CELLULAR MECHANISMS OF IMMUNE AND HEMATOPOIETIC DYSFUNCTION FOLLOWING RADIATION AND BURN INJURIES

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ABSTRACT

Brandon Michael Lee Linz: Cellular Mechanisms of Immune and Hematopoietic Dysfunction Following Radiation and Burn Injuries
(Under the direction of Bruce A. Cairns and Robert Maile)

The immune system has evolved to protect the body against damage from infection, disease, or injury. Severe injuries, such as large burns or radiation exposures, induce profound immune dysfunctions at the cellular and humoral levels that heighten the body’s susceptibility to infections. Despite progress made toward reducing the consequences of burn shock, translocation of intestinal bacteria and wound and pulmonary infections leading to sepsis are still major causes of mortality following a traumatic injury. Following a severe burn or radiation injury, the body must respond rapidly to activate or produce new immune cells to challenge the insult and to restore homeostasis also while preventing any bacteria from establishing an infection. Therefore, to improve patient outcomes, it is important to understand not only the immune but also the hematopoietic responses to injury and infection.

NLRP12 is a member of the NLR family of proteins that are responsible for coordinating inflammatory responses upon recognition of invading pathogens and damage signals. Mutations in human NLRP12 have been linked to atopic dermatitis and hereditary periodic fevers with skin, however the mechanisms by which NLRP12 affects these conditions remain to be fully elucidated. To better understand these mechanisms, Nlrp12 knockout mice were subjected to a model of radiation-thermal combined injury. Remarkably, Nlrp12 deficient mice failed to repopulate their peripheral immune compartments in addition to a significant reduction in bone marrow cellularity. Prolonged, elevated serum concentrations of TNF in injury Nlrp12-deficient
animals induced the stem cells responsible for the bulk of myeloid cell production to undergo apoptosis. This defect in repopulation of the peripheral immune system lead all Nlrp12 knockout animals to quickly succumb to an infectious challenge, thus highlighting the importance of Nlrp12 in responding to infection or injury.

Following a radiation-thermal combined injury, wild type myeloid progenitor cells underwent apoptosis at a low level. Administration of the glycoprotein granulocyte-monocyte colony stimulating factor was evaluated as a therapeutic to stimulate stem cell maturation and production of myeloid cells following injury. Treatment resulted in increased myeloid cell production: including increases in platelets, red blood cells, immature monocytes, dendritic cells, neutrophils, and macrophages. Notably, platelets and monocytes displayed increased function, in turn decreasing mortality and response to an infectious challenge. The innate immune response was then assessed early after only burn injury. Burn mice were susceptible to an early wound infection with Pseudomonas aeruginosa as shown with increased mortality and systemic bacterial colonization. The defective bacterial clearance was associated with a neutrophil anti-inflammatory polarization phenotype (N2; IL-10+ IL-12-). This work expands on our understanding of NLRP12 function in vivo as well as insights into the cellular mechanisms of immune dysfunction and hematopoiesis following burn and radiation injuries. Furthermore, our results reveal novel treatments for improved hematopoietic and innate immune response to reduce the impact of sepsis and subsequent bacterial infections after injury.
To my grandparents, whose lives and legacies still inspire me.
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LIST OF ABBREVIATIONS

ASC: apoptosis-associated speck-like protein containing a CARD domain

ATP: adenosine triphosphate

C-terminal: carboxy-terminal

cAMP: cyclic adenosine 3’,5’-monophosphate

CAPS: Cryopyrin-Associated Periodic Syndromes

CARD: caspase activation and recruitment domain

CATERPILLER: caspase activation and recruitment domains [CARD], transcription enhancer, R [purine]-binding, lots of leucine repeats

CDX: e.g. cluster of differentiation 3, cluster of differentiation 40

CD40L: CD40 ligand

CLP: common lymphoid progenitor

DAMP: damage associated molecular pattern

DC: Dendritic cell

FCAS: familial cold auto-inflammatory syndrome

GCSF: granulocyte colony stimulating factor

GM-CSF: granulocyte monocyte colony stimulating factor

GMP: granulocyte monocyte progenitor

H&E: hematoxylin and eosin
Hsp70: heat shock protein 70

Hsp90: heat shock 90

IFNα/β/γ: interferon alpha/beta/gamma

IL-X: e.g. interleukin 1-beta, interleukin-2, interleukin-18

IL1ra: interleukin 1 receptor-a

IκB: inhibitor of NFκB

IKK: inhibitor of NFκB kinase

IRAK1: interleukin-1 receptor-associated kinase

LPS: lipopolysaccharide

LRR: leucine-rich repeats

MAPK: mitogen-activated protein kinase

MEP: megakaryocyte erythrocyte progenitor

N-terminal: amino-terminal

NBD: nucleotide bind domain

NFκB: nuclear factor kappa B

NIK: NFκB inducing kinase

NK: Natural killer

NLR: nucleotide binding domain leucine-rich repeat
NLRC4: nucleotide binding domain leucine-rich repeat CARD protein 4

NLRP12: nucleotide binding domain leucine-rich repeat containing a pyrin domain 12

PRR: pattern recognition receptor

TAK1: transforming growth factor beta-activated kinase 1

TCR: T cell receptor

TGFβ: tumor growth factor-beta

TLRX: e.g. Toll-like receptor-2, Toll-like receptor-4

TNF: tumor necrosis factor

Wt: Wild type

Δ: deletion
CHAPTER 1: INTRODUCTION

1.1 Burn injury

Burn injuries occur when a hot liquid (scald), solid (contact burn), or flame are exposed to a tissue and cause subtotal or total destruction of the cells present in the skin or below. Burns can also be caused by exposure to radiation sources, electricity, and caustic chemicals (Gabrielsen, 2003). In 2007, there were 11 million cases of burns that required medical treatment, making it the fourth most common injury world-wide ("World Health Organization. The Global Burden of Disease: 2004 Update," 2008). In the US alone, there were 450,000 emergency room visits and 3,500 deaths from burn injuries in 2010 (Miller et al., 2008). The rapid onset of burn shock following injury necessitates immediate, specialized care to reduce morbidity and mortality. Aggressive fluid resuscitation, regulation of body temperature, analgesics, wound debridement, wound excision and closure, and preventative infection are measures in which certified American Burn Associated burn centers specialize (Association, 2015). The cost of treatment at one of these centers can be greater than $200,000 (Association, 2015). However, this does not include expenses from rehabilitation, occupational and physical therapies, and any chronic complications.

1.2 Grading of burn wounds

Burn injuries are graded based on size and how many layers of skin have been injured. A first-degree or superficial burn only destroys the first layer of the epidermis and heals quickly; the most common first degree burn experienced is a sunburn (Gabrielsen, 2003). A second-degree or partial thickness burn destroys both the epidermis and dermis tissues; this can result in
blisters that will often heal and scar over within 5 weeks (Roth & Hughes, 2015). A third-degree or full thickness burn extends through all layers of the skin and into the subcutaneous tissue. Since this depth includes the basement membrane and progenitor cells responsible for dermal maintenance and cell production, treatment of these wounds requires excision of the damaged tissue and a skin-graft to close the now open wound. Lastly, a fourth-degree burn involves other organ tissue below the skin and soft tissue, such as muscle, connective tissue, and bone. Burn injuries are also measured by size or area of injury (Gabrielsen, 2003). The percent total body surface area (TBSA) of an adult patient is typically assessed by the rule of nines where most areas of the body (i.e. an arm, back and front of the legs, half of the chest or back) can be assumed to be approximately 9% of the total body surface area of the adult. The overall burn size and depth help to guide fluid resuscitation and wound management within the burn unit (Roth & Hughes, 2015).

