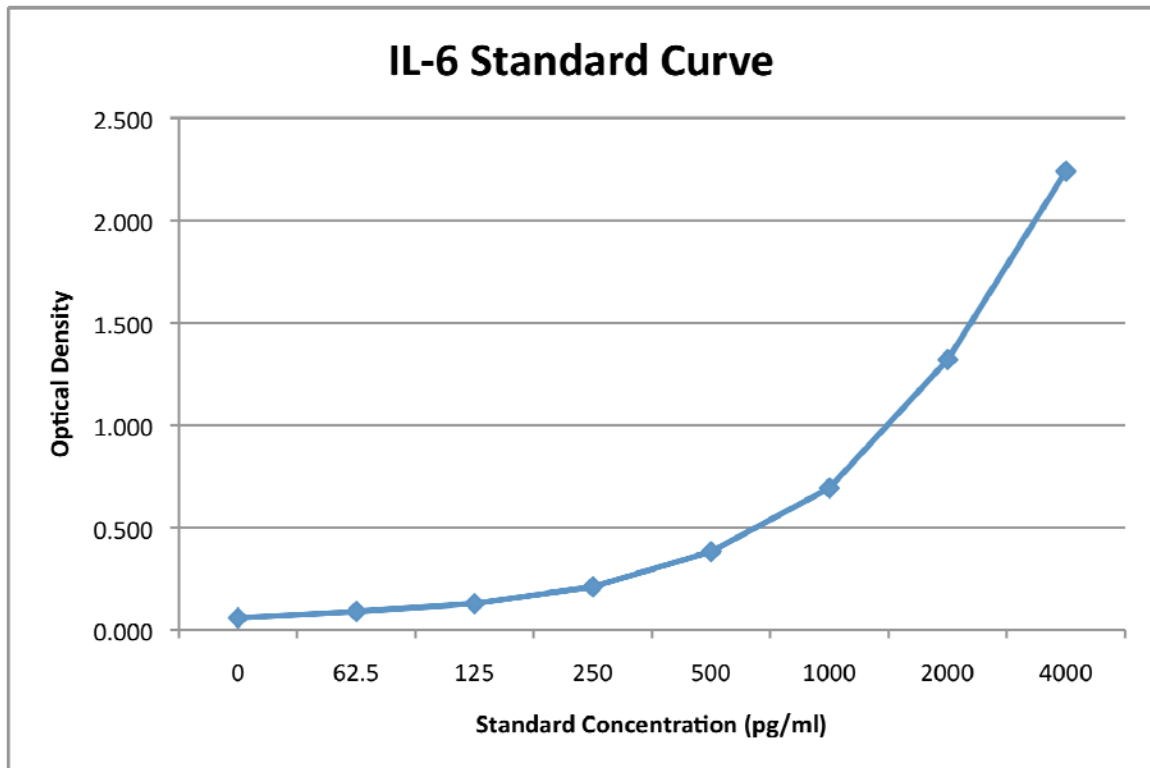


Figure 48: Standard Curve for IL-6 ELISA for Rat Group Beginning 258339

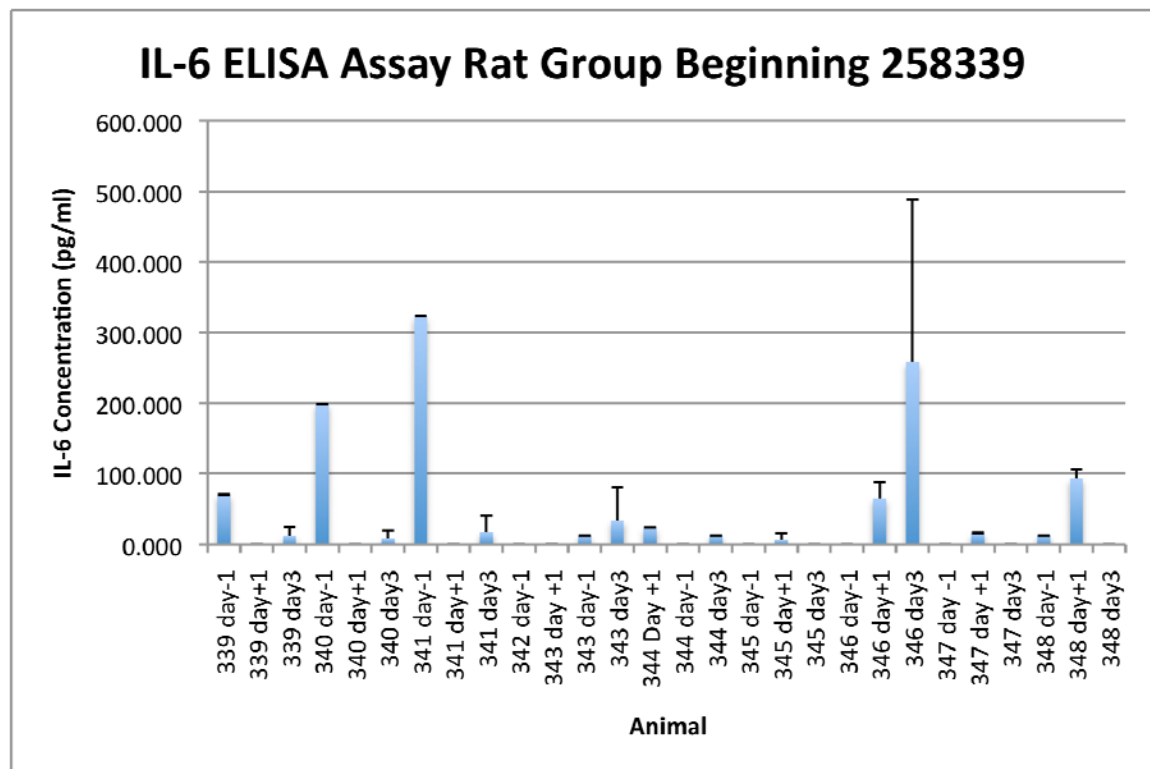


Figure 49: IL-6 ELISA Values for Days -1, +1, and Day3 for Rat Group Beginning 258339

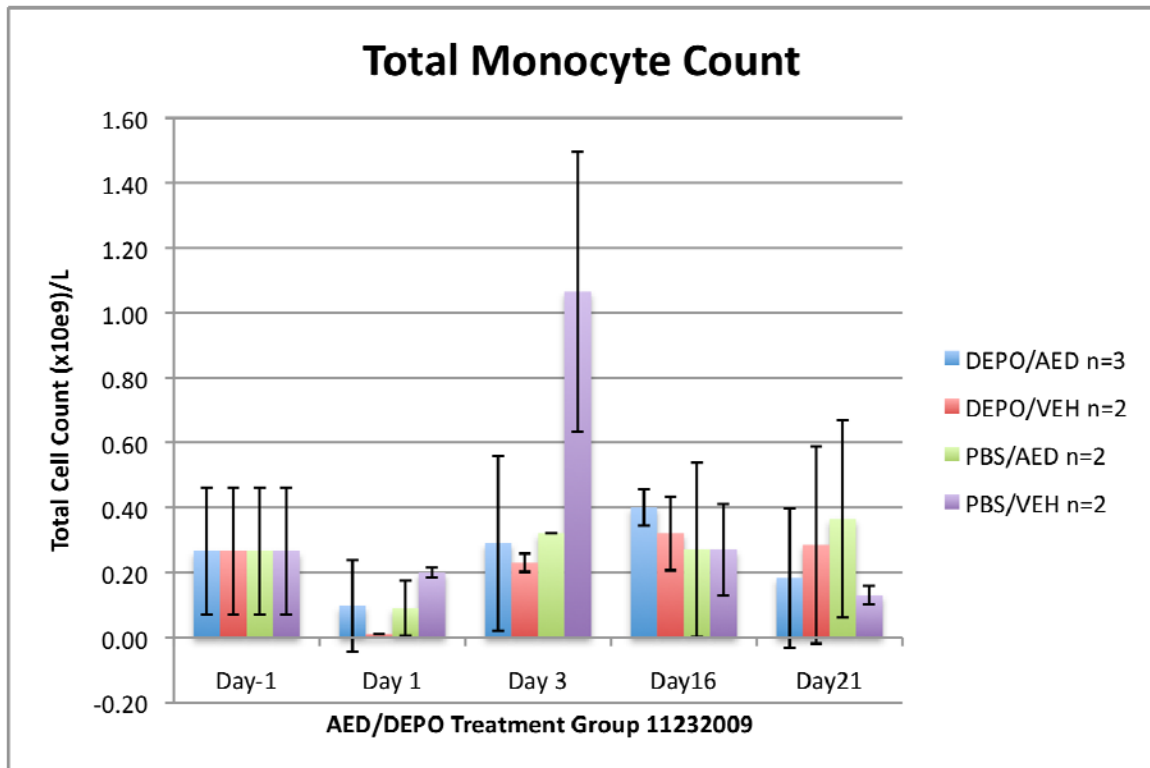


Figure 50: Total Monocyte Counts for Rat Group Beginning 258339

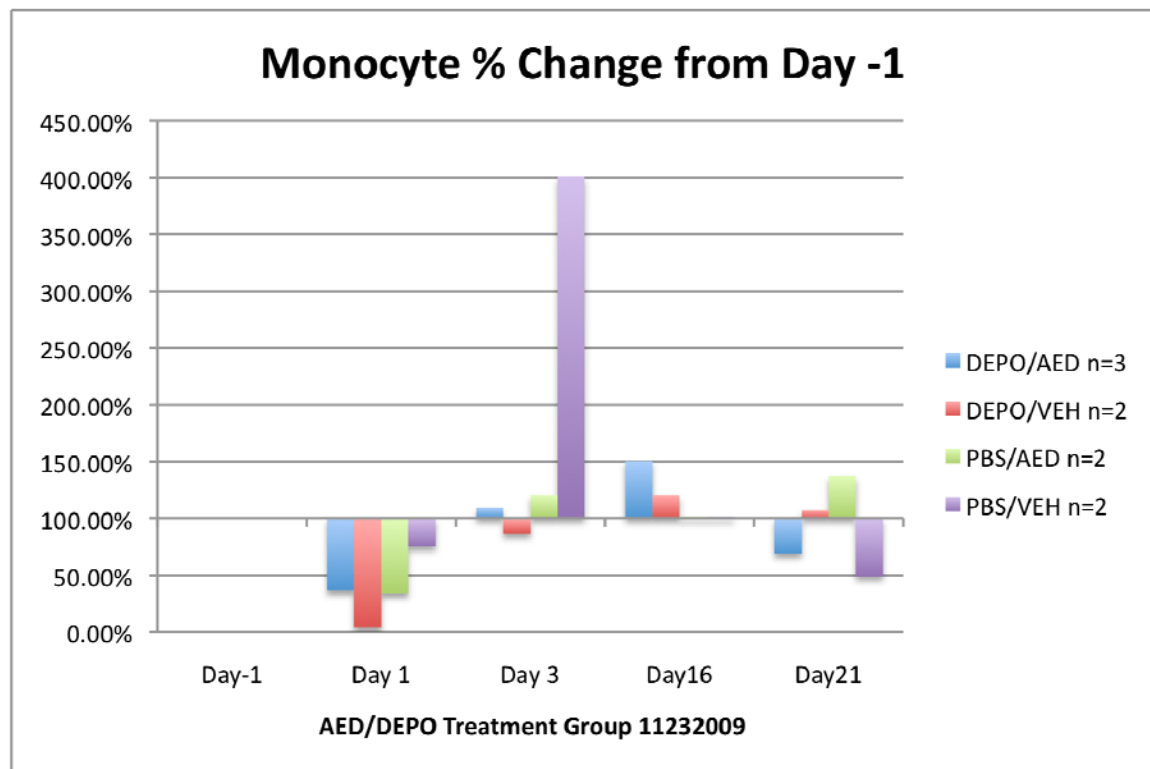


Figure 51: Changes in Monocyte Count from Day-1 Average in Rat Group Beginning 258339

