Cellular Mechanisms of Immune Dysfunction Following Severe Burn Injury

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ABSTRACT

CRYSTAL J. NEELY: Cellular Mechanisms of Immune Dysfunction
Following Severe Burn Injury
(Under the direction of Bruce A. Cairns)

The immune system protects the body against infection and disease. Severe burn injury induces profound immune dysfunction rendering patients extremely susceptible to infection. Although progress has been made in reducing the incidence of infection, burn wound infection and pulmonary sepsis are still major causes of mortality. Therefore, in order to improve patient outcome it is important to understand the immune response to infection after burn injury. Furthermore, the immune response following burn injury is dynamic and changes over time. Therefore, elucidating the immune response both early and late after burn is also crucial.

Using a murine model of thermal injury, we characterized the pro-inflammatory $CD4^+$ T cell response at various timepoints after burn. We detected Th17 cells in wound-draining lymph nodes at 3, 7, and 14 days post burn. Also, there was bimodal skewing of the Th1/Th17 T cell balance with an early predominance of Th1 cells Th17 cells late.

The innate immune response to a clinically relevant pathogen was then assessed early after burn injury. Burn mice were susceptible to an early wound inoculation with *Pseudomonas aeruginosa* as demonstrated with high mortality rates and bacterial spread systemically. Defective bacterial clearance of *P. aeruginosa* early after burn injury correlated

with polarization of neutrophils into an anti-inflammatory (N2; IL-10+ IL-12-) phenotype. Administration of flagellin after burn injury skewed the neutrophil response towards a pro-inflammatory neutrophil (N1; IL-10-IL-12+) phenotype resulting in increased bacterial clearance.

Additional studies evaluated susceptibility to pulmonary *P. aeruginosa* infection late after burn injury. In contrast to early wound infection, burn mice exhibited enhanced clearance of a delayed pulmonary P. aeruginosa challenge compared to non-burn controls. This appeared to result from to a burn-induced accumulation of neutrophils within the lungs. Collectively, these data suggests that neutrophil responses vary after burn injury where they exhibit an anti-inflammatory phenotype early after burn followed by a late and pro-inflammatory phenotype.

This work provides insights into the cellular mechanisms of immune dysfunction following severe burn injury, as well as identifies neutrophil polarization as a novel therapeutic target for the reversal of bacterial susceptibility after injury.

To my grandparents

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ALI: acute lung injury

B6: C57BL/6 mouse strain

BAL: brochoalveolar lavage

CD: cluster of differentiation

CFU: colony forming units

COPD: chronic obstructive pulmonary disease

DAMP: damage associated molecular pattern

DC: dendritic cell

DHR: dihydrorhodamine

Exo: exoenzyme

Foxp3: forkhead box P3

hpi: hours post infection

IC: immune complexes

IFN-γ: interferon gamma

IL: interleukin

IP: intraperitoneal

IT: intratracheal

KC: keratinocyte-derived cytokine

LPS: lipopolysaccharide

M1: pro-inflammatory macrophage

M2: anti-inflammatory macrophage

MCP-1: monocyte chemoattractant protein 1

mDC: myeloid dendritic cell

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

N1: pro-inflammatory neutrophil

N2: anti-inflammatory neutrophil

NET: neutrophil extracellular trap

NO: nitric oxide

NOI: neutrophil oxidative index

PAMP: pattern associated molecular pattern

pDC: plasmacytoid dendritic cell

PGE2: prostaglandin E2

PLN: peripheral lymph nodes

PMA: phorbol 12-myristate 13-actetate

RNI: reactive nitrogen intermediates

ROI: reactive oxygen intermediates

T2SS: type II secretion system

T3SS: type III secretion system

TBSA: total body surface area

TCR: T cell receptor

TGF-β: transforming growth factor beta

Th: T helper

TLR: toll-like receptor

TNF-α: tumor necrosis factor alpha

VAP: ventilator-associated pneumonia

CHAPTER 1

Introduction

1.1 Burn injury

The International Society of Burn Injuries defines burn as damage to the skin or other tissues primarily caused by thermal injury. This typically occurs when hot liquids (scalds), hot solids (contact burns), or flames (flame burns) induce subtotal or total destruction of cells within the localized area of exposure. The etiology of burn is not limited to the aforementioned modes of trauma; it also includes radiation, electric, friction and chemical injuries.

1.2 Incidence of burn

Burns are among the most common and devastating forms of trauma. In 2004, nearly 11 million individuals required medical treatment for burn injury making it the fourth most common injury world-wide[1]. The global incidence of burn injury is higher than the incidence of tuberculosis and HIV infections combined[2]. In the United States, approximately 2 million burn injuries occur annually[3] resulting in over 450,000 emergency room visits and 3,500 deaths[4].

1.3 Cost of burn care

Individuals with severe burn injury require immediate specialized care in order to minimize morbidity and mortality. This specialized care includes aggressive fluid resuscitation and prevention of fluid loss, regulation of body temperature, management of severe pain, wound debridement, early excision and wound closure, and prevention of infections[5]. The cost of initial hospitalization and the surgical care of a burn patient is approximately $200,000[6,7]. However, this estimate does not include expenses incurred by rehabilitation, vocational services, late complications, and disability. Although the overall hospitalization rates from minor burn injuries have significantly declined over the last 50 years, the proportion of patients admitted to burn centers has increased[7]. This has led to an annual cost of more than $18 billion for specialized burn care in the United States[7].

1.4 Grading of burn wounds

Burn injuries are classified by depth and size. The skin has 3 layers: the epidermis, dermis, and subcutaneous tissue. The degree of tissue injury resulting from burn determines the burn depth. A superficial or first-degree burn involves only the epidermis and typically heals within 3-4 days without scarring[5]. A partial thickness or second-degree burn extends through the epidermis and into the dermis. This type of burn can be further divided into superficial or deep depending on the depth of injury into the dermis. Blisters are often observed with partial-thickness burns, and they heal within 1-5 weeks with some scarring[5]. Full thickness burn, also known as a third-degree burn, extends through the entire dermis and into the subcutaneous tissue; therefore, they must be treated be excision and skin grafting[5].

Lastly, a fourth-degree burn involves other organ tissue below the skin and soft tissue, such as muscle and bone[5]. Burn injuries are also measured by size or area of injury. The percent total body surface area (TBSA) of an adult patient is typically assessed by the rule of nines, while the Berkow formula is used on children[5]. Taken together these classification schemes guide fluid resuscitation and wound management.

1.5 Systemic response to burn injury

Severe burn injuries covering greater than 30% TBSA are typically followed by a period of hypermetabolism, altered hemodynamics, vascular permeability and edema, decreased renal blood flow, and increased gut mucosal permeability[8]. The massive release of inflammatory mediators in the wound, as well as in other tissues, is believed to impact and/or trigger this multi-organ dysfunction. There is also immune suppression demonstrated by prolonged allograft skin survival on burn wounds[5]. Since burn injury impairs all parts of the immune system, patients are extremely susceptible to infection. With burns of more the 20% TBSA, the magnitude of immune impairment is proportional to the size of burn[5].

1.6 Wound infection

Since the skin is a barrier against invading pathogens, it is not surprising that the risk of subsequent burn wound infection also correlates with the size of the burn injury[9,10]. Immediately after admission the burn wound is cleaned and debrided, but it eventually becomes colonized with microbes[11]. These pathogens can come from the patient's normal gastrointestinal and upper respiratory flora, the hospital environment, or transferred by a

health care worker's hands[11-13]. Gram-negative bacteria are currently the most common infectious agents seen in burn centers due to their numerous virulence factors and antimicrobial resistance traits[14]. Burn wound infection delays epidermal maturation and leads to additional scar tissue formation[15,16]. Invasion of microbes into the tissue layers below the dermis may also result in bacteremia and sepsis where progression to multiple organ failure is usually fatal[17,18].

1.7 Pulmonary infection

Pulmonary bacterial infections are a major cause of mortality in burn patients. Hypostatic pneumonia is common in patients with large burns due to hyperventilation and decreased lung expansion[19]. Hematogenous pneumonia, where the infection is spread by the blood from another part of the body, is much less common in burn patients due to aggressive antibiotic therapies[20]. Nevertheless, both can be fatal. Inhalation injury, which occurs in approximately one-third of all major burns, damages the airway mucosa, impairs immune cell function, and requires intubation for airway protection[21,22]. This significantly increases a burn patient's risk of developing a pulmonary bacterial infection and in particular ventilator-associated pneumonia (VAP)[21,23]. VAP occurs when a mechanically ventilated patient develops pneumonia more than 48 hours after intubation. Invasive procedures such as bronchoalveolar lavage allow more appropriate administration of antibiotic therapy but have not been shown to change the overall mortality from VAP[24].

1.8 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a gram negative extracellular bacterium, is one of the most commonly encountered infectious agents in burn centers across the United States[25]. The organism can be isolated from environmental sources, such as freshwater and soil. It can also survive on surfaces contaminating the floors, bed rails, and sinks of hospitals[26,27]. Innate immune responses are essential for controlling *P. aeruginosa*. Clinically, the vast majority of cases of *P. aeruginosa* infection are found in patients with comprised immune systems[28].

1.9 *P. aeruginosa* virulence factors

P. aeruginosa has numerous virulence factors that contribute to its pathogenesis. Surface structures, including a single polar flagellum and polar Type IV pili, are important for efficient motility, adhesion, and formation of biofilms. During infection *P. aeruginosa* can also downregulate synthesis of flagellin, the major component of bacterial flagellum[29]. This appears to be advantageous to the invading bacteria, allowing them to avoid recognition by the host's Toll-like receptor 5 (TLR5), and therefore limiting inflammation. *P. aeruginosa* also expresses two Type II secretion systems (T2SS) and one Type III secretion system (T3SS). The T3SS, as well as the T2SS to a much lesser extent, has been associated with increased *P. aeruginosa* virulence. Several of the T3SS effector proteins, which are translocated across both the bacterial cell envelope and eukaryotic plasma membrane, can modulate innate immune recognition of bacteria or target effector mechanisms of the innate immune system. For example, Exoenzyme U (ExoU) inhibits inflammasome activation by *P.*

aeruginosa and induces rapid necrotic cell death[30,31]. Also, ExoS and ExoT can inhibit macrophage and neutrophil migration and phagocytosis[32,33].

1.10 Immune response to infection

The immune system is the body's defense against infection and disease. It detects a wide variety of antigens derived from invading pathogens and distinguishes them from the host's own tissue. The immune response can be divided into innate and adaptive immunity. The innate immune system is non-specific, meaning it recognizes and responds to pathogens in a generic way. More specifically, it depends upon germline-encoded receptors (ie. TLRs) to recognize features that are common to many microbes. Most pathogens are detected and destroyed within minutes to hours of invasion by innate immune cells, which includes the neutrophils and macrophages. However if a pathogen persists, the adaptive immune response ensues. The adaptive immune system is specific and consists of T and B lymphocytes. It targets a precise pathogen by utilizing pathogen-specific receptors, such as T cell receptors (TCR), which are acquired during the lifetime of the host. Induction of an adaptive immune response leads to immunological memory, which ensures a more rapid and effective response on subsequent encounters with the same pathogen.

1.11 Toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors that respond to pathogen-associated molecular patterns (PAMPs) and endogenous stress signals termed danger-

associated molecular patterns (DAMPs). In humans there are 10 known TLRs, whereas 12 have been characterized in mice[34]. Each TLR recognizes and is activated by a small assortment of microbe-derived molecules. For example, TLR2, TLR4, and TLR5 are involved in control of *P. aeruginosa* infection by recognizing outer membrane lipoproteins, LPS, and flagellin, respectively[35-37]. TLRs are expressed by immune cells and a wide variety of non-immune cells. TLR signaling induces the expression of hundreds of genes required for the inflammatory response, including inflammatory cytokines, chemokines, antimicrobial molecules, and major histocompatibility complex (MHC) and costimulatory molecules important for adaptive immune activation[34]. Although TLRs act primarily to initiate an innate immune response, some adaptive immune responses are elicited by TLR signaling.

1.12 TLR and burn injury

Over the last ten years, numerous laboratories have reported alterations in TLR expression and/or responsive following burn injury. Both *in vitro* and *in vivo* studies have shown that TLR2 and TLR4 responses are heightened between 1-7 days after burn injury[38]. Upon TLR stimulation, macrophages, dendritic cells, and γδ T cells from burn mice have increased cytokine production compared to non-burn controls[38-40]. The precise mechanism responsible for TLR hyper-responsiveness after burn injury is unknown. However, there is evidence to suggest that increased cell surface expression of TLR proteins[39, 41] and increased phosphorylation of p38 MAP kinase[42], a component of the TLR signaling cascade, both contribute. Our laboratory has also shown that at 14 days after burn injury, macrophages have a significant reduction in TLR2 and TLR4 expression, as well as diminished cytokine

secretion upon stimulation[39]. Conversely, there is upregulation in TLR4 expression on memory $CD4^+$ and $CD8^+$ T cells at 14 days after burn[41].

1.13 Neutrophils

Neutrophils are considered the first responders of the immune system since they are quickly recruited from the vasculature to the site of infection. Non-activated human neutrophils can survive up to 5 days, while activated neutrophils can survive 1-2 days in tissues[43]. During an inflammatory response, neutrophils are attracted to tissues by chemokines, such as human interleukin-8 (IL-8) or mouse keratinocyte-derived cytokine (KC), secreted by resident mast cells and macrophages[44]. There are three strategies that activated neutrophils use to directly attack invading pathogens. The first is phagocytosis, a cellular process in which receptor-bound pathogens are engulfed by the cell membrane to form an internal phagosome. The phagosome fuses with the lysosome leading to intracellular killing of the pathogen. Neutrophils also undergo an oxidative burst during phagocytosis which generates a variety of toxic products that help destroy the engulfed pathogen[45]. Degranulation, which is the release of antimicrobial cytotoxic molecules from secretory vesicles called granules, is another mechanism neutrophils use to eradicate invading pathogens[46]. Lastly, neutrophils can release neutrophil extracellular traps (NETs) that are composed of antimicrobial proteins bound to DNA to kill pathogens[47].

1.14 Neutrophils and burn injury

Neutrophil function is significantly impaired after burn injury although the timing and extent is still unclear. One study showed that neutrophils have decreased Fc receptor-mediated phagocytosis, as well as a 50% reduction in intracellular killing, after burn injury. This group also showed that the ability of circulating neutrophils to undergo oxidative burst gradually declines during the first two weeks after burn injury[48]. Another study reported that there is an increased number of neutrophils in the peritoneal cavity and an increase in neutrophil oxidative burst at one day after burn[49]. Neutrophils have also been reported to be immunosuppressive after burn injury as demonstrated by their secretion of IL-10, a potent anti-inflammatory cytokine, upon TLR2 stimulation[50].

1.15 Macrophages

Macrophages reside in every tissue of the body although they are sometimes called by other names such as microglia, Kupffer cells, and osteoclasts. Upon infection, circulating monocytes are rapidly recruited to the tissue, where they differentiate into tissue macrophages[51]. Macrophages play a central role in the initiation of inflammation, primarily through phagocytosis, release of inflammatory cytokines, oxidative burst, and antigen presentation[52]. During antigen presentation, the macrophage internally digests pathogens, binds resulting peptide fragments to MHC molecules, and then presents the peptide-MHC complexes on its surface to T lymphocytes[53]. Although macrophages are capable of antigen presentation, they are not particularly effective at this during a primary immune response because they do not migrate to secondary lymphoid organs[52]. Macrophages are also

remarkably plastic and can change their functional phenotype depending on the environmental cues they receive. For example, a macrophage can be polarized towards a pro-inflammatory phenotype (M1) marked by production of IL-12, as well as other pro-inflammatory mediators, when activated in the presence of interferon-gamma (IFN-γ)[54]. However, if a macrophage is later exposed to IL-10, glucocorticoids, or immune complexes in the presence of the TLR ligands, it can exhibit an anti-inflammatory phenotype (M2, IL-10^+ IL-12^-)[55,56].

1.16 Macrophages and burn injury

Macrophages are reported to be hyper-responsive following burn injury. Upon stimulation, macrophages exhibit exaggerated production of IL-1, IL-6, transforming growth factor (TGF-β), prostaglandin E2 (PGE2), and reactive nitrogen intermediates (RNI)[57-59]. Previous studies have implicated macrophage hyperactivity in the increased susceptibility to bacterial sepsis following burn injury[59,60]. In addition, recent evidence suggests that severe burn injury results in a shift of macrophage polarization towards an M2 phenotype[61]. These M2 macrophages have reduced capacity to kill bacterial pathogens. Soluble factors released by these cells inhibit macrophage conversion of resident macrophages to a M1 phenotype[61,62].

1.17 Dendritic cells

Similar to macrophages, dendritic cells (DCs) are capable of both phagocytosis and antigen presentation. However, DCs are not directly involved in immediate pathogen clearance; instead their primary function is antigen presentation[63]. Immature DCs are present in all peripheral tissue and constantly sample the surrounding environment for invading pathogens. When an immature DC phagocytoses a pathogen, it begins to mature by upregulating cell-surface receptors that act as co-receptors in T-cell activation. It also upregulates CCR7, a chemotactic receptor that directs DCs to secondary lymphoid organs[63]. Once inside a lymphoid organ, DCs act as antigen-presenting cells to initiate antigen-specific immune responses. DCs also secrete a variety of cytokines, such as IL-12, that are protective against a number of infections[64,65].

1.18 Dendritic cells and burn injury

A significant reduction in DCs has been reported after burn injury. More specifically, two DC subsets, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), are decreased in peripheral blood of burn patients[66]. Also, the number of circulating mDCs and pDCs appears to directly correlate with the severity of burn (depth and size) and incidence of infection[66]. In a murine model, antigen presentation by dendritic cells and subsequent activation of $CD4^+$ T cells was not impaired after burn[67]. However, reduced TLR9 expression and activation on DCs has been implicated in skewed T cell function after burn injury[68].

1.19 Th17 Cells

Until recently, $CD3^+CD4^+$ T cells were divided into pro-inflammatory Th1 (IFN-γ-producing) or anti-inflammatory Th2 (IL-4-producing) cells[69]. However, a newly discovered subset of $CD4^+$ T cells, called Th17 cells, have been shown to secrete IL-17, IL-21, and IL-22, but do not secrete IFN- γ or IL-4[70-72]. IL-17 activates macrophages and promotes the production of inflammatory cytokines, which includes tumor necrosis factor-α (TNF-α) and IL-1β. Subsequently, these cytokines recruit neutrophils to sites of infection[73]. For example, IL-17 receptor deficient mice have reduced neutrophil recruitment, increased bacterial loads, and high mortality rates after challenge with *Klebsiella pneumoniae*[74]. Furthermore, overexpression of IL-17 in the lungs results in a localized increase in TNF-α and IL-1β, as well as enhanced neutrophil accumulation and *K. pneumoniae* clearance[75]. IL-17 receptor deficient mice are also more susceptible to infection with *Candida albicans*[76]. IL-21, another cytokine secreted by Th17 cells, acts on epithelial cells and is involved with antimicrobial peptide production[77-79], tissue repair[80], and epithelial cell proliferation[81], differentiation[82], and survival[83]. Together, these findings suggests that the main function of Th17 cells is to promote antimicrobial immunity at mucosal interfaces.

1.20 Th17 cells and burn injury

Murine models have revealed local and systemic Th17 responses following burn injury. At the burn wound, Th17 cytokines IL-17 and IL-22 appear to be elevated within 3 hours of the insult[84]. Moreover, elevated levels of IL-17 have been observed in cardiac tissue at 3 hours[85] and in the circulation at 1 and 7 days after burn injury[86]. Also, analysis of adult

and pediatric burn patient serum has shown that IL-17 is elevated within 1 week after injury[87,88]. Another recent study examined Th17 cell development in patients with full thickness burns and revealed that peripheral blood mononuclear cells isolated from these patients had a decreased production of IL-17 in response to *C. albicans* antigen stimulation compared to healthy controls[89]. Since burn patients are highly susceptible to *C. albicans* infection, enhancing the IL-17 response after burn injury could be beneficial.

1.21 Remaining questions

The data outlined above describes the complexity of burn injury and in particular the profound immune impairment observed following burn. A better understanding of the basic mechanisms underlying burn-induced immune dysfunction may lead to development of novel therapeutic options. This body of work attempts to dissect specific aspects of the immune response following burn injury, specifically by determining their contributions to infection.

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CHAPTER 2

Th17 (IFNγ$^-$ IL-17$^+$) CD4$^+$ T cells generated after burn injury may be a novel cellular mechanism for post-burn immunosuppression

2.1 Summary

The mechanism responsible for initiating and controlling the immunosuppressive response following burn injury remains unknown. Interleukin-17 (IL-17) secreting Th17 (interferon (IFN)-γ$^-$ IL-17$^+$) cells are a novel subset of CD4$^+$ T cells associated with a weak pro-inflammatory response that antagonizes the pro-inflammatory Th1 (IFN-γ$^+$IL-17$^-$) response. Given that transforming growth factor (TGF)-β and IL-6 mediate Th17 cell development, we hypothesized that burn injury may generate Th17 cells that could mediate post-burn immunosuppression. Following a 20% total body surface area burn in female C57BL/6 mice, wound-draining lymph nodes were harvested 3, 7 or 14 days after injury. CD4$^+$ T cells were enriched by magnetic selection, and flow cytometry was used to identify intracellular IL-17 and IFN-γ in CD3$^+$CD4$^+$ T cells. Additional purified CD3$^+$CD4$^+$ T cells were cultured with Th17- polarizing IL-6 and TGF-β for four days, and flow cytometry was again used to identify intracellular IL-17 and IFN-γ in CD4$^+$ T cells. The number and percentage of preformed Th17 cells was significantly greater in burn mice compared to sham at all timepoints. In addition, the ratio of Th17 cells to Th1 cells was significantly higher in burn mice compared to sham at 14 days post burn. These differences were eliminated in Th17 polarizing conditions *in vitro*. CD4$^+$ T cells never generated both IL-17 and IFN-γ. These results demonstrate for the first time that Th17 cells (IFN-γ$^-$IL-17$^+$) are spontaneously generated after burn injury. Given that Th17 cells (IFN-γ$^-$IL-17$^+$) are antagonistic to Th1

(IFN-γ^+IL-17$^-$) cells, these results suggest a novel mechanism for initiating and controlling post-burn immunosuppression that deserves further investigation.

2.2 Introduction

CD4$^+$ T-helper (Th) cells have been classically divided into pro- (Th1) or anti-inflammatory (Th2) T cells based on the type of cytokines they produce after stimulation[1]. However, a newly discovered subset of CD4$^+$ T cells, called Th17 cells, has been shown to secrete interleukin (IL) -17 but not interferon (IFN)-γ or IL-4[2-4]. Recent studies have suggested that the main functions of IL-17 are to activate macrophages and to promote their production of inflammatory cytokines. Subsequently these cytokines, which include tumor necrosis factor-α (TNF-α) and IL-1β, recruit neutrophils to sites of infection[5]. For example, IL-17-receptor deficient mice have reduced neutrophil recruitment, increased bacterial loads, and high mortality rates following challenge with *Klebsiella pneumoniae*[6]. Furthermore, overexpression of IL-17 in the lungs results in a localized increase in TNF-α and IL-1β, as well as enhanced neutrophil accumulation and *K. pneumoniae* clearance[7]. IL-17-receptor deficient mice are also more susceptible to infection with *Candida albicans*[8]. Since these pathogens primarily cause infection in individuals with suppressed immune systems, this has led to the belief that Th17 cells are weak pro-inflammatory cells.