1.3 Acute radiation exposure

Exposure to high amounts of ionizing radiation can lead to numerous cellular and systemic damages. Ionization of DNA from radiation exposure leads to decreased ability for cells to divide due to ionization-induced mutation of key factors involved in cell division (Coleman, Stone, Moulder, & Pellmar, 2004). This elimination of rapidly dividing cells – namely hematopoietic stem cells, intestinal stem cells, and hair follicles – these symptoms and their consequences can start to be observed as early as one to two hours post exposure and may persist for weeks to months (Browne, 2013; Coleman et al., 2004; Gaugler, 2014). Acute exposure to high levels of ionizing radiation initially causes nausea, vomiting, diarrhea, headaches and a mild fever typically within 24 hours. However, the ionizing radiation also induced DNA-damage repair mechanisms that lead to the apoptosis or necrosis of dividing cells causing neurological,
gastrointestinal, and hematopoietic distress (Chua et al., 2012; Gaugler, 2014). The apoptosis of the majority of hematopoietic progenitor cells leads to severe leukopenia and subsequent increased susceptibility to infections (Chua et al., 2012; Heslet, Bay, & Nepper-Christensen, 2012; Heylmann, Rödel, Kindler, & Kaina, 2014).

1.4 Radiation-thermal combined injury

   Greater than 30%, and predicted to potentially be as high as 65-70%, of accidental or incidental exposures to high doses of radiation are coupled with a secondary burn injury either from the heat from the primary radiation exposure source or from a secondary fire created by an industrial accident or incidental exposures (Fushiki, 2013; Hasegawa et al., 2015; Shaw, 2014). Patients who receive a radiation-thermal combined injury (RCI) undergo physiologic changes and display cytokine profiles consistent with burn shock, but uniquely show immune and hematopoietic cell destruction consistent with total body radiation injury ablation of rapidly dividing cells (Cherry, Williams, O'Banion, & Olschowka, 2013; Chua et al., 2012; Coleman et al., 2004; Hasegawa et al., 2015). This combination of unique injuries leads to unique challenges for medical interventions that have been utilized for the individual injuries which may be counter-indicated in RCI in the absence of research into RCI-relevant treatments (Basile et al., 2012; Browne, 2013). Despite no to low numbers of cases each year, the heightened security concerns of today coupled with the lack of clinically proven treatments necessitate inquiry.

1.5 Systemic response to burn injury

   Severe burn injuries covering greater than 20% TBSA are typically followed by a period of hypermetabolism, altered hemodynamics, vascular permeability and edema, decreased renal blood flow, and increased gut mucosal permeability (Baker, Miller, & TRUNKEY, 1979; Hettiaratchy & Dziewulski, 2004; Miller et al., 2008). With burns of more the 20% TBSA, the
magnitude of immune impairment is proportional to the size of burn (Baker et al., 1979). The massive release of cellular debris and damage associated molecular patterns (DAMPs) trigger a systemic immune response (Finnerty, Przkora, Herndon, & Jeschke, 2009). This massive release of inflammatory mediators from the wound and other tissues is believed to impact and/or trigger multi-organ dysfunction (Santaniello et al., 2004). Following the initial systemic inflammatory response, there is also long-term immune suppression demonstrated by prolonged allograft skin survival on burn wounds (Lagus, Sarlomo-Rikala, Böhling, & Vuola, 2013; Mowlavi, Andrews, Milner, Herndon, & Heggens, 2000). Since burn injury impairs all parts of the immune system – hematopoietic, innate, and adaptive systems – patients are extremely susceptible to infection (Church, Elsayed, Reid, Winston, & Lindsay, 2006; Manson, Pernot, Fidler, Sauer, & Klasen, 1992).

1.6 Nucleotide-binding oligomerization domain family of receptors

Pattern recognition receptors (PRR) play a key role in regulating acute and chronic innate immune responses to tissue damage or infection (Broz & Monack, 2013). In addition to toll-like receptors (TLRs), the more recently discovered family of intracellular receptors, the nucleotide-binding oligomerization domain-like receptors (NLRs), have been found to not only cooperate with TLRs, but also both positively and negatively regulate inflammatory responses, initiate enzymatic cleavage of cytokines, as well as regulate apoptotic responses (Duran, Alvarez-Mon, & Valero, 2014). The majority of study has focused on a key members’ function as inflammasomes: namely NLRP3, NLRC4, NLRP1, and putatively NLRP12 (Jacobs, Tabor, Burks, & Campbell, 1989; Jeru et al., 2008; Mariathasan et al., 2004; Vladimer et al., 2012). These proteins, upon binding of their ligand (or by other mechanisms which remain to be fully elucidated), will undergo a conformational change that typically induces interaction with the
adaptor protein apoptosis-associated speck-like protein containing a c-terminal caspase recruitment domain (ASC) (Franklin et al., 2014; Hara et al., 2013). Following binding to ASC, the inchoate inflammasome will recruit either caspase-1 or caspase-11 (Aachoui et al., 2013; Guey, Bodnar, Manié, Tardivel, & Petrilli, 2014; Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Kuida et al., 1995). Then, utilizing either its own caspase activation and recruitment domain (CARD) or the CARD adaptor on ASC, will oligomerize into a heptamer. This now active inflammasome will then begin to catalytically cleave pro-interleukin (IL)-1β (IL-1β) and pro-IL-18 into their active forms (Aachoui et al., 2013; Groß et al., 2012; Guey et al., 2014; Hagar et al., 2013; Kuida et al., 1995; Pilla et al., 2014). Active IL-1β is a pleiotropic cytokine that initiates cellular proliferation, cytokine production and secretion, induction of proptosis, and an anti-viral state (Ali, Karin, & Nizet, 2015); whereas IL-18 serves to increase interferon-γ (IFNγ) production by natural killer cells (NK cells) and T cells (Takeda et al., 1998; Wong, Muthuswamy, & Kalinski, 2012). In addition to the inflammasome subfamily, other NLRs have been primarily found to be involved in regulation of innate immune responses, most significantly through regulation of the NFκB signaling cascade proteins: NOD1 and NOD2 have been found to activate the kinase RIP2 that in turn activates IκB kinase, which in turn leads to NFκB activation (Caruso, Warner, Inohara, & Núñez, 2014; Lich & Ting, 2007); NLRX1 amplifies NFκB signaling and JNK signaling by increasing production of radical oxygen species (Irving C Allen et al., 2011; Tattoli et al., 2008); NLRP12 has been shown to interact with heat shock protein 90 (Hsp90) and in turn with NFκB inhibitory kinase (NIK), this complex then in turn suppresses NFκB signaling (Arthur, Lich, Aziz, Kotb, & Ting, 2007; Krauss et al., 2015; Vitale et al., 2013; Ye et al., 2008).
1.7 NLRP12