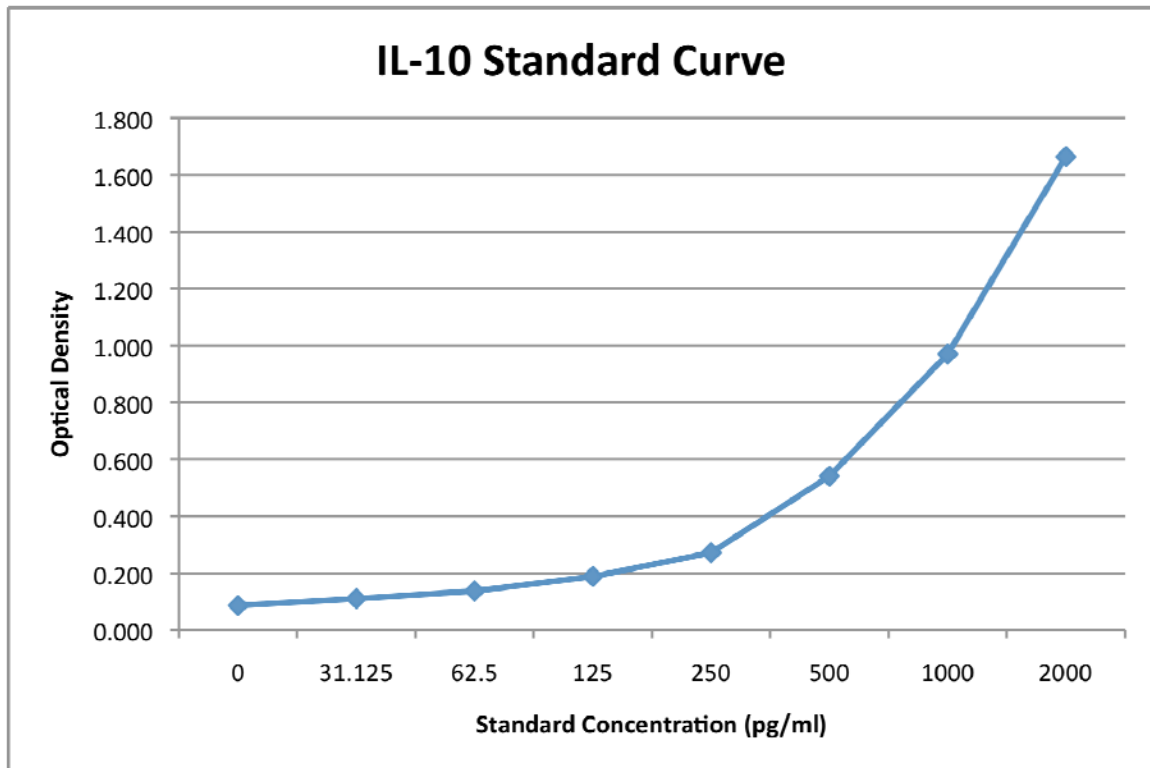


Figure 52: Standard Curve for IL-10 ELISA for Rat Group Beginning 258339

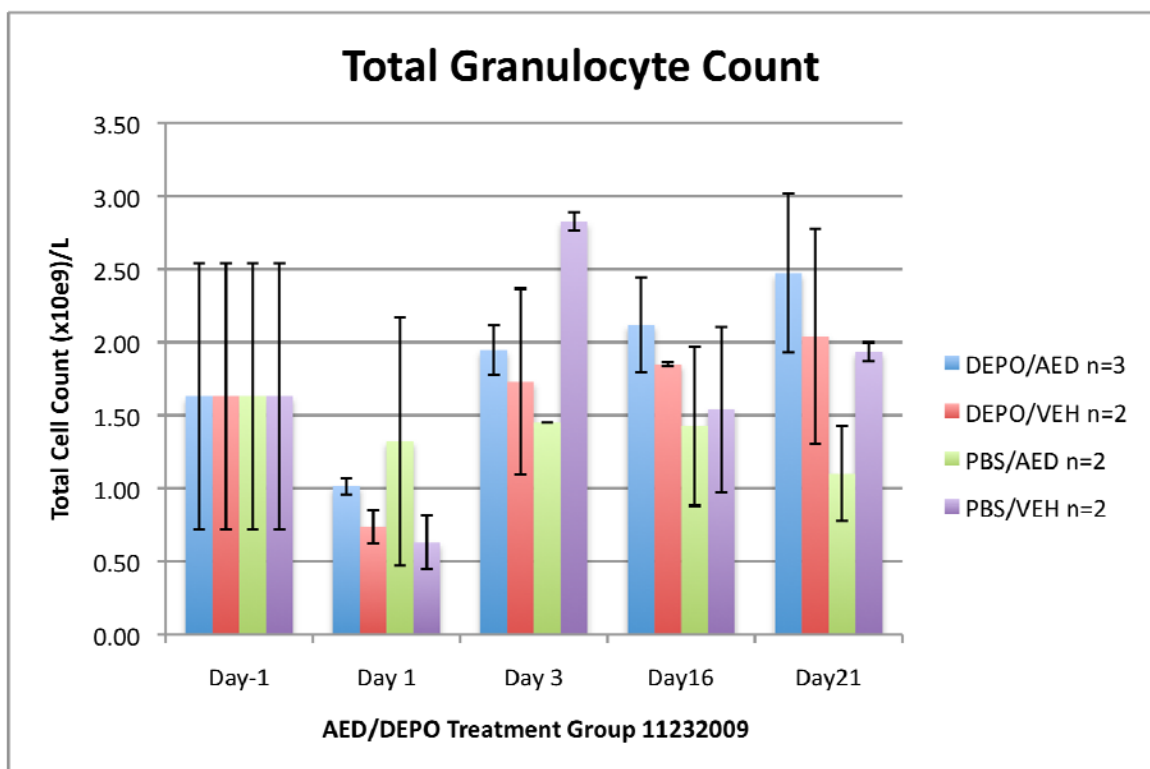


Figure 53: Total Granulocyte Counts for Rat Group Beginning 258339

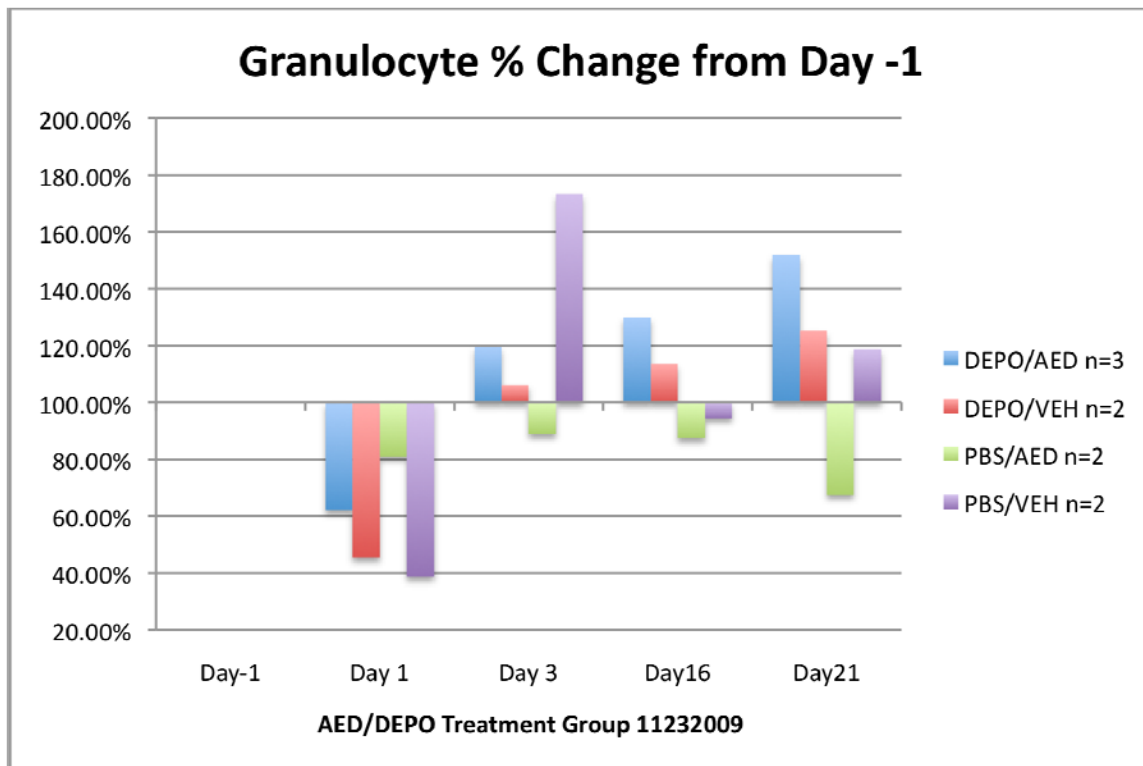


Figure 54: Changes in Granulocyte Count from Day-1 Average in Rat Group Beginning 258339

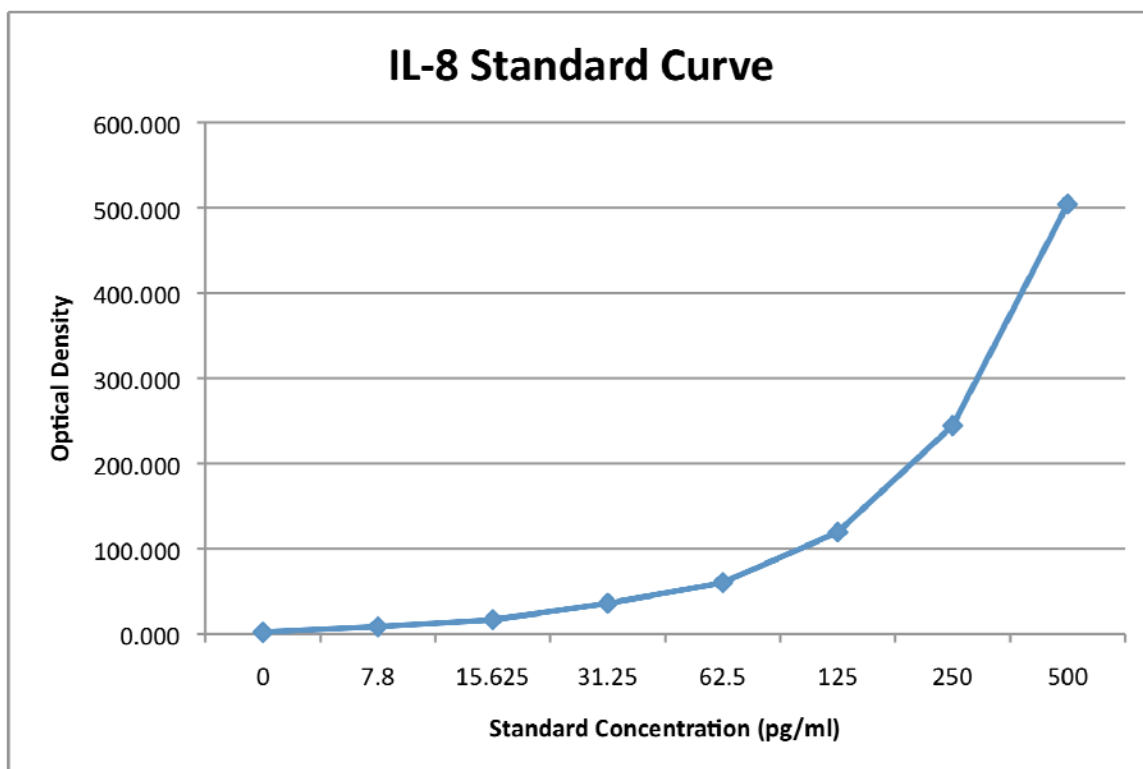


Figure 55: Standard Curve for IL-8 ELISA for Rat Group Beginning 258339

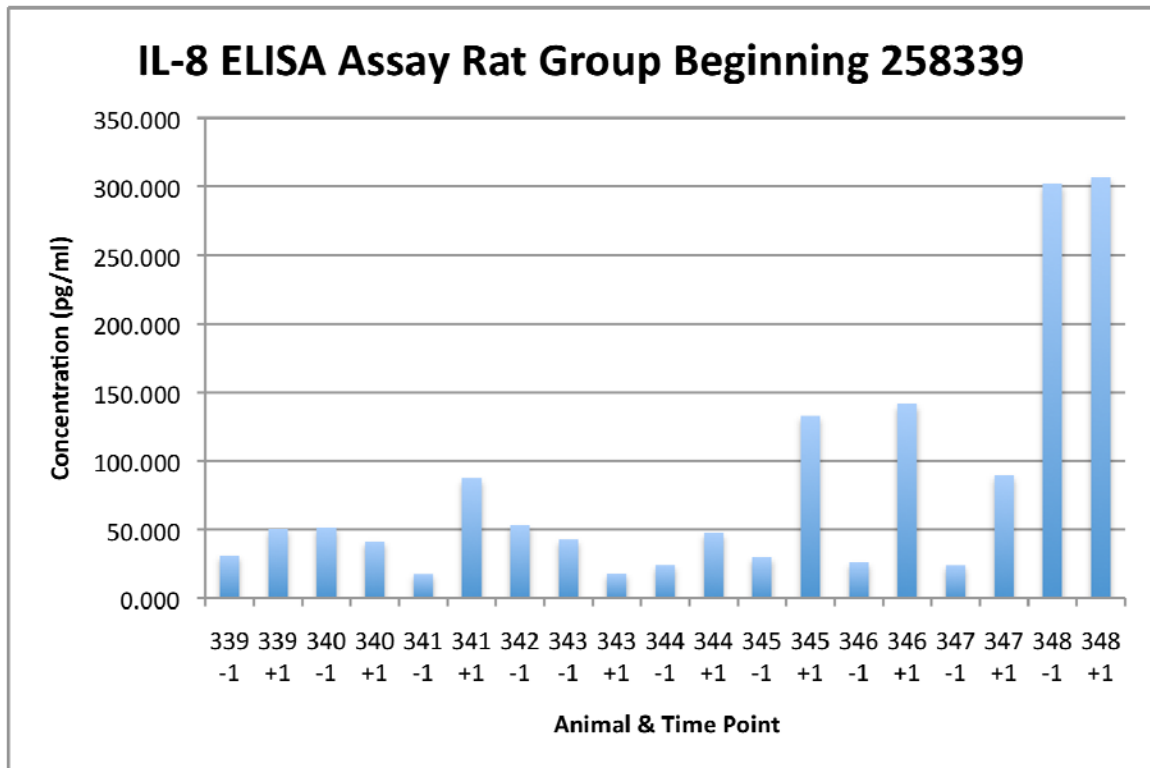


Figure 56: IL-8 ELISA Values for Days -1 and Day+1 for Rat Group Beginning 258339

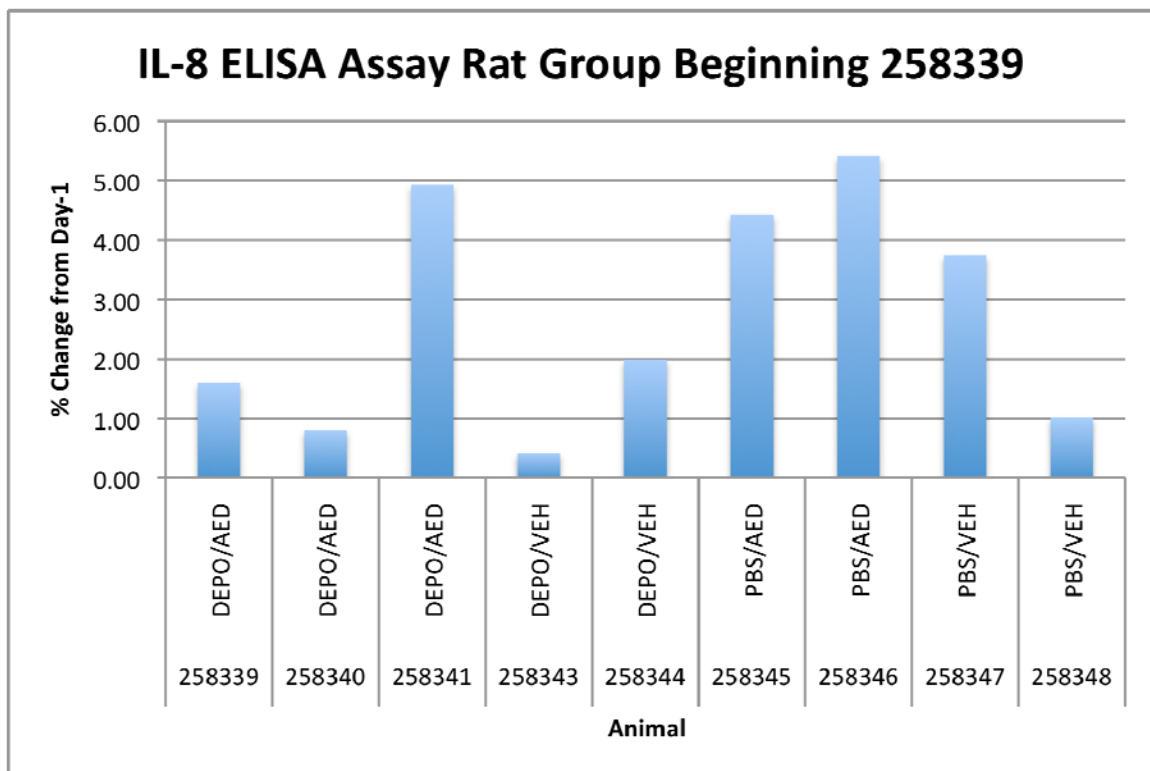


Figure 57: IL-8 ELISA Illustrating Changes from Day-1 to Day+1 for Rat Group Beginning 258339