Naïve CD4$^+$ T cells are regarded as being able to differentiate towards one of four lineages; Th1, Th2, Treg or Th17 depending on many factors such as strength of antigen stimulation and cytokine microenvironment. Differentiation of naïve CD4$^+$ T cells towards a Th17 phenotype requires the presence of transforming growth factor (TGF)-β and IL-6, while IL-23 is believed to play a role in the maintenance of Th17 effector function[9-11]. This

differentiation process requires a unique transcription factor, ROR-γt, to induce transcription of the IL-17 gene[12]. Additionally, cells genetically deficient in the master transcription factors required for Th1 and Th2 differentiation have either undiminished or enhanced Th17 development[2]. Together these findings indicate that Th17 cells are a lineage distinct from Th1 and Th2 cells. Furthermore, cytokines secreted by differentiated Th1 and Th2 cells inhibit formation of Th17 cells (Figure 2.1). Conversely, when naïve $CD4^+$ T cells are exposed to TGF-β in the absence of IL-6, they express forkhead box P3 (Foxp3), which is the master transcription factor that drives induction of Foxp3+ regulatory T cells (Treg)[13]. Therefore, the presence of IL-6 switches the development of naïve $CD4^+$ T cells from a Treg pathway to a Th17 pathway.

Severe burn injury causes a dangerous immune dysfunction. Much research effort has been expended into defining the immune consequence of burn in terms of lymphocyte cytokine identity[14-33]. One model of burn injury suggests there is a rapid onset of a systemic inflammatory response characterized by the production of pro-inflammatory cytokines[23, 34]. If this pro-inflammatory state is uncontrolled, patients can experience early multiple organ dysfunction syndrome and death[35]. Patients surviving this period are then thought to develop a compensatory anti-inflammatory response characterized by immune suppression and decreased resistance to infection[36].

We and others have defined $CD4^+$ and $CD8^+$ T cell population changes both early and late after burn in patients and animal models. After burn injury, there is an early (hours to days) pro-inflammatory response followed by a shift towards an anti-inflammatory phenotype in both the $CD4^+$ and $CD8^+$ T cell compartments[15, 22, 23]. More specifically, $CD4^+$ T cells begin to produce the pro-inflammatory cytokines IFN-γ and IL-2 on stimulation,

which are defining characteristics of Th1 cells. In addition, macrophages produce TNF-α, IL-1β, and IL-6 as well as other pro-inflammatory cytokines[37]. Furthermore, the level of TGF-β increases gradually in the burn wound throughout this phase[36, 38]. Following this phase after burn, the pro-inflammatory state of the immune system switches to an anti-inflammatory response characterized by over-production of TGF-β[36, 38], as well as a switch in $CD4^+$ T cell responses from a Th1 to a Th2 (IL-4) phenotype[39]. This manifests itself as decreased antigen-specific proliferation, diminished cytokine secretion and cytotoxic T lymphocyte activity[18, 22, 40, 41].

Since most patients develop immune failure days to weeks after injury, we have been interested in both $CD8^+$ and $CD4^+$ T cell function during the first two weeks after injury. We have previously demonstrated profound alterations in the cytokine profile late after burn injury. $CD8^+$ T cells, crucial for anti-pathogen and anti-allograft immune responses, are impaired immediately post-burn but experience increased proliferation and have a unique and dramatic altered cytokine profile later after burn (14 days)[18, 22, 40, 41]. This functional enhancement is characterized by a selective peripheral T cell lymphopenia that drives a homeostatic increase in "spontaneous" memory-like $CD8^+$ and $CD4^+$ T cells in the periphery[14, 22]. Since elevated TGF-β and IL-6 levels are found following bury injury, we hypothesize that $CD4^+$ Th17 cells are generated after burn injury in a murine model of burn.

2.3 Methods and materials

Animals

Wildtype C57Bl/6 (B6) mice were purchased from Taconic Farms, USA. All mice used in the study were maintained under specific pathogen-free conditions in the American

Association of Laboratory Animal Care-accredited University of North Carolina Department of Laboratory Animal Medicine Facilities.

Mouse Burn Injury

Six to eight week old (15-20g weight) female B6 mice were used as subjects in all experiments. All protocols were performed in accordance with the National Institutes of Health guidelines and approved by the University of North Carolina IACUC as previously described[15]. Briefly, animals were anesthetized with inhalation of isoflurane vapor (Pitman-Moore, Washington Crossing, NJ) and their dorsal and flank hair clipped. A full-contact burn of approximately 20% total body surface area (TBSA) was produced by applying a copper rod, heated in boiling water, to the animal's dorsum and flank for 10 seconds. Four applications of a 65g rod (1.9 cm in diameter) were used to produce the wound, and previous biopsies of the wounds demonstrate full-thickness cutaneous burn with visible unburned muscle beneath. Mice were resuscitated with an intraperitoneal (IP) injection of Lactated Ringer's solution (0.1 ml/g body weight) and were given a subcutaneous injection of buprenorphine (2 mg/kg body weight) for pain control immediately after burn injury. Animals were returned to individual cages and were provided food and morphinated water *ad libitum* throughout the experiment. Sham controls with the 0% TBSA burn underwent all the described interventions except for the actual burn injury. There is a negligible mortality (<1%) after burn injury with this protocol. Animals were sacrificed where indicated, splenocytes and cells from axillary, brachial, inguinal, lumbar and caudal peripheral lymph node (PLN) were prepared for culture or CD4+ T cell purification.

CD4+ T cell purification

Cell suspensions were prepared from PLN of mice. $CD4^+$ T cells were negatively selected by depletion of $CD8^+$, MHC Class II^+, $CD11b^+$ and other cell types using the BD iMag Mouse $CD4^+$ T Lymphocyte Enrichment Set according to the manufacturer's instructions (Becton Dickinson, San Diego, CA). This method routinely provides us with greater than 90% pure $CD4^+$ T cell populations.

T cell stimulation for cytokine analysis

Splenocytes, bulk PLN cells or purified $CD4^+$ T cells from burn and sham mice (1 x 10^6 cells/ml) cells were stimulated with PMA (1ug/ml; Simga Aldrich, St. Louis, MO) and ionomycin (1ug/ml; Sigma Aldrich, St. Louis, MO) for a total of 4 hours in 1ml of complete RPMI (10% fetal calf serum) in 24 well flat-bottom plates. Brefeldin A (3.0ug/ml; eBioscience, San Deigo, CA) was added for the final 2 hours of culture to retain cytokines within the cell.

Th17* in vitro *cell polarization

Purified $CD4^+$ T cells from burn and sham mice (1 x 10^6 cells/ml) cells were stimulated with plate bound anti-mouse CD3 antibody (1ug/well; Becton Dickinson, San Diego, CA) and soluble anti-mouse CD28 antibody (5ug/ml; BD Pharmingen, San Diego, CA) in the presence of IL-6 (50ng/mL; BD Pharmingen, San Diego, CA) and TGF-β (1ng/ml; PeproTech, Rocky Hill, NJ) cytokines for a total of 4 days in 0.5ml of complete RPMI (10% fetal calf serum) in 48 well flat-bottom plates. Serum was collected from burn mice and added to cultures where indicated at a final dilution factor of 1/10.

Flow cytometric analysis

The panel of monoclonal antibodies used for flow cytometric analyses were anti-ROR-γt (AFKJS-9), anti-CD8α (53-6.7), anti-CD3ε (145-2C11), anti-CD4 (L3T4), anti-IFN-γ (XMG-1.2) (BD Pharmingen, San Diego, CA) and anti-IL17 (eBio17B7) (eBioscience, San Diego, CA). Intracellular staining for cytokines and ROR-γt was performed using standard methods [15]. Four and five-color analysis was performed using standard methods. List mode data were collected on a FACS Cyan (Dako, Ft. Collins, CO) and analyzed using Summit software (Dako, Ft. Collins, CO).

Statistical Analysis

Data were analyzed using Student's t-test for intracellular cytokine, absolute number and ratio differences. Statistical significance was defined as $p<0.05$ unless indicated otherwise.

2.4 Results

Th17 cells exist in wound-draining lymph nodes but not in spleen following burn injury

We and others have previously shown a dynamic change in the $CD4^+$ and $CD8^+$ T cell compartments at various timepoints following severe burn injury with the most dramatic changes occurring at 3 and 14 days after burn[14-33]. In order to examine whether pre-formed Th17 cells exist within peripheral immune sites at these timepoints, we utilized a model of contact burn injury in wildtype female B6 mice. Cells were harvested from spleen, inguinal and axillary PLN at 3 or 14 days after a 20% TBSA contact burn or sham injury. Wound-

draining PLN have been previously shown to exhibit marked lymphocyte alterations after burn injury[25, 42, 43]. T cells were stimulated with mitogen, and standard intracellular cytokine staining employed to identify $CD3^+$ $CD4^+$ IL-17^+ (Th17), $CD3^+$ $CD4^+$ IFN-γ^+ (Th1), $CD3^+$ $CD8^+$ IL-17^+ (Tc17) and $CD3^+$ $CD8^+$ IFN-γ^+ (Tc1) cells using flow cytometry. At 3 days following burn or sham injury, we found an increased percentage of Th1 cells in the spleen (as reported previously[15]) and inguinal PLN but no increase in IL-17 producing $CD4^+$ T cells (Figure 2.2). In contrast, there was a significant increase in the amount of Th17 cells found in the axillary PLN of the burn mice when compared to sham mice (Figure 2.3). We did not observe any expression of IL-17 by $CD8^+$ T cells (data not shown). At 14 days post-burn and sham injury, we found a burn-dependent increase in the percentage of Th17 cells present in both the axillary and inguinal PLN but not the spleen (Figure 2.3). The percentage of Th1 cells in all sites studied was significantly lower at day 14 versus day 3, corresponding to the suppressed pro-inflammatory state observed late after burn injury. Again, we did not observe any expression of IL-17 by $CD8^+$ T cells at day 14 (data not shown). At all timepoints and in all organs studied, we did not observe at dual positive $CD3^+$ $CD4^+$ IL-17^+ IFN-γ^+ T cells.

Stimulation of CD4+ T cells by mitogen, such as PMA and ionomycin, is well known to quickly and dramatically downregulate CD4 co-receptor expression [44]. In order to better quantify the proportion and absolute numbers of Th17 cells in the wound-draining lymph nodes, we repeated the above experiment but utilized a $CD4^+$ T cell purification step prior to mitogenic stimulation. At 3, 7 and 14 days following burn or sham injury, we harvested cells from various wound-draining PLN and pooled cells from each mouse to ensure enough cells were present for subsequent $CD4^+$ T cell purification and stimulation. At all timepoints there was a statistically significant burn-dependent increase in the percentage of viable $CD4^+$ T

cells that were Th17 cells, as well as in the absolute number of Th17 cells, when compared to sham (Figure 2.4).

We also confirmed ROR-γt expression using an anti-ROR-γt antibody at these timepoints. ROR-γt staining was concurrent with IL-17 expression, but not with IFN- γ expression at day 7 post burn, further confirming these are Th17 cells (Figure 2.5). ROR-γt was also expressed in Th17 cells at day 14, as expected, but there was also significant expression within Th1 cells. The plasticity of the Th1/Th17 lineage is an emerging concept in the current literature (12), and we are actively pursuing this as a mechanism for Th17 production within burn mice.

Burn injury induces a dynamic alteration in the Th1/Th17 balance

Current studies demonstrate that strong inflammation mediated by cytokines, such as IL-6 and TGF-β, results in Th17 differentiation of $CD4^+$ pre-T cells (Figure 2.1). In order to define the kinetics of Th17 to Th1 differentiation after burn injury, we calculated the ratio of the absolute number of Th17 cells ($CD3^+$ $CD4^+$ IL-17^+) to the absolute number of Th1 cells ($CD3^+$ $CD4^+$ IFN-γ^+) for each timepoint after sham or burn injury. For comparative purposes, we defined the sham mice having a Th17/Th1 ratio of 1.0 at each timepoint and normalized each burn mouse ratio to the sham. At day 3, we observed a significant shift of the pro-inflammatory T cell phenotype towards a Th1 response in burn mice compared to sham, in agreement with previous studies[15, 22]. This shift was lost at day 7 after burn when there appeared to be equilibrium of Th17/Th1 balance similar to the sham mice. At day 14, there was a statistically significant difference between burn and sham with skewing of the pro-inflammatory $CD4^+$ T cell response to a Th17 phenotype in burn mice compared to sham

(Figure 2.6). These data suggest that although we observe an increase in Th17 cell number and percentage at all timepoints, there are increases of Th1 cells at day 3 and day 7 after burn, with day 3 having an overt burn-dependent Th1 response. We predict that day 14 represents a period after burn where the pro-inflammatory $CD4^+$ T cell response is beginning to bias towards the weaker pro-inflammatory Th17 phenotype.

$CD4^+$ T cells from burn and sham mice have a similar ability to polarize towards a Th17 phenotype

Various cytokines are known to polarize a pro-inflammatory response towards a Th17 phenotype, such as IL-6 and TGF-β. IL-23 is known to "fix" this phenotype in place. As a possible cellular mechanism for increased Th17 development, we assessed the ability of $CD4^+$ T cells isolated from burn and sham mice to polarize *in vitro* towards a Th17-phenotype. We also tested whether serum collected from burn mice was able to aid in the ability to polarize. Serum was collected and $CD4^+$ T cells were purified from PLN 3 and 14 days following burn or sham injury. The $CD4^+$ T cells were activated *in vitro* via T Cell Receptor (TCR) ligation with anti-CD3 antibody and anti-CD28 costimulation in presence of IL-6 and TGF-β for four days, which has been previously shown to promote Th17 differentiation[2, 3]. Sham and burn T cells were also stimulated in the presence or absence of burn serum. Th17 and Th1 $CD4^+$ T cells were identified after each culture condition utilizing intracellular cytokine staining and flow cytometry. At both 3 and 14 days, CD4+ T cells from burn mice had an equivalent ability to polarize to Th17 cells when compared to sham in the presence of know polarizing conditions (Figure 2.7). We predicted that as the Th17 phenotype was maximal at day 14 and the presence of IL-23 or some other unidentified serum factor would also be maximal at this timepoint. Addition of 14 day burn serum had no

effect on Th17 polarization / stabilization of sham or burn $CD4^+$ T cells (Figure 2.8), nor was there no significant difference between the response to serum alone and to any of the treatments shown in Figure 2.8. These data suggest that burn injury has no inherent effect on the ability of $CD4^+$ T cells to bias towards a Th17 phenotype under *in vitro* polarizing cytokine conditions. For example, serum from burn mice may not contain any soluble factors, such as IL-23, which affects Th17 polarization[2, 9-11].

2.5 Discussion

Many variables contribute to the development, regulation, and effector functions of $CD4^+$ Th1, Th17, and regulatory T cells. We hypothesized that burn injury would have a profound effect on the balance of Th1/Th17 T cells. The main reasoning behind this was that certain cytokines known to promote Th17 polarization are present after burn injury, namely IL-6 in the serum and TGF-β in the burn wound.

When examining bulk and $CD4^+$-purified T cell populations, we observed an increase in percentage of $CD4^+$ cells that were expressing IL-17 (Figures 2.2-2.3) in PLN draining the burn wound. However, these cells were not detected in the spleen. This localization of the Th17 cells might be due to higher concentrations of TGF-β in the draining PLN, or cells trafficking to the PLN from the wound where they were initially formed. The increase at day 14 compared to day 3 of Th17 cell number in the bulk T cell cultures from axillary and inguinal PLN (Figures 2.2-2.3) suggests that the signals generated from burn accumulate over time or increase later after burn, with a possible spread into more distal PLN (but not into the spleen). The Th17 response is observed bilaterally in the axillary and inguinal PLN. However, it should be noted that because of the way we perform our burn injury,

corresponding lymph nodes are approximately equidistant to the burn wound. However, it does appear that proximity to the wound corresponds to Th17 expansion (Figure 2.2).

To eliminate the possibility of underestimating the absolute number of Th17, due to likely downregulation of the CD4 co-receptor during T cell stimulation, we purified the $CD4^+$ T cell population prior to culturing. In order to generate enough cells for this selection process, various wound-draining PLN were collected and pooled together for each mouse. With the addition of the $CD4^+$ T cell-purification, we also saw a significant increase in the number of Th17 T cells in wound draining PLN across all timepoints compared to sham (Figure 2.4). While the percentage and cell number differences between burn and sham are statistically significant, the values are modest. This is likely due to the inefficiency and low sensitivity of intracellular cytokine staining at revealing polarized T cell subsets. We identified ROR-γt staining within these cells to confirm their phenotype.

While the proportion and absolute number of Th17 cells are important to study, the role of the Th1/Th17 balance has been postulated as being key for overall pro-inflammatory status in human and animal studies. Indeed, after burn we observed a dynamic shift in this ratio. To account for variability between intracellular cytokine staining between experiments, we defined the sham as having a Th1/Th17 ratio of 1.0 at each case so that data from different experiments could be compared. In agreement with previous studies[15, 22], we observed an early significant shift of the pro-inflammatory T cell phenotype towards a Th1 response in burn mice compared to sham. This shift was lost at day 7 after burn, but at the day 14 timepoint, there was a clear skewing of the pro-inflammatory $CD4^+$ T cell response to a Th17 phenotype in burn mice compared to sham (Figure 2.6). These data suggest that an increase of Th1 cells at day 3 and day 7 after burn offsets the increased Th17 differentiation

we observed meaning that Th1-promoting effects of burn over-ride the Th17-polarizing effects of IL-6 and TGF-β produced in response to burn injury.

These results suggest that day 3 represents a period of overt burn-dependent Th1 responses to antigen. Since IFN-γ is known to inhibit naïve $CD4^+$ T cells development towards a Th17 pathway, this would likely impact the ability of the patient to recruit neutrophils to sites of infections. Likewise, IL-12 is another cytokine driving differentiation into Th1 cells. While it has been shown that inducing a stronger Th1-response by IL-12 administration can protect against cecal ligation and puncture sepsis in mice[45], such a response has not been specifically studied with respect to the role of Th17 cells and pathogens that require rapid neutrophil clearance (e.g. *Pseudomonas, Klebsiella* and *Candida*) mediated effectively by Th17 cells. Indeed, when burn mice are challenged with *Pseudomonas aeruginosa*, they experience high levels of mortality within 2 to 3 days following injury correlated with an overt pro-inflammatory response[46]. Therefore, driving differentiation at these early tim points towards a Th17 differentiation pathway could be a potential benefit for patients.

In contrast to the early pro-inflammatory Th1 phase, it appears that day 14 in our mouse model represents a period after burn where the pro-inflammatory $CD4^+$ T cell response is tempered, appearing to bias towards the Th17 phenotype on stimulation. This is a period of time we have previously defined as having unique and dramatic altered cytokine profile after burn injury, with exaggerated cytokine responses but no clear dominant Th1 or Th2 phenotype. Since elevated TGF-β and IL-6 levels are found following bury injury, we hypothesize that $CD4^+$ Th17 cells are generated late after burn injury and become the dominant Th phenotype.

The mechanism for this Th17-dominance is currently unclear. The driving force for the Th1/Th2 cytokine imbalance at day 14 after burn injury appears to be intense homeostatic expansion of "spontaneous" memory-like $CD8^+$ and $CD4^+$ T cells as a consequence of lymphopenia early after burn injury. Th17 cells, also often defined as possessing a memory-like phenotype, have not yet been studied as being a consequence of homeostatic proliferation. Another potential mechanism is that Th17 polarization can be driven in vitro and in vivo by innate stimulation through Toll-like Receptors. (TLR)[47]. We have shown altered Toll-like Receptor (TLR) expression on innate cells (e.g. macrophages express lower TLR) and adaptive cells (T cells express higher levels of TLR) late after burn injury[48, 49]. Therefore, we predict that T cells are stimulated directly or indirectly via innate cells to various innate stimuli produced by burn injury, such as endogenous innate signaling molecules[50-58] released from the burn wound. While the results suggest that burn serum did not affect Th17 polarization of sham $CD4^+$ T cells during *in vitro* stimulation under TGF-β and IL-6 conditions, the serum levels of such endogenous innate signaling molecules might not be high enough to detect any differences. Experiments to test the role of innate stimulation after burn in affecting adaptive T cell responses are underway.

Attempts to improve the T cell response to burn injury by manipulating cytokines has been largely been unsuccessful and are not generally employed in the clinic. Our data suggests why cytokine manipulation may be unsuccessful. For example, the $CD4^+$ T cell phenotype after burn injury is dynamic and may already be set along a certain pro-inflammatory phenotype when pro-inflammatory cytokines, such as IFN-γ or IL-2, are being administered. In addition, we have shown previously that immune dysfunction is clearly more of a late, rather than early, problem after injury as infection, sepsis, and multiple system

organ failure develop weeks to months after burn injury. Thus, the majority of lymphocyte studies that focus solely on the early response to burn injury (less than 10 days) or have been given an additional insult, such as cecal ligation and puncture, may have missed an important characteristic of the T cell response to injury.

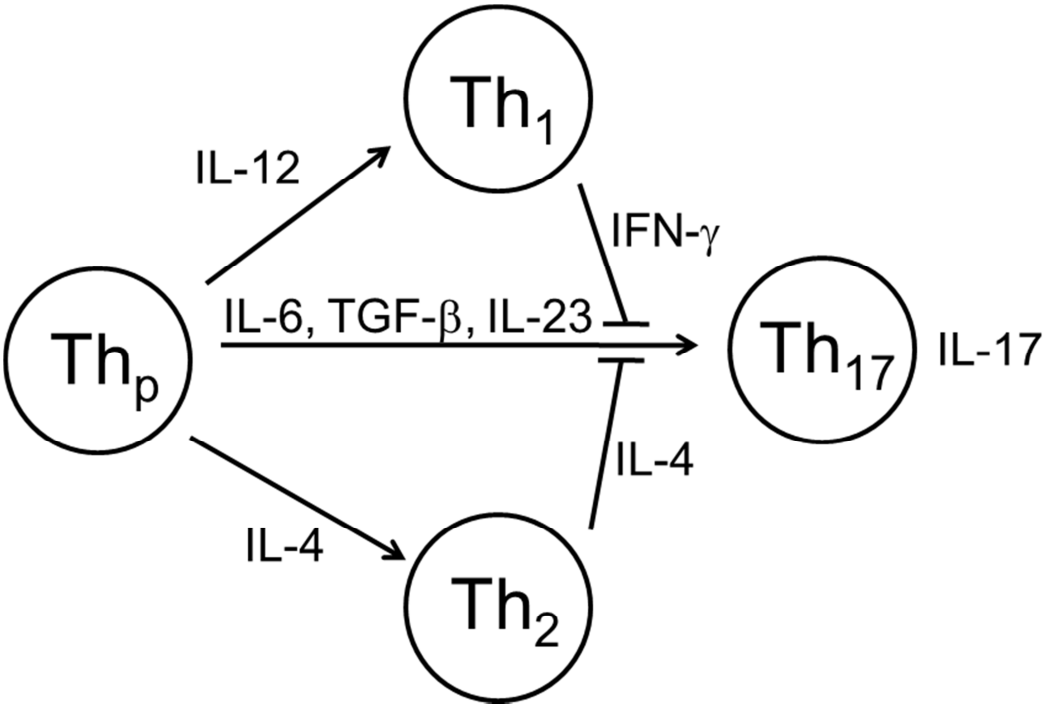

Figure 2.1. Th17 cells are a distinct lineage of CD4$^+$ T cells. Naïve CD4$^+$ T precursors (Thp) can differentiate towards either Th1, Th2 or Th17 phenotype. Differentiation of naïve CD4$^+$ T cells towards a Th17 phenotype requires the presence of transforming growth factor (TGF)-β and IL-6, while IL-23 is required for the maintenance of Th17 effector function. Unique cytokines secreted by differentiated Th1 (IFN-γ) and Th2 (IL-4) cells inhibit formation of Th17 cells.