Mutations in Nlrp12, formerly known as Monarch-1 or Pypaf-7, have been shown to be associated with familial cold auto-inflammatory syndrome (FCAS), an extremely rare autosomal dominant disease that results in recurrent fever and skin urticarial due to cold conditions (Lich & Ting, 2007; Liu et al., 2013; Vitale et al., 2013; Xia et al., 2016). NLRP12 is expressed in innate immune cells (specifically monocytes and macrophages), intestinal cells, bone and bone marrow cells, as well as liver cells (Lech, Avila-Ferrufino, Skuginna, Susanti, & Anders, 2010). This is achieved by inducing proteasome-mediated degradation of NF-κB inducing kinase (NIK) in response to pathogens and activation through pro-inflammatory receptors (Arthur et al., 2007; Lord et al., 2009; Zaki et al., 2011). NLRP12 is stabilized by interaction with Hsp90, thus allowing for suppression of NFκB inhibitory kinase (NIK) (Arthur et al., 2007; Ataide et al., 2014; Vladimer et al., 2012). Because NLRP12 functions to dampen these signals, it is clear that NLRP12 must be controlled in order to mount an adequate cellular response to such insults. However, NLRP12 has also been found to act as an inflammasome by oligomerizing with ASC and Caspase-1 during Yersinia pestis and malaria infections and mediate cleavage of IL-18 into its active form (Ataide et al., 2014; Vladimer et al., 2012). Additionally, molecular analysis reveals that in the absence of NLRP12, dendritic cells display an inappropriate activation of NIK, resulting in high levels of NIK dependent gene expression (Arthur et al., 2010; Krauss et al., 2015). Taken together, NLRP12 function within the immune system has yet to be fully explained.

1.8 NFkB signaling

NFkB is a critical transcriptional regulator of cytokine production and cell survival as one of the first responding molecules to a harmful cellular stimulus. Induction of NFkB can be
caused by reactive oxygen species (ROS), lipopolysaccharides (LPS) from Graham – bacteria, interleukin-1β (IL-1β), ionizing radiation, and tumor necrosis factor (TNF) (Van Antwerp, Martin, Kafri, Green, & Verma, 1996; Ward-Kavanagh, Lin, Šedý, & Ware). Canonically, NFkB signaling cannot be achieved without activation by IκB kinase (IKK) composed of the two subunits IKKα and IKKβ in addition to the master regulatory IKKγ. IKK phosphorylates IκB, altering its quaternary structure and allowing ubiquitination and subsequent destruction through the proteasome (S.-C. Sun, 2011). Non-canonical signaling begins when the lymptoxin β-receptor (LTβR), BAFF, or RANK activates NFkB inducing NIK (NIK) allowing IKKA to cleave p100 into the mature p52 subunit (S.-C. Sun, 2011).

1.9 Tumor necrosis factor

Following tissue damage, infection, or trauma, initial responses are initiated by the cytokine tumor necrosis factor (TNF, also known as TNFα) (Beg & Baltimore, 1996; Peschon et al., 1998). TNF can be secreted by macrophages, monocytes, CD4+ T cells, neutrophils, mast cells, and eosinophils (Schindler et al., 1990). Primarily, TNF acts as a pyrogen, mediates acute liver responses to damage, acts as a chemoattractant for neutrophils, stimulates increased macrophages phagocytosis, and pro-inflammatory cytokine production and expression (Kapas et al., 1992). High concentrations of TNF induce shock-like symptoms, with prolonged exposure resulting in cachexia, as typified by latent tuberculosis infections (Croft, Benedict, & Ware, 2013; Di Paolo et al., 2016; Hayden & Ghosh, 2014). Moreover, high serum or tissue concentrations of TNF will act upon TNF Receptor 2 (TNFR2) that is uniquely expressed on leukocytes to recruit c-IAP1 and c-IAP2 (Vince et al., 2007; Zarnegar et al., 2008). Recruitment of these anti-apoptotic proteins likely functions by inducing degradation of TNFR2 and initiating and anti-apoptotic state (Croft et al., 2013; Hayden & Ghosh, 2014; Wei, Yue, & Qingwei,
Following prolonged, high concentrations of serum TNF, leukocytes will downregulate their expression of TNFR2 and maintain or increase expression of TNFR1 (Wicovsky et al., 2009). Prolonged signaling through TNFR1 on myeloid and myeloid progenitor cells will cause the cell to no longer signal through TNF receptor associated factor 2 (TRAF2) and (nuclear factor k B) NFkB signaling cascade, but rather to recruit TNFRSF1A-Associated via Death Domain (TRADD) and fas-associated protein with Death Domain (FADD) which in turn recruits the cysteine protease procaspase 8 (Micheau & Tschopp, 2003; Van Antwerp et al., 1996). This recruitment will activate proteolytically cleave caspase 3 into its active form. From which, the now active caspase 3 and 9 will lead to Bid-associated apoptosis (Cai et al., 2014; Croft et al., 2013; Di Paolo et al., 2016; Hayden & Ghosh, 2014; Micheau & Tschopp, 2003; Wei et al., 2014; Zhao et al., 2012).

### 1.10 Hematopoiesis following injury

In steady-state adult hematopoiesis, most hematopoietic stem cells (HSCs) are in the resting phase of the cell cycle (Panopoulos & Watowich, 2008). Upon enhanced hematopoietic demand, HSCs can be induced to divide and self-renew or differentiate (Kovtonyuk, Manz, & Takizawa, 2016). Following RCI, pro-inflammatory cytokines are elevated, such as IL-1β, TNF, IL-4, IL-6, IL-12, and IL-15 (Mendoza et al., 2012; Palmer et al., 2013). These cytokines act to promote increased progenitor cell production and maturation in order to repopulate the vacuum created by the radiation and burn induced ablation of peripheral and expanding immune cells (T. Chen et al., 2007; Giron-Michel et al., 2005; Kominato, Galson, Waterman, Webb, & Auron, 1995; Kopf et al., 1995; Musashi, Clark, Sudo, Urdal, & Ogawa, 1991). Following RCI, we have noted that the lymphocytic compartment is much slower to respond; however, two weeks after injury, the predominate cell type present in the bone marrow and peripheral tissues – namely
blood, spleen, lungs, and liver – is a cell type that by its cell surface protein expression (Ly6G+Ly6C+CD11b+) resembles myeloid derived suppressor cells (MSDCs) (Carter et al., 2013; Mendoza et al., 2012; Palmer et al., 2013). However, upon further study, the cells promote, albeit weakly, T cell proliferation and IFNγ production (Mendoza et al., 2012). We have identified these cells as inflammatory monocytes and have shown that they are vital to responding to an infectious challenge after injury (Mendoza et al., 2012).