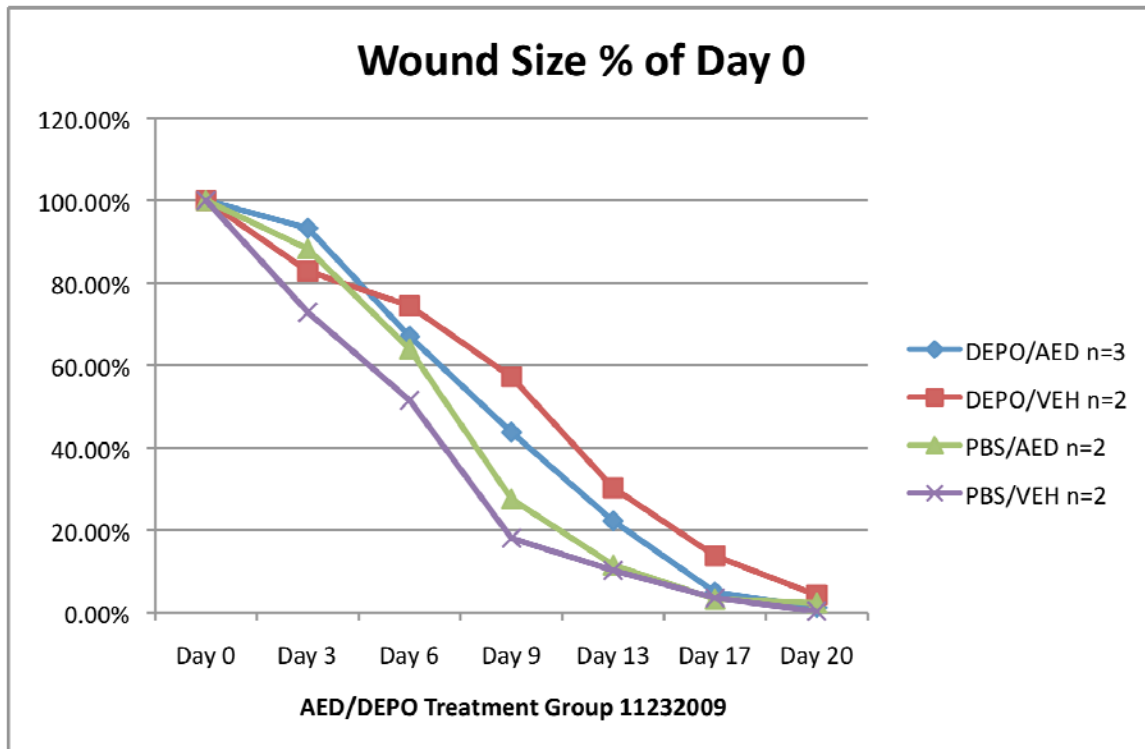


Figure 58: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 258339

Cortisone/AED Group Beginning 261328

The last rat group in this survey of study groups, beginning 261328 was measured and given Day-1 treatment on January 27, 2010. During the treatment course, blood samples were taken on days -1, 3, 6, and day 9, all referencing the day of wounding (Day 0). Animals were also photographed at these time points. As we had become that the immunosuppression of methylprednisolone could not become using the current administration of AED and with the current model for trauma, we elected to test one group using the less potent glucocorticoid, cortisone. In contrast to the immediately preceding groups, this steroid appeared to provide no reduction in the total white blood cell count, in fact resulting in higher values by Day 6 (Figure 59). When compared to average Day-1 values, the data indicates that although this

group started with slightly lower values when compared to the average Day-1 counts, they reached levels over 150% by Day 6, and then began to trend back down. There appears to be no discernable difference between the treatment groups.

T-lymphocyte levels appear to set the aforementioned features in the total white blood cell count (Figure 60). While initially presenting at low levels, below 70% of normal (Figure 61), these groups recover and then exceed normal levels before dropping. The monocyte counts provide a welcome deviation from the previous two cell groups, indicating the same trending as was seen before, but showing that the AED treatment group had higher levels of circulating monocytes than did the vehicle group (Figure 62). When referencing the average Day-1 values, we can see that while the monocytes in the vehicle group indicated twice the normal levels or monocytes, the AED treatment group nearly quadrupled. While the granulocyte data is fairly unimpressive, the fact that there is so little difference between groups is in fact interesting. The changes in granulocyte levels reflect those seen in both the lymphocyte and total WBC counts, however, with absolutely no difference between the treatment groups (Figure 63). Both groups also show increases in granulocyte count doubling, and then tripling before beginning to drop again.

While in this group we saw differences in cell counting that were not patently obvious in other treatment groups, there again failed to be any apparent difference in the manner or timeline in which the wound healed (Figure 64). In fact, these

treatment groups presented more similar healing timelines than an of the other study models.

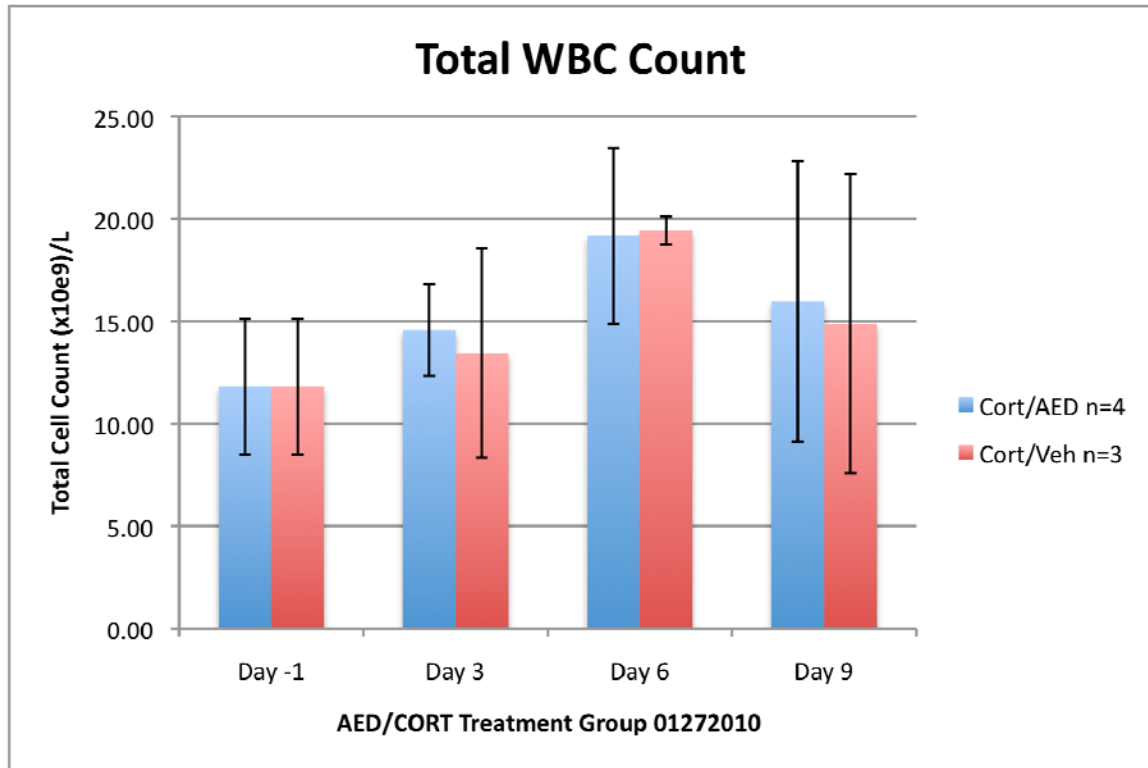


Figure 59: Total White Blood Cell Counts for Rat Group Beginning 261328

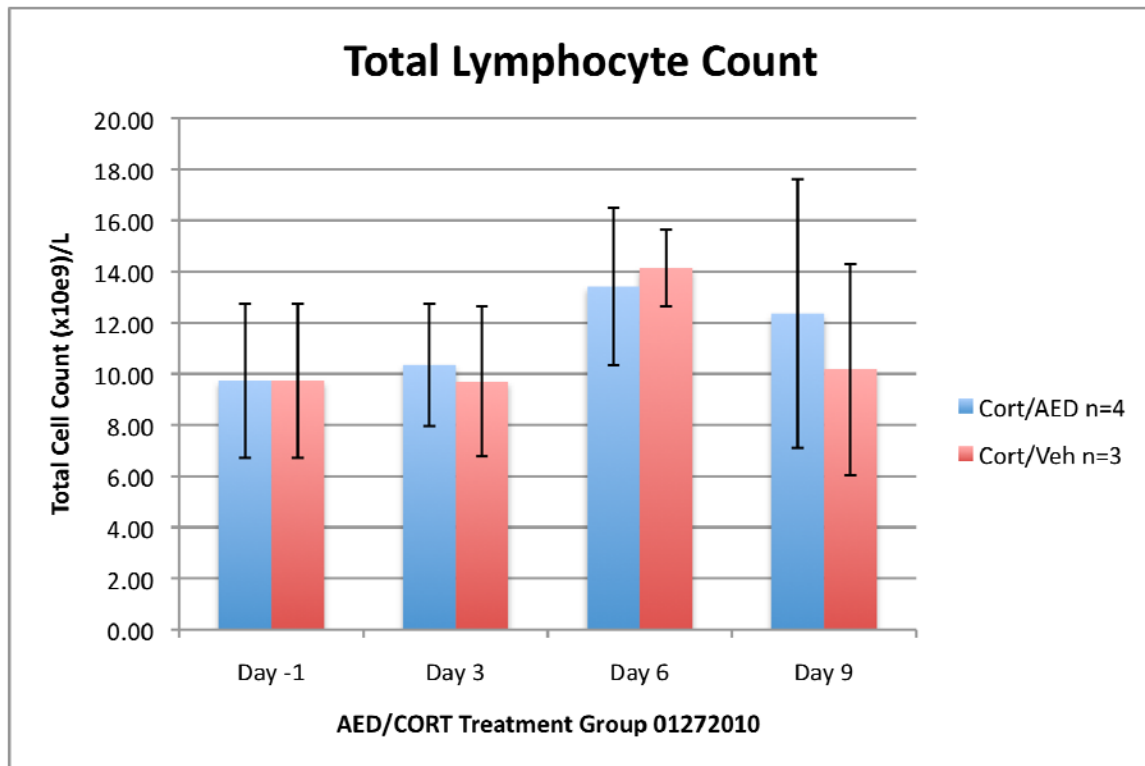


Figure 60: Total Lymphocyte Counts for Rat Group Beginning 261328

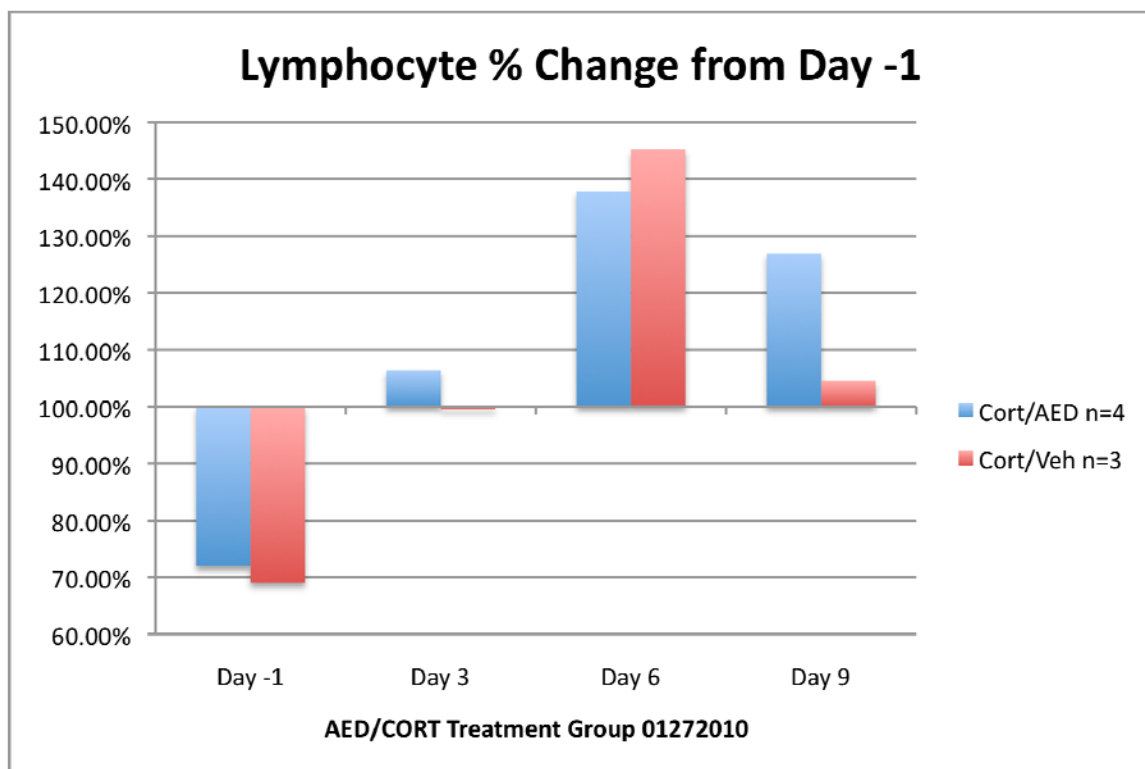


Figure 61: Changes in Lymphocyte Count from Day-1 Average in Rat Group Beginning 261328