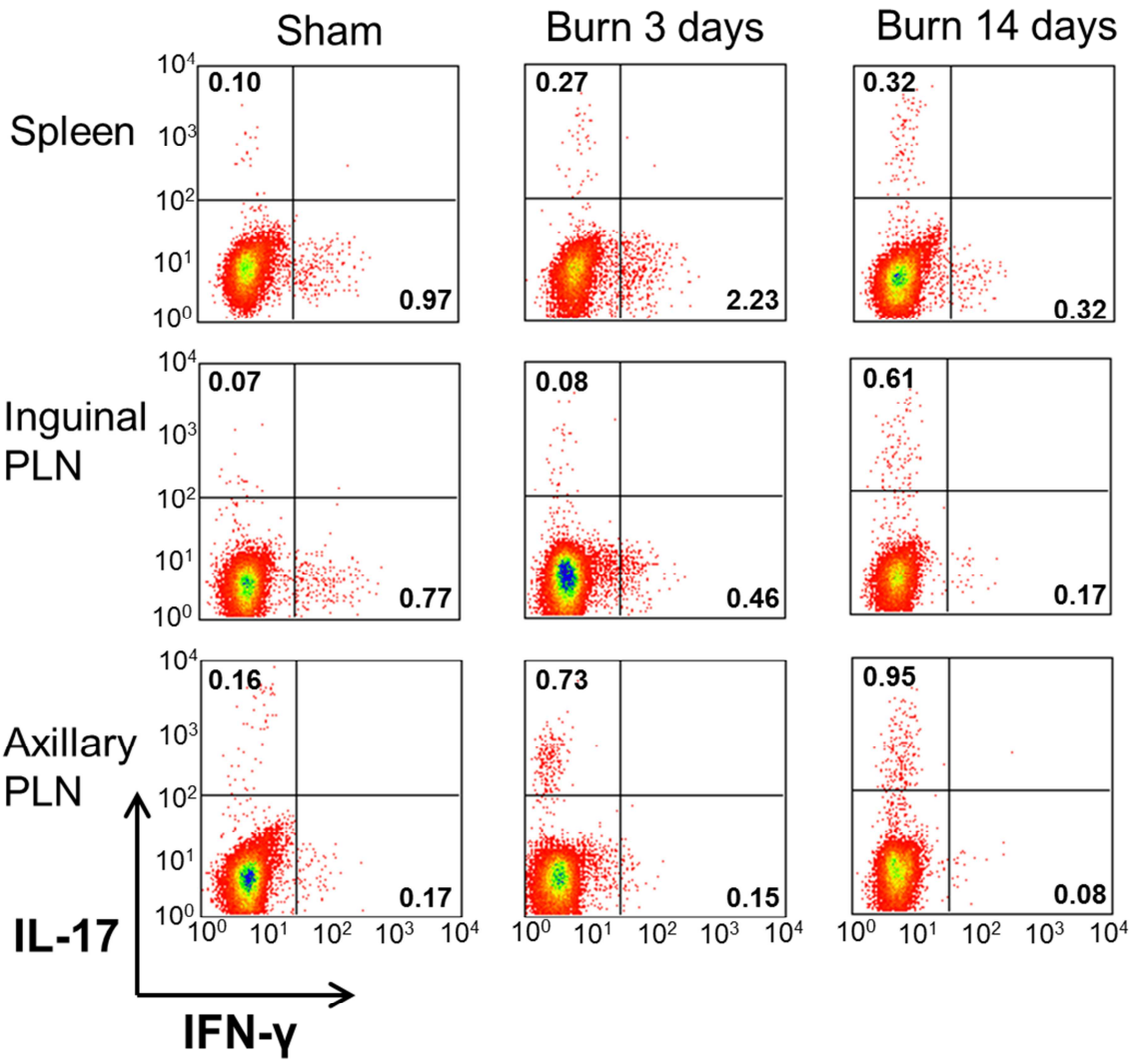

Figure 2.2. Wound-draining lymph nodes and the spleen contain Th17 cells 14 days after burn injury. Cells were harvested from spleens, as well as inguinal and axillary peripheral lymph nodes (PLN), 3 or 14 days following a 20% total burn surface area, full-thickness burn or sham injury. Bulk PLN cells were stimulated in vitro and intracellular cytokine staining was performed and flow cytometry used to measure IL-17 and IFN-γ production by $CD3^+$ $CD4^+$ cells. Representative examples of the flow cytometry histograms for each lymphoid organ are provided.

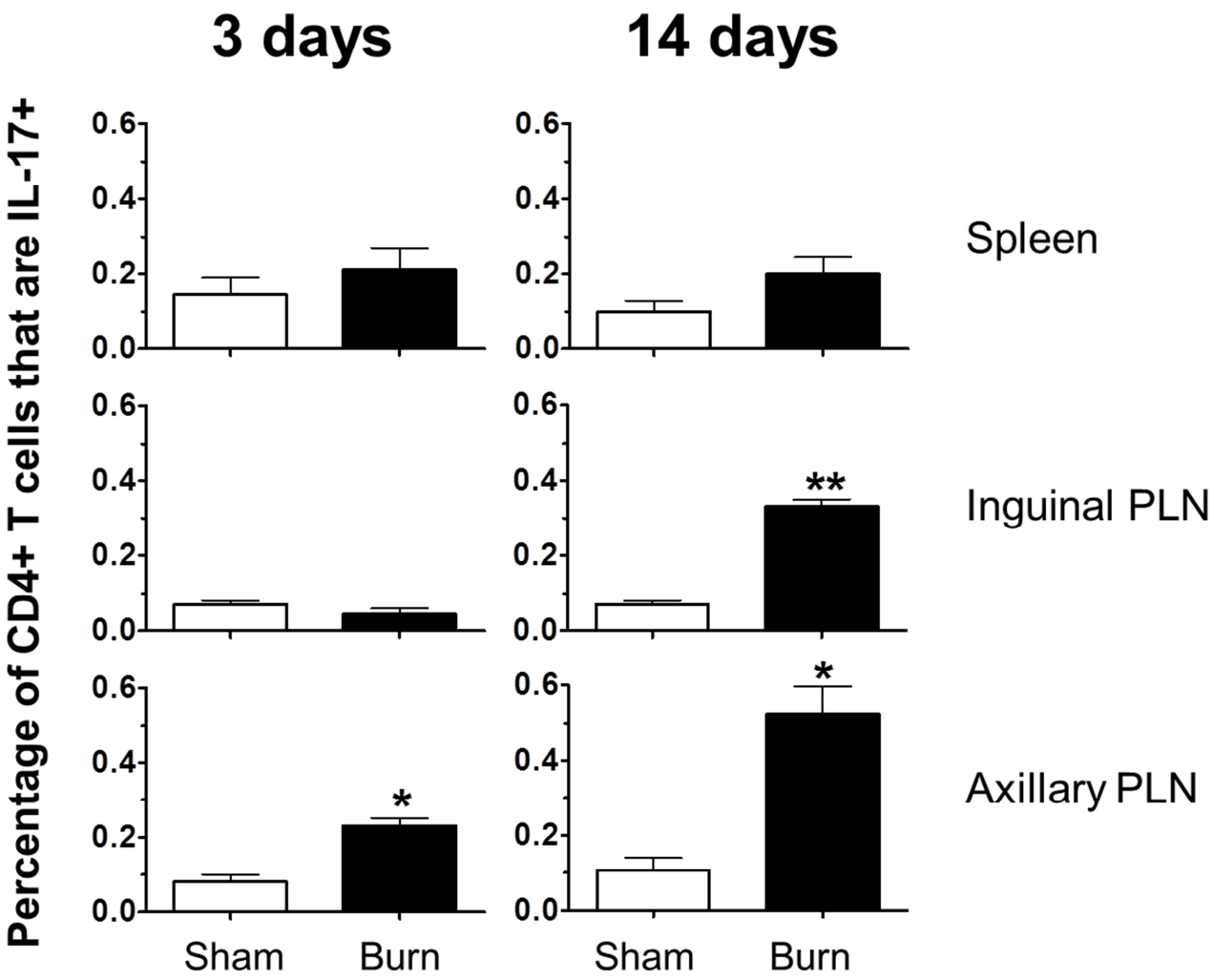

Figure 2.3. Percentage of $CD4^+$ T cells that produce IL-17 are significantly higher in burn compared to sham. Cells were harvested from spleens, as well as inguinal and axillary peripheral lymph nodes (PLN), 3 or 14 days following a 20% total burn surface area, full-thickness burn or sham injury. Bulk PLN cells were stimulated in vitro and intracellular cytokine staining was performed and flow cytometry used to measure IL-17 and IFN-γ production by $CD3^+$ $CD4^+$ T cells. Data are expressed as mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$ compared to matched sham controls by Student's t test (n=4-6 mice/group).

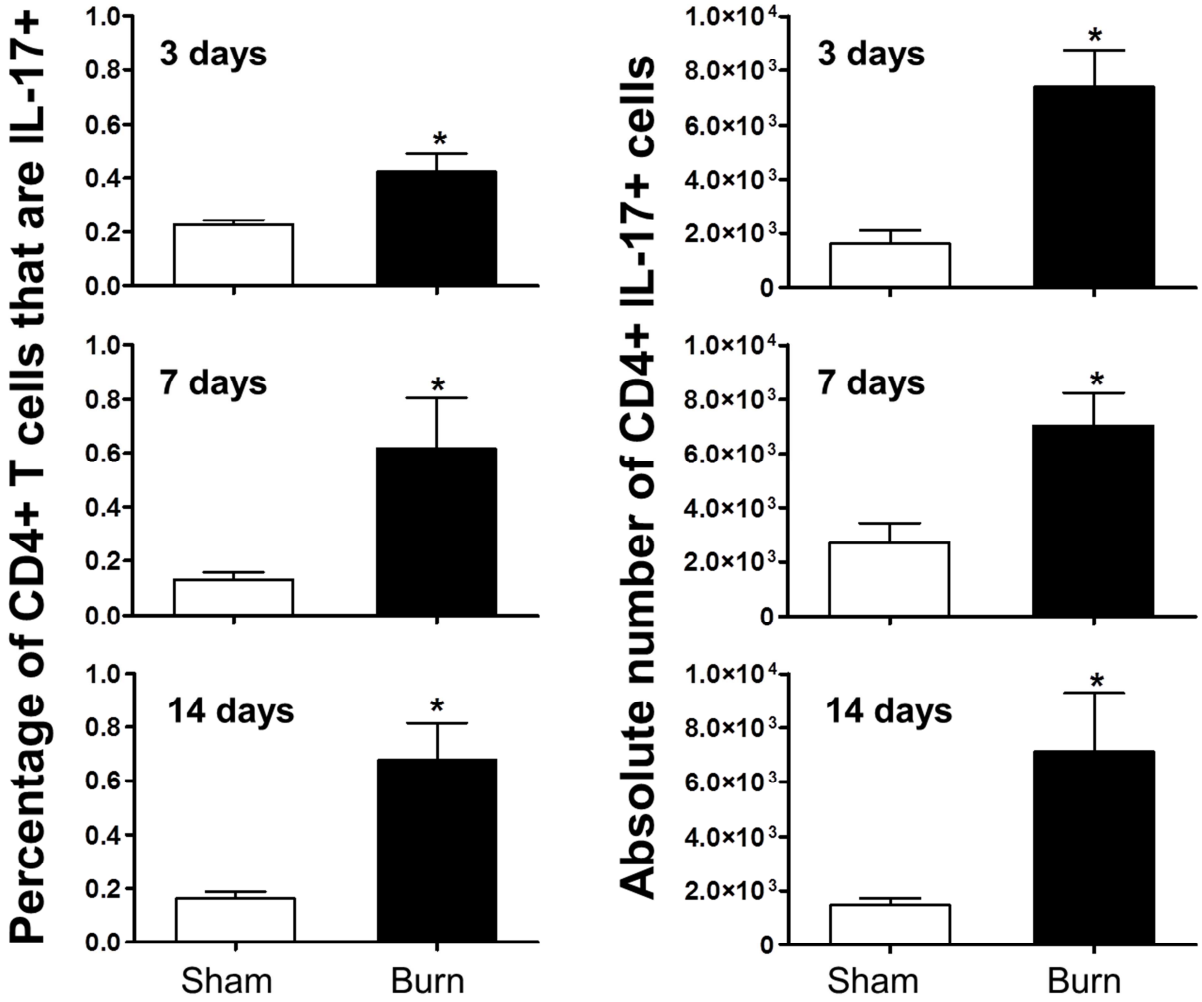

Figure 2.4. Frequency and absolute number of Th17 cells is significantly greater in the wound-draining lymph nodes of burn mice at all timepoints examined. Splenocytes and peripheral lymph node (PLN) cells were harvested at 3, 7 or 14 days following a 20% total burn surface area, full-thickness burn- (black bar) or sham- (white bar) injury. $CD4^+$ T cells were enriched by negative magnetic selection and stimulated in vitro. Intracellular cytokine staining was performed and flow cytometry used to quantify $IL\text{-}17^+$ $CD4^+$ T cells. Data are expressed as mean ±SEM, * $p \leq 0.05$ compared to matched sham controls by Student's t test (n = 4-6 mice/group).

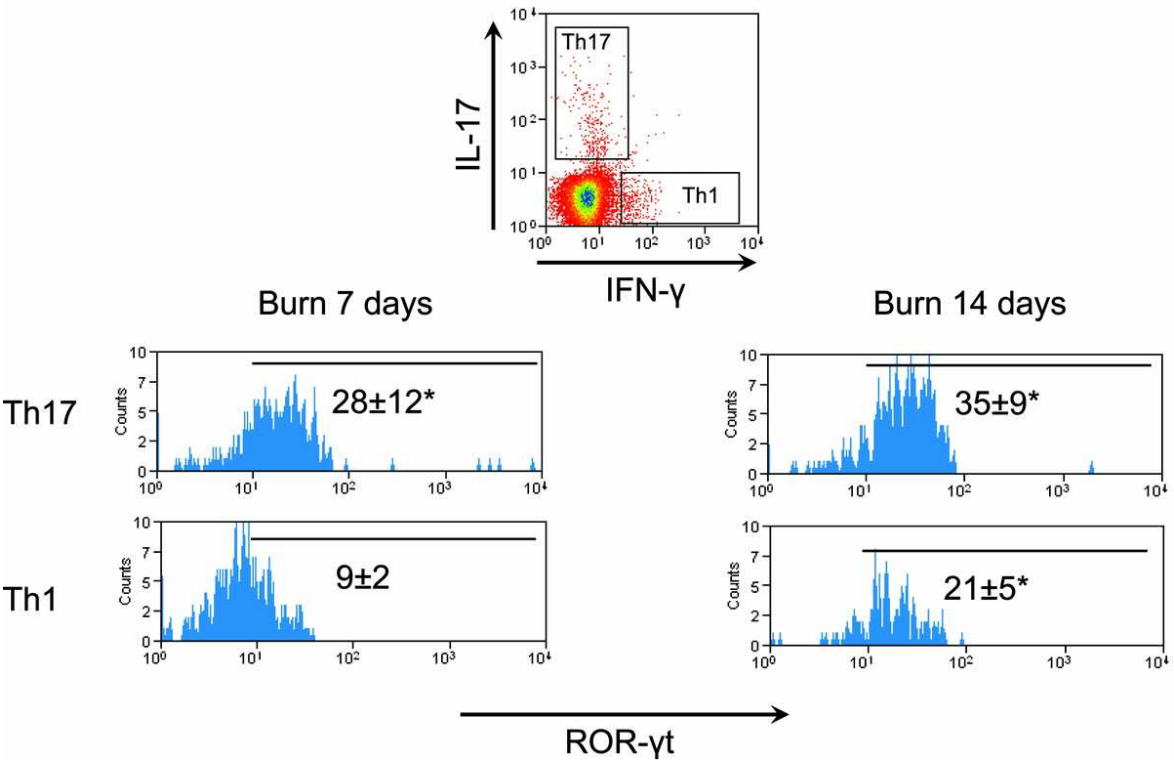

Figure 2.5. CD4+ T cells that produce IL-17 also express ROR-γt. Cells were harvested from inguinal and axillary PLN, 7 days or 14 days after a 20% TBSA, full-thickness burn injury. CD4+ T cells were enriched by negative magnetic selection and stimulated in vitro. Intracellular cytokine and ROR-γt staining was performed, and flow cytometry was used to gate Th17 (IL-17+ CD4+) and Th1 (IFN-γ+ CD4+) T cells. Representative ROR-γt staining is shown in each gated population. Numbers represent mean ROR-γt fluorescent units ± standard error of the mean, *p <= 0.05; compared with matched isotype control by Student's t test (n = 4–6 mice/group).

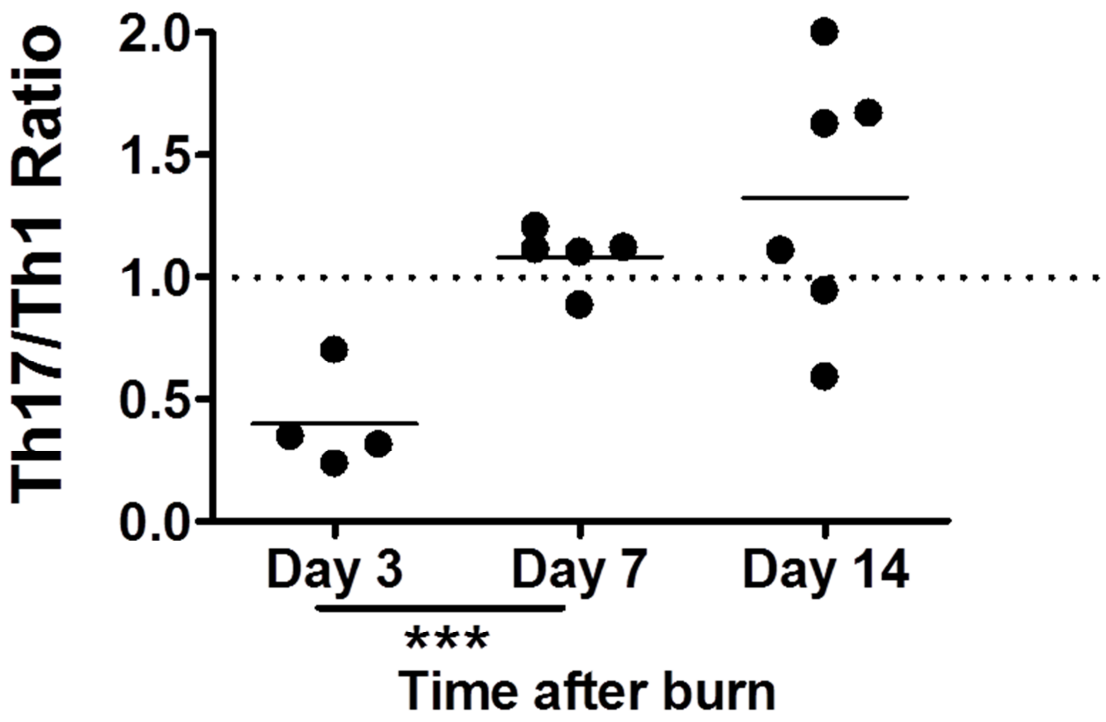

Figure 2.6 Th17/Th1 ratio is dynamic following burn injury. Cells were harvested from various peripheral lymph nodes at 3, 7 or 14 days following a 20% total burn surface area, full-thickness burn or sham injury. Negative magnetic selection was performed to enrich the $CD4^+$ T cell population. $CD4^+$ cells were stimulated *in vitro* before intracellular cytokine staining was performed, and flow cytometry used to quantify IL-17^+ $CD4^+$ T cells. For each timepoint, the average sham Th17/Th1 ratio was set at 1.0. For each burn mouse, its Th17/Th1 ratio was normalized to the corresponding sham ratio. A ratio above 1.0 represents a skew in the pro-inflammatory response to a Th17 phenotype, while a ratio below 1.0 indicates that a Th1 phenotype is dominant. ***$p \leq 0.0005$ compared to matched sham controls by Student's t test (n = 4-6 mice/group).

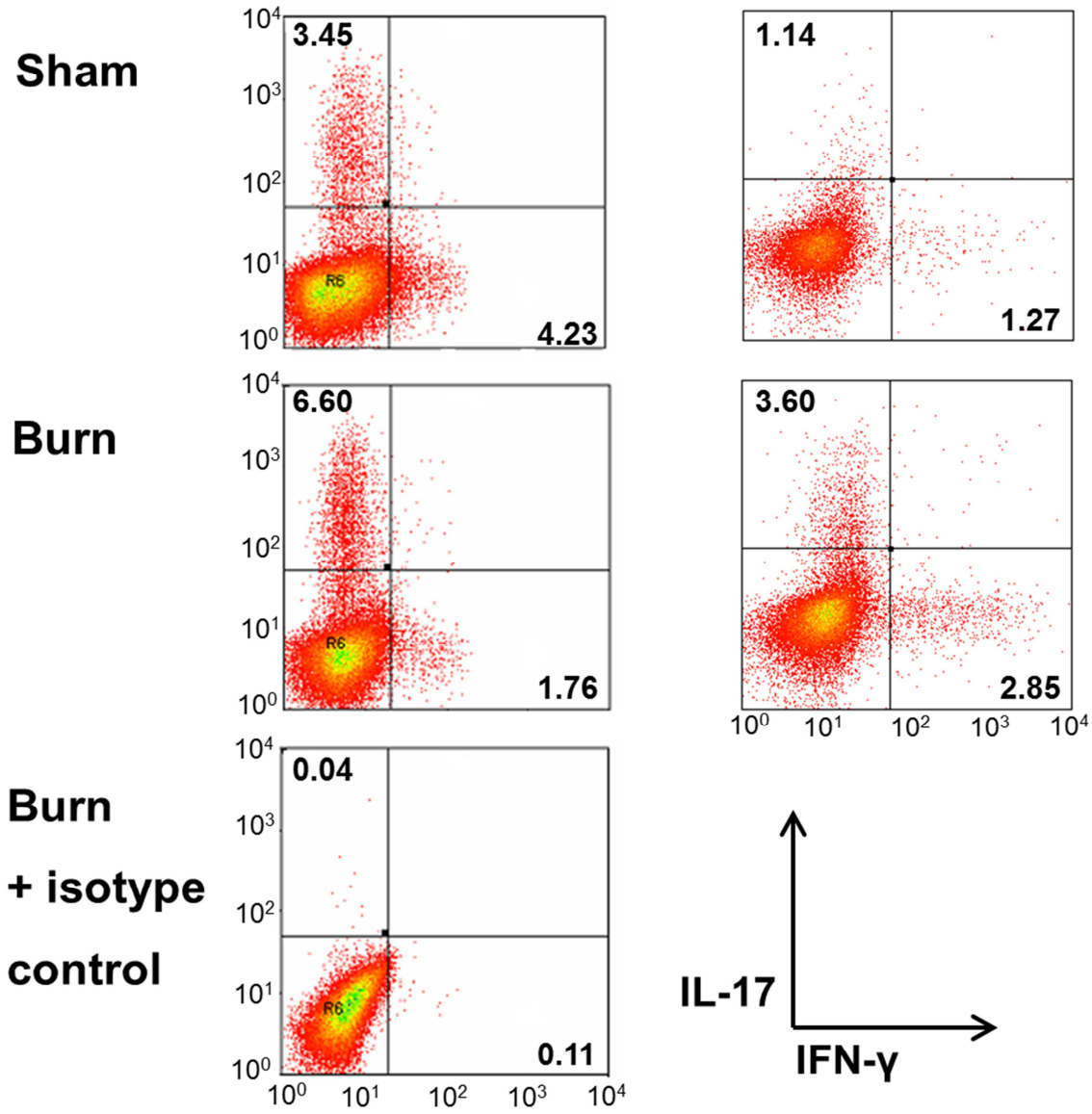

Figure 2.7. CD4$^+$ T cells from burn and sham mice have similar abilities to polarize to Th17 cells. Cells were harvested from various peripheral lymph nodes at 3 or 14 days following a 20% total burn surface area, full-thickness burn or sham injury. CD4$^+$ T cells were enriched by negative magnetic selection and cultured with plate bound anti-CD3, soluble anti-CD28, IL-6, and TGF-β for 4 days. IL-17 and IFN-γ production by CD3$^+$ CD4$^+$ cells was analyzed using flow cytometric staining. Representative examples of the histograms for each timepoint are provided.