1.11 Pulmonary *Pseudomonas aeruginosa* infections

A major cause of mortality for patients who survive the initial shock of a burn injury are pulmonary bacterial infections. Prolonged ICU stays and ventilation increase a patient’s risk of developing a pulmonary infection, often termed ventilator-associated pneumonia (VAP). VAP typically occurs when a patient is mechanically ventilated for more than 48 hours (Chastre & Fagon, 2007; Fabian, 2000; Hollaar et al., 2016; Shorr, Sherner, Jackson, & Kollef, 2005). The Gram negative saprophyte, *Pseudomonas aeruginosa*, is one of the most common infectious agents in United States’ burn centers due to its ability to survive on many hospital surfaces (Lyczak, Cannon, & Pier, 2000). While the innate immune system of a healthy adult is able to easily clear *P. aeruginosa* infections, immunocompromised hosts are more susceptible to infection and mortality (Chitkara & Feierabend, 1980; Lavoie, Wangdi, & Kazmierczak, 2011; Lyczak et al., 2000). During infection, *P. aeruginosa* will downregulate synthesis of the motility protein flagellin, the major component of bacterial flagellum. This allows the bacterium to avoid detection by host Toll-like receptor 5 (TLR5), thus limiting humoral and cellular immune responses (Santangelo, Shoup, Gamelli, & Shankar, 2000; Sutterwala et al., 2007).
1.12 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 (e.g. TLRs) to recognize features that are common to many microbes (Duran et al., 2014). 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 (Angus & Van der Poll, 2013; Baker et al., 1979). 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 (Ohkura et al., 2012). 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 (McHeyzer-Williams, Okitsu, Wang, & McHeyzer-Williams, 2012; Mueller, Gebhardt, Carbone, & Heath, 2013).

1.13 Granulocyte-monocyte colony stimulating factor

Granulocyte-monocyte colony stimulating factor (GM-CSF) is a monomeric glycoprotein produced by macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts (Egea et al., 2013). GM-CSF acts to bring myeloid stem cells (including mono-, myeloid-, proerythro-, and megakaryoblasts) out of their steady state in order to produce more stem cells as well as fully differentiated cells (platelets, neutrophils, macrophages, dendritic cells, monocytes, eosinophils, and basophils (J. G. Noel et al., 2002; Singh, Newman, & Seed, 2015). In addition
to stimulating stem cell function, GM-CSF inhibits neutrophil migration, increases reactive oxygen species production, and acts as an embryokine. Administration of GM-CSF to mice and humans results in the production of leukocytes following chemotherapy to prevent neutropenia (Dragon, Saffar, Shan, & Gounni, 2008; Dugan et al., 2002; Gardner et al., 2014; J. G. Noel et al., 2005; J. G. Noel et al., 2002; Reeves, 2014). Additionally, GM-CSF has been used clinically under the name Sargramostin as a therapeutic for inflammatory bowel disease, leukemia, and acute lung injury (Campo et al., 2012; J. B. Cohen et al., 2015; Danese, 2012).

1.14 Platelets

Small fragments of megakaryocyte cytoplasm, platelets, or thrombocytes, play important roles in primary and secondary hemostasis as crucial steps of the coagulation cascade occur on their cell surfaces (Hess et al., 2014; Vieira-de-Abreu, Campbell, Weyrich, & Zimmerman, 2012). Despite their primary function as hemostatic regulators, platelets can also act as inflammatory cells through the release of cytokines, chemokines, expression of pro-inflammatory surface markers, interactions with leukocytes and endothelial cells, and release of inflammatory mediators through degranulation (Vieira-de-Abreu et al., 2012).

1.15 Neutrophils

The first immune cell type recruited to sites of infection and injury are neutrophils that extravasate through the vasculature to the site of injury following a chemokine gradient such as human IL-8 or murine keratinocyte-derived cytokine (KC) secreted by resident mast cells or macrophages (Dragon et al., 2008; Gil et al., 2012). Once activated, these cells can survive anywhere from 1-5 days (Pillay et al., 2010). During this time the cells will phagocytose pathogens or cellular degree for internal phagosome processing and destruction; phagosome-lysosomal fusion will digest and kill the pathogen (Nordenfelt & Tapper, 2011). In addition to
phagocytosis, activated and recruited neutrophils will generate a variety of toxic byproducts to help destroy invading pathogens through an oxidative burst. Degranulation of secretory vesicles called granules releases cytotoxic molecules to aid in bacterial killing (Borregaard, 1997). One study showed that neutrophils have decreased Fc receptor mediated phagocytosis, as well as a 50% reduction in intracellular killing, after burn injury (Adediran et al., 2010; Bjerknes, Vindenes, & Laerum, 1989). This group also showed that the ability of circulating neutrophils to undergo oxidative burst gradually declines during the first two weeks after burn injury. 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 (Bjerknes et al., 1989; L. W. Chen, Huang, Lee, Hsu, & Lu, 2006). 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 (S. W. Jones et al., 2013; G. Noel et al., 2010; Greg Noel et al., 2011).

1.16 Macrophages

Macrophages are capable of phagocytosis, cytokine production, oxidative bursts, and antigen presentation (Fujiwara & Kobayashi, 2005). With their ability to rapidly respond to infection as circulating monocytes recruited to tissues, or as resident macrophages, these cells play central roles in the initiation and resolution of injury and infection. Antigen presentation consists of the macrophages internally digesting pathogens or antigens and processing these proteins for presentation as fragments on MHC-complexes, to in turn. Upon activation, macrophages can produce significant amounts of IL-1β, IL-6, and transforming growth factor-β (TFGβ) (Fujiwara & Kobayashi, 2005; J. G. Noel et al., 2005). Macrophage activation represents a spectrum from fully pro-inflammatory macrophages (M1) that produce IL-12 in response to
interferon-gamma (IFN-γ) compared to anti-inflammatory macrophages (M2) that produce IL-10. This shift toward an M2 phenotype is achieved by exposure to IL-10, glucocorticoids, or TLR ligation (N. Wang, Liang, & Zen, 2015). Macrophage hyperactivity and shifts in macrophage polarization toward M2 phenotypes have been suggested as a cause of increased bacterial susceptibility following injury (Greg Noel et al., 2011; J. G. Noel et al., 2005).

1.17 Dendritic cells

Similar to macrophages, dendritic cells (DCs) are capable of both phagocytosis and antigen presentation. The primary function of DCs are to bridge innate immune cell activation and responses to adaptive and humoral efforts (Auffray, Sieweke, & Geissmann, 2009; Savina & Amigorena, 2007). DCs achieve this by presenting foreign antigens to stimulate T cell activation directly as well as through secretion of a variety of cytokines, namely IL-12 (Auffray et al., 2009). A significant reduction in the total number of DCs have been reported after burn injury, specifically myeloid DCs (mDCs) and plasmacytoid DCs (pDCs); this reduction is directly correlated with burn size and depth.