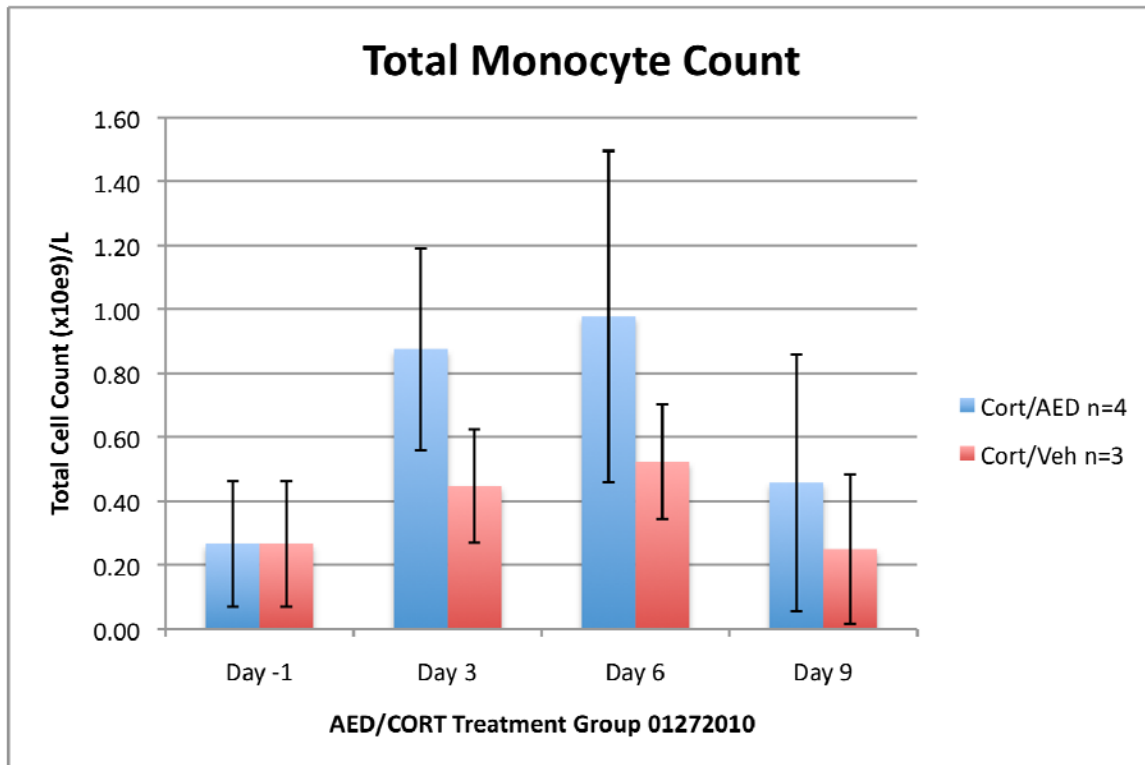


Figure 62: Total Monocyte Counts for Rat Group Beginning 261328

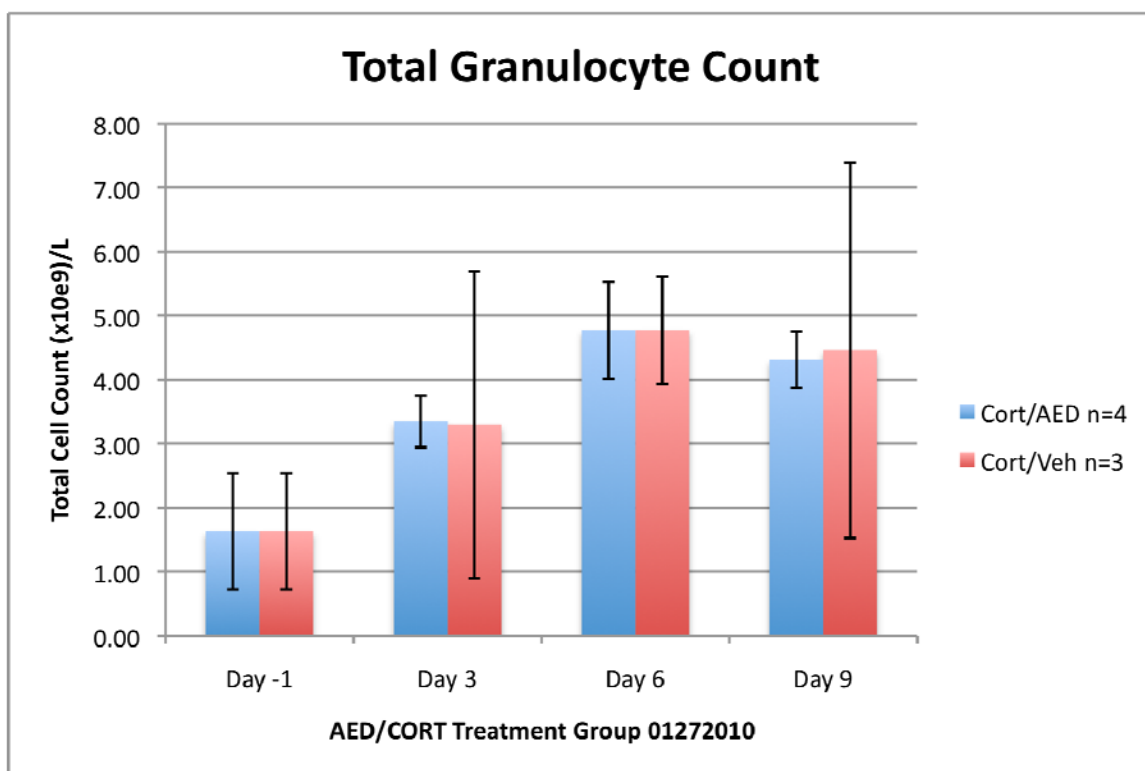


Figure 63: Total Granulocyte Counts for Rat Group Beginning 261328

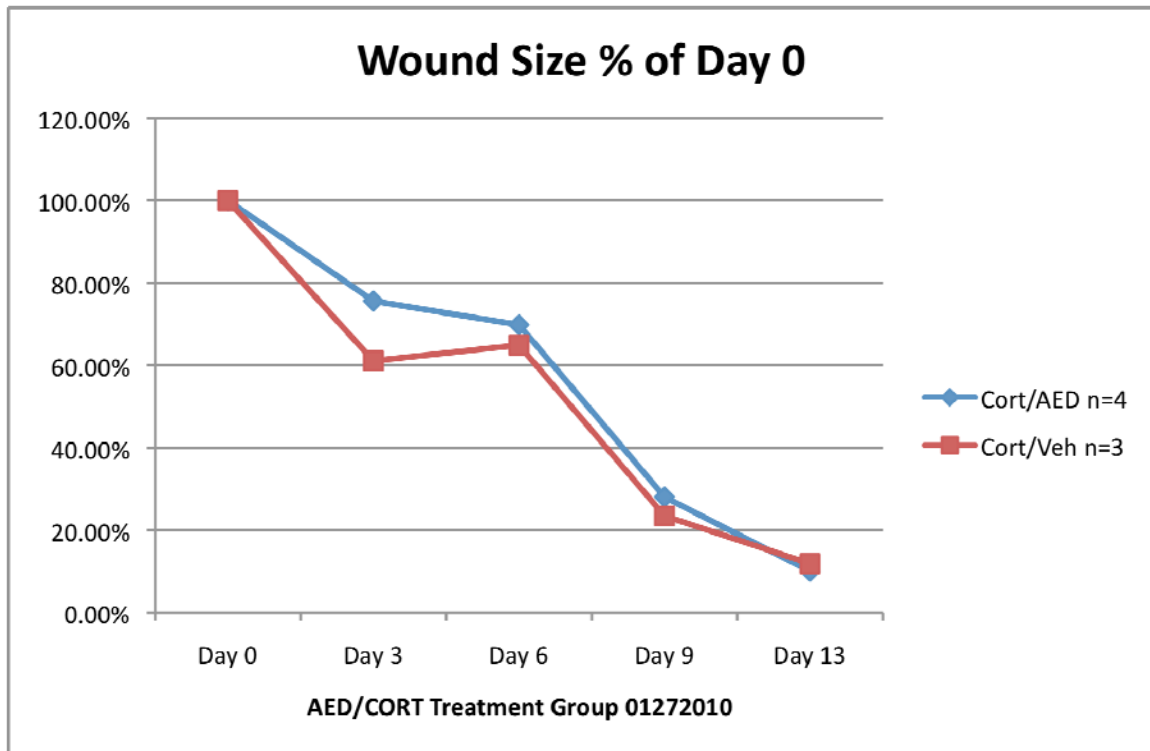


Figure 64: Wound Size Data as a Percentage of Day-1 Wound Size in Rat Group Beginning 261328

Discussion

As stated previously, the results provided above represent a select subset of the data obtained in approximately twenty study groups. Through a close examination, we can see that through the process of induced trauma, administration of immunosuppressive glucocorticoids, and immunomodulatory sex-steroid derivatives, we have indeed modified the presentation of white blood cells counts, and also altered some cytokine profiles. These processes also provided some data reflecting an altered healing process in groups corresponding with the treatment they received. Again however, while we did see some trends reflecting differences between these groups, as well as some significant differences during the healing process, the final result(s) were not different enough, if at all, to recognize a real distinction in the healing process in response to the experimental drugs. The question then remains: why?

Our expectation was that the administration of androstenediol would relieve immunosuppression induced by the prior administration of methylprednisolone, thus enabling the host to heal full-thickness wounds at a rate faster than those not receiving androstenediol. As stated previously, this hypothesis was based on data obtained in a previous murine model in this lab [6]. There are numerous reasons, perhaps too many to examine, that could account for the difference in results obtained between these two studies. There are however, several factors that are more easily identifiable which play a significant role in these differences.

The first and perhaps most significant possibility is the immunomodulatory drug itself. The original source of the drug *and* the vehicle it was administered in were a proprietary mixture then and are now unavailable. We were of course able to procure the Δ^5 -androstene- $3\beta,17\beta$ -diol from alternate sources. However, we had no assurances that the procedures that were utilized allowing the drug to become soluble in vehicle were the same as previously employed. Additionally, we did not have the formulation for the original vehicle, and while we experimented with several vehicles, we were challenged with using vehicle components that are known to modulate the immune system themselves. Some, in fact (at least in the rat) induced skin ulcers similar to those seen in chemical burns. Furthermore, some vehicle solutions appeared to disallow the drug to enter either the general circulation of the lymph system, as encapsulated deposits of drug were found at the site of injection. While we found great success in the use of β -cyclodextrin, the victory was incomplete. The β -cyclodextrin allowed us to produce a completely soluble form of the drug in solution. However, we struggled to discern how much of the drug was taken up by the starch-derived cages, as well as how much of the drug was made available to the host. These results illustrate the difficulties in formulating a vehicle that would allow the drug into solution while still remaining bioavailable and non-toxic.

Ignoring for a moment the possibility that the drug administered may have had no effect due to an altered formulation or an incompatible vehicle, we did see some impressive changes in the quantities of white blood cells, as well as some changes in the expression of inflammatory cytokines (Table 2).

	Trend Difference				
Group ID	Lymph.	Mon.	Gran.	IL-8	Wound Healing
248376A	↓	↑	↑	N/A	--
248680	↓	↑	↑	N/A	--
252251	↓	↑↓	--	N/A	↑-
252968	↓↑	↑↓	--	N/A	↓
254682	↓	--	↑	N/A	--
258339	↓	--	↑	↑	--
261328	--	↑	--	N/A	--

Table 2: Changes in Cell Count and Cytokine Levels in AED/AET Animals vs. Immunosuppressed Animals Only

Perhaps most surprising is the fact that some of these white blood cells, namely monocytes, are some of the most potent mediators of the healing process available. However, while there appears to be little difference in the healing of groups with increased monocyte levels compared to those with flat or biphasic changes in monocyte counts. Similarly, changes in the inflammatory cytokine IL-8 also did not seem to present any difference in the healing process. So then, why are there changes associated with these drug administrations reflected in mediators of the healing process, with no actual changes in healing? We are assuming that the expression of systemic cell types and protein levels are indicative of a changed process at the site of injury or inflammation. While indeed this may be the case, the opposite may in fact be true where as the relationship between local and circulating cells may be inverted. When speaking of cells recruited from the periphery, the introduction of glucocorticoids or sex steroids such as androstenediol may in fact

restrict cellular migration to the site of injury. Glucocorticoids have been shown to decrease levels of activated white blood cells, including the reduced expression of glycosylated proteins responsible for the migration of cells from the systemic circulation into the site of injury. This effect would thus increase circulating levels of white blood cells by allowing the synthesis as part of a natural or pathological process while preventing their access to injury. In fact, one may be able to produce a relationship between increased levels of circulating cells and reduced local counts. In addition, while the activity of white blood cells may or may not be a systemic indication of local activity, there remains the question of cytokine and other protein messenger expression. IL-8 is a well known recruiter of neutrophils and its expression certainly correlates with increased systemic neutrophil counts. While perhaps not true of other chemokines, IL-8 surely must act systemically in order to recruit circulating granulocytes. Other cytokines failed to present themselves at detectable levels, which may be a result of trauma below the threshold for the expression of circulating cytokines or activation of the immune system, still to be discussed. Other mediators not discussed here may not be expressed systemically, acting in a paracrine or autocrine manner locally. Thus increased levels of circulating white blood cells may not be an indicator of local activity, and in some cases, in fact the inverse may be predicted.

Whether or not the assays utilized served as accurate predictors of activity within the immune system or healing, the fact remains that healing within the AED or AET groups failed to exceed that of the methylprednisolone only groups. Most other studies illustrating the difference made by the administration of β -

androstenes are within models with multiple insults such as trauma and hemorrhage, trauma and infection, or burn and infection etc. These previous studies have provided positive data, while this one did not, suggesting that a significant upregulation of the immune system by either trauma or infection may be required in order to provide an improved outcome. Within these study groups, the rats were specified to be between two hundred twenty-five grams and two hundred fifty grams upon delivery. Rats normally lost weight during the course of the experiment, presumably due to increased metabolism following administration of the methylprednisolone. This of course means that the rats were at their maximum weight upon delivery to the lab, between two hundred twenty-five grams and two hundred fifty grams. This is relevant in calculating their body surface area. Calculating the body surface area of rodents can be very difficult, however, it is typically measured by inputting the mass into a standard calculation, which is adjusted for the specific species by a prefactor. The equation given for the Norway Rat is:

$$\text{Surface Area (sq.cm.)} = 9.1 \times (\text{Mass (g)})^{2/3}$$

This equation results in a body surface area of 324.4cm², or 32,470mm². When this is related to the wound sizes applied, it produces wound sizes totaling 0.17% for the total of both 6mm wounds, 0.7% for the total of both 12mm wounds, and 0.95% of total body surface area for both 14mm wounds. Needless to say, these wounds represent minimal trauma, and may not be significant enough to induce the immune system into either a state requiring correction, or a state that can make good use of the immunomodulatory androstenes. However, the previous murine study utilized

6mm wounds in mice with approximately one quarter of the body surface area of these rats. The wounds in these mice then approach 0.7% of the body surface area, comparable to the 12mm wounds in the rat. The subject may however be more complex as the increased wound size in the rat failed to produce the desired results.