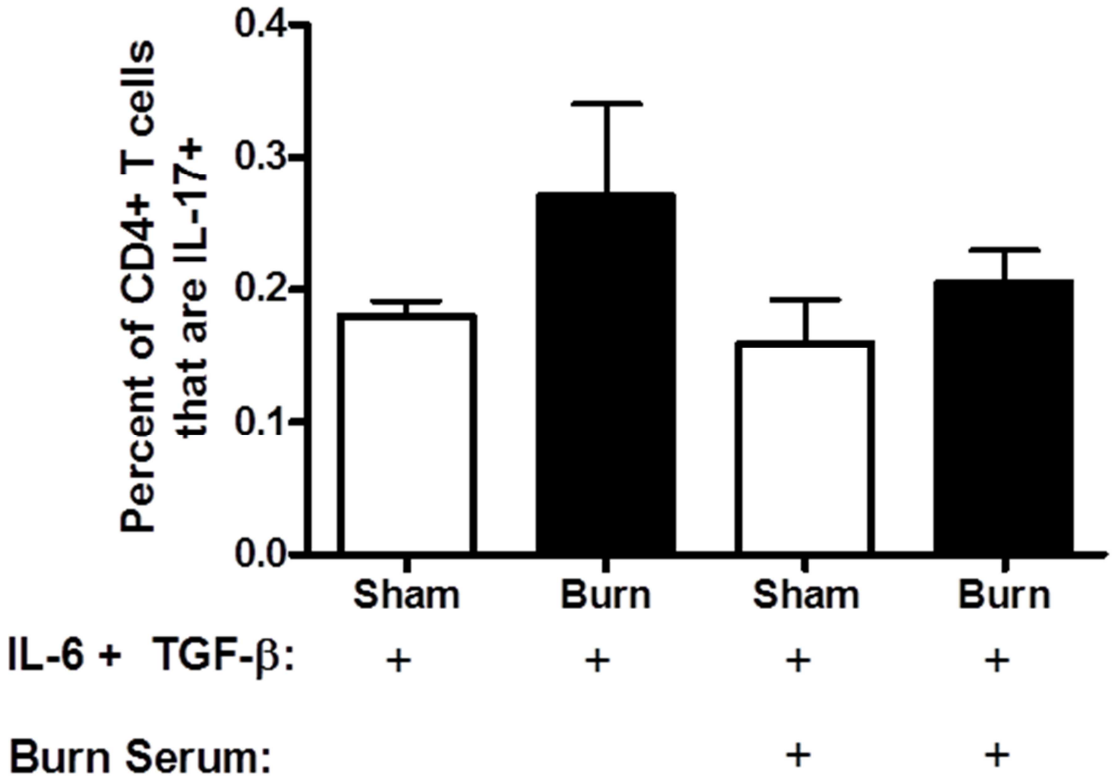

Figure 2.8. Burn serum does not contain a soluble factor that increases that ability of $CD4^+$ T cells to polarize to a Th17 phenotype. Cells were harvested from various peripheral lymph nodes at 14 days following a 20% total burn surface area, full-thickness burn or sham injury. Burn serum was also collected at 14 days following burn injury. $CD4^+$ T cells were enriched by negative magnetic selection. Cells were then cultured with plate bound anti-CD3, soluble anti-CD28, IL-6, and TGF-β in the presence or absence of burn serum for 4 days. IL-17 and IFN-γ production by $CD3^+$ $CD4^+$ cells was analyzed using flow cytometric staining. Isotype refers to staining with isotype control antibodies. Data expressed at mean ±SEM (n=4-6 mice/group).

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CHAPTER 3

Flagellin treatment prevents increased susceptibility to systemic bacterial infection after injury by inhibiting IL-10^{+} IL-12^{-} neutrophil polarization

3.1 Summary

Severe trauma renders patients susceptible to infection. In sepsis, defective bacterial clearance has been linked to specific deviations in the innate immune response. We hypothesized that the innate immune modulations observed during sepsis also contribute to increased bacterial susceptibility after severe trauma. A well-established murine model of burn injury, which was used to replicate infection following trauma, showed that wound inoculation with *Pseudomonas aeruginosa* quickly spreads systemically. This correlated with apoptosis of dendritic cells and memory CD8^{+} T cells, as well as differential Toll-like receptor expression on a variety of innate immune cells. The systemic IL-10/IL-12 axis was also skewed after burn injury with infection as indicated by a significant elevation in serum IL-10 and polarization of neutrophils into an anti-inflammatory (N2; IL-10^{+} IL-12^{-}) phenotype. Infection with an attenuated *P. aeruginosa* strain (Δ*cyaB*) was cleared more efficiently than the wildtype strain and was associated with an increased pro-inflammatory neutrophil (N1; IL-10^{-}IL-12^{+}) response in burn mice. This suggests that neutrophil polarization influences bacterial clearance after burn injury. Administration of a TLR5 agonist, flagellin, after burn injury skewed the neutrophil response towards a N1 phenotype resulting in an increased clearance of wildtype *P. aeruginosa* after wound inoculation. These

findings, for the first time, detail specific alterations in innate cell populations after burn injury that contribute to increased susceptibility to bacterial infection. In addition, it identifies neutrophil polarization as a therapeutic target for the reversal of bacterial susceptibility after injury.

3.2 Introduction

Each year traumatic injury accounts for over 40 million emergency room visits and 2 million hospital admissions across the United States[1]. Severe trauma predisposes patients to infection resulting in an overall infection rate of 37%[2]. Infectious complications, such as sepsis and pneumonia, increase the length of hospitalization and cost of treatment [3, 4]. Furthermore, infection increases the mortality rate of trauma patients by 5-fold[5].

In healthy individuals, the innate immune system sufficiently clears most invading bacteria. Neutrophils, which are considered the first-responders of the innate immune system, have a wide variety of anti-microbial functions including phagocytosis, oxidative burst, release of granule proteins, and generation of neutrophil extracellular traps (NETs)[6-8]. Macrophages and dendritic cells are also phagocytic, and antigen presentation and pro-inflammatory cytokine secretion (such as tumor necrosis factor [TNF]-α and interleukin [IL]-12) by these cells shape the adaptive immune response[9, 10]. Toll-like receptors (TLRs), which recognize conserved microbial products, are vital for detection of invading pathogens, and their signaling leads to the induction or suppression of hundreds of inflammatory genes that further influences the developing immune response[11, 12]. Specific populations of adaptive cells, for example memory $CD8^+$ T cells, also have innate anti-bacterial functions that operate independently of T Cell Receptor engagement[13]. Collectively, these innate immune responses lead to clearance of the invading bacteria.

During sepsis, defective bacterial clearance has been linked to alterations in the innate immune response. More specifically, apoptosis-induced depletion of immune cells leads to immunosuppression during sepsis[14-16]. TLR expression and signaling is often altered leading to hypo- or hyper-responsiveness[17, 18]. In addition, macrophages and neutrophils tend to be polarized into an anti-inflammatory phenotype due to TLR-signaling by danger-associated molecular patterns (DAMPS) released from damaged tissue[19-23]. These polarized macrophages (M2) and neutrophils (N2) secrete high amounts of IL-10, a potent anti-inflammatory cytokine. IL-10 can limit tissue damage by dampening the exaggerated production of pro-inflammatory cytokines observed during sepsis and induce tissue healing[24, 25]. However, excessive IL-10 has been shown to be detrimental for bacterial clearance by attenuating protective pro-inflammatory cytokines, such as IL-12[26-28]. We hypothesized that these innate immune modulations observed during sepsis also contribute to increased bacterial susceptibility after severe trauma.

Burn injury is a very common form of trauma world-wide with long lengths of hospital stay and substantial patient mortality due to a high prevalence of infection[29]. Utilizing a well-established murine model of burn injury to replicate infection following trauma, we found that burn mice were highly susceptibility to systemic wildtype *P. aeruginosa* infection after wound inoculation. A rigorous study of systemic innate cell types revealed significant burn-mediated apoptosis of dendritic cells and memory $CD8^+$ T cells, as well as differential TLR expression on a variety of innate immune cell populations. We also found that the systemic IL-10/IL-12 axis was skewed after burn injury and infection demonstrated by a substantial elevation in serum IL-10. Furthermore, a significant number of neutrophils, but not macrophages, were polarized into an $IL\text{-}10^+$ $IL\text{-}12^-$ N2 phenotype. To

confirm if neutrophil polarization played a role in bacterial clearance after burn injury, mice were then infected with an attenuated *P. aeruginosa* strain (Δ*cyaB*). We found that better clearance of Δ*cyaB* compared to the wildtype strain was associated with an increased N1 response in burn mice. Also, we were able to skew the neutrophil response towards a pro-inflammatory N1 phenotype by the administration of a TLR5 agonist, flagellin, immediately after burn injury. This correlated with an increased clearance of wildtype *P. aeruginosa* after wound inoculation. These findings, for the first time, detail specific alterations in innate cell populations after burn injury that contribute to increased susceptibility to bacterial infection. Furthermore this work reveals neutrophil polarization as a potential therapeutic target for the reversal of bacterial susceptibility after injury.

3.3 Methods and materials

Animals

Wildtype C57BL/6 (B6) mice were purchased from Taconic Farms (Hudson, NY). All mice used in the study were maintained under specific pathogen-free conditions in the Animal Association of Laboratory Animal Care-accredited University of North Carolina Department of Laboratory Animal Medicine Facilities. All protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines.

Mouse burn injury

Eight to 12 week old (>18 grams), female B6 mice were used for all experiments. Animals were anesthetized by inhalation of vaporized isoflurane (Baxter Healthcare, Deerfield, IL) and had their dorsal and flank hair clipped. A subcutaneous injection of

morphine (3mg/kg body weight; Baxter Healthcase) was given prior to burn injury for pain control, and an intraperitoneal injection of lactated Ringer's solution (0.1mL/g body weight; Hospira, Lake Forest, IL) was given immediately after burn injury for fluid resuscitation. To create a full-contact burn of approximately 20% total body surface area (TBSA), a 65-g rod copper rod (1.9 cm in diameter), heated to 100°C was used. Four applications of the rod, each for 10 seconds, to the animal's dorsal/flank produced the wound. Previous studies analyzing skin biopsies of the burn wound have demonstrated full-thickness cutaneous burn with visible unburned muscle beneath when following this procedure. Animals were returned to individual cages, provided food and morphinated water *ab libitum*, and monitored twice a day. Sham controls with 0% TBSA underwent all described interventions except for the actual burn injury. There was negligible mortality (<1%) after burn injury alone.

Bacterial strains and preparation

Wildtype *P. aeruginosa* strain PAK and an attenuated isogenic mutant strain lacking the adenylate cyclase gene, *cyaB*, (Δ*cyaB*) were obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC)[30]. Bacteria were grown from frozen stock at 37°C overnight in Luria-Bertani (LB) broth then transferred to fresh medium and grown for an additional 2 hours until they reached mid-log growth phase. Cultures were centrifuged at 12,000 x g for 30 seconds, and the pellet washed with 1mL of 1 % protease peptone in phosphate buffered saline (PBS +1%PP). Following a second wash, the bacterial concentration was determined by assessing optical density at 600nm. After diluting the bacteria to obtain the desired concentration, the inoculum was verified by plating serial 10-

fold dilutions on LB agar plates containing 25ug/mL irgasan (Sigma-Aldrich, St. Louis, MO), and colony forming units (CFUs) were enumerated after incubation at 37°C overnight.

Animal infections

Twenty-four hours following burn or sham injury, mice were anesthetized by intraperitoneal injection of Avertin (0.475mg/g body weight; Sigma-Aldrich). A subcutaneous injection of bacteria was injected in the mid-dorsal region. For burn mice, this was in unburned skin surrounded by burn wound. Initial experiments monitored survival until 120 hours post infection (hpi). In subsequent experiments, mice were sacrificed at 48hpi to enumerate bacterial load and analyze immune responses. In select experiments, mice were administrated flagellin two hours prior to infection. Ultrapure flagellin from *Salmonella typhimurium* (InvivoGen; San Diego, CA) was given intraperitoneally at a concentration of 0.125ng/100ul per mouse.

Determination of **P. aeruginosa** *Organ Burden*

At time of sacrifice, a 5mm skin biopsy of the initial injection site, the left lobe of the liver, and the lungs were aseptically removed and placed in 0.5mL of LB broth. The tissues were homogenized using 3.2mm stainless steel beads and a BulletBlender (Next Advance; Averill Park, NY). Serial dilutions of tissue homogenates were plated on LB agar containing irgasan (*P. aeruginosa* selective media), and CFUs were enumerated after overnight incubation at 37°C.

$CD11b^+$ Cell Enrichment

Cell suspensions were prepared from spleens of mice. $CD11b^+$ cells were positively selected using the BD Imag Mouse CD11b Magnetic Particles according to manufacturer's instructions (BD Biosciences). This method routinely provided a highly enriched population of $CD11b^+$ cells.

In vitro *stimulation*

Following the CD11b enrichment, both $CD11b^+$ and $CD11b^-$ cells were resuspended in RMPI containing 10% fetal bovine serum to achieve 10^6cells/mL. Cells were plated in a 48 well plate and cultured for 6 hours with 0.1ul/mL of lipopolysaccharide (LPS; Sigma-Aldrich) at 37°C at 5% CO_2. During the last 4 hours of the stimulation, 3.0ul/mL of brefeldin-A solution (eBioscience; San Diego, CA) was added to block protein secretion.

Flow cytometric analysis

Splenocytes were incubated with anti-mouse CD16/32 (BD Biosciences; San Jose, CA) at a concentration of 1ug per million cells for 5 min at 4°C to block Fc receptors. The panel of mAbs used for flow cytometric analyses were anti-Gr1 (RD-8C5), anti-CD11b (M1/70), anti-Ly6C (AL21) , anti-Ly6G (1A8), anti-CD11c (N418) , anti-F4/80 (BM8), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-NK1.1 (PK136), anti-TLR2 (6C2), anti-TLR4 (MTS510), anti-TLR5 (85B152.5), anti-IL-10 (JES5-E16E3), and anti-IL-12 (p40/p70), which were purchased from BD Biosciences, eBiosciences, and BioLegend (San Diego, CA). Intracellular staining was performed using BD Cytofix/Cytoperm Kit (BD Bioscience). Apoptosis was evaluated by LIVE/DEAD

(Fixable Dead Cell Kit; Invitrogen, Grand Island, NY) and CaspACE™ FITC-VAD-FMK staining (Promega; Madison, WI). Data were collected on a Dako CyAN and analyzed using Summit software (Dako; Carpinteria, CA).

Serum Cytokine assays

Submandibular bleeds were performed on mice prior to organ harvest. Serum was collected using MicroTubes with gel and following manufacturer's protocol (IRIS International, Westwood, MA). Serum IL-10 and IL-12 levels were determined using the BD Cytometric Bead Array (Becton Dickinson, San Diego, CA).

Statistical analysis

Data were analyzed using Student's t test for differences in CFU recovery, cell staining, and cytokine assays; log-rank analysis was used to test differences in mouse survival. GraphPad Prism version 5 was used for the analyses. Statistical significance was defined as $p \leq 0.05$ unless indicated otherwise.

3.4 Results

Burn mice, but not sham mice, developed a systemic infection following wound inoculation with wildtype *P. aeruginosa*

Initial studies assessed survival of burn and sham mice following wound infection with wildtype *P. aeruginosa* strain PAK. At 24 hours following burn or sham procedure, mice were anesthetized and given a subcutaneous injection of bacteria ($2x10^3$, $2x10^4$, or $2x10^5$ CFUs/mouse) at the mid-dorsum. There was 100% survival of sham mice, even with the highest dose of $2x10^5$ CFUs (Figure 3.1A). Burn mice, however, exhibited mortality that

was dose dependent (Figure 3.1A). Mortality of infected burn mice was seen as early as 1 day after inoculation. To evaluate bacterial clearance in burn and sham mice, animals were inoculated $2x10^4$ CFUs of wildtype *P. aeruginosa* and various tissues were harvested 48 hours post-infection before significant mortality occurred. As shown in Figure 3.1B, sham mice had no bacteria recovered from skin biopsies of the injection site, while all burn mice had bacteria detected in their skin samples. Furthermore, the amount of bacteria recovered from the skin of burn mice was 1-4 logs higher than the initial inoculum. This suggests bacterial recovery was not solely due to a lack of clearance, but that bacteria actively replicated in the skin. Distal organs were also analyzed to examine bacterial dissemination. The liver and lungs of sham mice had no detectable bacteria, whereas the organs of burn mice had a high bacterial load (Figures 3.1C-F). These data show that burn mice develop a systemic infection by 48 hours following wound inoculation with wildtype *P. aeruginosa*.

Splenic dendritic cells and memory $CD8^+$ T cells preferentially underwent apoptosis after burn and infection

Since innate immune cells, such as neutrophils, macrophages, and dendritic cells, are essential for control and clearance of *P. aeruginosa*[31], we hypothesized that these specific cell populations would be diminished in the spleen following burn and infection. In order to perform a systemic and detailed quantification of various immune cell populations after burn injury and infection, we devised a flow cytometric staining panel and gating scheme (Table 3.1). The absolute number of splenic neutrophils and macrophages, as well as a $Ly6G^+$ $CD11b^+$ myeloid population, were similar in all treatment groups (data not shown). However, infected burn mice had a significant reduction in the absolute number and frequency of splenic dendritic cells compared to uninfected burn and infected sham mice (Figure 3.2A and

3.2B). Memory $CD8^+$ T cells were also reduced in number and frequency following the combination of burn injury and infection (Figure 3.2C and 3.2D).

To determine if the reduced number of splenic dendritic cells and memory $CD8^+$ T cells in infected burn mice was due to increased cell death by apoptosis, a hallmark of burn injury and sepsis[15, 32-34], cell viability and caspase activation were evaluated. All dead cells were positive for caspase activation suggesting they had undergone apoptosis rather than necrosis (Representative histogram, Figure 3.3A). Uninfected burn mice had a higher percentage of apoptotic dendritic cells than uninfected shams. Infection following burn injury led to a significant increase in apoptosis that was not observed in the infected sham mice (Figure 3.3B). A similar response was observed for splenic memory $CD8^+$ T cells (Figure 3.3C). Burn injury and/or infection did not increase the frequency of apoptosis for all other immune cells analyzed (data not shown). These data suggest that burn injury alone causes a significant increase in apoptosis of splenic dendritic cells and memory $CD8^+$ T cells and that bacterial infection after burn causes an even greater increase in the apoptosis of these cells.

Innate cell populations had altered TLR expression with the combination of burn injury and infection

We and others have shown that Toll-like receptor (TLR) mRNA[35, 36] and protein[35] levels change after burn with an increase observed in $CD11b^+$ cells at 3 days following thermal injury. Since TLR2, TLR4, and TLR5 are involved in control of *P. aeruginosa* infection by recognizing outer membrane lipoproteins, LPS, and flagellin, respectively[37-39], we hypothesized that decreased bacterial clearance after burn injury was due to reduced expression of these TLRs on innate immune cells. Using flow cytometric analysis, there was

no detectable change in the relative expression of the TLR2, TLR4, or TLR5 after burn injury alone. Likewise, all treatment groups had similar TLR expression on their splenic dendritic cells and memory $CD8^+$ T cells with or without infection (data not shown). Upon bacterial infection, splenic neutrophils and $Ly6G^+$ $CD11b^+$ myeloid cells from burn mice had significantly reduced TLR2, TLR4 and TLR5 expression compared to uninfected burn and infected sham mice (Figure 3.4A and 3.4B). In contrast, splenic macrophages from infected burn mice had increased TLR2 and TLR4 but unchanged TLR5 expression compared to uninfected burn and infected sham mice (Figure 3.4C). These data demonstrate that the innate cell populations that survive the burn-induced apoptosis have specific alterations in TLR expression.

Infection following burn injury resulted in a systemic increase in IL-10

Macrophages and neutrophils can be polarized into pro- (M1/N1) and anti-inflammatory states (M2/N2)[19-22]. Formation of anti-inflammatory $IL\text{-}10^+$ $IL\text{-}12^-$ M2 and N2 cells has been shown to occur after TLR stimulation, particularly in the context of injury where there is release of tissue DAMPs[23]. Many studies have shown that IL-10 is deleterious whereas IL-12 is beneficial for clearance of *P. aeruginosa*[26-28]. Therefore, we hypothesized that infected burn mice would have a skewing in the IL-10/IL-12 axis towards an IL-10 response. Three days following burn or sham treatment, there was no detectable IL-10 in the serum (data not shown). Infection of burn mice resulted in a substantial elevation of serum IL-10 while infection of sham mice did not induce an IL-10 response (Figure 3.5A). Burn and sham mice also had no detectable IL-12 at three days post treatment (data not shown). However, infection caused an increase in serum IL-12 for both groups of mice (Figure 3.5B).

In summary, infection following burn injury led to a predominant systemic IL-10 response, while infection after sham treatment induced an IL-12 response.

Infected burn mice had an increased polarization of neutrophils, but not macrophages, into an IL-10$^+$ IL-12$^-$ phenotype

Infected burn mice had a systemic anti-inflammatory response following infection, which was marked by elevated serum IL-10 levels; therefore, we hypothesized that the innate cells were polarized towards an anti-inflammatory phenotype (IL-10$^+$ IL-12$^-$) following burn and infection. Splenocytes were harvested at 48 hours post infection and underwent intracellular staining for cytokine analysis without further stimulation *in vitro*. IL-10 producing Gr1$^+$ CD11b$^+$ cells were readily detected in the spleen of the infected burn mice (representative histogram, Figure 3.7A). Due to these data along with previous reports about IL-10 production by innate cells following burn injury[40, 41], we focused our subsequent studies on these cell types. Splenocytes were harvested at 48 following infection then underwent CD11b enrichment by magnetic selection. CD11b$^+$ (macrophages, neutrophils, inflammatory myeloid cells, and dendritic cells) cells, as well as cells from the CD11b$^-$ portion (memory CD8$^+$ T cells and other immune cells), were cultured in the presence of LPS and brefeldin-A to measure intracellular accumulation of IL-10 and IL-12. Cell surface and intracellular staining indicated that neutrophils were the major immune cell type producing IL-10 in the spleen. Furthermore, infected burn mice had a significantly higher percentage of splenic neutrophils producing IL-10 than infected sham mice (Figure 3.6B). As for IL-12 production, infected burn mice had a significantly lower percentage of splenic neutrophils, dendritic cells, and macrophages producing this cytokine than infected sham mice (Figure 3.6C-E). These data, along with the serum cytokine response, suggest that following burn

injury, the immune system mounts an inappropriate systemic IL-10 response with neutrophils exhibiting a N2 phenotype upon bacterial infection.

Increased resistance of burn mice to systemic infection with an attenuated strain of *P. aeruginosa* correlated with reduced N2 polarization of neutrophils

P. aeruginosa strain PAK, lacking the adenylate cyclase encoding gene *cyaB,* (ΔcyaB) has been previously reported to be attenuated in an adult mouse model of acute pneumonia, despite wildtype growth *in vitro*[42]. We predicted that burn mice could control infection with the Δ*cyaB* strain better than wildtype PAK. Also, we hypothesized that differences in the innate immune response to Δ*cyaB* and wildtype *P. aeruginosa* infection would reveal mechanisms that contribute to enhanced bacterial clearance and thus identify potential targets for immune modulation. Twenty-four hours following burn or sham treatment, mice were given a subcutaneous injection ($2x10^4$ CFUs/mouse) of wildtype *P. aeruginosa* (PAK) or the isogenic attenuated mutant (Δ*cyaB*). At 48 hours following infection, skin biopsies at the injection site were harvested to measure localized bacterial clearance. Distal organs were also harvested to assess bacterial dissemination from the injection site. Regardless of *P. aeruginosa* strain, infected sham mice had no bacteria recovered from their skin, liver, and lung samples (data not shown). Infection with wildtype PAK or Δ*cyaB* following burn injury resulted in a similar bacterial load at the injection site (data not shown). In contrast, burn mice infected with Δ*cyaB* had significantly less bacterial recovery in the distal organs than burn mice infected with wiltype PAK (Figure 3.7A and 3.7B). These data indicate that burn mice are more resistant to developing systemic infection with an attenuated strain of *P. aeruginosa* than with wildtype. Therefore, burn mice retain some antibacterial activity which allows for improved control of the attenuated strain.