1.18 Toll-like receptors

TLRs are PRRs that respond to pathogen associated molecular patterns (PAMPs) and endogenous stress signals termed danger associated molecular patterns (DAMPs) (Matzinger, 2012; O'Neil, Golenbock, & Bowie, 2013). TLRs, expressed by both immune and non-immune cells, are activated by binding to their specific ligand: TLR2 binds membrane bound lipoproteins, TLR4 binds lipopolysaccharide (LPS), and TLR5 binds flagellin. TLR signaling through the transcription factors nuclear factor k B (NFkB) and activator protein-1 (AP1) to induce expression of cytokines, chemokines, antimicrobial molecules, major histocompatibility complex, and T cell receptor (TCR) costimulation molecules, all of which are required to mount
an inflammatory response (Buechler, Teal, Elkon, & Hamerman, 2013; L. W. Chen et al., 2006; Duran et al., 2014; Lee, Avalos, & Ploegh, 2012; Moore et al., 2007; O'Neil et al., 2013; Paterson et al., 2003). Both in vitro and in vivo studies have shown that TLR2 and TLR4 responses are heightened between 1-7 days after burn injury (B. A. Cairns, C. M. Barnes, S. Mlot, A. A. Meyer, & R. Maile, 2008). Upon TLR stimulation, macrophages, dendritic cells, and γδ T cells from burn mice have increased cytokine production compared to non-burn controls (Neely et al., 2011). 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 and increased phosphorylation of p38 MAP kinase, a component of the TLR signaling cascade, both contribute (B. A. Cairns et al., 2008; Hagar et al., 2013; Moresco, LaVine, & Beutler, 2011).

1.19 Remaining questions

The data outlined above describes the complexity of burn and radiation combined injury and in particular the profound immune impairment observed following RCI in the absence of NLRP12. 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 radiation and thermal combined injuries, specifically by determining their contributions to hematopoiesis and modulation of inflammation.
CHAPTER 2: INNATE IMMUNE CELL RECOVERY IS POSITIVELY REGULATED BY NLRP12 DURING EMERGENCY HEMATOPOESIS

2.1 Summary

With enhanced concerns of terrorist attacks, dual exposure to radiation and thermal combined injury (RCI) has become a real threat with devastating immunosuppression. NLRP12, a member of the NOD-like receptor family, is expressed in myeloid and bone marrow cells and has been implicated as a checkpoint regulator of inflammatory cytokines as well as an inflammasome activator. We show that NLRP12 has a profound impact on hematopoietic recovery during RCI by serving as a checkpoint of TNF signaling and preventing hematopoietic apoptosis. Using a mouse model of RCI, increased NLRP12 expression was detected in target tissues. Nlrp12/- mice exhibited significantly greater mortality, inability to fight bacterial infection, heightened levels of pro-inflammatory cytokines, overt granulocyte/monocyte progenitor cell apoptosis and failure to reconstitute peripheral myeloid populations. Anti-TNF antibody administration improved peripheral immune recovery. These data suggest that NLRP12 is essential for survival after RCI by regulating myelopoiesis and immune reconstitution.

2.2 Introduction

The hematopoietic system is capable of rapidly increasing myeloid cell production in response to tissue damage and is critical for wound healing and infection clearance (Baldridge, King, & Goodell, 2011; Dugan et al., 2002; Gardner et al., 2014; Manz & Boettcher, 2014; J. G. Noel et al., 2005; J. G. Noel et al., 2002; Santangelo et al., 2000; Serafini et al., 2007; Toliver-

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1 This chapter is currently under review at the Journal of Immunology
Kinsky, Lin, Herndon, & Sherwood, 2003; H. Zhang et al., 2010). While the factors that initiate emergency myelopoiesis are not fully elucidated, it is generally accepted that emergency myelopoiesis is tightly coupled with cytokine and growth factor production, namely TNF and IL-6, and is mediated by NF-κB and other immune regulatory transcription factors.

Rare mutations in Nlrp12, a nucleotide-binding leucine rich repeat and pyrin domain-containing receptor (NLR, also known as NOD-like receptor), have been associated with periodic fevers in humans although the association needs to be further studied. Nonsense and splice mutations within human-Nlrp12 have been shown to diminish suppression of NF-κB signaling (Jeru et al., 2008), however some variants do not exhibit such activity but are associated with modestly enhanced or more rapid inflammasome activation (Borghini et al., 2011). The different functions observed with NLRP12 may be consistent with NLRP12 exhibiting an inflammasome function in certain infections (Ataide et al., 2014; Vladimer et al., 2012) but not other infections or inflammatory conditions (Zaki, Man, Vogel, Lamkanfi, & Kanneganti, 2014). While the pyrin-domain containing members of the NLR family have largely been studied in the context of the inflammasome (Wen, Miao, & Ting, 2013), there is growing evidence that a few play an important role in regulating inflammatory signaling. Some NLR proteins have been shown to be positive regulators of NF-κB, while NLRP12 has been implicated as a negative regulator of both the canonical and non-canonical pathways of NF-κB (I. C. Allen et al., 2012; Arthur et al., 2010; Savic, Dickie, Battellino, & McDermott, 2012; Ye et al., 2008; Zaki et al., 2011). NLRP12-mediated NF-κB suppression has been implicated in colonic inflammation and tumorigenesis (I. C. Allen et al., 2012) and osteoclast differentiation (Krauss et al., 2015).
The cytokines that regulate hematopoietic stem cell (HSC) function, such as IFNα/β, IFNγ, IL-12, and TNF, are tightly controlled elements of cell expansion. Type I IFNs and TNF, induced by TLR signaling, can act upon myeloid progenitors to promote the expansion of granulocyte/monocyte progenitors (GMP), leading to systemic myeloid expansion (Buechler et al., 2013). Alternatively, excessive TNF signaling reduces myelopoiesis by inducing caspase-3/caspase-8-dependent progenitor cell apoptosis (Wei et al., 2014). Excessive TNF, TLR signaling, and deficiencies in negative regulation of NF-κB lead to apoptosis of HSCs and defects in myeloid progenitor function (Buechler et al., 2013; Stein & Baldwin, 2013).

We and others have shown that burn and radiation injuries lead to increased susceptibility to infection within survivors (Dugan et al., 2002; Mendoza et al., 2012; J. G. Noel et al., 2005). This is a pressing clinical problem in the face of nuclear accidents and possible incorporation of nuclear materials within explosives. This susceptibility has been attributed to a loss of inflammatory regulation, incomplete immune restoration and a systemic anti-inflammatory response following sepsis and shock (S. W. Jones et al., 2013; Neely et al., 2014; Neely et al., 2011). Following a radiation-thermal combined injury (RCI), an immature monocyte population (iMo) rapidly expands and predominates the periphery (Mendoza et al., 2012). Using this model, we observed that TNF is significantly increased in RCI compared to burn, radiation, and sham alone.

Given that NLRP12, which is known to suppress a number of cytokines, is present in bone marrow and myeloid cells (Baldridge et al., 2011; Stein & Baldwin, 2013), we tested NLRP12-mediated regulation of TNF signaling within the context of emergency myelopoiesis. Unexpectedly, we demonstrate that NLRP12-deficient mice are vulnerable to RCI due to decreased myelopoiesis.
2.3 Methods and Materials

*Mice and Combined Irradiation and Burn Injury procedure*

The *Nlrp12*<sup>−/−</sup>, *Caspase1/11*<sup>−/−</sup>, *Asc*<sup>−/−</sup> and *IL-1Ra*<sup>−/−</sup> mouse strains have been described (Arthur et al., 2007; Hirsch, Irikura, Paul, & Hirsh, 1996; Kuida et al., 1995; Takeda et al., 1998). All experiments were conducted with female mice housed under SPF conditions that were age-matched and backcrossed for at least nine generations onto the C57BL/6 background. All studies were conducted in accordance with the IACUC guidelines of the University of North Carolina at Chapel Hill and NIH Guidelines for the Care and Use of Laboratory Animals. Our model of RCI has been previously described; briefly, mice received a subcutaneous injection of morphine (3mg/kg body weight) for pain control immediately before burn injury. A full-thickness contact burn of 20% total body surface area (TBSA) was produced and within 1 hour, mice received a 5Gy (dose rate of 0.98 Gy/min) whole-body dose of ionizing radiation and were maintained on oral morphine for the duration of the experiment. Sham controls with 0% TBSA underwent all described interventions except for the burn and γ-irradiation exposure.