Some of the last identifiable and more complex issues to be examined as complicating factors for these experiments involve the catabolism and interaction of the administered as well as the endogenous steroids. Namely, is the catabolism of androstenediol and/or methylprednisolone different in the rat than in the murine model? Secondly, could the metabolites of these drugs interfere or otherwise alter the effects of the other? Lastly, is there a relationship between trauma, inflammation and the catabolism of these drugs and the subsequent metabolites? The catabolism of both endogenous and exogenous steroids, including mineralocorticoids, glucocorticoids, and sex steroids, is commonly known to be completed primarily in the liver. This is not to say that these steroids are not catabolized at other sites in the body, as they are. Studies seeking to compare the existence and activity of p450 enzymes (also known as CYP enzymes) have long sought to draw relationships between human and animal models. Recent studies in fact, have characterized both the mouse and the male rat as the species most similar to man in their inhibition and enzymatic activities when examining the catabolism of testosterone [118]. This is of course a broad generalization both due to the difference in the species as well as the wide range of various human cytochrome p450 levels due to inter-individual variation and genetic polymorphisms. Due to the broad diversity of p450 enzymes and the common redundancy witnessed in

important biological systems, it seems unlikely that a single enzyme is wholly responsible for the catabolism of any of the steroid species administered during the course of the experiments. However, some common enzymes have come to be recognized across the animal species we are examining here. P450-7B1, a potent 7- α hydroxylase has been observed operating in the brain, skin, prostate, and the liver, all frequent targets of steroid messengers [119]. However, and perhaps most interesting, while mice are able to carry out 7 α -hydroxylation in lymphocytes, human are not [120]. Research into whether rats express the ability to conduct 7 α -hydroxylation in lymphocytes remains unanswered. In addition, this enzyme is responsible for the 7 α -hydroxylation of circulating 3 β -hydroxysteroids, including DHEA, 5-androstene-3 β , 17 β -diol (AED), and estradiol [121]. Expressed at human chromosome eight, rat chromosome 2, and mouse chromosome 3, the enzyme has been found to produce the expected metabolites from these precursors in the skin, spleen, thymus, prostate and joints. However, earlier work had identified 6 β -hydroxylation as the common mechanisms responsible for the catabolism of testosterone, androstenedione, and progesterone in both rat and human studies [122]. There is some likelihood that this enzyme identified by Waxman et al. [122] is also known as CYP3A4, characterized by its 6 β -hydroxylation of testosterone and inhibition by itraconazole [123]. Thus it is very likely that there are multiple mechanisms for the metabolism of the various steroid species, perhaps even redundant mechanisms for the same steroid. There appear to be some similarities as well as some differences among these enzymes when considering the different animal species examined in this paper. Therefore, we must be careful when

comparing the metabolism of steroid species between these organisms and be mindful of the differences between them.

While the identification of these enzymes is not *terribly* important for this work, it is important to recognize that there are likely separate enzymes processing the drugs introduced to our subjects. And while this difference seems clear, it is also possible that the structural nature of both drugs may mean that there is also some cross-interaction between these enzymes and their substrates. At some undetermined level of concentration, it is possible to force an enzyme to metabolize a less preferable structure over the primary substrate. Beyond the possible overlap between steroid species and enzymes due to structural similarities, there is also some evidence that the administration of steroids can also alter the expression of P450 enzymes. Studies examining the metabolites of common precursors in rats, including 7α -hydroxytestosterone, illustrated increased catabolism following high dose androstenedione [34]. Conversely, a human study examined the effects of methylprednisolone administration on the activity of the CYP3A4 enzyme and found that no alteration in activity could be found in short term high dose or long term low dose administration [124]. Add to these equations any endogenous substrates created by wounding and the experimental process and there is at least the possibility that levels of one steroid may affect the catabolism of the other(s) by either the interference with enzyme binding or the increased expression of the enzyme itself.

Lastly, one of the more important questions with regard to this study is how the events of injury and inflammation interact with the two steroidal agents

(primarily methylprednisolone and AED), presumably to the benefit of the organism? As we have illustrated, there are separate but perhaps overlapping mechanisms for the catabolism of these compounds, and the mechanisms for the elimination of these compounds appear to be in place in both models, though perhaps to differing degrees. In addition, any perceived inequality due to the differences in wounding between the murine and rat models should not have any quantifiable difference, as the scale of wounding between the groups appears similar. However, it bears noting that there have been established relationships between the activity of the aforementioned p450 enzymes and a state of inflammation. Dulos et al. has shown that the introduction of pro-inflammatory cytokines increases mRNA levels of CYP7B, thus increasing catabolism of DHEA into the 7- α metabolite, which itself may contribute to the maintenance of chronic inflammation [125]. In contrast, many more studies have indicated that infection and inflammation result in the reduction in capacity to eliminate drugs [126]. This hypothesis has been reinforced many times, particularly in the rat model. No single mechanism has been attributable to this outcome; in fact reductions have been tied to repression of gene transcription, as well as protein turnover of cytochrome P450 enzymes. These results are further attributable to exogenous as well as endogenous sources, including interferons, interleukins, and tumor necrosis factors. Therefore any difference in the expression of these signaling factors so prevalent within the immune system could exercise some effects on the catabolism of either the immunosuppressor methylprednisolone, the immunomodulator androstenediol, or both.

Conclusion

These mini-studies, as part of defining the parameters of a larger experiment, did in fact show some physiologic response to the drugs that were administered. While in some cases these differences were significant, we did not find any real difference in healing downstream of these changes. As discussed, there are many potential reasons that these results were different than those of previous experiments. Despite the disappointment of failing to show a difference in the rate of wound healing between the AED/DEPO and the DEPO only groups, it is perhaps from failure that we learn the most about how these agents exercise their influence. Ultimately, it seems that the levels of cytokine expression during these experiments gave the clearest indication that the model of trauma utilized here was not severe enough to induce expression of some of the most commonly measured pro-inflammatory cytokines. While this threshold may be different than was necessary in earlier models, the rat appears to be more well positioned to resist a pro-inflammatory state, and thus perhaps was not as well equipped to receive, or in need of, the immunomodulatory agents administered. Furthermore, the interaction between different exogenous and endogenous steroids raise significant questions with regard to how these compounds effects each other, as well as how they effect the metabolism of themselves and similar compounds. In addition, one of the most important lessons may be that systemic expression of inflammatory markers may not always be a good marker of local activity. These questions all bear consideration, however, they are perhaps only worth examining in a model in which there exists an inflammatory state in need of modulation.

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Appendix B

DOD Animal Use Protocol Cover Sheet

I. Name of Facility: US Army Institute of Surgical Research, Fort Sam Houston, TX

II. Protocol Number: A-10-013

III. Title: Effectiveness of Silver Based Wound Dressings to Inhibit Infection in Rat (*Rattus norvegicus*) Contact Burn Wounds

IV. Principal Investigator/Division/Phone/E-mail

a. Printed Name: Shanmuganathan Seetharaman, PhD

Title: PTEI Post-Doctoral Fellow

Division: Research, Regenerative Medicine

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V. Task Area Manager/Division/Phone/E-mail (*This signature verifies that the proposed research is consistent with the goals of the task area and will therefore have appropriate resources applied to it.*)

a. Printed Name: Joseph Wenke, PhD

Title: Task Area Manager

Division: Extremity Trauma Research

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VI. Scientific Review/Division/Phone/E-mail (*This signature verifies that the animal use proposal received appropriate scientific peer review and is consistent with good scientific practice.*)

a. Printed Name: David G. Baer, PhD

Title: Director, Office of Research

Division: Research

-original signed-

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b. Signature: _____ **Date:** _____

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Appr 14 Jun 10

VII. Statistical Review/Division/Phone/E-mail (*A person knowledgeable in biostatistics is required to review all proposals to ensure that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.*)

a. Printed Name: John A. Jones, BS

Title: Statistician

Division: Research

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VIII. Attending Veterinarian/Division/Phone/E-mail (*The Animal Welfare Act Regulations require that an attending veterinarian must be consulted in the planning of procedures/manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.*)

a. Printed Name: Kevin R. Armstrong, DVM, LTC

Title: Chief, Laboratory Animal Medicine Services

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2 Jun 10

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DOD Animal Use Protocol

PROTOCOL TITLE: Effectiveness of Silver Based Wound Dressings to Inhibit Infection in Rat (*Rattus norvegicus*) Contact Burn Wounds

PRINCIPAL INVESTIGATOR:
Shanmuganathan Seetharaman, PhD

ASSOCIATE INVESTIGATOR(S):

Robert J. Christy, PhD
David G. Baer, PhD
Shanmugasundaram Natesan, PhD
David O. Zamora, PhD

I. NON-TECHNICAL SYNOPSIS:

Burn wounds sustained under battlefield conditions are considered to be contaminated with bacteria and initial treatments focus on decreasing this contamination, thereby reducing the possibility of future infection. Unfortunately, conditions on the battlefield often do not always allow for the rapid evacuation of a casualty to surgical care and this delay increases the chances of infection/sepsis resulting in higher morbidity and mortality of our military personnel. Traditional methods for prophylaxis and treatment of wound infections, such as dressing soaks and burn cream application, are time and resource consuming and are not suited for the conditions on the battlefield and in field hospitals. There is a need to develop new first-line treatments to prevent burn wound infection and sepsis. In this protocol anesthetized rats will be administered a partial thickness contact burn wound and exposed to infective bacteria. We will then evaluate the effectiveness of newly developed wound dressings for the prevention of infection, sepsis and improved wound healing.

II. BACKGROUND

II.1.1 Influence of Infection on the Healing Process

Local factors such as infection, hypoxia or foreign bodies are important in the healing wound and should be corrected or controlled. Various extracellular virulence factors such as elastase, exotoxins and exozymes are shown to influence directly or indirectly the healing process. Since thermal injury induces an immune compromised state, infection due to microbial pathogens further exacerbates the normally occurring array of events (1-4). Notably, infections associated with injuries observed in military combat have not been fully established, and most importantly, burns complicate approximately 5% to 10% of all combat casualties. An average evacuation time is 7 days from the combat zone, throughout the chain to definitive rehabilitative care in the United States. During this period of time, injured army personnel have been treated by 4-5 different physicians and this causes a fragmentation in continuous standard care (5,6). Infectious risks associated with these injuries include both those from initial wound contamination and those from nosocomial infections associated with long-term care. The role of bacteria has been dealt with importance and its influence in the healing process is well documented. Heavy colonies of bacteria are the actual cause of exudation at the wound site and have been shown to have a number of deleterious effects on the healing process, including the breakdown of

extracellular matrix proteins, growth factors and tissue inhibitors of matrix metalloproteinases. In burn wounds, **burned dead skin or eschar** promotes bacterial colonization and imparts a considerable reduction in oxygen tension, followed by epidermal cell detachment, and it eventually prevents wound closure. The bacterial colonies potentiate scarring either by stimulation of inflammation or by causing Extracellular Matrix (ECM) degradation (wound dehiscence) (7-9). Inflammation is a crucial localized protective tissue response and is the host's first self-defense against a physiological insult. It serves to destroy, dilute or wall off both the injurious agents and the injured tissues (10). The response includes the early induction of proinflammatory cytokines, activation of the complement cascade, activation of coagulation factors, late production of anti-inflammatory cytokines, and subsequent down-regulation of cell mediated immunity. When the wound is colonized by bacteria, the inflammatory response is aggravated, leading to deleterious effects on subsequent healing phases (11,12). Critical injury and sepsis are also correlated with dysfunction of many immune cells that are generated by functional changes in neutrophils, monocyte/macrophages, dendritic cells, T lymphocytes, and B cells (13). During the normal healing process, infiltration of inflammatory cells ceases within a few days after injury but is expressed for a prolonged time in contaminated wounds. This excessive extravasation and accumulation of cellular components continues, and it becomes chronic, resulting in additional recruitment of inflammatory leukocytes associated with production of proinflammatory cytokines, mainly Interleukin-1 β (IL-1 β), Tumor Necrosis Factor- α (TNF- α) and Interleukin -6 (IL-6). There exists a balance of inflammatory cytokines related to severity and mortality. In particular, IL-1 β and TNF- α are commonly reported to exhibit synergism, and the net effect should be considered when correlating cytokines levels and severity of disease. These cytokines also play a major role in regulation of immune response, hematopoiesis and inflammation. Among these cytokines IL-6 has a short peak time in the normal healing process, while IL-1 β and TNF- α are shown to persist for longer times (14-16). There is considerable evidence to suggest that chronic inflammation and angiogenesis are closely linked. Angiogenic blood vessels at the site of inflammation are enlarged and hyperpermeable in order to maintain the blood flow and to meet the increased metabolic demands of the tissue. During severe infection, the mechanical stability of the provisional matrix is diminished with accumulation of inflammatory cells. This hinders the development of new blood vessels (17). Since fibrin is a natural provisional matrix required for cell migration and angiogenesis, the current dressing supplies the need of scaffolding architecture where it is depleted due to infection. Moreover, in a non-infected wound, up-regulation of proangiogenic factors such as vascular endothelial growth factor has been previously reported in inflamed tissue. Whereas in an infected wound either there is a low level of VEGF expression or destruction of the growth factor occurs due to bacterial exotoxins and high levels of inflammatory cytokines. The growth and chemotactic factor secretion by neutrophils and macrophages plays a vital role in the transition of the inflammatory phase to the proliferative phase of wound repair (18). These events collectively prolong the remodeling phase, especially formation of granulation tissue. Increased levels of endopeptidases, mainly the matrix metalloproteinases (MMPs), result in an imbalance in matrix synthesis and matrix degradation leading to delayed healing (19). Hence therapeutic intervention to control infection using an active wound dressing (like fibrin scaffold) will positively regulate inflammation and induce angiogenesis to achieve faster healing of infected burn wounds.

II.1.2 Silver Based Antimicrobials

The use of topical dressing and/or ointment/creams is fundamental to prevent infections in deep and superficial burns or extensive intermediate burns. Increasingly, antibiotics, due to widespread indiscriminate prescription, are becoming less effective as pathogens are becoming more resistant to their action. Silver may be a useful prophylactic or therapeutic agent for the prevention of wound colonization by organisms that impede healing, including antibiotic-resistant bacteria (20). It is a well-known bactericidal agent routinely used in clinical settings and the antimicrobial activity of silver ion is well defined. Silver is a broad-spectrum antimicrobial agent that controls yeast, mold, and bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). As a metal, silver is relatively inert and is poorly absorbed by mammalian or bacterial cells. However, in the presence of wound fluids or other secretions, it readily ionizes and becomes highly reactive in binding to proteins and cell membranes (21). Similar to other heavy metals, silver is toxic to microorganisms by poisoning respiratory enzymes and components of the microbial electron transport system as well as impairing some DNA function. The inhibitory action of silver can be attributed to its strong interaction with thiol groups present in cell respiratory enzymes in the bacterial cell. Additionally, silver has been shown to interact with structural proteins and preferentially bind with DNA bases to inhibit replication. *In vitro* studies provided evidence that the bactericidal effect of silver is attributable largely to the binding of the silver ion to free sulphhydryl groups in the bacterium or on its surface leading to inactivation of the enzyme phosphomannose isomerase. More substantive information on the bactericidal action of silver relates to its accumulation in the bacterial cells and its opportunity to interact with the cytosolic proteins, mitochondrial enzymes and nuclear DNA or RNA synthesis (22).