Infection of burn mice, regardless of bacterial strain, caused an elevation of serum IL-10 compared to sham mice (Figure 3.7C). In contrast, infection with the Δ*cyaB* mutant following burn injury resulted in a significant increase in serum IL-12 levels relative to wildtype PAK (Figure 3.7D). In both treatment groups, the main source of IL-10 in the spleen was neutrophils. Also, the percentage of splenic neutrophils producing IL-10 was similar in burn mice infected with Δ*cyaB* and those infected with wiltype PAK (Figure 3.7E). As for IL-12 production, Δ*cyaB* infection resulted in a higher percentage of IL-12$^+$ neutrophils within the spleen (Figure 3.7F). Hence, infection with Δ*cyaB* following burn injury results in a higher percentage of IL-12$^+$ cells within the spleen and an increase in serum IL-12. We also analyzed other aspects of the innate immune response, but found no significant difference between the two infections. Specifically, infection with the Δ*cyaB* caused a similar reduction in the number of splenic dendritic cells and memory CD8$^+$ T cells in burn mice (data not shown). Also, TLR2, TLR4, and TLR5 expression on the various immune cells was comparable between wildtype PAK and Δ*cyaB* infected burn mice (data not shown). These data suggest that the reduced susceptibility to Δ*cyaB* in the burn mice is due to a skewing of the IL-10/IL-12 balance to a protective IL-12 response.

Treatment of mice with flagellin after burn injury enhanced clearance of wildtype *P. aeruginosa*

Flagellin, the ligand of TLR5, has been shown to increase IL-12 production[23, 43]. Therefore, we hypothesized that flagellin administration prior to infection could improve clearance of wildtype *P. aeruginosa* in burn mice by increasing the protective IL-12 response. Burn mice received an intraperitoneal injection of flagellin (.125ng/mouse) two hours prior to subcutaneous infection with wiltype PAK. Forty-eight hours following

infection with or without pretreatment with flagellin, various organs were harvested to determine bacterial load. Pretreatment with flagellin did not affect bacterial recovery from skin biopsies at the injection site (data not shown). However, there were significantly less bacteria recovered from the liver and lungs of burn mice pretreated with flagellin compared to untreated controls (Figure 3.8A and 3.8B). The reduced bacterial load in the periphery correlated with an increased percentage of IL-12 producing neutrophils whereas IL-10 production by neutrophils was unchanged (Figure 3.8C and 3.8D). These data suggest that a single treatment with flagellin after injury is sufficient to reduce the systemic infection of wildtype *P. aeruginosa* by skewing more neutrophils towards a pro-inflammatory phenotype.

3.5 Discussion

Severe trauma results in a period of immune impairment that predisposes patients to infectious complications, such as sepsis. However, the specific mechanisms that contribute to diminished bacterial clearance are not clearly defined. In this study, we utilized a murine model of severe burn injury and challenged mice with a clinically relevant pathogen to reveal specific trauma-induced deviations in the innate immune response that contribute to increased susceptibility to infection. Within 48 hours of wound inoculation with a wildtype strain of *P. aeruginosa* (PAK), the bacteria replicate to a high titer and spread to distal organs resulting in bacterial sepsis. In this model, burn injury and subsequent bacterial infection induces rapid apoptosis of splenic dendritic cells and memory $CD8^+$ T cells. Neutrophils and $Ly6G^+$ $CD11b^+$ myeloid cells, which escape apoptosis, have decreased TLR expression. In addition, neutrophils are profoundly polarized into an anti-inflammatory (N2; IL-10^+ IL-12^-) phenotype.

Furthermore, we hypothesized that some antimicrobial effector functions are retained after severe burn injury and that amplifying these responses therapeutically can enhance bacterial clearance even if in face of overt immune suppression. To identify these potential targets, mice were infected with an attenuated mutant of *P. aeruginosa* strain PAK (Δ*cyaB*). We found that burn mice have greater control of Δ*cyaB* infection than wildtype PAK infection, which is exhibited by reduced bacterial recovery systemically. By comparing various aspects of the innate immune response, it appears that increased neutrophil polarization towards a pro-inflammatory phenotype (N1; IL-12^{+} IL-10^{-}) contributes to improved clearance of Δ*cyaB* in the periphery. We next investigated the effectiveness of flagellin, a natural TLR5 ligand that can induce IL-12 production, as a therapeutic agent in our model[23, 43]. We found that treatment with flagellin after burn injury enhances clearance of wildtype *P. aeruginosa* in the periphery and increases the percentage of IL-12 producing neutrophils in the spleen. Nevertheless, IL-10 production by splenic neutrophil remained elevated compared to sham controls. These data suggest that although infection following burn injury polarizes neutrophils towards an anti-inflammatory phenotype, flagellin administration can tilt this back towards a pro-inflammatory response that is beneficial for bacterial clearance.

Previous studies have attempted to delineate cellular mechanisms underlying the increased susceptibility to infection after injury. This study utilized a very precise panel of antibodies for the flow cytometric identification of specific innate cell populations so that their role in infection after burn injury could be better assessed. Using cell surface markers CD11b, CD11c, F4/80, Gr1, Ly6C, and Ly6G, we can clearly define neutrophils, macrophages, a Ly6G^{+} CD11b^{+} myeloid population, and dendritic cells (Table 3.1). A

distinct $Gr1^+$ $CD11b^-$ sub-population was found to exist that we defined as memory $CD8^+$ T cells (Table 3.1). Using such an in depth staining panel and gating scheme allowed for quantification of various immune innate cell populations after injury that has not been reported to date.

It is apparent that the immune system is very sensitive to stress as seen by large-scale apoptosis of immune cells after sepsis, infection, and trauma. Depletion of splenic dendritic cells is characteristic of sepsis and many types of bacterial infections[15, 44, 45]. Here we reveal that splenic dendritic cells are susceptible to apoptosis after burn injury alone, and subsequent bacterial infection magnifies apoptosis of these cells. A similar response was seen for the memory CD8+ T cells after burn injury with and without infection. While glucocorticoid-mediated apoptosis of activated T cells occurs within the first two days after injury[32-34], we demonstrate for the first time that, coupled with bacterial infection, there is also significant loss of the memory CD8+ T cell compartment early after injury. Collectively, these data suggest that burn injury "primes" dendritic cells and memory CD8+ T cells to be more susceptible to apoptosis upon bacterial challenge. Further studies are required to define the role of dendritic cells and innate memory CD8+ T cells in controlling bacterial infection after injury.

Macrophages, neutrophils, and $Ly6G^+$ $CD11b^+$ myeloid cells survive the apoptotic event observed after burn injury and acute infection. In various models of trauma, a $Gr\text{-}1^+$ $CD11b^+$ cell population, also defined as an immature inflammatory monocyte, has been shown to arise. Some controversy exists as to whether these $Gr\text{-}1^+$ $CD11b^+$ cells are analogous to the Myeloid-derived Suppressor Cells (MDSC) that mediate T cell suppression in the tumor microenvironment[46]. Our laboratory has recently described that burn-induced

$Gr1^+$ $CD11b^+$ cells indeed suppress T cell proliferation[47] and polarize T cells towards a Th2-anti-inflammatory response[48] suggesting they mimic aspects of MDSC function. MDSCs employ various mechanisms, such as arginase, IL-10, and nitric oxide production, to inhibit T cell proliferation and activation[49-51]. This study did not reveal IL-10 secretion by $Ly6G^+$ $CD11b^+$ myeloid cells after burn injury and/or an acute bacterial infection, but we predict that these cells become a predominant population at later timepoints after burn injury[47] due to continuous myelopoeisis. Further work is required to determine if and how these cells impact both the innate and adaptive arms of the immune system later after burn injury.

As for the remaining innate cells populations, we observed neutrophil, but not macrophage, polarization in our model system. Polarization of adaptive immune cells, such as naïve $CD4^+$ T cells into a Th1 or Th2 phenotype, is well established[52]. However, the polarization and plasticity of innate immune cell populations has only been recently recognized. Most of the information within the field originates from tumor research and focuses on macrophage polarization[53]. Although the details are still unclear, the literature suggests that the local microenvironment in which a cell is activated determines the cell's subsequent phenotype and that changing this microenvironment can skew polarization of the cell population. For example, a macrophage can be polarized towards a pro-inflammatory phenotype (M1) marked by production of IL-12, as well as other pro-inflammatory mediators, when activated in the presences of interferon-γ[54]. However, if a macrophage is then exposed to IL-10, glucocorticoids, or immune complexes in the presence of the TLR ligands, it can exhibit an anti-inflammatory phenotype (M2, $IL\text{-}10^+$ $IL\text{-}12^-$) [55, 56]. In our model system, we find that neutrophils, not macrophages, are the main innate immune cell population that is polarized. It appears that infection following burn injury skews neutrophils

towards an anti-inflammatory phenotype. Yet when mice are administered flagellin, they exhibit a mixed N1/N2 phenotype that correlates with enhanced bacterial clearance in the periphery. In the context of sepsis, a predominant M1 response is detrimental since the robust pro-inflammatory cytokine production by the macrophages can exacerbate tissue damage[20]. Also, an overt M2 response is believed to deleterious by significantly impairing bacterial clearance[57]. Thus, a mixed M1/M2 response appears to be ideal during sepsis. Our data support the idea that a mixed N1/N2 response is also beneficial after sepsis; however, future research is needed to delineate this correlation in more detail.

Since infectious complications are a main cause of mortality after traumatic injury, it is essential to identify biomarkers of infection and drug targets to improve control of invading pathogens. Numerous studies have linked high circulating levels of IL-10 with poor outcome following burn injury, sepsis, and a wide variety of bacterial infections[28]. In our model system, serum IL-10 is elevated in infected burn mice, regardless of strain, but not in uninfected controls. Collectively, this supports the use of IL-10 as a useful biomarker of bacterial infection.

Taken together these data detail specific changes in innate cell populations following burn injury that contribute to increased susceptibility to bacterial infection and reveal neutrophil polarization as a therapeutic target for the reversal of bacterial susceptibility after injury. Future experiments should examine other aspects of neutrophil function, such as phagocytosis and NET formation, after burn injury and infection and determine if flagellin administration impacts these antimicrobial activities. Furthermore, the timing of treatment should be investigated to determine if flagellin administration could improve clearance of an established bacterial infection, which would be extremely valuable in the clinical setting.

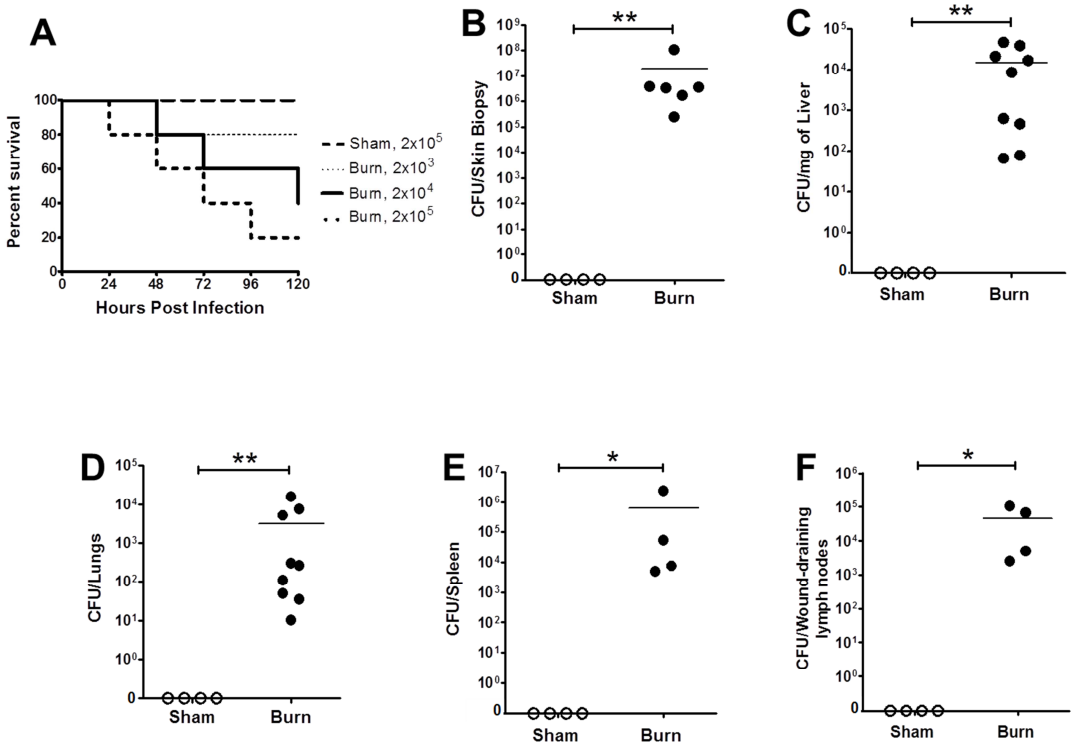

Figure 3.1. Burn mice, but not sham mice, exhibit dose-dependent mortality and develop a systemic infection following a *P. aeruginosa* wound inoculation. Wildtype *P. aeruginosa* strain PAK was administered subcutaneously at 24 hours after burn or sham treatment. A) Various doses of bacteria ($2x10^3$, $2x10^4$, or $2x10^5$ CFUs) were given, and survival was monitored for 120 hours post infection (hpi). B-F) Using a dose of $2x10^4$ CFUs, bacterial load at the injection site and distal organs was assessed at 48hpi in sham (open circles) and burn (closed circles) mice. (n = 4-9 per group) *, $p \leq 0.05$. **, $p \leq 0.005$. These experiments were repeated three times with similar results.

Cell Type	Gr1	Ly6C	Ly6G	CD11b	CD11c	F4/80	CD3	CD8	CD4	CD44	NK1.1
Neutrophils	+	+	+	+	+	-	-	-	-	-	-
Macrophages	+	+	-	+	-	+	-	-	-	-	-
$Ly6G^+$ $CD11b^+$ Myeloid Cells	+	+	+	+	-	+	-	-	-	-	-
Dendritic Cells	-	-	-	+	+	-	-	-	-	-	+/-
Memory $CD8^+$ T Cells	+	+	-	-	-	-	+	+	-	+	-

Table 3.1. Five distinct immune cell populations were identified in spleen. Antibody panels were devises to define these cell populations.

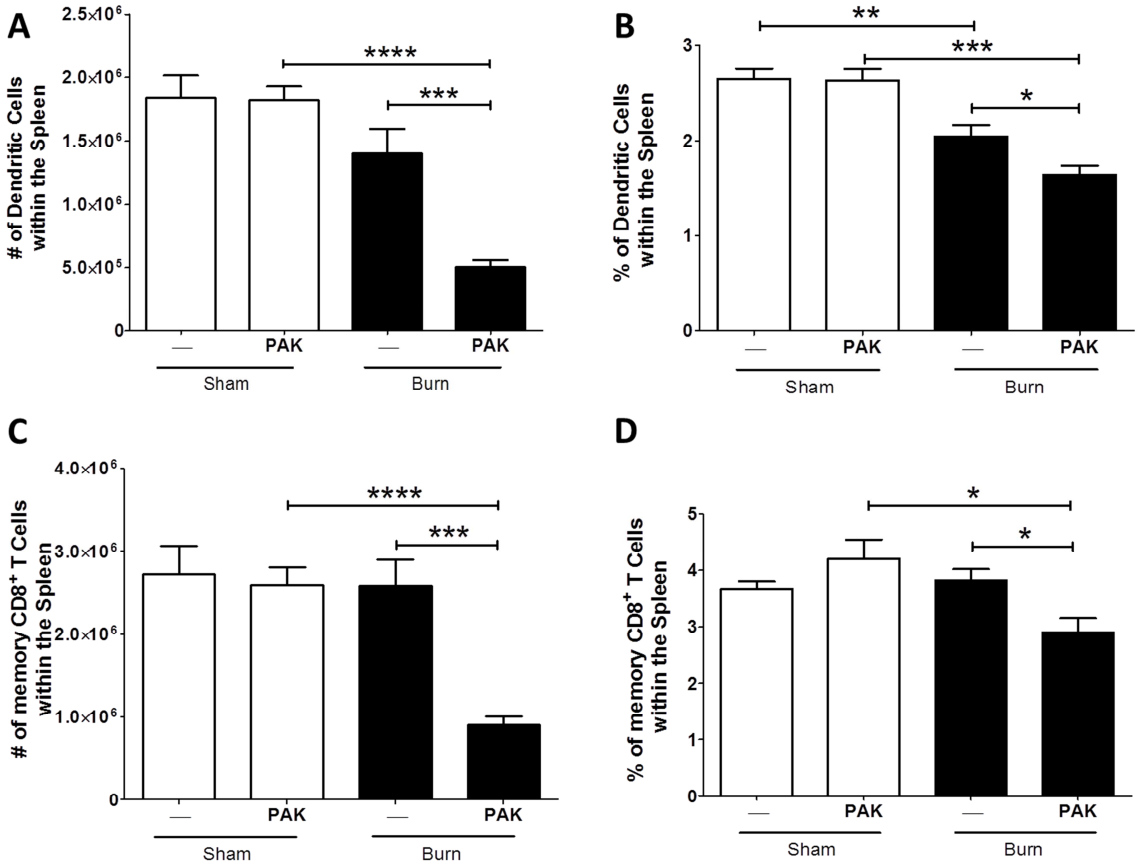

Figure 3.2. Infection following burn injury results in a reduced frequency of splenic dendritic cells and memory CD8$^+$ T cells within the spleen. Splenocytes were harvested from sham (open bars) and burn (solid bars) mice with (PAK) and without (-) *P. aeruginosa* wound inoculation. The absolute number and percentage of (A-B) splenic dendritic cells and (C-D) splenic memory CD8$^+$ T cells was determined using FACS analysis. Data expressed as mean ± SEM. (n = 4, 4, 6, 8) *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$. ****, $p < 0.0001$. These experiments were repeated three times with similar results.

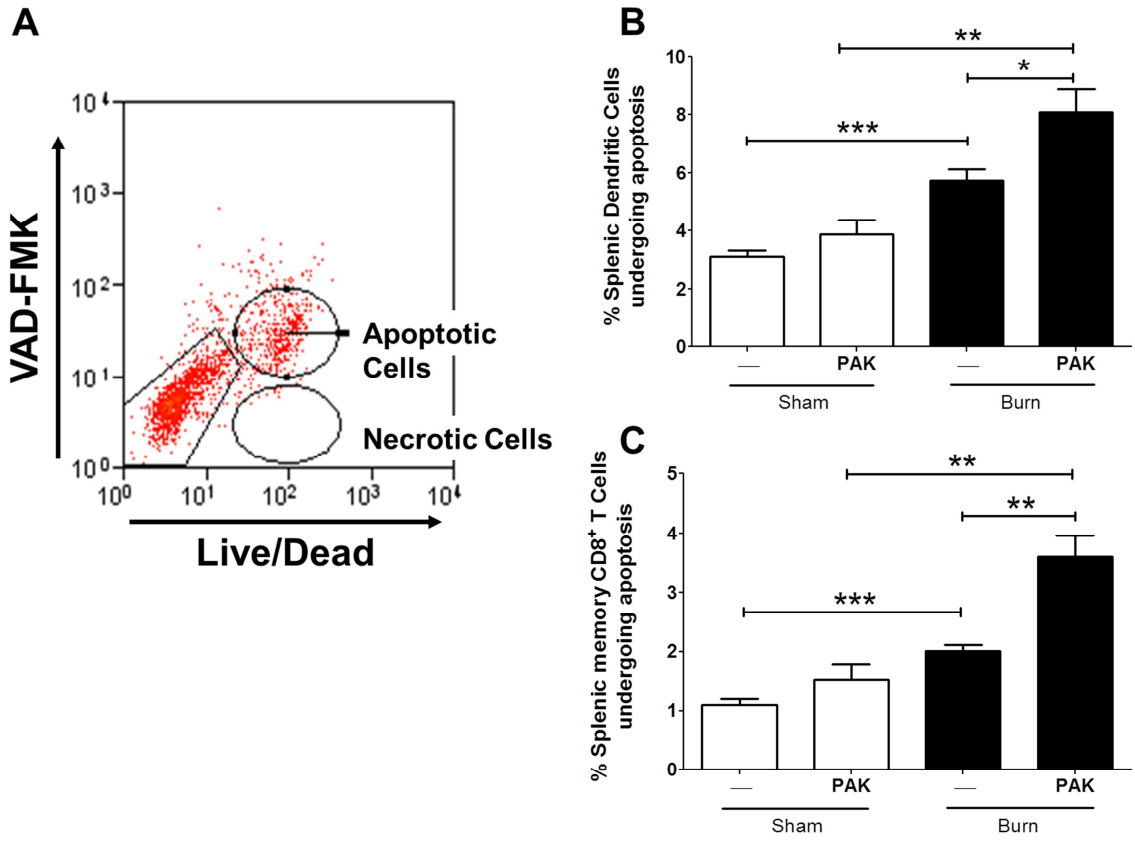

Figure 3.3. Splenic dendritic cells and memory CD8$^+$ T cells preferentially undergo apoptosis after burn injury alone or with infection. A) Shown is a representative histogram of Live/Dead and VAD-FMK staining, which was used to assess cell death and caspase activation. All dead cells were VAD-FMK positive suggesting they had undergone apoptosis rather than necrosis. B) The percentage of apoptotic dendritic cells and C) the percentage of apoptotic memory CD8$^+$ T cells was elevated in sham (open bars) and burn (solid bars) mice with (PAK) and without (-) *P. aeruginosa* wound inoculation. Data expressed as mean ± SEM. (n = 4, 4, 7, 10) *, p≤ 0.05. **, p≤ 0.005. ***, p≤ 0.0005. These experiments were repeated three times with similar results.

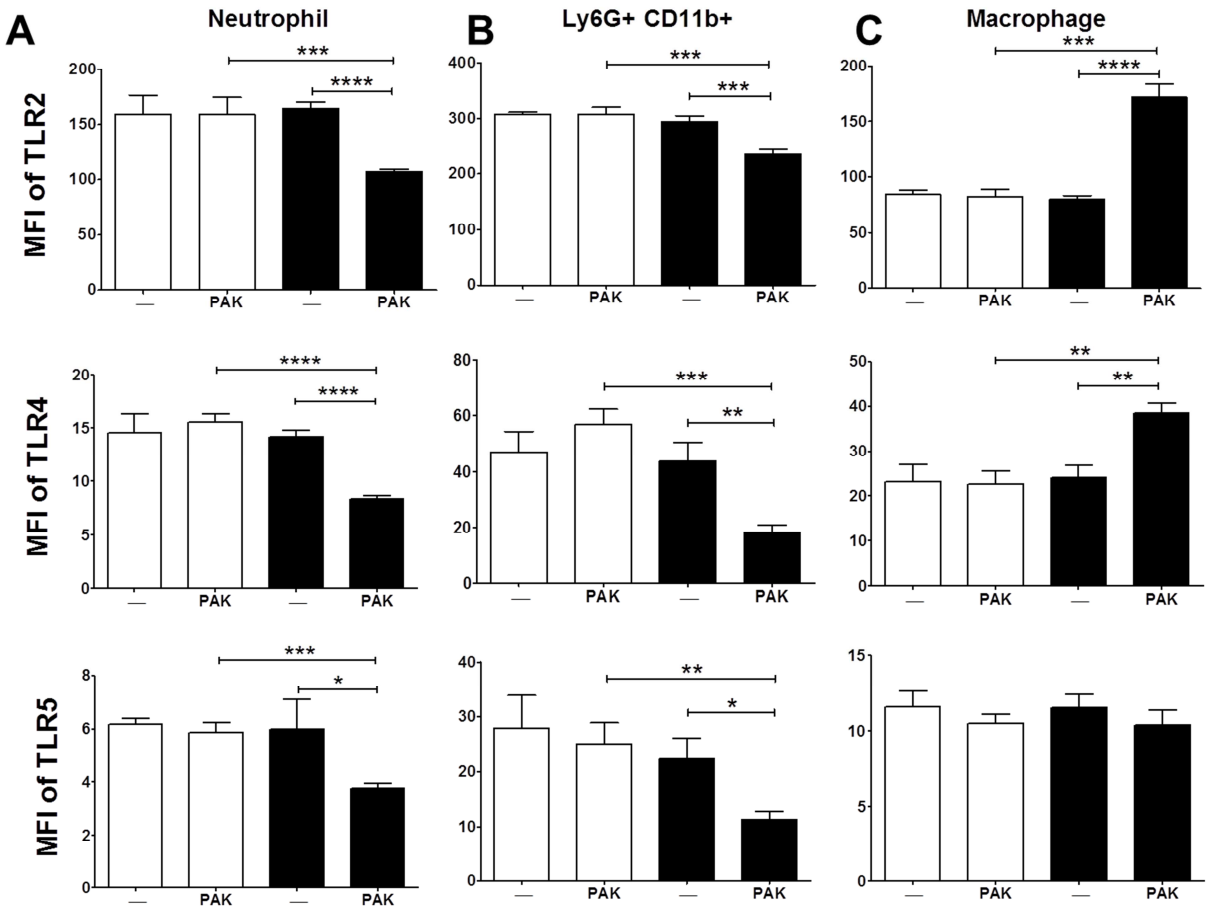

Figure 3.4. TLR expression is decreased on splenic neutrophils and Ly6G$^+$ CD11b$^+$ myeloid cells, but not macrophages, after burn injury with infection. Splenocytes were harvested and mean fluorescence intensity (MFI) of TLR2, TLR4, and TLR5 expression was elevated on splenic A) neutrophils, B) Ly6G$^+$ CD11b$^+$ myeloid cells, and C) macrophages at 3 days post burn (solid bars) or sham (open bars) treatment combined with (PAK) or without (-) *P. aeruginosa* wound inoculation. Data expressed as mean ± SEM. (n = 4-10) *, p≤ 0.05. **, p≤ 0.005. ***, p≤ 0.0005. ****, p< 0.0001. These experiments were repeated three times with similar results.