*Quantitative RT-PCR*

RNA was extracted from organ homogenates, suspended in TRIzol and isolated according to the manufacturer’s protocol (Life Technologies, Carlsbad, CA). qPCR was performed using the Verso 1-step RT-qPCR SYBR Green Fluorescein Kit (Thermo Fisher, San Jose, CA). The expression of mouse mRNA encoding NLRP12 and GAPDH was assessed using the SYBR kit and analyzed on an Applied Biosystems machine; results were normalized to expression of the gene encoding GAPDH and were quantified by the change-in-threshold method (ΔΔC<sub>T</sub>) using primers previously described (Arthur et al., 2010).
Histology

Mouse femurs were extracted and muscle and connect tissue were removed and initially preserved in 10% formalin. Femurs were then decalcified with Immunocal, water washed, and paraffin infused. Following sectioning and processing, sections were then stained by hematoxylin and eosin. Samples were processed using ImageJ to determine area of cell loss within each femur.

Pseudomonas aeruginosa infection

A wildtype strain (PAK) of *P. aeruginosa* was obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC). 10⁶ bacteria were then aerosolized intratracheally as described previously.

Serum Collection and Cytokine ELISA

Animals underwent a submandibular bleed and systemic cytokines were measured by single-plex ELISA (eBioscience, CA, USA or Biolegend, CA, USA) according to the manufacturer’s instructions or by Cytokine Mouse 20-Plex Panel (Life Technologies, Carlsbad, CA) on Luminex Bead Array technology.

Flow Cytometry

All fluorescence-conjugated FACS antibodies were purchased from BD Biosciences or Biolegend. Antibody panel used to identify neutrophils and macrophages are described in the figures. The antibody panel for monocyte and neutrophil analysis was comprised of CD11c-PerCPCy5.5, CD11b-PECy7, Ly6G-APC, Ly6G-PE, and F4/80-FITC. The antibody panel for progenitor analysis was comprised of CD3, CD8, NK1.1, CD19, CD45RA, TER-119 (Ly-76) as a lineage negative gate with all antibodies conjugated to FITC, CD127-PE/CF594, Sca1-APC,
cKit-BUV395, FcγR-BV605, CD34-Alexa647, and Annexin V-Pacific Blue. In each case, a million cells per organ were used for flow cytometric analysis.

**Intracellular Staining and Phospho-flow**

Intracellular staining was performed using a BD Bioscience Cytofix/Cytoperm kit. Antibodies used were TNF-PE (BD Biosciences), phosphor-p65 S528 (BD Biosciences), phospho-IκBa S32/536-eFlour 660 (eBiosciences), phospho-p38 ST180/Y182-PE and phospho-IKKα/β S176/180-PE (Cell Signaling Technologies). In each case, a million cells per organ were used for flow cytometric analysis.

**TNF-Depletion**

Immediately following combined irradiation and burn injury, mice were given 25mg/kg of rat IgG1, kappa anti-mouse TNF, clone MP6-XT3 or ratIgG1 isotype control (eBioscience, CA, USA) intraperitoneally dissolved in PBS (Sigma, CA, USA).

**Statistical Analysis**

Analysis was carried out with Prism 7.0 for Windows. All data are presented as the mean +/- standard error of the mean (SEM). Complex data sets were analyzed by analysis of variance (ANOVA) with a Tukey-Kramer post-test HSD for multiple comparisons. Single data points were assessed by the Student’s two-tailed t test. The product limit method of Kaplan-Meier was utilized for generating the survival curves, which were compared using the log rank test. A p value less than 0.05 was considered statistically significant for all data sets.

**2.4 Results**

**NLRP12 limits morbidity and mortality following RCI**

Previous work has implicated NLRP12 in suppression of canonical and non-canonical NF-κB, a key driver of inflammatory cytokine signaling (I. C. Allen et al., 2012; Arthur et al.,
2010; Moore et al., 2007; Vladimer et al., 2012; Zaki et al., 2014; Zaki et al., 2011). We therefore investigated whether NLRP12 was acting to limit excessive inflammatory signaling and consequently promote peripheral immune reconstitution in our model of emergency myelopoiesis.

Wild type and Nlrp12<sup>−/−</sup> mice received a 20% TBSA burn and were irradiated with 5-Gy of γ-irradiation within an hour of burn injury. In wild type mice, we observed elevated NLRP12 expression in spleen, bone marrow and lung tissues early (3, 7, and 14 days post-injury) after RCI (Figure 2.1A) compared to burn or radiation alone and sham controls. Mortality among NLRP12-deficient mice was significantly elevated following RCI, but not following burn or radiation alone (Figure 2.1B). While RCI-wild type animals lost weight initially, they were able to return to a baseline weight by seven days after injury and exceed their baseline weight by 14 days post injury; RCI-Nrlp12<sup>−/−</sup> animals lost more weight and did not fully recover weight in comparison to wild type animals (Figure 2.1C). These data suggest that NLRP12 protected against morbidity after RCI.

**Splenic and pulmonary immune repopulation is impaired following RCI in Nlrp12<sup>−/−</sup> mice**

During events that induce enhanced myelopoiesis and inflammation, specifically RCI, we have shown that immature monocytes with high granularity comprise the majority of the peripheral immune system (Mendoza et al., 2012). We examined the splenic compartment in the Nlrp12<sup>−/−</sup> mice after RCI. NLRP12 deficiency resulted in a significant decrease in the total number of splenocytes by 14 days post-injury (Figure 2.2A). Using flow cytometry, with representative staining in Figure 2.2B, we observed a decreased number of splenic neutrophils (CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> F4/80<sup>+</sup>) and immature monocytes (iMOS; CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>hi</sup>F4/80<sup>hi</sup>) post-injury in Nlrp12<sup>−/−</sup> mice (Figure 2.2C). We also investigated the contribution of NLRP12 to
the repopulation of lung immune cells—common sites of opportunistic infection in burn patients—after RCI. *Nlrp12*−/− mice displayed a reduced ability to repopulate the lung after RCI. This inability was characterized by a decrease in total CD45+ leukocytes and by the absence of the immature monocyte accumulation normally observed following RCI at two weeks post-injury (Figure 2.3A-B). There were no differences in macrophage (CD11b+ Ly6C+Ly6GloF4/80hi) accumulation in *Nlrp12*−/− mice when compared to wild type (Figure 2.4A). The total number of pulmonary macrophages, B and T cells were similar in *Nlrp12*−/− and wild type mice (Figure 2.4B-D). These data implicate a role for NLRP12 in regulating emergency hematopoiesis following RCI.