Antimicrobial Delivery

The importance of drug delivery has been emphasized not only with respect to physiological response but also to the mechanism of drug release, especially as a carrier for on-site delivery of antibacterial agents. To accomplish a controlled release, drugs can be entrapped in micro-carriers like micro/nano spheres. The advantages of microspheres are, it can protect and control the release of the drug. Among the recent advancements in wound care management, the timed release of drugs provides scope for developing novel wound dressings. A wide range of materials (both synthetic and natural) are available for developing controlled release systems. Among these polymers, natural polymers have gained special interest over synthetic polymers mainly because of better biocompatibility, degradation products being biocompatible, cost effectiveness, and bulk availability (23,24). Some of the natural polymers include chitosan, alginate, collagen, gelatin, chondroitin sulfate and others. In this project natural polymers will be used to deliver drugs at the wound site. The kinetics of the drug release will be optimized according to the pathophysiology of the wound or wound status. Chitosan has been chosen as a carrier of choice to deliver antimicrobial agents. We have previous experience in developing wound dressings with alginate, chitosan and gelatin which can deliver drugs topically in a controlled manner and fibrin based hydrogel for angiogenesis (25,26).

Fibrin Based Dressings

Natural polymers over the years have gained more importance due to their excellent biocompatibility and ability to interact with cells *in vitro* and *in vivo*. Fibrin is a versatile biopolymer for mediating thrombin-mediated cleavage of fibrinopeptide A from monomeric fibrinogen. Fibrin and fibrinogen have critical roles in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia. Fibrin has been

used clinically as an FDA-approved hemostatic agent and as a sealant in a variety of clinical applications, including procedures such as soft tissue dissection (27,28). Fibrin hydrogels from commercially purified allogeneic fibrinogen and thrombin have been used widely in the last decade in a variety of tissue engineering applications, and these include tissue engineering of adipose, cardiovascular, ocular, muscle and skin tissue types. In addition, fibrin hydrogels have applications for promoting angiogenesis. Major disadvantages with fibrin hydrogel as a potential scaffold are the shrinkage of the gel, low mechanical stiffness, and rapid degradation before the proper formation of tissue engineered structures (29). To overcome these problems, fibrin needs to be modified before use to serve as a better three dimensional tissue engineering scaffold. One such approach would be copolymerizing the fibrin with polyethylene glycol. Our preliminary work has demonstrated several unique features of PEGylated fibrin that make it advantageous in wound healing over other hydrogel dressings (30). PEGylated fibrin exhibits unique features of both synthetic hydrogels and natural materials. Specifically, (1) the presence of PEG provides a highly hydrated (>90% water) moist environment for managing exudate. (2) The presence of fibrin confers biodegradability to the material; however, our prior results show that it is significantly more stable *in vitro* than fibrin alone. Finally, (3) the inherent biologic activity of fibrin encourages healing by stimulating tissue and blood vessel in-growth.

Androstenediol (AED) and Androstenetriol (AET)

A recently discovered class of immune-regulating hormones (IRH), AED and AET have been shown to have remarkable anti-inflammatory properties, yet can potentiate the cellular and humoral immune response to a number of local and lethal systemic insults. By regulating the uncontrolled and chronic inflammation associated with infection and sepsis, the morbidity and mortality that normally results should be reduced or prevented. A number of recent studies cited below have demonstrated that AED and AET mediate a significant increase in the levels of the nuclear receptor factor Peroxisome Proliferator Activated Receptor-gamma (PPAR γ). Similar effects were also observed with NF-k β . It is recognized that PPAR and prostaglandins have a major role in controlling immune response¹, inflammation^{2,3} and wound healing^{4,5,6,7}.

AED & AET (supplied by Dr. Robert Diegelmann, Virginia Commonwealth University) will be administered either subcutaneously on the dorsal area away from the burn site or administered intraperitoneally in a dose range of 40, 80 or 320 mg/Kg in either 0.25ml in DMSO:Ethanol, 1:1 or 0.2ml in sulfobutyl ether β (beta) cyclodextrin, sodium salt. In some studies Androstenetriol (AET) will be administered subcutaneously on the dorsal area away from the burn site in a dose of 30 to 40 mg/Kg 0.25ml in DMSO:Ethanol, 1:1 or 0.2ml in sulfobutyl ether β (beta) cyclodextrin, sodium salt. AET is more soluble than AED and is 10 fold more bioactive. The time line for AED or AET administration will be on day 0, the time of burn and every day up to day nine.

II.2. Literature Search for Duplication:

II.2.1. Literature Source(s) Searched: BRD, REPORTER, DTIC, PubMed

II.2.2. Date of Search: May 2010

II.2.3. Period of Search: January 2000 to May 2010

II.2.4. Key Words of Search: Contact burn, wound infection in rat, burn wound healing animal models.

II.2.5. Results of Search: Literature review provided information and procedures for partial contact burn procedures and the wound healing response. The search also supplied data and information that allowed us to propose feasible experiments for the effect of antimicrobial agents on the infected wound after burn injury. No unnecessary duplication of effort was found by the Principal Investigator.

III. OBJECTIVE/HYPOTHESIS:

Objectives:

Phase 1) **Develop infected contact burn wound model and to determine the bacterial load (challenging inoculums) in rats at the Institute of Surgical Research.**

Phase 2) Determine the effectiveness of silver based antimicrobial dressings in an infected contact burn wound.

We hypothesize that the use of silver based topical antimicrobial dressings will prevent infection and accelerate wound healing.

IV. MILITARY RELEVANCE:

Thermal injuries are common to all military conflicts and historically constitute approximately 5 to 10% of all warfare military casualties (31). As a result of the improvised explosive devices being used in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF), burns are the primary injury in approximately 5% of the personnel being evacuated with infection being the leading cause of mortality (32). Typically, these burn patients face many complications including; supplementary injuries from multiple fragment wounds, high rate of infection, and long rehabilitation and hospitalization time (33,34). Even relatively small burns can be incapacitating, disfiguring, and painful and can strain the logistical and manpower resources of military medical units, overwhelming medical-resource allocation requirements. Moreover, they cause serious psychological implications for both combat and medical personnel.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures:

This protocol will use a two phase experimental design. Phase 1 will develop an infected burn injury of specific size and depth in rats using a contact burn inflicting device. We will induce a burn wound using the procedure developed in protocol A-09-010 that creates reproducible burn wounds in the rat model. We will then infect the wound with ATCC standard pathogenic strains of *Pseudomonas aeruginosa* or *Staphylococcus aureus* at three different concentrations. **The objective of phase 1 is to develop infected contact burn wound model and to determine the bacterial load (challenging inoculums) that will be used in Phase 2.** The objective for Phase 2 is to test the antimicrobial dressings in an infected burn wound and determine the biocompatibility of the test products.

V.1.1 Phase 1 – Infected Wound Model Development and bacterial load determination Contact burn model (Protocol # A-09-010)

Wounds sustained under battlefield conditions are considered to be contaminated, and their initial treatment should focus on decreasing this contamination and thus reducing the possibility

of infection. Burn injury may be inflicted by various methods, including; scald, thermal, chemical etc. In this protocol an infected contact burn injury model will be developed based on our previous protocol (A-09-010) which developed a precise partial thickness burn injury to skin.

Instrument for burn induction

The instrument was designed using a commercially available soldering unit and was used as a controlled heat source. The soldering iron was modified by welding a 17mm diameter, 2.5mm thick brass disk on the tip and weighted with 500 gram to induce sufficient pressure for efficient heat transfer as shown in Figure 1.

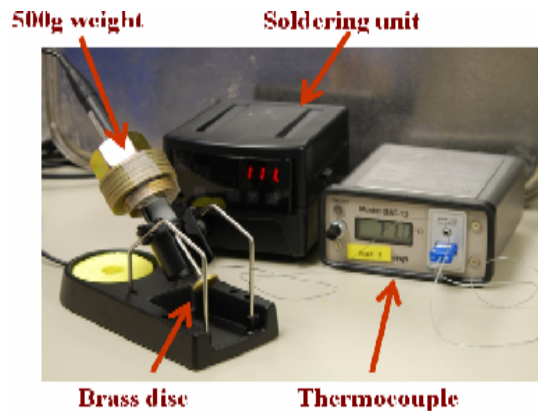


Fig 1. Contact Burn Unit.

On the non-contacting surface of the disc a thermal sensor was soldered, with an electrical lead connected to a digital temperature read-out to accurately control temperature without extensive operator involvement. The temperature control system accepts a temperature sensor, such as a resistance temperature detector (RTD), as input and it compares the actual temperature to the desired control temperature, or set-point, and provides an output to a control element, such as a proportional controller with digital read out. The desired temperature will be attained by heating the solder rod electrically.

Creation of contact burn wound in rats (partial thickness)

The animals undergo general anesthesia and the dorsal skin is shaved and disinfected. The circular disc is heated to 85°C and allowed to stabilize for 20 minutes. The external contacting surface of the circular iron/brass disc is then placed on the shaved dorsal side of the rat for 10 seconds without exerting any external pressure. An example of the resulting burn wound (approximate wound area of 226.8 mm²) is shown in Figures 2. After 12hrs the depth of the wound was histologically assessed by staining the skin biopsy (5-7 µm thick sections) with Haematoxylin and Eosin (H&E), Masson's trichrome (Figure 3) and/or for collagen IV immunoreactivity (Figure 4).

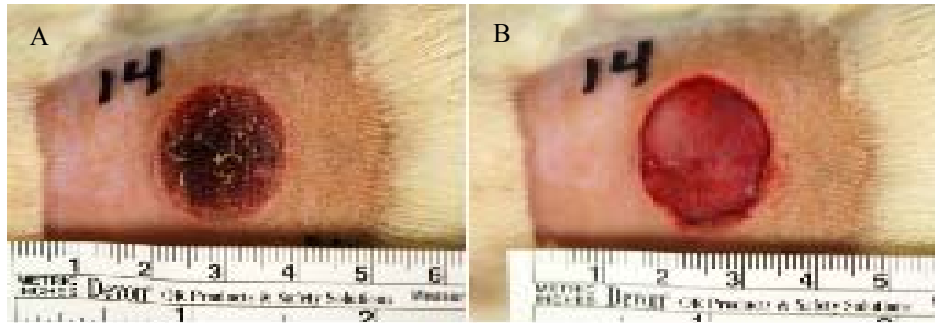


Figure 2. A representative image of the injury created in rats after 12 hours. Panel A shows the wound with eschar and panel B shows the wound with eschar removed.

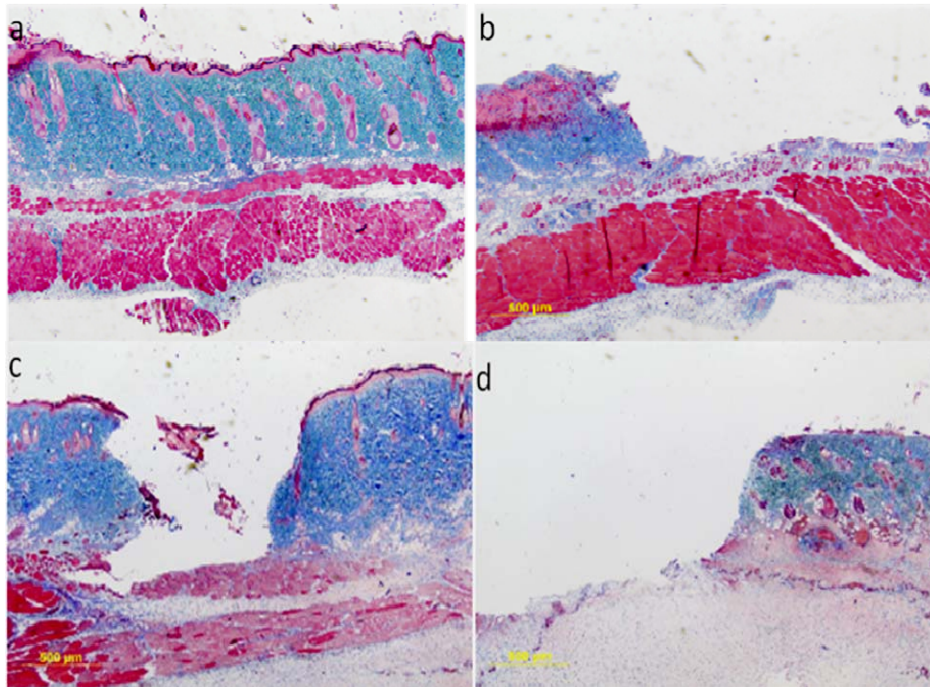


Figure 3. The depth of the wound was histologically assessed after 12 hours. Skin biopsies (5-7 μ m thick sections) were stained with Masson's Trichrome a) Control - All layers of skin is intact and viable. b) 10s - Epidermis and most of dermis are lost (partial thickness burn). c) 15s - Damage extending to the subcutaneous-hypodermal junction (full thickness). d) 20s - Burn injury with the burn depth reaching the muscular tissue.