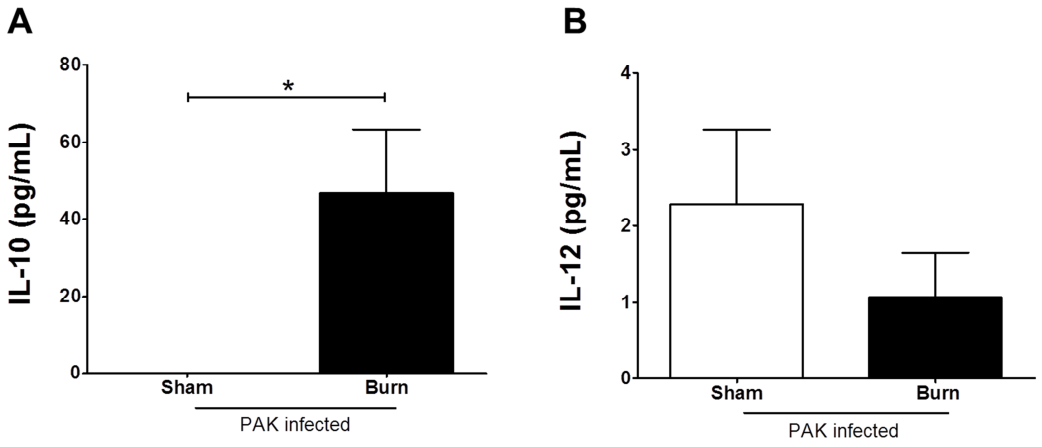

Figure 3.5. Burn mice, but not sham mice, mount a robust serum IL-10 response after *P. aeruginosa* wound inoculation. Twenty-four hours after sham (open bars) or burn (solid bars) treatment, mice were given a subcutaneous injection of wild-type *P.aeruginosa*. Forty-eight hours following infection, serum was collected to determine circulating levels of A) IL-10 and B) IL-12 by cytometric bead array. Data expressed as mean ± SEM. (n =10-15) *, $p \leq 0.05$.

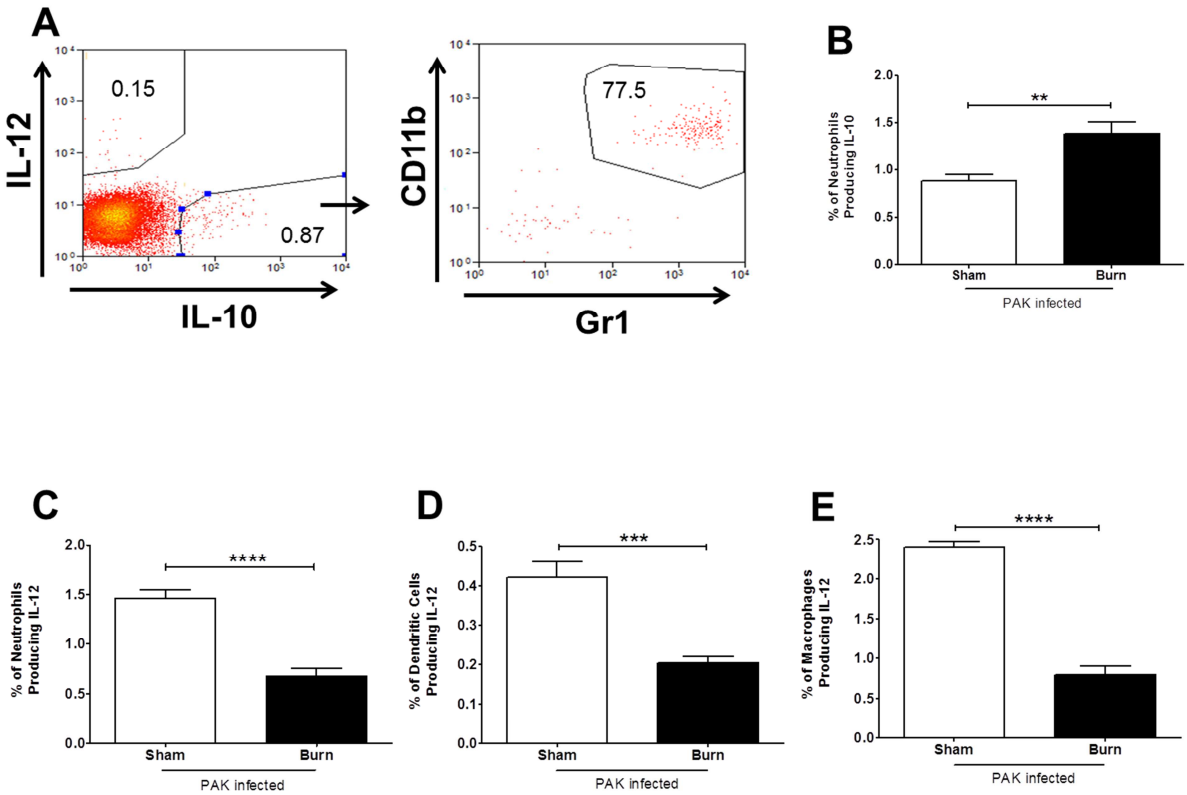

Figure 3.6. Infected burn mice have a higher percentage of IL-10$^+$ neutrophils and a lower percentage of IL-12$^+$ neutrophils, dendritic cells, and macrophages than infected sham mice. A) Splenocytes were harvested at 48 hours post infection and underwent intracellular staining for cytokine analysis without further stimulation *in vitro*. Shown is a representative histogram from an infected burn mouse, which indicates that IL-10 is being produced by Gr1$^+$ CD11b$^+$ cells within the spleen. B-E) Splenocytes were harvested at 48 following infection and underwent CD11b enrichment by magnetic selection. CD11b$^+$ cells were cultured in the presence of LPS and brefeldin-A then were subjected to cell surface and intracellular staining. The percentage of B) neutrophils producing IL-10, as well as the percentage of C) neutrophils, D) dendritic cells, and E) macrophages producing IL-12 were measured for infected sham (open bars) and burn (solid bars) mice. Data expressed as mean ± SEM. (n = 6, 7) **, $p \leq 0.005$. ***, $p \leq 0.0005$. ****, $p < 0.0001$. These experiments were repeated three times with similar results.

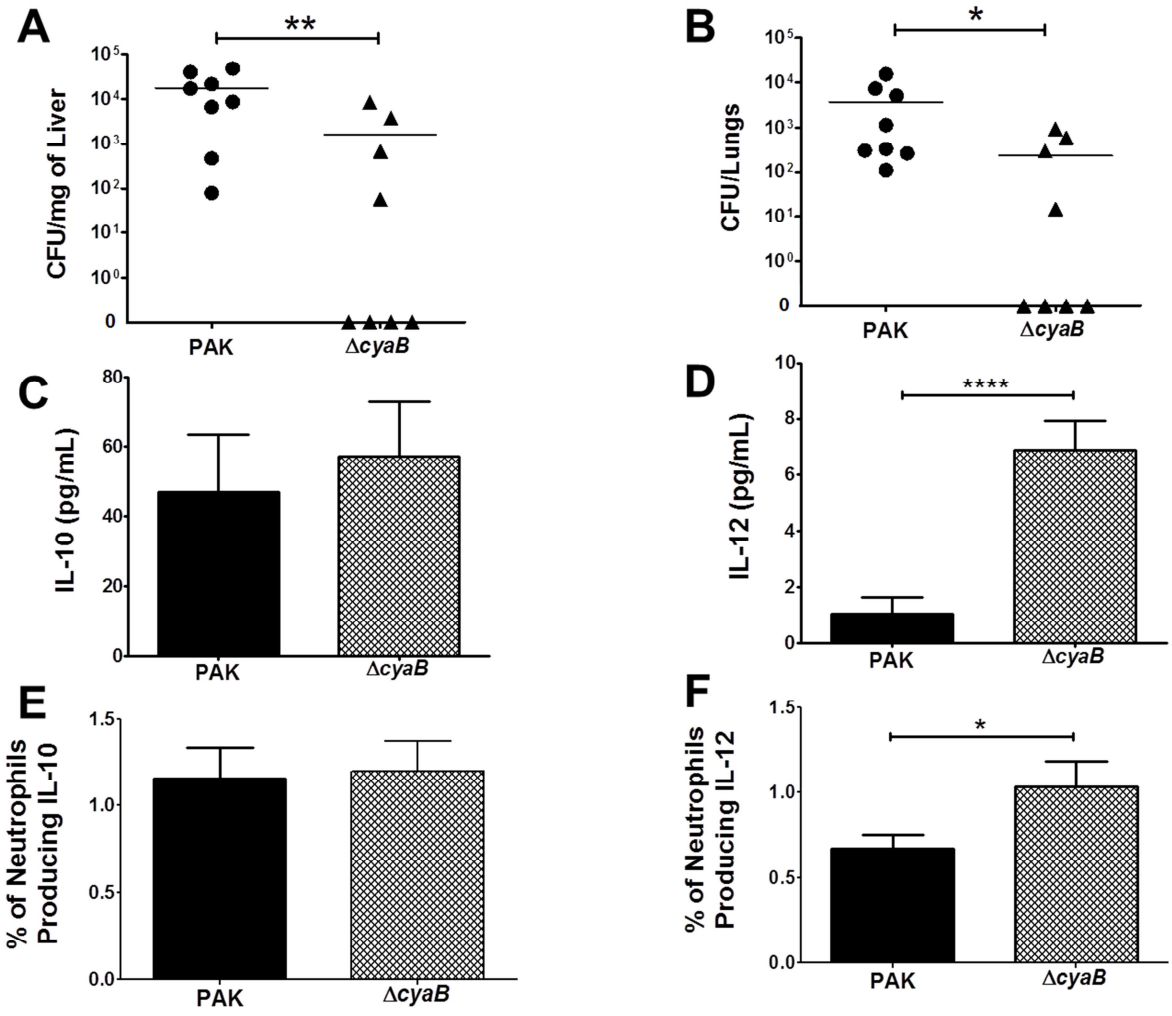

Figure 3.7. Reduced bacterial load at distal organs following wound inoculation with an attenuated *P. aeruginosa* strain (Δ*cyaB*) is associated with an increased serum IL-12 and a pro-inflammatory neutrophil (N1; IL-10$^-$IL-12$^+$) response in burn mice. Forty eight hours following wildtype PAK (circles/solid bars) or Δ*cyaB* (triangles/checkered bars) wound infection, various organs were harvested from burn mice. Bacterial load in A) liver and B) lung samples was determined based on the recovery and enumeration of bacterial CFUs. Serum C) IL-10 and D) IL-12 levels were assessed by cytometric bead array. Also, the percentage of splenic neutrophils producing E) IL-10 and F) IL-12 was determined by flow cytometric analysis. Data expressed as mean ± SEM. (n = 8, 8) *, p≤ 0.05. **, p≤ 0.005. ****, p< 0.0001. These experiments were repeated three times with similar results.

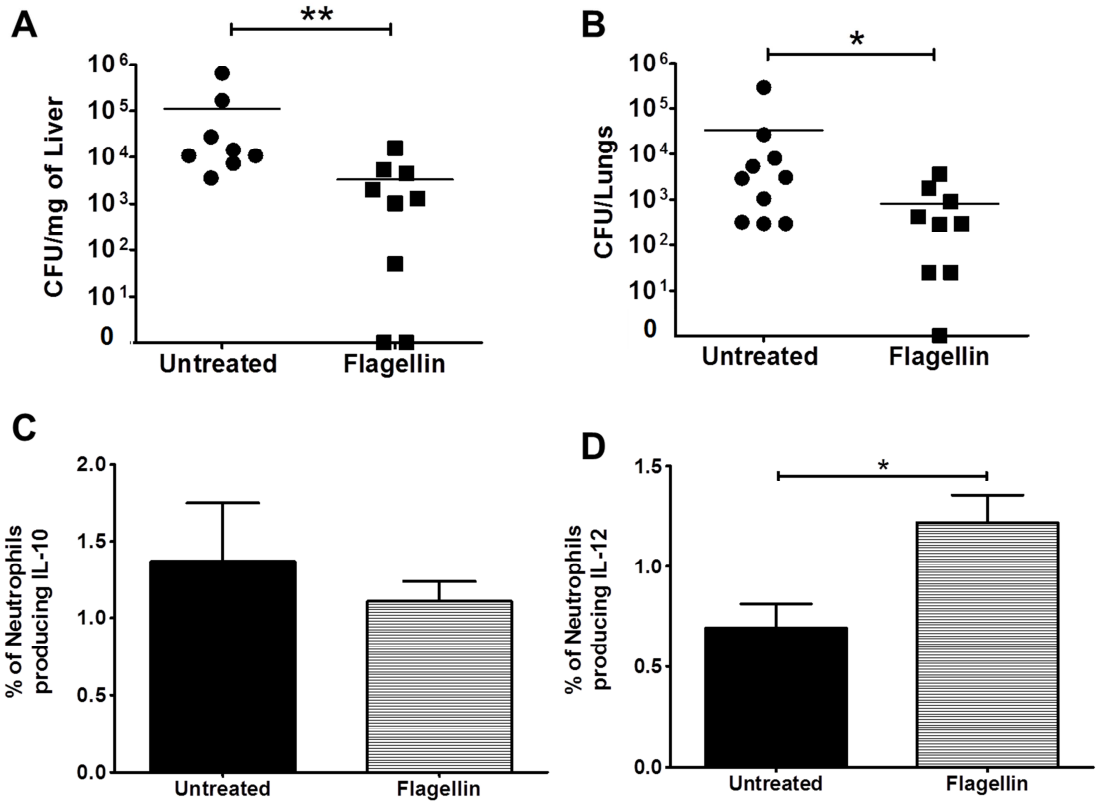

Figure 3.8. Administration of flagellin prior to wound infection with wildtype *P. aeruginosa* (PAK) reduces bacterial load in the periphery and increases the percentage of IL-12 producing neutrophils within the spleen. Two hours prior to wound infection with PAK, burn mice were given an intraperitoneal injection of flagellin (circles/solid bars) or left untreated (squared/striped bars). Forty-eight hours following bacterial challenge, various organs were harvested. Bacterial load in A) liver and B) lung samples was determined by CFU assay. The percentage of splenic neutrophils producing C) IL-10 and D) IL-12 was determined by flow cytometric analysis. Data expressed as mean ± SEM. (n = 8-10) *, $p \leq 0.05$. **, $p \leq 0.005$. These experiments were repeated three times with similar results.

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CHAPTER 4

Burn-induced "innate-priming" enhances clearance of bacterial challenge late after injury

4.1 Summary

Severely burned patients have a high incidence of delayed pulmonary bacterial infection. Using a well-established murine model of thermal injury, we investigated clearance of a clinically relevant pathogen, *P. aeruginosa*, late after burn in an attempt to uncover immune deviations that increase susceptibility in this patient population. Contrary to our hypothesis, we found that late after burn injury mice have enhanced clearance of pulmonary *P. aeruginosa* challenge compared to non-burn controls. This appeared to be due to a burn-induced accumulation of neutrophils within the lungs. Mice are also more resistant to an intraperitoneal challenge of *P. aeruginosa* late after burn. To better mimic the clinical setting wherein patients are repeatedly exposed to bacteria through invasive devises such as central lines, catheters, and ventilators, mice were then given two sequential bacterial infections. The exposure to a second bacterial infection appeared to have no impact on *P. aeruginosa* clearance in burn mice. Collectively, these data show that late after burn injury there is pulmonary inflammation, marked by neutrophil accumulation. This appears to prime burn mice for better control of subsequent bacterial infections; however, this prolonged inflammation may be responsible for other complications such as local tissue damage and decreased pulmonary function.

4.2 Introduction

Severe thermal injury is one of the most devastating physical and psychological injuries a person can suffer. In the United States, approximately 2 million burn injuries occur every year[1]. Of these, over 450,000 individuals require medical treatment and more than 3,500 patients die annually[2]. Burn injury induces a state of immunosuppression leaving patients vulnerable to fatal infections[1, 3]. Advancements in burn care, such as early eschar excision, rapid wound closure, and aggressive antibiotic therapies, have significantly reduced the incidence of local wound infection[4]. However, the incidence at remote organ sites like the lungs has remained unchanged[5, 6].

Pseudomonas aeruginosa, a gram negative extracellular bacterium, is one the most commonly encountered infectious agents observed in burn centers across the United States[4, 6]. Most research on *P. aeruginosa* infection after burn injury has focused on early wound infection, rather than delayed bacterial pneumonia. Due to the emergence of antibiotic-resistant bacterial strains[7], it is important to understand the immune response to pulmonary infection after burn injury in order to improve treatment. Furthermore, the immune response following burn injury is dynamic and changes over time[8, 9] so understanding the immune response to infection late (days to weeks) after burn is also crucial.

In this study, we investigated clearance of *P. aeruginosa* late after burn using a murine model of thermal injury. Although we hypothesized that burn mice would have an increased susceptibility to infection compared to sham controls, they exhibited enhanced bacterial clearance which correlated with a burn-induced accumulation of neutrophils within the lungs. Mice were also more resistant to an intraperitoneal challenge of *P. aeruginosa* late after burn. However, the mechanism of enhanced bacterial clearance in the periphery is

under investigation. To better mimic the clinical setting wherein patients are repeatedly exposed to bacteria through invasive devises such as central lines, catheters, and ventilators, mice were then given two sequential bacterial infections. The exposure to a second bacterial infection appeared to have no impact on *P. aeruginosa* clearance in burn mice, whereas sham mice displayed greater clearance after the second bacterial challenge. Collectively, these data show that late after burn injury there is pulmonary inflammation, marked by neutrophil accumulation. This appears to prime burn mice for better control of bacterial infections; however, this prolonged inflammation may be responsible for other complications such as local tissue damage and decreased pulmonary function.

4.3 Methods and materials

Animals

Wildtype C57BL/6 (B6) mice were purchased from Taconic Farms (Hudson, NY). All mice used in the study were maintained under specific pathogen-free conditions in the Animal Association of Laboratory Animal Care-accredited University of North Carolina Department of Laboratory Animal Medicine Facilities. All protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines.

Mouse burn injury

Eight to 12 week old (>18 grams), female B6 mice were used for all experiments. Animals were anesthetized by inhalation of vaporized isoflurane (Baxter Healthcare,

Deerfield, IL) and had their dorsal and flank hair clipped. A subcutaneous injection of morphine (3mg/kg body weight; Baxter Healthcare) was given prior to burn injury for pain control, and an intraperitoneal injection of lactated Ringer's solution (0.1mL/g body weight; Hospira, Lake Forest, IL) was given immediately after burn injury for fluid resuscitation. To create a full-contact burn of approximately 20% total body surface area (TBSA), a 65-g copper rod (1.9 cm in diameter), heated to 100°C was used. Four applications of the rod, each for 10 seconds, to the animal's dorsal/flank produced the wound. Previous studies analyzing skin biopsies of the burn wound have demonstrated full-thickness cutaneous burn with visible unburned muscle beneath when following this procedure. Animals were returned to individual cages, provided food and morphinated water (0.4mg/mL; Roxane Laboratories, Columbus, OH) *ad libitum*, and monitored twice a day. Sham controls with 0% TBSA underwent all described interventions except for the actual burn injury. There was negligible mortality (<1%) after burn injury alone.

Bacterial strains and preparation

Wildtype *P. aeruginosa* strain PAK and an isogenic strain containing a plasmid that constitutively expresses green fluorescent protein (PAK-GFP) were obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC). Bacteria were grown from frozen stock at 37°C overnight in Luria-Bertani (LB) broth then transferred to fresh medium and grown for an additional 2 hours until they reached mid-log growth phase. Cultures were centrifuged at 12,000 x g for 30 seconds, and the pellet washed with 1mL of 1 % protease peptone in phosphate buffered saline (PBS (+1%PP)). Following a second wash, the bacterial concentration was determined by assessing optical density at 600nm. After diluting the

bacteria to obtain the desired concentration, the inoculum was verified by plating serial 10-fold dilutions on LB agar plates. Wildtype PAK was grown on LB plates containing 25ug/mL of irgasan (Sigma-Aldrich, St. Louis, MO) while PAK-GFP was grown on LB plates containing irgasan and 150ug/mL of carbenicillin (American Bioanalytical, Natick, MA)). Colony forming units (CFUs) were enumerated after overnight incubation at 37°C.

Animal infections

For intratracheal (IT) infections, mice were anesthetized by intraperitoneal injection of Avertin® (0.475mg/g body weight; Sigma-Aldrich), and 10^6 CFUs/50uL of wildtype PAK was administered using a MicroSprayer® Aerosolizer – Model IA-1C (Penn-Century, Wyndmoor, PA) at 14 days post burn or sham procedure (14d). For intraperitoneal (IP) infections, mice were given an intraperitoneal injection of 10^5 CFUs/1mL of wildtype PAK at 14d. In experiments containing sequential infections, mice were given an IT infection with wildtype PAK at 14d then given an IP infection of PAK-GFP three days later (17d).

Determination of* P. aeruginosa *Organ Burden

Twenty-four hours following infection the spleen, the left lobe of the liver, and the lungs were aseptically removed. Spleen and liver samples were placed directly into 1.5mL safe lock tubes, while the lungs were finely minced in petri dishes using sterile razor blades. Each lung sample was divided in half where one portion was placed in a pre-weighed 1.5mL safe lock tube and reweighed while the rest was used for collagenase digestion. Weights were used to calculate the proportion of lung in each sample. Tissues were homogenized in 0.5mL of LB broth using 3.2mm stainless steel beads and a BulletBlender (Next Advance, Averill

Park, NY). Serial dilutions of tissue homogenates were plated on LB agar containing irgasan (*P. aeruginosa* selective media) without or with carbenicillin (selective media for PAK-GFP strain). CFUs were enumerated after incubation overnight at 37°C.

Collagenase digestion of lungs

For each lung sample, the remaining portion of the minced tissue was placed in a pre-weighed tube containing 5mL of PBS with 10% fetal bovine serum (PBS+10%FBS). Tubes were reweighed to determine the proportion of lung in each sample, and then 300U/mL of *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Lakewood, NJ) and 50U/mL of DNAse I (Worthington Biochemical) were added. The samples were incubated on a roller drum (New Brunswick Scientific, Enfield, CT) for 1 hour at 37°C. Digested lungs were filtered through 100uM nylon mesh strainers, pelleted, and resuspended in Ack lysis buffer to remove red blood cells. Following a wash step, cell concentration was determined using a hemocytometer.