**Nlrp12−/− mice show decreased bone marrow and peripheral cell numbers following RCI**

NLRP12 has been shown to be expressed constitutively in bone marrow cells (Arthur et al., 2010; Savic et al., 2012; Vitale et al., 2013; Zaki et al., 2014). We hypothesized that reduced immune repopulation in the periphery of injured *Nlrp12*+/− mice was due to reduced cell generation and output by the bone marrow. To test this, we investigated the impact of NLRP12 deficiency on bone marrow populations after RCI. *Nlrp12*−/− mice had reduced total bone marrow cells compared to wild type mice after RCI. We also observed a decrease in total iMO and neutrophils (Figure 2.3C) within the bone marrow of *Nlrp12*−/− mice as early as seven days post injury compared to wild type mice. Additionally, we observe decreases in the total numbers of monocytes and neutrophils in the peripheral blood (Figure 2.3D). These data suggest that peripheral immune repopulation defects after RCI are likely attributed to decreased bone marrow cell numbers, which appear to be regulated by NLRP12.
Defects in myelopoiesis following RCI are not observed in inflammasome-deficient animals

NLRP12 is also found to form an inflammasome complex or regulate caspase-1 activity (I. C. Allen et al., 2012; Vladimer et al., 2012; Ye et al., 2008) and regulates IL-1β processing by complexing with ASC during infection with Yersinia or malaria (Vladimer et al., 2012). To examine whether NLRP12 is important for inflammasome activation following RCI, we assessed IL-1β levels after RCI in wild type or Nlrp12/- mice. There were no detectable levels of IL-1β at any time point measured (Figure 2.5A) nor was there any differences in serum IL-18 (data not shown). Due to inability to capture IL-1β levels in serum because of its high turnover, we examined the role of the inflammasome in RCI. We applied the RCI model to various mice strains lacking key components of genes encoding proteins that encode common shared components of the inflammasome. These include Caspase1/11/- which lacks both canonical and noncanonical inflammasome caspases, Asc/- which lacks the common adaptor shared by multiple inflammasome NLRs and AIM2, or Il1r/- which lacks the IL-1 receptor protein. Following RCI, Caspase1/11/-, Asc/- and Il1r/- mice had a similar immune repopulation in the lung and spleen as wild type mice (Figure 2.5B-C). We also saw no significant differences in bone marrow populations in Caspase-1/11/-, Asc/- or Il1r/- mice following RCI (Figure 2.5D). Additionally, injured Caspase-1/11/-, Asc/- or Il1r/- animals did not show an increase in mortality compared to wild type (Figure 2.5E). Together, these results suggest that NLRP12 controls myelopoiesis in an inflammasome-independent pathway.

Nlrp12/- mice display increased serum TNF, IL-6 and IL-12 cytokine and bone marrow TNF-receptor expression

Cytokines that are attenuated by NLRP12, in particular TNF, have been shown to enhance hematopoietic stem cell (HSC) expansion (Gardner et al., 2014; Manz & Boettcher, 2014; Stein & Baldwin, 2013; Toliver-Kinsky et al., 2003). We therefore examined NLRP12-
dependent production of selected cytokines and their receptors after RCI. In wild type animals, serum TNF expression increased early following injury and declined over time (Mendoza et al., 2012). In sham-treated Nlrp12-/- animals, the TNF level was similar to sham wildtype controls. However, during RCI in Nlrp12-/- animals, the TNF increased initially and was maintained over time – a significant elevation when compared to wild type mice (Figure 2.6A). In addition, Nlrp12-/- bone marrow cells displayed increased TNFR expression (Figure 2.6B) as well as CD40 and RANK (Figure 2.6C). Using intracellular straining, we observed that monocyte production of TNF after RCI is increased when compared to burn and radiation controls; however, in the absence of NLRP12, monocyte production of TNF is significantly elevated compared to wild type controls (Figure 2.6D). As well as TNF, other cytokines such as IL-6, IL-12, IFNα, and IFNγ were increased in Nlrp12-/- mice compared to wild type mice but less so than TNF (Figure 2.6A). This elevation is potentially derived from the initial shock and the selective apoptotic environment induced by the absence of NLRP12 and necessary myelopoiesis. Heightened levels of IL-6 following trauma have been shown to be the major predictor of poor outcome (bacterial infection) following a traumatic injury (Gebhard, Pfetsch, Steinbach, & Strecker, 2000). Other cytokines and growth factors (IL-4, IL-10, and GM-CSF) were measured but showed no significant differences between wild type and Nlrp12-/- animals (Figure 2.6B).

**IκBa activity is increased in CD34+ cells Nlrp12-/- animals after RCI**

Both the canonical and non-canonical pathways of NFκB have been shown to be negatively regulated by NLRP12 (I. C. Allen et al., 2012; Ataide et al., 2014; Krauss et al., 2015). We therefore examined NLRP12-dependent activation of key regulators of each pathway. No changes were seen in phosphorylation levels in sham, burn, or radiation controls; however, RCI NLRP12-deficient animals showed greater levels of pIκBα as well as pp65 (Figure 2.6D).
Increased phosphorylation is indicative of increased canonical NFκB signaling in the absence of NLRP12. However, pIKKa/β and the downstream p38/MAPK showed no changes in activity when comparing wild type to Nlrp12−/− injured animals. Taken with the increased TNFR expression on marrow cells, these results suggest that NLRP12 is negatively regulating the canonical NFκB signaling cascade.

**RCI of Nlrp12−/− animals leads to increased granulocyte/monocyte progenitor apoptosis**

We observed that NLRP12 regulates reconstitution of granulocytic and monocytic bone marrow and peripheral cells in Nlrp12−/− mice following RCI. We therefore hypothesized that NLRP12 regulates bone marrow granulocyte/monocyte progenitors (GMP), the source of granulocytes and immature monocytes. To test this, we utilized flow cytometric analysis to evaluate the number of GMP (Lin− IL7R− Sca1− cKit+ FcγRhi CD34+) in Nlrp12−/− mice following RCI.

We detected a similar number of bone marrow GMP in Nlrp12−/− and wild type mice at 3 days after injury. However, at 7 and 14 days after injury, wild type GMP expanded and increased in numbers while Nlrp12−/−GMP expansion was attenuated (Figure 2.8A). There were no measured differences in lymphoid lineage progenitors (Figure 2.9A). We tested the hypothesis that the significant decrease in GMP in Nlrp12−/− mice is due to increased apoptosis. To distinguish apoptotic cells from necrotic cells, 7-AAD and Annexin V staining was performed. While sham control revealed no difference in WT and Nlrp12−/− mice, a significant percent of Nlrp12−/− GMP underwent apoptosis compared to wild type GMP during RCI. This increase in apoptosis was detected as early as 3-days post injury (Figure 2.8B). Representative flow gating is shown in Figure 2.7C.
Increased apoptosis and decreased bone marrow cellularity was confirmed by histological staining. H&E femur sections were obtained at 14 days post injury. There were no histological changes from wild type to Nlrp12-/- mice in sham, burn, or radiation alone animals. However, RCI-Nlrp12-/- mice displayed medial patches of cell loss within the femurs, which was not present in RCI-wild type femurs (Figure 2.8D). Collectively, our findings imply that NLRP12 prevents progenitor cell apoptosis, thus allowing myelopoiesis and peripheral immune cell reconstitution to occur in wild type animals.