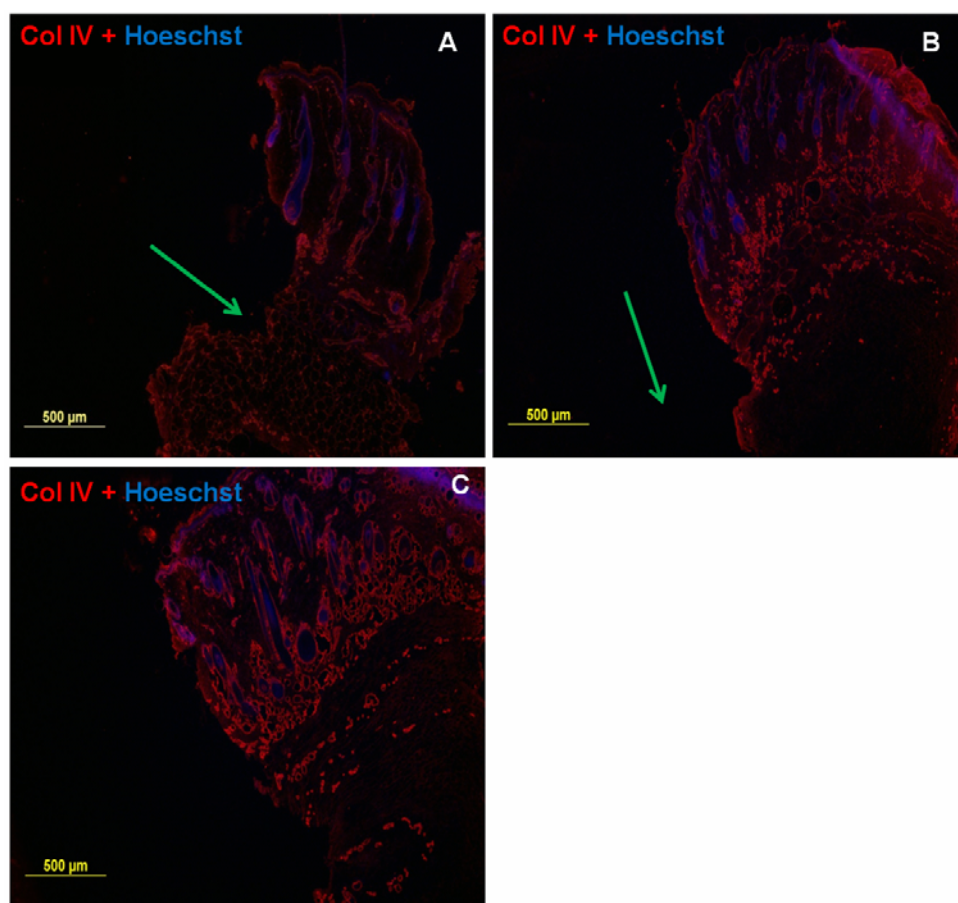


Fig 4. Skin biopsies (5-7 μm thick sections) were stained for Collagen IV (red) and Nuclei (blue). A) 10s burn – Collagen IV along the hair follicle and surrounding tissue extending to the subcutaneous-hypodermal region, indicating a partial thickness burn. B) 15s burn – No visible Collagen IV staining in the dermal region indicating a full thickness burn. C) 20s burn – complete loss of Collagen IV staining indicating the burn depth reached the muscular tissue.

Induction of infection in burn rats

Initial inoculums of *Pseudomonas aeruginosa* / *Staphylococcus aureus* will be prepared by inoculating five colonies from a freshly cultured plate into 10mL of MHB (Muller Hinton Broth) or TSB (Trypticase Soy Broth) and incubated at 37 °C until an OD₆₀₀ of 0.1 (approximately 3-6 hrs). From this culture 100 μL is transferred to 10mL of fresh MHB and incubated to attain exponential phase OD₆₀₀ of 0.1 (approximately 3hrs, 10⁸cfu/mL) (35). Appropriate dilutions will be made to prepare bacterial challenge inoculum (10⁶, 10⁷, 10⁸cfu/100μL of saline) for inducing infection. **The 17mm partial thickness contact burn injury will be done as described above (Day -2). Twelve hours after injury the wound will be infected with bacteria (Day -1). The suspension will be injected underneath the eschar and left overnight (12 - 14 hours) to establish the infection (36,37). Twelve hours after bacterial infection the eschar will be removed and a semi-occlusive bandage applied (Day 0).** The injection will be carefully administered at one site between the subcutaneous skin and paraspinus muscular layer. Based on our previous experience using Wistar rats (36), we have observed 10⁷ CFU to be the challenging inoculums (challenging inoculums was determined based on the presence of visible

inflammatory response and a bacterial load $>10^6$ CFU after 12 hour of induction). In this study we will be utilizing Lewis rats and it is necessary to determine the challenging inoculums for this species exactly. Therefore, an initial load of 10^6 , 10^7 , 10^8 cfu/100 μ l of saline will be used to determine the exact challenging concentration that produces a similar inflammatory response and bacterial load.

Positive bacterial colonization will be confirmed by taking wound biopsies (0.25-1cm²), mincing in 1mL of sterile saline and vortexing vigorously. The sample will be serially diluted and number of colonies determined by plating on MHA (Mueller Hinton Agar). The experimental time course is shown in Figure 5 and treatment groups are shown in Table 1. **The endpoint to determine adequate infection is the presence of visible inflammatory response and a bacterial load $>10^6$ CFU after 12 hour of induction. At each time point (Day 1 and Day 4) the complete wound area will be removed for biopsy (1 biopsy per rat).**

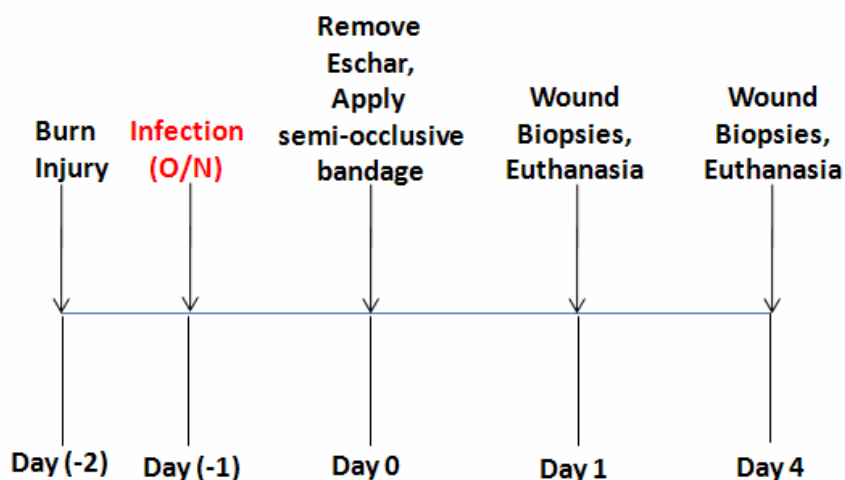


Figure 5. Phase 1 Experimental Time Course

Days	<i>Pseudomonas aeruginosa</i>			<i>Staphylococcus aureus</i>		
	Bacterial Load (cfu/100 μ l)			Bacterial Load (cfu/100 μ l)		
	10^6	10^7	10^8	10^6	10^7	10^8
Day 1	4 4		4	4	4	4
Day 4	4 4		4	4	4	4
Total Animals	24			24		

Table 1. Experimental groups for Phase 1

V.1.2 Phase 2: Effectiveness of antimicrobial dressings.

The experimental time-course is shown in Figure 6 and treatment groups are shown in Table 2. In Phase 2, we will use the contact burn injury with the bacterial load to induce infection determined in Phase 1 to produce an infected partial thickness wound. The 17mm injury was chosen as the dressings can be reproducibly prepared at this size so they will fit in to the wound bed **and will be done as described above (Day -2)**. Twelve hours after injury the wound will be infected with bacteria (Day -1) at the concentration determined in Phase I. Twelve hours after bacterial infection the eschar will be removed and the experimental dressing applied to the wound and a semi-occlusive bandage applied (Day 0). The dressings will be re-applied on Day 3, 6, and 9 following injury. This treatment regime was chosen because our test products are designed to deliver antimicrobial drug having sustained release for over 72 hours. In this experiment, the animals will be **euthanized** on Day 1, Day 4, Day 7, Day 14, Day 21 and Day 30. These time points will allow us to compare the effects of the dressings on the early infection response (Day 1, 4, 7 and 14) and later healing outcomes (Day 21, and 30). Aquacel Ag (Group 3) will be used as our clinical standard of care control. **To determine healing we will measure the amount of wound closure and bacterial load in the wound biopsy. The expected outcome would be prevention of infection and accelerated wound healing by the developed silver based dressing.**

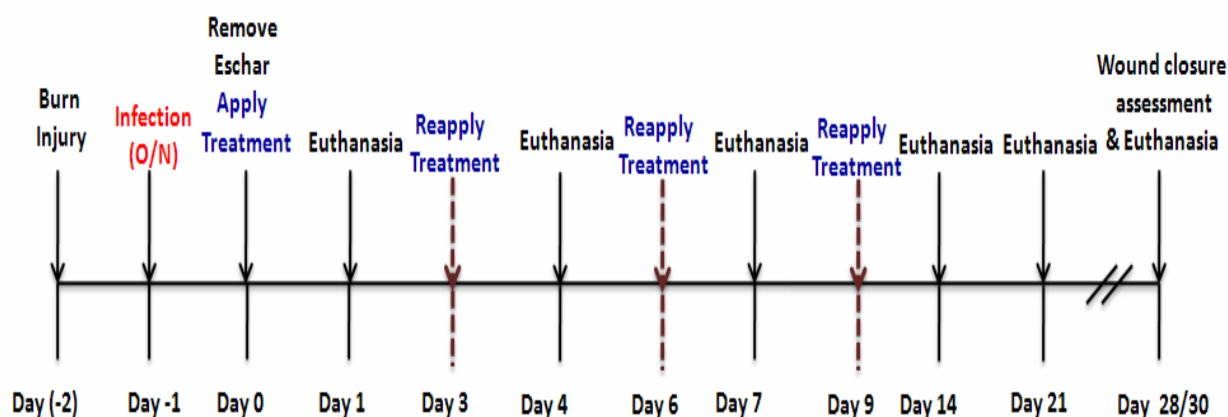


Figure 6. Phase 2 Experimental Time Course

410

Group	Description	Day 1	Day 4	Day 7	Day 14	Day 21	Day 30
1	Burn Only	6	6	6	6	6	6
2	Burn, infection	6	6	6	6	6	6
3	Burn, infection, control dressing- Aquacel Ag	6	6	6	6	6	6
4	Burn, FibrinAg dressing	6	6	6	6	6	6
5	Burn, infection, FibrinAg dressing	6	6	6	6	6	6
	Total Animals	30	30	30	30	30	30

411

412

Table 2. Experimental Groups for Phase 2.

413

V.2. Data Analysis: The following measurements will be taken in all control and treatment groups: Body weight (grams), reduction in wound size (% healing) – measured by tracing the wound area and photographing, Values will be expressed as mean +/- standard error. Number of bacterial colonies expressed as CFU/mg of tissue biopsy harvested at different day during healing. Two-way ANOVA with repeated measure, followed by suitable post-hoc tests accounting for multiplicity of comparisons will be used for continuous variables.

For sample analysis, a power analysis was performed in order to determine the minimum group size which produces statistical power congruent with sound scientific design. Sample-size requirement estimates are based on an overall F test for healing rate, a two-tailed alpha threshold of 0.05, and a power of 80% (program StatXact4).

424

425 **V.3. Laboratory Animals Required and Justification**

426

V.3.1. Non-animal Alternatives Considered: Since the effects of infection on wounds will be evaluated, an intact animal is required. Cell culture models cannot be used to evaluate the local and systemic effects of infection and treatments. A literature search was conducted and no other alternatives to a surgical model were identified by the primary investigator that will achieve the objectives of this study.

432

V.3.2. Animal Model and Species Justification: Rats were chosen as the least sentient animal model. Procedures for the contact burn in rat have been developed and are routinely used at this Institute.

436

437 **V.3.3. Laboratory Animals**

438

V.3.3.1. Genus and Species: *Rattus norvegicus*

440

V.3.3.2. Strain/Stock: Inbred Lewis rats (LEW/Crl)

442

V.3.3.3. Source/Vendor: Charles River Laboratories

444

V.3.3.4. Age: Age appropriate for weight range specified

445

V.3.3.5. Weight: 200-225 g

V.3.3.6. Sex: Male

V.3.3.7. Special Considerations: viral antibody free animals

V.3.4. Number of Animals Required (By Species): 409 - Phase I – 53 (48+5 replacement); Phase II – 356 (324 experimental+32 replacement). Replacement animals will only be used if needed and only after notifying the IACUC Chair and a staff veterinarian in writing.

V.3.5. Refinement, Reduction, Replacement

V.3.5.1. Refinement: The procedures described above will be conducted in a state of the art, AAALAC accredited animal facility. We have experienced individuals who have previously conducted partial thickness burn studies using this device. Pre-burn anesthesia will be confirmed by testing for lack of response to momentary painful stimulus, and the animal burned only when such a response has been identified to be absent. All personnel responsible for assessment of animal condition will be properly trained in recognizing pain and distress in rodents. Animals will be anesthetized for the duration of the treatment and receive post-operative analgesia for 48hr after surgery.

V.3.5.2. Reduction: A power analysis was performed to determine the minimum number of animals required to give meaningful results. In addition, prior to the start of this project, rats, immediately following euthanasia, will be obtained from ISR investigators (Dr s. Christy, Walters) to practice and refine the procedure; thereby also decreasing the number of animals required for the experiment.

V.3.5.3. Replacement: These animals will be used to simulate the human wound healing response. *In vitro* experiments were done for the determination of drug load, MI/C and M/BC following the NCCLS guidelines (35) and characterization of the gel loaded with drug (silver-based). It is not possible to test for systemic and dynamic effects using these methods; therefore, a living system must be used. This information is anticipated to benefit human health in general and the health and welfare of soldiers who have burn injuries.

V.4. Technical Methods

Histology: The recovered wound and surrounding tissue will be fixed in 10% neutral-buffered formalin, dehydrated, and embedded for histological analysis. For histological observations, different staining techniques are available to preferentially color components of interest in tissues. Some frequently used stains are: Haematoxylin-eosin (H&E), to identify the distribution of cells within the wound and Masson's Trichrome is a three-color stain that stains cell nuclei red, collagen green or blue, and the cell cytoplasm red. Histologic examination of sectioned samples stained with any of the stains described above will be performed by a veterinary pathologist. A specific grading system for analyzing the specimens will be developed in collaboration with the veterinary pathologist. An example of the histological scoring is shown in Table 3.

Grade	Inflammation
0	Normal Tissue
1	Rare to scattered neutrophils
2	Low number of neutrophils throughout
3	Moderate neutrophils throughout
4	Microabscess formation

Table 3. Example of histological scoring.

V.4.1. Pain/Distress Assessment: Animals will be assessed for pain and distress every 12 hours for the first 48 hours post-op. using the following criteria: abnormal activity (reluctance or inability to move), abnormal behavior (abnormal vocalization, or posture, unresponsiveness, aggressiveness), or physiologic signs (increased respiratory rate). Individuals conducting these assessments will be trained to recognize pain and distress in rodents.

Pain/distress assessment scoring sheets (similar to those used previously at this Institute, Appendix F) will be used (days 1-5) for post-operative assessment of weight, activity, wound cover, and signs of pain and distress. Any animals noted to have a score ≥ 5 on post-operative day 5 will continue to be clinically evaluated (using the pain/distress assessment scoring sheets) until a score < 5 is noted for 3 consecutive days. Thereafter, animals will continue to be evaluated daily until their respective study endpoint is reached. Any animal found to meet the endpoint criteria specified in IACUC policy A-04 will be immediately euthanized. The pain rescue plan includes consultation with a staff veterinarian for additional measures of pain relief.