Flow cytometric analysis

Collagenase-digested lung samples were incubated with anti-mouse CD16/32 (BD Biosciences, San Jose, CA) at a concentration of 1ug per million cells for 5 min at 4°C to block Fc receptors. The panel of mAbs used for flow cytometric analyses were anti-Gr1 (RD-8C5), anti-SiglecF (E50-2440), anti-CD11b (M1/70), anti-MHCII (M5/114.15.2), and anti-F4/80 (BM8) which were purchased from BD Biosciences, eBiosciences, and BioLegend (San Diego, CA). Data were collected on a Dako CyAN and analyzed using Summit software (Dako, Carpinteria, CA).

Neutrophil oxidative burst assay

Following cell surface staining, 35uM of dihydrorhodamine (DHR; Invitrogen, Grand Island, NY) was added to lung samples. Each sample was then divided into two equal portions where one half was unstimulated and the other stimulated with 98nM of phorbol-12-myristate-13 acetate (PMA). After 30 minutes, cells were fixed with a 4% paraformaldehyde solution. DHR oxidation to rhodamine was detected by a flow cytometer and reported as mean fluorescence intensity (MFI). The neutrophil oxidative index (NOI) was then calculated as the ratio of stimulated MFI versus unstimulated MFI.

Statistical analysis

Data were analyzed using Student's t test for differences in CFU recovery and cell staining; log-rank analysis was used to test differences in mouse survival. GraphPad Prism version 5 was used for the analyses (La Jolla, CA). Statistical significance was defined as $p \leq 0.05$ unless indicated otherwise.

4.4 Results

Enhanced clearance of pulmonary *P. aeruginosa* challenge late after thermal injury

We hypothesized that late after burn injury there is an increased susceptibility to pulmonary bacterial infection. To test this, mice were given an intratracheal (IT) injection with 10^6 CFUs of wildtype *P. aeruginosa* at fourteen days after burn or sham treatment. Following pulmonary challenge, burn and sham mice had a 100% survival (Figure 4.1A).

Therefore, we assessed bacterial load at 24 hours post infection to determine if there was a difference in *P. aeruginosa* clearance. Contrary to our hypothesis, burn mice had significantly less bacteria recovered from their lung samples than sham mice (Figure 4.1B). In addition, bacterial dissemination to distal organs, such as the spleen and liver, was observed less frequently in burn mice than sham mice (Figure 4.1C and data not shown). These data suggest that burn mice have better control of pulmonary *P. aeruginosa* infection later after burn injury than sham controls.

Increased frequency of pulmonary neutrophils late after burn

Neutrophils play an essential role in the clearance of *P. aeruginosa* during acute pulmonary infection, as demonstrated by the extreme susceptibility of neutropenic mice to this pathogen[10]. Therefore, we evaluated neutrophil responses following burn injury and/or pulmonary challenge. Flow cytometric analysis was used to quantify neutrophils within collagenase-digested lung samples (representative gating scheme, Figure 4.2). Fourteen days after burn, there was a 4-fold increase in the percentage and absolute number of neutrophils within the lungs compared to sham controls (Figure 4.3A and 4.3B). Infection of sham mice resulted in a significant increase in neutrophil migration into the lungs resulting in a similar frequency observed with burn injury alone (Figure 4.3A and 4.3B). This suggests that late after burn injury there is an accumulation of neutrophils within the lungs which leads to enhanced clearance of *P. aeruginosa* infection upon pulmonary challenge.

Neutrophil oxidative burst was not affected late after burn injury

Next we evaluated neutrophil antimicrobial activity by measuring their oxidative burst potential using a dihydrorhodamine flow cytometry based assay. At 14 days after burn injury, there was not change in the neutrophil oxidative index (NOI). Furthermore, pulmonary infection led to a significant increase in the NOI of both burn and sham mice (Figure 4.4). Although infected burn mice had a higher NOI than infected sham mice, the difference was no statistically significant (p=0.0736, Figure 4.4). These data suggest that augmented neutrophil migration, but not function, impacts clearance of pulmonary *P. aeruginosa* infection late after burn injury.

Mice were less susceptible to *P. aeruginosa* intraperitoneal challenge later after burn injury

To determine if enhanced bacterial clearance late after burn injury occurs with other routes of infection, mice were given an intraperitoneal (IP) injection with 10^5 CFUs of wildtype *P. aeruginosa* at fourteen days following burn or sham procedure. With this dose of bacteria, burn mice had approximately a 20% increase in overall survival compared to sham mice (Figure 4.5). Furthermore, burn mice had significantly less bacterial recovered from their liver and lung samples at 24 hours following infection than sham mice (Figure 4.6A and 4.6B). Together, these data imply that burn mice are more resistant to a single *P. aeruginosa* IT or IP infection than sham mice.

Enhanced bacterial clearance observed late after burn injury is maintained during a secondary bacterial infection

While delayed bacterial infections are a commonly observed in severely burned individuals, we found that late after burn injury mice had enhanced clearance of *P. aeruginosa* regardless of the route of infection. Since burn patients are repeatedly exposed to bacteria during their hospital stay, we hypothesized that burn mice would be more susceptible to a second bacterial challenge due to neutrophil exhaustion. To test this hypothesis, mice were given an IT injection with 10^6 CFUs of wildtype *P. aeruginosa* at fourteen days after burn or sham procedure then an IP infection with 10^5 CFUs of PAK-GFP three days later (Figure 4.7). We found that survival of burn mice following IP challenge was not impacted by pre-exposure to an IT infection (Figure 4.8A). However, sham mice had a notable improvement in survival following IP infection when they were pre-exposed to an IT infection (Figure 4.8B). To determine if the increase in sham survival was due to an increase in bacterial clearance, bacterial loads were assessed 24 hours after the second infection. Sham and burn mice had comparable bacterial burdens in their liver and lungs tissues (Figure 4.9A and 4.9B). Also, these bacterial loads were similar to those observed in burn mice that received just a single IP infection (Figure 4.6A and 4.6B). These data suggest that in sham mice the first bacterial infection leads to a "primed" neutrophil response upon a second bacterial challenge. In burn mice, the burn itself leads to a "primed" neutrophil response resulting in enhanced clearance of a single bacterial infection. This burn priming produces a maximal neutrophil response which is not further amplified nor exhausted by a second bacterial infection.

4.5 Discussion

Since severely burned patients have a high incidence of delayed pulmonary infection, we hypothesized that burn mice would also have an increased susceptibility compared to sham controls. Conversely, we found that late after burn injury there is an accumulation of neutrophils within the lungs which primes mice for enhanced bacterial clearance upon pulmonary challenge. Mice are also more resistant to an intraperitoneal challenge of *P. aeruginosa* late after burn, and the role of neutrophils in enhanced bacterial clearance in the periphery is currently being investigated. To better mimic the clinical setting wherein patients are repeatedly exposed to bacteria through invasive devises such as central lines, catheters, and ventilators, mice were then given two sequential bacterial infections. The repeated bacterial challenge did not impact *P. aeruginosa* clearance in burn mice suggesting that neutrophils are not functionally exhausted in our model system. Although there are still many aspects of neutrophil migration and function that still need further research, these data indicate that neutrophils contribute to pulmonary inflammation late after burn injury which renders burn mice more resistant to delayed bacterial infection.

While neutrophils are an important component of the inflammatory response, inappropriate and excessive neutrophil recruitment into the lungs can cause localized tissue damage. In Acute Lung Injury (ALI), neutrophils are sequestered in the pulmonary capillaries causing permeability changes and edema formation[11, 12]. Furthermore, the neutrophils that infiltrate the lungs and migrate into the airways express pro-inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α. This neutrophil-

mediated inflammation is believed to contribute to oxidant-induced injury and loss of the epithelium[13, 14]. Neutrophils have also been shown to aggravate lung injury during mechanical ventilation[15, 16]. Therefore, the increased pulmonary neutrophil infiltration observed late after burn injury could be contributing to localized tissue damage and/or impaired lung function and should be investigated further.

The mechanism responsible for neutrophil accumulation in the lungs late after burn injury is currently unknown. Monocyte chemoattractant protein 1 (MCP-1), a chemokine that regulates both monocyte and neutrophil migration, is significantly elevated in the serum after burn injury[17]. IL-17, another cytokine that regulates neutrophil migration, is also elevated in the serum after burn injury[18, 19]. It was found that bronchoalveolar lavage (BAL) cells collected at 7 days after burn injury have increased MCP-1 and IL-17 production following TLR2 or TLR4 stimulation compared to sham controls[20]. This suggests that MCP-1 and/or IL-17 could be playing a role in the influx of neutrophils into the lungs late after burn.

Infectious complications are the predominant cause of death for thermally injured patients. However, we found that mice are more resistant to *P. aeruginosa* infection late after burn injury. There are many potential reasons for this discrepancy. For example, one third of all severely burned patients have inhalation injury[22]. Smoke inhalation significantly damages normal respiratory physiology causing patients to require mechanical ventilation[23]. During intubation, bacteria can easily pass to lower airways, and in patients with both severe burn injury (systemic immune dysfunction) and inhalation injury (local immunosuppression[24] and tissue injury[22]) this creates an ideal environment for development of ventilator-associated pneumonia (VAP). Studies have also shown that the pneumonia rate is two times higher in

burn patients with inhalation injury as opposed to burn injury alone[25]. Our laboratory is working to develop a murine model of inhalation injury to better understand the interplay of these two insults and their impact on infection.

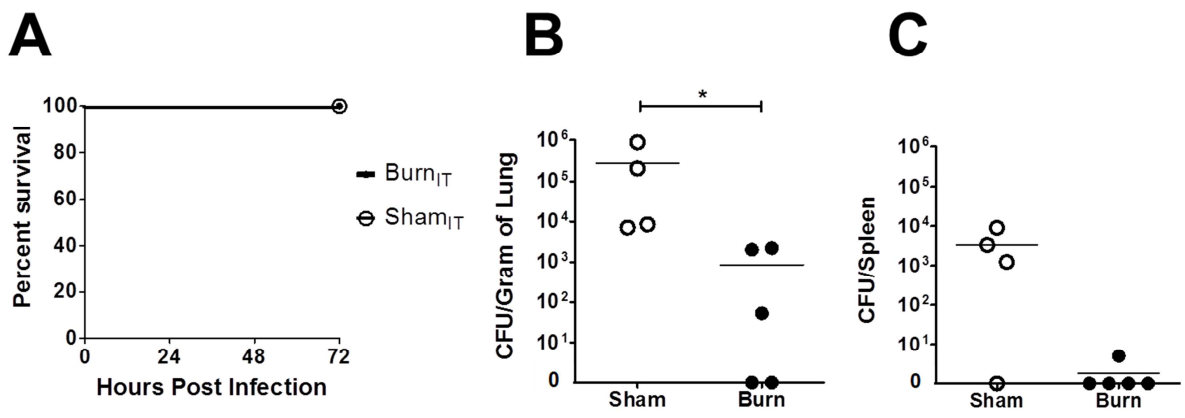

Figure 4.1. Late after burn injury mice have increased bacterial clearance following pulmonary *P. aeruginosa* challenge. At 14 days after burn or sham treatment, mice were given an intratracheal (IT) injection of 10^6 CFUs of *P. aeruginosa*. A) Survival was monitored for 72 hours post infection (hpi). B-C) Bacterial load was assessed in various organs at 24hpi in sham (open circles) and burn (closed circles) mice. (n = 4-5 per group) *, $p \leq 0.05$. These experiments were repeated two times with similar results.

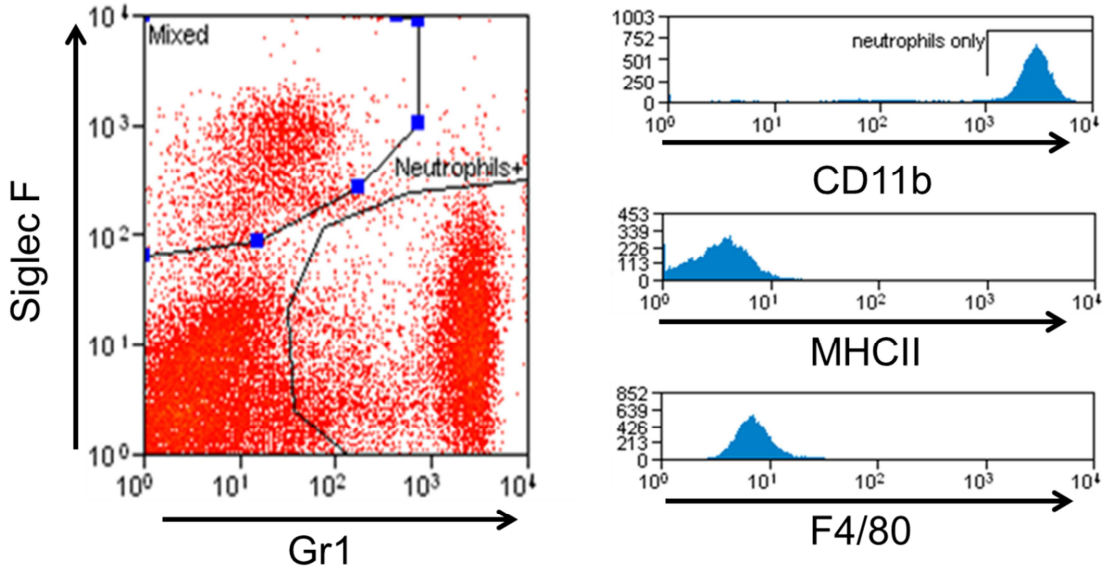

Figure 4.2. Neutrophils are detected in collagenase-digested lung samples using flow cytometric analysis. Cells that were $Gr1^{hi}$ $SiglecF^{-/lo}$ and $CD11b^{hi}$ were defined as neutrophils. These cells were also $MHCII^{-}$ and $F4/80^{-}$. Shown is a representative histogram from an infected burn mouse.

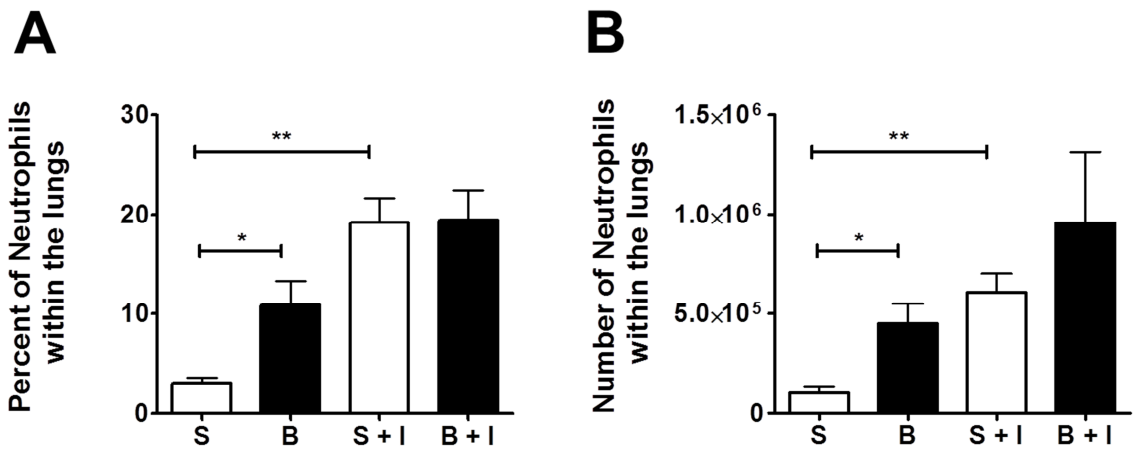

Figure 4.3. Late after burn injury, there is a significant increase in the percent and absolute number of neutrophils within the lungs. Fourteen days after burn ("B") or sham ("S") treatment, mice were given an intratracheal injection of *P. aeruginosa* (" + I"). Twenty-four hours later, cells were harvested from lungs. A) The percentage and B) the absolute number of neutrophils was determined using FACS analysis. Data expressed as mean ± SEM. (n = 3-5) *, $p \leq 0.05$. **, $p \leq 0.005$. These experiments were repeated with similar results.

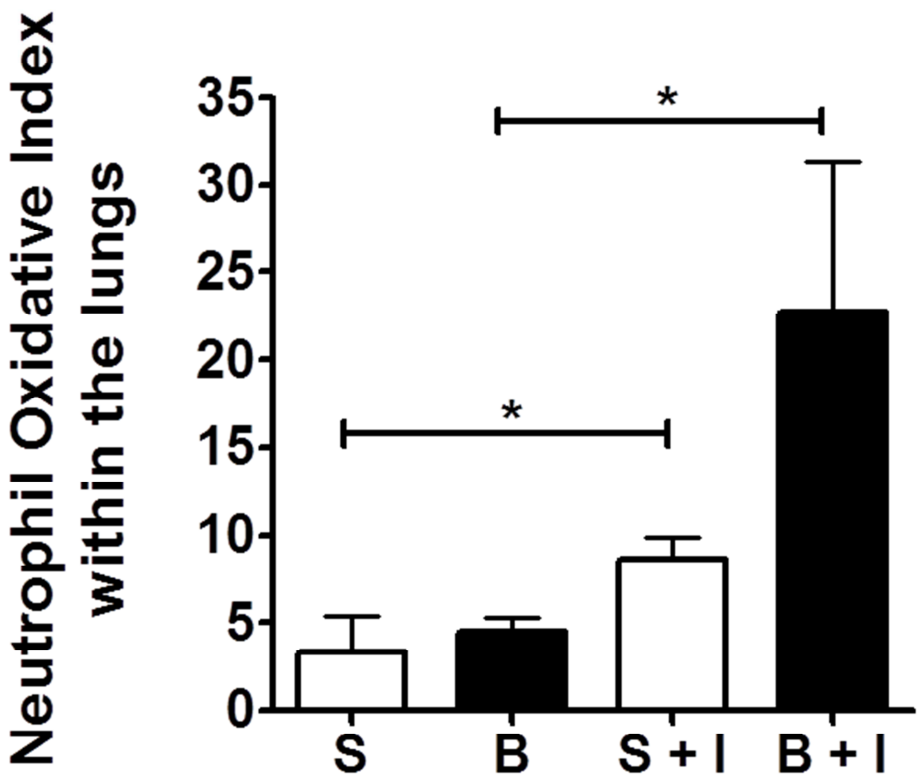

Figure 4.4. The ability of neutrophils to undergo oxidative burst is not impaired late after burn injury. Fourteen days after burn ("B") or sham ("S") treatment, mice were given an intratracheal injection of *P. aeruginosa* (" + I"). Twenty-four hours later, cells were harvested from lungs. Neutrophil oxidative burst potential was measured using a dihydrorhodamine (DHR) flow cytometry based assay. DHR oxidation to rhodamine was reported as mean fluorescence intensity (MFI).The neutrophil oxidative index (NOI) was calculated as the ratio of stimulated MFI versus unstimulated MFI. Data expressed as mean ± SEM. (n = 3-5) *, $p \leq 0.05$. These experiments were repeated with similar results.

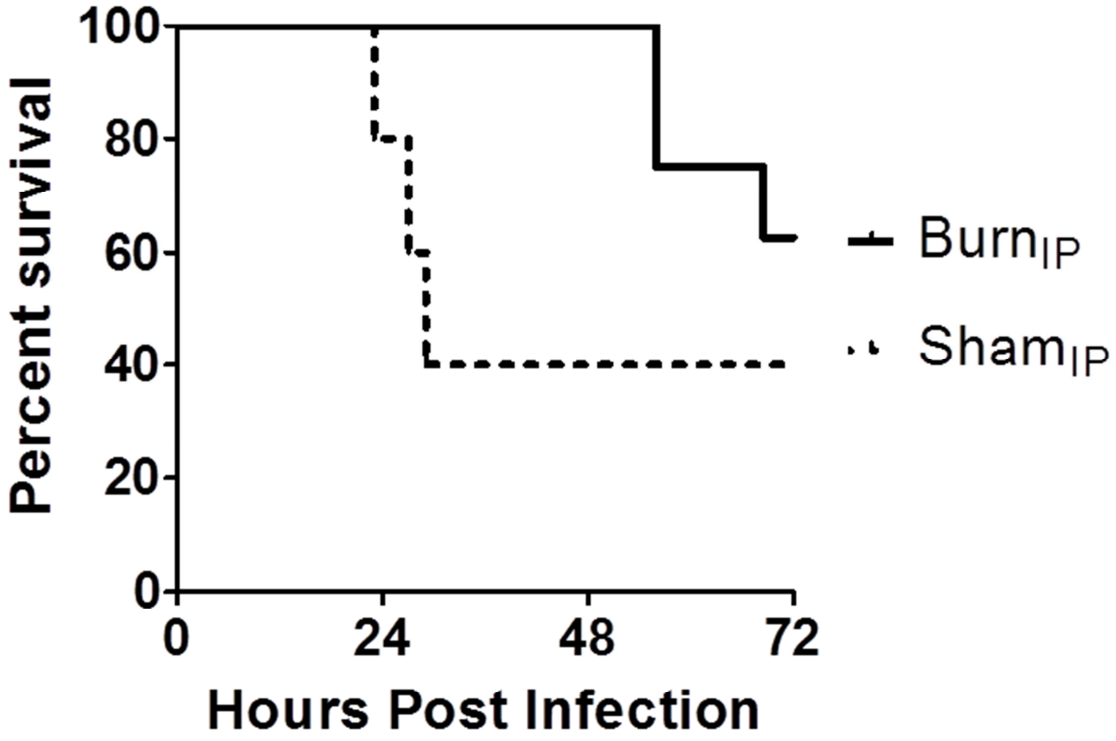

Figure 4.5. Late after burn injury mice have increased survival following intraperitoneal *P. aeruginosa* challenge. Mice were given an intraperitoneal (IP) injection of 10^5 CFUs of *P. aeruginosa* at 14 days after burn (solid line) or sham (dashed line) procedure. Survival was monitored for 72 hours post infection (hpi). (n = 5-8 per group) These experiments were repeated with similar results.

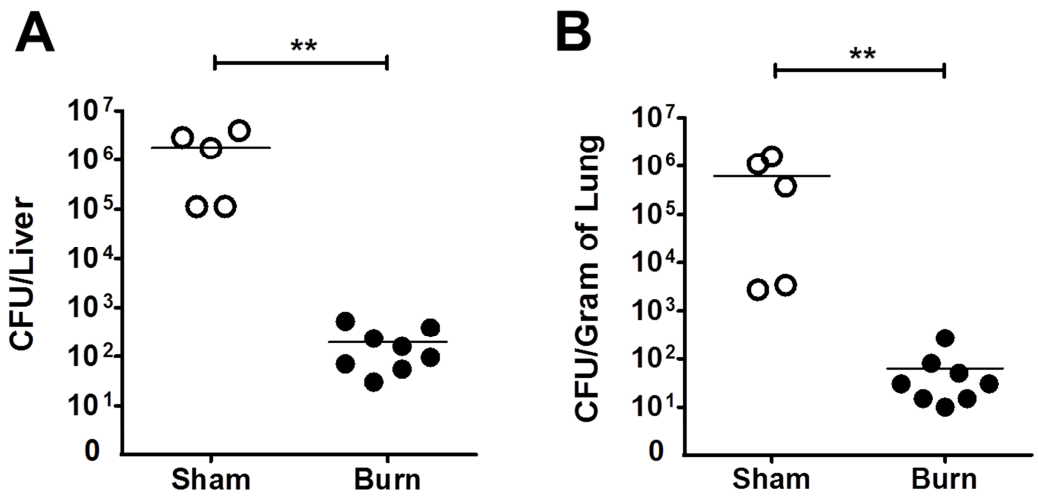

Figure 4.6. Late after burn injury mice have increased bacterial clearance following intraperitoneal *P. aeruginosa* challenge. Fourteen days after burn (closed circles) or sham (open circles) treatment, mice were given an intraperitoneal (IP) injection of 10^5 CFUs of *P. aeruginosa*. Twenty-four hours later, bacterial load in A) liver and B) lung samples was determined based on the recovery and enumeration of bacterial CFUs. (n = 5-8 per group) **, $p \leq 0.005$. These experiments were repeated two times with similar results.