V.4.1.1. APHIS Form 7023 Information (see attending veterinarian for assistance)

V.4.1.1.1. Number of Animals:

V.4.1.1.1.1. Column C: __ (Animal #)

V.4.1.1.1.2. Column D: 409 (100% of Animal #)

V.4.1.1.1.3. Column E: __ (Animal #)

V.4.1.2. Pain Relief/Prevention:

V.4.1.2.1. Anesthesia/Analgesia Tranquilization: Buprenorphine will be given as pre-emptive analgesia (0.1- 0.2mg/kg IP or SQ) and Isoflurane (2%-5% in O₂ via facemask) given to effect anesthesia. Analgesia, buprenorphine (0.1 to 0.2mg/kg IP or SQ) will be administered every 12 hours for 48 hours post surgery and continued as necessary upon the observation of signs of continued pain based on clinical assessment (using the pain/distress assessment scoring sheets).

Depth of anesthesia will be monitored by a combination of mechanical and observational monitoring techniques including but not limited to: respiratory monitoring, response to stimulus, presence or absence of vocalization or movement during injury, time to recovery, and progress in the study. Criteria for adequacy of anesthesia will be: (1) no conscious response to painful

stimuli, (2) no muscle twitching or withdrawal action in response to pain, (3) depression of reflex response and (4) no conscious vocalization. If any of the criteria listed as 1-4 are exhibited the anesthetic regime is considered a failure. If failure is determined, the animal will be induced under isoflurane for a longer time period until the desired plane of anesthesia (criteria 1-4) is met. Prior to any injury rats are evaluated by the standard toe pinch method.

Possible modifications to the above analgesic methods may be made by the staff veterinarians if necessary to ensure that the animals do not feel pain or distress.

V.4.1.2.2. Pre- and Post-procedural Provisions: All animals will be acclimated for at least four days prior to use in experiments. They will be allowed food, water and cage activity *ad libitum* throughout the experiments. On the day of injury, animals will be given pre-op analgesic (buprenorphine 0.1 to 0.2 mg/kg, IP or SQ) and anesthetized with isoflurane via facemask (2%-5% in O₂).

Efficacy of postoperative analgesic will be assessed at 30 to 60 minutes after administration, and supplemented as necessary to relieve pain and/or discomfort. Animals will be assessed at least daily with a minimum of determination of temperature, behavior and body weight. Animals displaying evidence of pain and/or discomfort as determined by abnormal activity (reluctance or inability to move), abnormal behavior (abnormal vocalization, or posture, unresponsiveness, aggressiveness), or physiologic signs (increased respiratory rate), or as determined by veterinary staff will be administered analgesic (buprenorphine 0.1 to 0.2 mg/kg, IP or SQ).

Euthanasia will be used as an alternative to death as an endpoint with a drop in body temperature >4°C from baseline or loss of mobility resulting in the inability to reach food or water. Using the pain/distress assessment scoring sheets any animals noted to have a score ≥ 5 will continue to be clinically evaluated. Any animal found to meet an endpoint or criteria specified in IA CUC policy A-04 will be immediately euthanized.

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures:

V.4.1.3.1. Sources Searched: AGRICOLA, BRD, REPORTER, DTIC, PUBMED

V.4.1.3.2. Date of Search: May 2010

V.4.1.3.3. Period of Search: May 2000 to 2010

V.4.1.3.4. Key Words of Search: rat skin wound pain, rat partial burn pain

V.4.1.3.5. Results of Search: The literature search found no alternatives or minimally invasive procedures that would cause us to alter the experimental model, proposed surgical techniques, and anesthesia or pain control.

V.4.1.4. Unalleviated Painful/Distressful Procedure Justification: N/A

V.4.2. Prolonged Restraint: None

V.4.3. Surgery: N/A

V.4.3.1. Pre-surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: None

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections: Buprenorphine (0.1-0.2 mg/kg IP or SQ every 12 hours; 25 gauge needle) will be administered to alleviate pain and/or distress. Anesthetized animals will be euthanized using commercial euthanasia solution (e.g., Fatal-Plus[®] or similar) given IV, IC or IP, 25 gauge needle, at the label dose (0.1 mL/ 450 grams body weight).

V.4.4.2. Biosamples: At the experimental endpoints and different time intervals (Day 1, Day 4, Day 7, Day 14, Day 21 and Day 30) the wound area will be measured, rats will be euthanized (appropriate number of subjects specified in table 2), and the wound bed with portion of intact skin surrounding the wound will be excised and processed for histological analysis. The wound area measurement provides a simple and reliable measure of wound repair and will be the initial endpoint measurement.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Animals will be identified by cage cards and/or tail numbers

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing: None planned at this time, however, tissue may be shared with other investigators upon request.

V.4.5. Study Endpoint: After procedure and treatment as described, animals will be observed up to 30 days to determine the efficacy of treatment on wound closure. Animals will be assessed at least daily. Animals will be euthanized on an appropriate day determined in a random manner. In the event a drop in body temperature $>4^{\circ}\text{C}$ from baseline and a loss of body weight and/or loss of mobility resulting in the inability to reach food or water used as the criteria to euthanize an animal and be considered an alternative to death.

V.4.6. Euthanasia: Animals that experience any pain and/or distress that cannot be promptly relieved, will immediately be euthanized IAW the 2007 AVMA Guidelines on Euthanasia. Euthanasia will be accomplished with a commercial euthanasia solution (e.g., Fatal-Plus[®] or similar) given IV, IP or IC at the label dose (0.1 mL/ 450 grams body weight).

V.5. Veterinary Care

V.5.1. Husbandry Considerations: All animals will be housed and cared for IAW the ILAR *Guide for the Care and Use of Laboratory Animals* and all applicable Federal, DoD, state, and local regulations. The BAMC/ISR joint facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl.). Husbandry will be provided by trained animal care staff in accordance with standard operating procedures. They will be held a minimum of 72 hours after receipt for acclimation/stabilization purposes prior to being used for research. Animal housing environmental conditions are controlled by a dedicated HVAC system engineered and maintained to meet ILAR Guide recommendations. Conditions are monitored with the use of the Watchdog[®] Environmental Monitoring System. All animals will receive laboratory grade commercial feed. Water is provided *ad libitum* to all animals via an automated water delivery system (lixits).

V.5.1.1. Study Room: Study room will be located in the lower level of building 3611. Vivarium procedure room(s) L87-2 or L87-7, or as assigned by veterinary services.

V.5.1.2. Special Husbandry Provisions: Animals will be allowed food and water *ad libitum*. If the injury impedes the rats ability to access food placed in the cage lid, food will be provided on the cage floor following injury.

V.5.1.3. Exceptions: N/A

V.5.2. Veterinary Medical Care:

V.5.2.1. Routine Veterinary Medical Care: During the 72-hour acclimation period, prior to experimental manipulations, animals will be evaluated at least twice daily, including weekends, by both animal caretakers and veterinary technical personnel. If an animal appears ill or debilitated, a staff veterinarian will be immediately notified and appropriate action will be taken to diagnose and alleviate the problem. If an animal is determined to be experiencing pain and / or distress that cannot be promptly alleviated, it will be immediately removed from protocol and euthanized by a staff veterinarian or veterinary technician.

V.5.2.2. Emergency Veterinary Medical Care: In the event of emergency, the VSD Facility Manager and/or a staff veterinarian will be immediately contacted. They can be reached by pager

or cellular telephone. A veterinary personnel point of contact roster is maintained on the VSD bulletin board outside the veterinary technician office (Room L86-5) and is also available at the USAISR security desk which is staffed 24-hours/day, 7 days/week. In the event the Principal Investigator or research personnel must be reached, a protocol personnel point of contact roster is maintained in the Facility Manager's office (Room L86-7).

V.5.3. Environmental Enrichment

V.5.3.1. Enrichment Strategy: All rats will be maintained in conventional filter-top micro isolator solid-bottom caging, or in individually ventilated cages (IVC). They will be individually housed but kept within sight, sound and smell of their conspecifics. Nestlets®, Nylabones®, Shepherd Shacks®, Tunnels or Rodent Retreats™ will be used for environmental enrichment, if they do not affect the bandage covering the wound area.

V.5.3.2. Enrichment Restriction: Shepherd Shacks®, Tunnels or Rodent Retreats™ will be removed if they affect the bandage covering the wound area.

690 **VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:**

ACTIVITY List specific procedures to be performed	NAME (PI/AI Research Assistant Technical Staff Key Animal Handler, if any)	QUALIFICATIONS Professional Degrees Certifications	TRAINING Relevant experience and specialized training for species and procedures listed under “ACTIVITY” column; Years of experience ex. >25 years of animal research/handling (pigs, rats, mice)
Principal Investigator Coordinate details of study Induce Wound Perform treatments and analgesia injections	Mani Seetharaman	PhD, Postdoctoral Fellow	>10 year rodent handling experience Has experience in partial contact burn model.
Co-Investigator Oversee experimental details of the study	Robert J. Christy	PhD, Research Physiologist	>20 yr experience with rodent research
Co-Investigator Oversee experimental details of the study	David Zamora	PhD, Postdoctoral Fellow	>10 yr experience with rodent research
Co-Investigator Oversee experimental details of the study	Shan Natesan	PhD, Postdoctoral Fellow	>10 yr experience with rodent research. Has experience in partial contact burn model.
Co-Investigator Oversee experimental details of the study	David G. Baer	PhD, Director, Office of Research	>10 yr experience with rodent research
Research Assistant Induce Wound Perform daily assessments Perform treatments and analgesia injections Coordinate details of study	Janet Roe	BS, LATG	Institutional experience in rat models, including burn, and experienced in rodent research for >15 years. Trained to recognize pain and distress in rodents.
Assist in treatments Perform daily assessments Perform treatments and analgesia injections	Melissa Sanchez	BS	>1 year in rodent handling and research
Assist in treatments Perform daily assessments Perform treatments and analgesia injections	Nicole Wrice	MS	>5 years in rodent handling, research and surgery
Assist in treatments Perform daily assessments Perform treatments and analgesia injections	Sharanda Hardy	BS	<1 year experience in rodent handling.

All investigators / research personnel involved in this study are trained and qualified to perform the specific procedures outlined in this protocol and have demonstrated an understanding of the humane care and use of research animals. In addition, they have taken part in discussions of pertinent laws and regulations concerning the use of animals in biomedical research. They are familiar with the concept of the 3R's, including refinement, reduction and replacement, and have concluded that there is an absolute need for the use of animals in this study. They are familiar with the proper methods for minimizing and/or alleviating pain and/or distress in the animal species selected. They will either have a veterinary technician assigned to assist them who is trained and qualified to perform all the techniques required for this study or have exhibited sufficient proficiency themselves to justify unsupervised work without direct guidance from the veterinary staff. They have been advised on Institutional Animal Care and Use policy and are aware of the established reporting mechanisms for any observed deficiencies or concerns regarding animal use.

VII. BIOHAZARD/SAFETY: All personnel participating in this protocol are currently enrolled in an Occupational Health and Safety Program, have received a risk assessment relative to protocol related hazards, and have been cleared to conduct all proposed activities listed. All personnel will be advised of the potential for zoonotic disease transmission. This is considered minimal risk as animals are procured from USDA-licensed vendors and are only used if determined to be healthy by the veterinary staff. There is potential for sharps injury from needles and surgical instruments. This risk will be minimized by using standard surgical techniques, sharps safety and standard precautions. There is potential for animal bites that will be reduced by using standardized animal handling procedures as well as appropriate personal protective equipment (PPE). There is potential for exposure to waste anesthetic gases that will be minimized by using an appropriate waste anesthetic gas scavenging system.

VIII. ENCLOSURES

APPENDIX A – Impact Statement(s) – (if applicable)

APPENDIX B – References

APPENDIX C – Budget Statement

APPENDIX D – Curricula vitae (*on file with protocol coordinator*)

APPENDIX E – Literature Searches (*on file with protocol coordinator*)

APPENDIX F – Semiquantitative Assessment Sheet

APPENDIX A
VETERINARY IMPACT STATEMENT

PROTOCOL TITLE: Effectiveness of Silver Based Wound Dressings to Inhibit Infection in Rat (*Rattus norvegicus*) Contact Burn Wounds

PRINCIPAL INVESTIGATOR: Shanmuganathan Seetharaman, PhD

LOCATION OF STUDY: Fort Sam Houston, Texas

FACILITY TO BE USED: U.S. Army Institute of Surgical Research

VETERINARY SUPPORT REQUESTED: Housing of animals for study

NUMBER OF SUBJECTS: No more than 60 animals will be housed at one time

NUMBER OF SAMPLES: N/A

ESTIMATED LENGTH OF STUDY: 30 days

WILL ADDITIONAL FUNDING BE REQUIRED TO MEET THIS REQUEST? No

ESTMATE OF ADDITIONAL FUNDING: N/A

=====

☒ **Approved**

☐ **Approved with Comment:**

☐ **Unable to Support Request.**

=====

SIGNATURE COORDINATION / APPROVAL

-original signed-

1 Jun 10

DATE: _____

 Kevin R. Armstrong, DVM, LTC
 Chief, Laboratory Animal Medicine Services

Appr 14 Jun 10

APPENDIX B REFERENCES

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APPENDIX C

Budget Estimate FY 10 Infected Contact Burn - Rat Model			
☐ Proposal?		CHRISTY, Robert J., PhD	
Military Labor	Time %	Cost	
Military Labor Total =			
Civil Service Labor	Time %	Cost	Benefits
CHRISTY, Robert J., PhD	5	\$4,975	\$1,741
HARDY, Sharanda	10	\$3,311	\$1,159
Labor Total =	\$8,286	Benefits Total =	\$2,900
		Civil Service Cost Total =	\$11,186
Contract Labor	Time %	Cost	
NATESAN, Shan, PhD	10	\$824	
Shanmuganathan, Mani	10	\$824	
		Contracts Total =	\$1,648
Equipment			
		Equipment Total =	
Travel =		\$4,000	
Transportation of Things =			
Rent/Communication =			
Printing/Reproduction =		\$1,000	
Supplies and Materials =		\$15,354	
		Indirect Cost = \$23,564	
		Direct Cost Total = \$33,188	
<i>Date Prepared: 27/May/10</i> <i>Date Printed: 27/May/10</i>		BUDGET ESTIMATE TOTAL \$56,752	