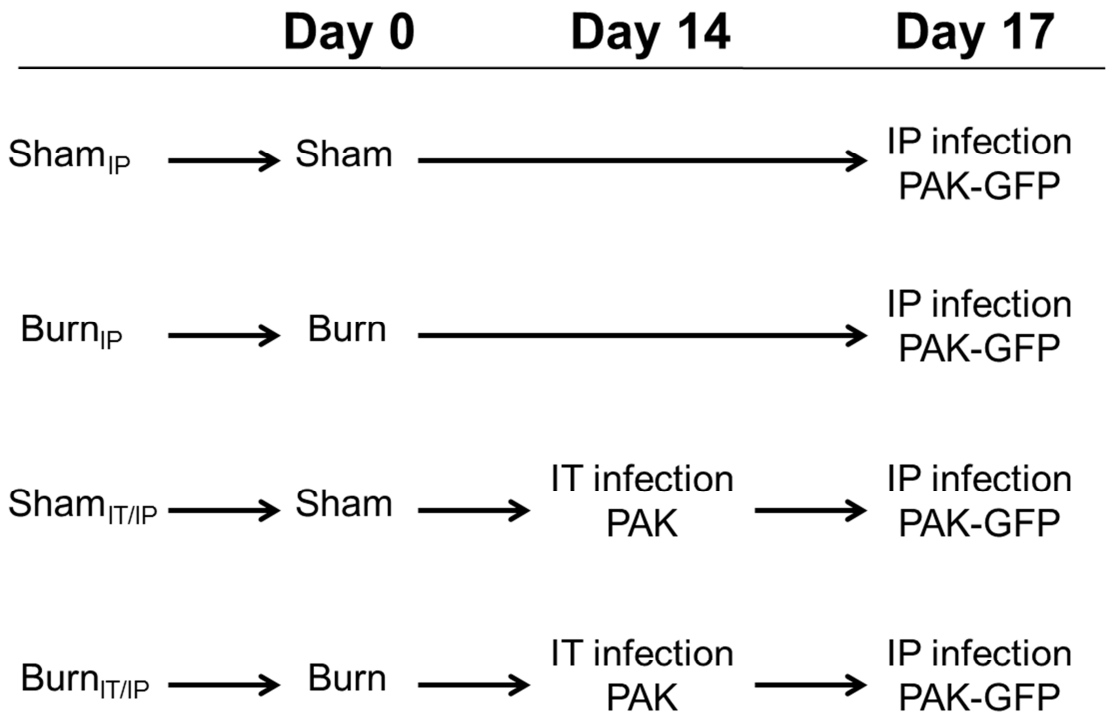

Figure 4.7. Flowchart of time and route of infection following burn or sham treatment. Mice underwent a burn or sham procedure. Fourteen days later, a cohort of mice was given an intratracheal (IT) injection of 10^6 CFUs of wildtype *P. aeruginosa* strain PAK. Three days later, all mice received an intraperitoneal (IP) injection of 10^5 CFUs of an isogenic strain containing a plasmid that constitutively expresses green fluorescent protein (PAK-GFP).

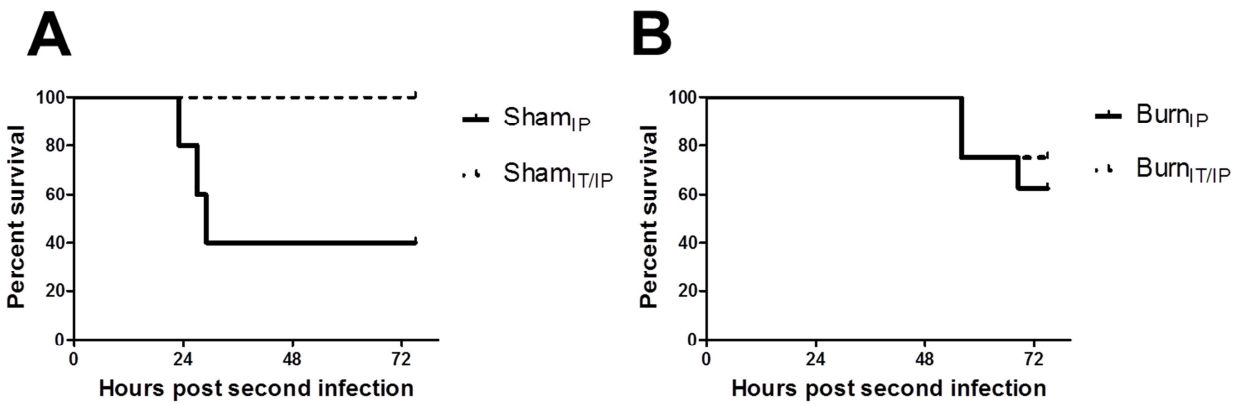

Figure 4.8. Survival of sham mice, but not burn mice, following intraperitoneal challenge is impacted by pre-exposure to an intratracheal infection. A cohort of mice was given an intraperitoneal (IT) injection with 10^6 CFUs of wildtype *P. aeruginosa* at fourteen days after A) sham or B) burn procedure. Three days later, all mice received an intraperitoneal (IP) infection with 10^5 CFUs of PAK-GFP. Survival was monitored for 72 hours post IP infection. (n=5, 8 per group) These experiments were repeated with similar results.

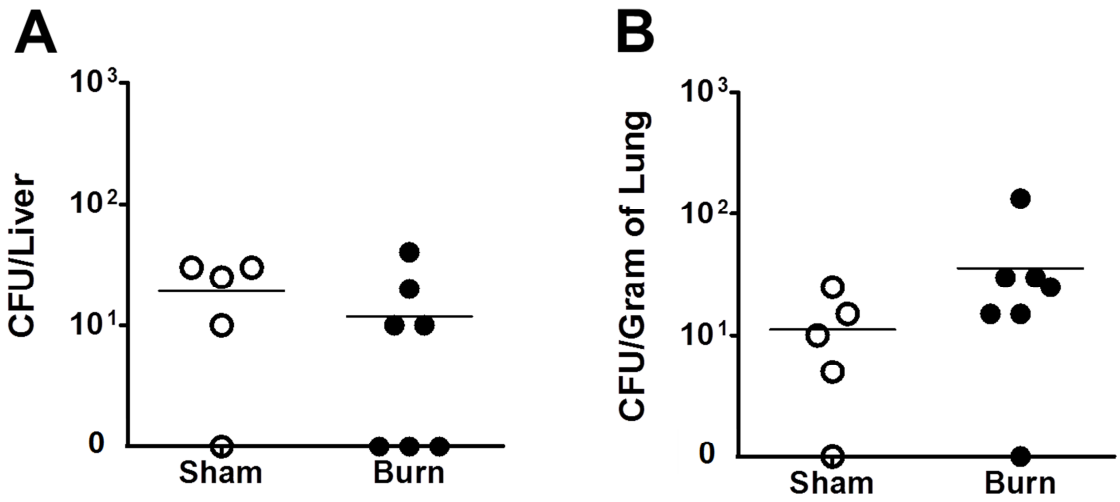

Figure 4.9. Sham and burn mice had comparable bacterial burdens following sequential *P.aeruginosa* challenge. Mice were given an intraperitoneal (IT) injection at 14 days following sham (open circles) or burn (closed circles) procedure then an intraperitoneal (IP) infection three days later. Twenty-four hours after the IP infection, various organs were harvested and bacterial load in A) liver and B) lung samples was determined based on the recovery and enumeration of bacterial CFUs. (n=5-7) These experiments were repeated with similar results.

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CHAPTER 5

Conclusions and Future Directions

5.1 A clinical need

The immune system protects the body against infection and disease. Severe burn injury induces profound immune dysfunction rendering patients extremely susceptible to infection. Although progress has been made in reducing the incidence of infection through wound debridement and aggressive use of antibiotics[1], burn wound infection and pulmonary sepsis still are major causes of mortality. Therefore, in order to improve patient outcome it is important to understand the immune response to infection after burn injury. Furthermore, the immune response following burn injury is dynamic and changes over time[2] thus understanding the immune response both early and late after burn is also crucial.

5.2 Characterization of $CD4^+$ T cell response after burn injury

We and others have defined $CD4^+$ T cell population changes both early and late after burn (Figure 5.1). More specifically, early (hours to days) after burn injury $CD4^+$ T cells begin to produce the pro-inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-2 upon stimulation[3,4], which are defining characteristics of Th1 cells. At the same time,

macrophages produce tumor necrosis factor (TNF)-α, IL-1 and IL-6 as well as other pro-inflammatory cytokines[5]. Transforming growth factor (TGF)-β is immediately detected in the wound, and serum levels progressively increase beginning at day 3[6-9]. This early pro-inflammatory phase is followed by a switch to an anti-inflammatory response characterized by over-production of TGF-β[6,8], as well as a switch in $CD4^+$ T cell responses from a Th1 to a Th2 (IL-4) phenotype [10].

A newly discovered subset of $CD4^+$ T cells, called Th17 cells, have been shown to secrete IL-17 but not IFN-γ or IL-4 [11-13]. Differentiation of naïve $CD4^+$ T cells towards a Th17 phenotype requires the presence of TGF-β and IL-6[14-16]. Since elevated TGF-β and IL-6 levels are found following bury injury, we hypothesized that Th17 cells are generated after burn in a murine model of thermal injury. We detected Th17 cells in wound draining lymph nodes at 3, 7, and 14 days after burn injury, as well as in the spleen at day 14. Nevertheless, the Th17/Th1 balance was significantly shifted towards a Th1 response at 3 days post burn. This shift was lost at day 7, but at day 14 there was a clear skewing of the response to a Th17 phenotype in burn mice compared to sham. These findings demonstrate bimodal skewing of Th17 lymphocytes following burn injury.

5.3 Future directions: defining the role of Th17 cells after burn injury

Our laboratory was the first to characterize the presence of Th17 cells after burn injury. Subsequently, there have been reports of elevated IL-17 in the serum of burn patients[17, 18]. IL-17 contributes to effective clearance of numerous pathogens (e.g. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans*) through its indirect

recruitment of neutrophils[19-21]. Since infection is a major complication for burn patients, understanding the role Th17 cells play in neutrophil recruitment and bacterial clearance after burn injury would be advantageous. Early (0-3 days) after burn injury when the Th1 response predominates, mice are extremely susceptible to wound infection with *P. aeruginosa* (Chapter 3). Promoting differentiation of naïve CD4+ T cells towards a Th17 phenotype early after burn injury may lead to increase neutrophil migration to sites of infection and therefore better bacterial clearance. Furthermore, late (14 days) after burn injury when the Th17 response prevails, mice have enhanced clearance of pulmonary *P. aeruginosa* challenge due to a burn-induced accumulation of neutrophils within the lungs (Chapter 4). The secretion of IL-17 by Th17 cells may be involved in this neutrophil influx, which could be easily assessed mice deficient in IL-17 receptor.

5.4 Cellular mechanisms of increased susceptibility to early wound infection

The immune response to early wound infection was investigated using a murine model of thermal injury. Mice were challenged with a clinically relevant pathogen, *Pseudomonas aeruginsoa*, at 24 hours after burn or sham procedure. Within 48 hours of wound inoculation with a wildtype strain of *P. aeruginosa* (PAK), the bacteria replicated to a high titer and spread to distal organs resulting in bacterial sepsis. This correlated with apoptosis of dendritic cells and memory CD8+ T cells, as well as differential TLR expression on a variety of innate immune cells. The systemic IL-10/IL-12 axis was also skewed after burn injury with infection as indicated by a significant elevation in serum IL-10 and polarization of neutrophils into an anti-inflammatory (N2; IL-10+ IL-12-) phenotype.

Infection with an attenuated isogenic mutant strain of *P. aeruginosa*, lacking the adenylate cyclase gene *cyaB*, (Δ*cyaB*) was cleared better than the wildtype strain and was associated with an increased pro-inflammatory neutrophil (N1; IL-10-IL-12+) response in burn mice. Furthermore, administration of flagellin after burn injury skewed the neutrophil response towards a N1 phenotype resulting in an increased clearance of wildtype *P. aeruginosa* after wound inoculation. These findings identify neutrophil polarization as a therapeutic target for the reversal of bacterial susceptibility after burn injury.

5.5 Future directions: characterization of neutrophil response early after burn injury

In our model system, neutrophil phenotype directly correlated with bacterial clearance. However, a deeper understanding of neutrophil function, such as phagocytic capacity and oxidative burst potential, is still needed. Furthermore, the mechanisms responsible for neutrophil polarization after burn injury have yet to be defined. Delineating these aspects of the neutrophil response early after burn injury could help with modulating the response to improve patient outcome following infection.

TLRs can regulate neutrophil function. For example, TLR2 and TLR4 stimulation has been shown to directly induce neutrophil oxidative burst[22]. We find that early after burn injury and subsequent wound infection neutrophils have decreased TLR2, TLR4, and TLR5 expression compared to infected sham controls. This decreased TLR expression may be resulting in diminished neutrophil function or hypo-responsiveness and should be investigated.

We find that neutrophils, not macrophages, are the main innate immune cell population that is polarized early after burn injury. More specifically, wound inoculation with wildtype *P. aeruginosa* early after burn injury shifts neutrophils towards an anti-inflammatory (N2; IL-10+ IL-12-) phenotype, which is detrimental for bacterial clearance. Polarization of neutrophils has recently been described in tumor literature, where TGF-β is believed to drive N2 responses at the tumor microenvironment[23]. Immediately after burn injury, TGF-β levels begin to increase at the wound site [7,6]. We believe that this cytokine promotes differentiation of naïve CD4+ T cells towards a Th17 phenotype in wound draining lymph nodes, and it is possible that TGF-β is also driving the N2 response after burn injury. Both genetic (overexpression of sTβRII, a soluble TGF-β type II receptor) and pharmacologic (1D11, a TGF-β neutralizing antibody) approaches to block TGF-β signaling could be used to determine if TGF-β is influencing neutrophil polarization after burn injury. In addition, glucocorticoid exposure promotes an anti-inflammatory macrophage (M2) phenotype[24], and the massive release of glucocorticoids after burn injury induces lymphocyte apoptosis[25]. Therefore, it is conceivable that burn-induced glucocorticoid release is contributing to the development of the N2 response, which can be tested by blocking glucocorticoid receptor binding with RU-486.

As previously mentioned a single treatment with flagellin after burn injury skewed the neutrophil response towards a pro-inflammatory (N1) phenotype. Flagellin is detected by both TLR5 and the NLRC4 inflammasome[26,27]. It is currently unclear which immune sensor is mediating the polarization of neutrophils towards a N1 response. Furthermore, we do not know if flagellin is acting on neutrophils directly or indirectly. In order for flagellin to be a viable therapeutic, it is essential to delineate this process as well as understand how flagellin

impacts other aspects of neutrophil function, such as phagocytosis and NET formation, after burn injury and infection.

5.6 Neutrophil accumulation in the lungs late after burn injury

Since severely burned patients have a high incidence of delayed pulmonary bacterial infection, we investigated bacterial clearance late after burn using a murine model of thermal injury. More specifically, mice were given an intratracheal injection of wildtype *P. aeruginosa* at 14 days after burn or sham procedure. Although we hypothesized that burn mice would have an increased susceptibility to infection compared to sham controls, they exhibited enhanced bacterial clearance which correlated with a burn-induced accumulation of neutrophils within the lungs. Mice were also more resistant to an intraperitoneal challenge of *P. aeruginosa* late after burn. To better mimic the clinical setting wherein patients are repeatedly exposed to bacteria through invasive devises such as central lines, catheters, and ventilators, mice were then given two sequential bacterial infections. The exposure to a second bacterial infection appeared to have no impact on *P. aeruginosa* clearance in burn mice whereas sham mice displayed enhanced clearance after the second bacterial challenge. Collectively, these data show that late after burn injury there is pulmonary inflammation, marked by neutrophil accumulation. This appears to prime burn mice for better control of bacterial infections.

5.7 Future directions: characterization of neutrophil response late after burn injury

The mechanism responsible for neutrophil accumulation in the lungs late after burn injury is currently unknown. One possible mechanism is through increased IL-17 (via Th17 cells) in the lungs late after burn injury (detailed in 5.2). Another potential mechanism is by increased levels of monocyte chemoattractant protein 1 (MCP-1). MCP-1 regulates neutrophil migration and is significantly elevated in the serum after burn injury. In addition, bronchoalveolar lavage (BAL) cells collected at 7 days after burn injury have increased IL-17 and MCP-1 production following TLR2 or TLR4 stimulation compared to sham controls[28]. Therefore, it is likely that IL-17 or MCP-1 expression is elevated in the lungs late after burn injury and mediates the increase in neutrophil influx, which our laboratory is currently investigating.

While neutrophils are an important component of the inflammatory response, inappropriate and excessive neutrophil recruitment into the lungs can cause localized tissue damage. In chronic obstructive pulmonary disease (COPD), neutrophil infiltration into the lungs has been implicated in disease progression[29]. More specifically, neutrophil elastase and other neutrophil-derived proteases are believed to be the key mediators of tissue damage and decline in lung function. A similar response is observed in Acute Lung Injury (ALI) where neutrophil-induced inflammation contributes to oxidant-induced injury and loss of the epithelium[30,31]. Neutrophils have also been shown to aggravate lung injury during mechanical ventilation[32,33]. Therefore, the increased pulmonary neutrophil infiltration observed late after burn injury may be contributing to localized tissue damage and/or impaired lung function. Our laboratory is collaborating with Dr. Stephen Tilley to examine the impact that burn injury, infection, and inhalation injury have on pulmonary function.

5.8 Closing remarks

Collectively, the studies described here confirm that the immune response following burn injury is dynamic and changes over time. However, our findings also indicate the overall state of the immune response cannot be categorized as pro-inflammatory or anti-inflammatory at any given moment. We find that early after burn, which is traditionally thought of as pro-inflammatory phase, there is shift in the neutrophil response towards an anti-inflammatory (N2; IL-10+ IL-12-) response (Figure 5.3) Then late after burn injury, which is believed to be a period of overt immune suppression, neutrophil accumulation is the lungs appears to “prime” burn mice for enhanced bacterial clearance and Th17 cells persist. Nevertheless, this work has provided a better understanding of immune response following burn injury, as well as revealed potential therapeutic targets for immunomodulation after burn injury.

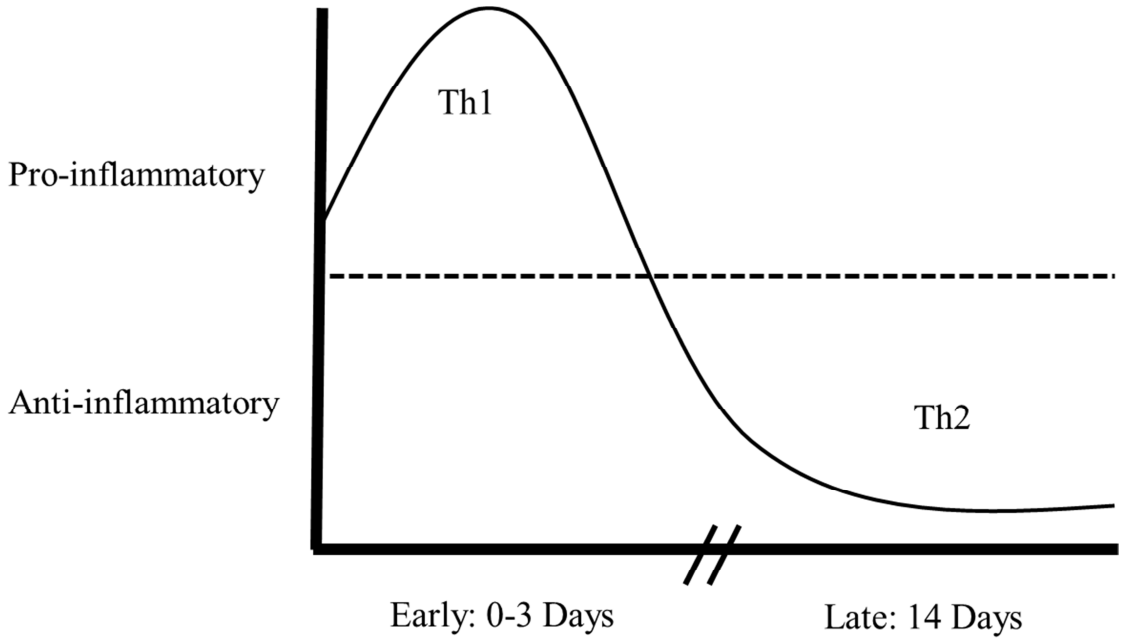

Figure 5.1 Burn-induced immune dysfunction in dynamic and changes over time. The immune response after burn injury has been classically divided into an early (0-3 days) pro-inflammatory phase followed by a late (7-14 days) anti-inflammatory. During this early pro-inflammatory phase, CD4+ T exhibit a Th1 phenotype on stimulation. Late after injury, there is a switch to an anti-inflammatory response characterized the presence of an anti-inflammatory Th2 response.

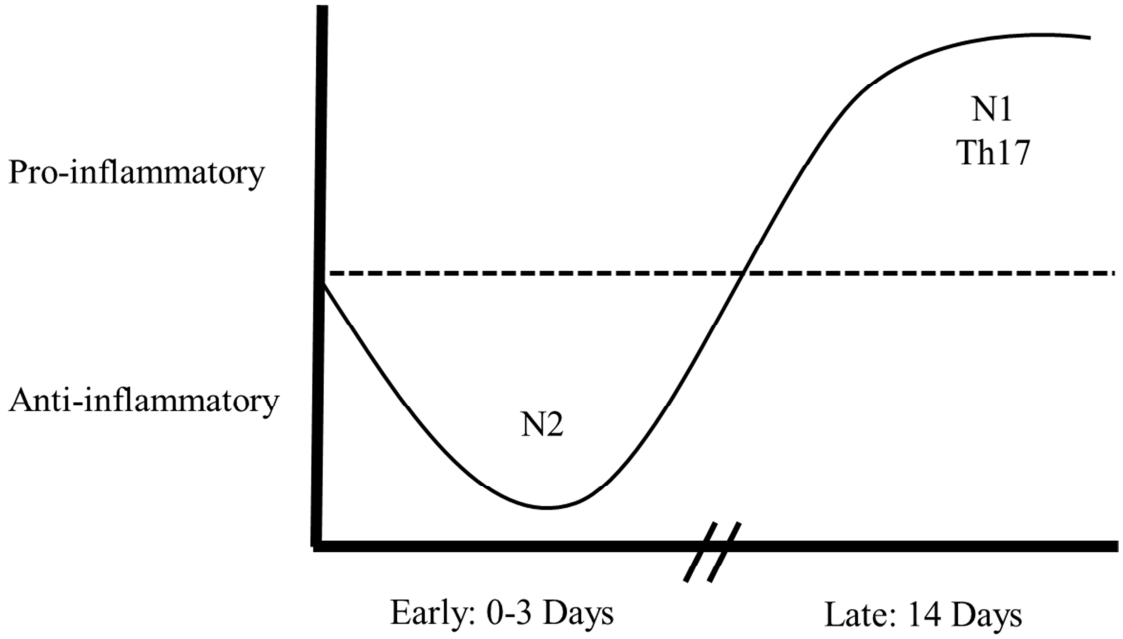

Figure 5.2 The neutrophil response following burn injury does not match the classical paradigm. Our data suggests that early after burn injury, neutrophils have an anti-inflammatory (N1) phenotype marked by high production of IL-10. Late after burn injury, neutrophil appear to have a pro-inflammatory (N2) phenotype as demonstrated to effective bacterial clearance and oxidative burst. Furthermore, Th17 cells are present in wound-draining lymph nodes and the spleen, and these cells are known to induce neutrophil recruitment.

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