Leukopenia can have complex etiologies in both inflammatory and non-inflammatory conditions, many of which involve alterations in HSC steady-state hematopoiesis (T. D. Jones, Morris, Young, & Kehlet, 1993; Serafini et al., 2007; Toliver-Kinsky et al., 2003). Our data show that NLRP12 limits TNF following RCI, resulting in expansion of myeloid precursors and monocyte populations throughout the periphery. Previous studies showed increased hematopoiesis following total body irradiation; however, our results are novel because we have shown that NLRP12 promotes hematopoiesis of specific lineages during RCI (Baldridge et al., 2011; Mendoza et al., 2012).

**Ant-TNF antibody administration prevents NLRP12-associated GMP apoptosis after combined injury**

After observing significantly elevated levels of TNF and reduced myelopoiesis in injured Nlrp12-/- mice, we hypothesized that increased levels of TNF were leading to pathology through TNF-mediated apoptosis of immune progenitor cells as seen in other models of excessive TNF (Micheau & Tschopp, 2003). Specifically, we hypothesized that GMP were undergoing TNF-mediated apoptosis with reduced peripheral neutrophil and inflammatory monocyte accumulation. To test this, wild type and Nlrp12-/- mice received a single administration of anti-TNF or isotype control antibody immediately following RCI.
We observed significantly fewer GMP in the Nlrp12/- mice given the isotype control compared to wild type mice. However, Nlrp12/- mice given the anti-TNF antibody had similar numbers of GMP compared to isotype and anti-TNF treated wild type mice (Figure 2.9A). Additionally, the proportion of GMP actively undergoing apoptosis was higher in the Nlrp12/- isotype treated animals compared to Nlrp12/- mice treated with anti-TNF (Figure 2.9B). This is correlated with a decrease in the total CD45+ pulmonary cells as well as pulmonary iMO (Figure 2.9C-D). These data indicate that in the absence of NLRP12, TNF mediates the enhanced bone marrow death during RCI and resultant incomplete restoration of the peripheral immune system.

**Nlrp12/- mice lack control of pulmonary infection following radiation-thermal combined injury**

In a clinical setting, patients that are able to survive initial shock from a burn or radiation-thermal-combined injury will often succumb to a pulmonary infection associated with the prolonged hospital stay (Moore et al., 2007). We sought to evaluate the role NLRP12 deficiency plays in a clinically relevant model of a lung infection following injury. Wild type and Nlrp12/- animals were subjected to either sham, burn, or radiation only, and RCI. Mice were then sustained for two weeks in individual housing wherein they were infected intratracheally with 1x10⁶ CFU of *Pseudomonas aeruginosa* (PAK).

RCI-Nlrp12/- infected animals displayed a significant increase in mortality, with animals starting to succumb to infection after as few as 12 hours. All Nlrp12/- animals became moribund within 36 to 48 hours after infection (Figure 2.10A). Lung and liver from infected animals that survived until 48 hours post infection were collected and plated to enumerate bacterial load locally and systemically. RCI-Nlrp12/- mice lungs and liver showed a 10-fold increase in bacteria compared to injured, wild type animals (Figure 2.10B). These data suggest that NLRP12 plays a vital role in response to an infection insult following a traumatic injury.
We next sought to determine the immune response to infection following RCI. Nlrp12-/mice showed a decrease in innate, pulmonary immune cell populations following RCI and infection (Figure 2.10C), leading us to conclude that NLRP12 results in increased hematopoietic recovery which is likely crucial to the effective control of infection after traumatic injury.

2.5 Conclusions

Our study demonstrates that NLRP12 suppresses TNF signaling in vivo during inflammation-induced emergency myelopoiesis. Most importantly, our research indicates a role for NLRP12 in hematopoietic progenitor cells by limiting TNF-induced apoptosis of these cells. TNF inflammation initiated by RCI without NLRP12 leads to the apoptosis of progenitor cells and a defective peripheral immune reconstitution, associated with increased mortality and inability to control an infectious challenge.

In addition to inhibiting inflammation, defects in NF-κB signaling lead to weakened hematopoiesis (Stein & Baldwin, 2013). There is no single mechanism that has been defined for immune suppression in the response to traumatic injury, but hematopoietic stem cell (HSC) expansion and immune repopulation have been shown to be important factors (Gardner et al., 2014; T. D. Jones et al., 1993; Manz & Boettcher, 2014; Stein & Baldwin, 2013; Toliver-Kinsky et al., 2003). We propose that NLRP12 suppression of immune signaling pathways leading to attenuated cytokines contributes to homeostatic proliferation of granulocytes and monocytes following induction of severe leukopenia. Moreover, this NLRP12-mediated suppression limits overt TNF-induced inflammation that could lead to HSC apoptosis by limiting canonical NFκB signaling. Our findings add to the studies that suggest that NLRP12 acts as a cellular rheostat to limit inflammation, and is emerging as a “checkpoint” or inhibitor (I. C. Allen et al., 2012; Krauss et al., 2015) of canonical NFκB signaling. This immune inhibition is mediated, at least in
part, by suppression of the non-canonical NFκB pathway and cross talk with the canonical pathway (I. C. Allen et al., 2012; Arthur et al., 2007; Lich & Ting, 2007; Lich et al., 2007). NLRP12-mediated NFκB suppression likely limits TNF and cellular death during inflammation and hematopoiesis. Our data in NLRP12-deficient mice shows compromised hematopoiesis due to enhanced TNF production, leading to flagrant HSC/GMP apoptosis. This lack of HSC function leads to global leukopenia and correlates with increased mortality compared to wild type mice. These studies would be applicable in instances of increased myelopoiesis, TNF-driven inflammation, and induced apoptosis.
Figure 2.1. NLRP12 expression is increased after combined injury, and acts to limit mortality and weight loss. Wildtype C57BL/6 mice were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI). (A) mRNA was isolated from spleen, bone marrow, and whole lung at 3, 7, and 14 days post injury. Relative Nlrp12 expression was determined by qRT-PCR. (n=6/timepoint). Wildtype C57BL/6 or Nlrp12-/- mice were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or RCI. (B) Survival and (C) weight loss were quantified. Data represented as mean ± SEM, with statistical significance compared to sham defined as *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test, with experiments performed in triplicate.
Figure 2.2. NLRP12 regulates peripheral immune repopulation after combined injury. Wildtype C57BL/6 or Nlrp12-/- mice were subjected to sham or combined radiation and burn injury (RCI). Spleens were harvested 3, 7 and 14 days post injury and the total number of (A) splenocytes, neutrophils (CD11b+ Ly6Cint Ly6G+ F4/80-) and immature monocytes (iMOS; CD11b+ Ly6C+ Ly6Ghi F4/80hi) were quantified by flow cytometry analysis, (B) representative flow cytometric gating from an RCI mouse after gating on CD45+ and F4/80 expression level). Data represented as mean ± SEM, with statistical significance defined as *, p<0.05 and **, p<0.005 by Student’s t test with n=10 mice per group, with experiments performed in triplicate.
Figure 2.3. NLRP12 regulates pulmonary immune repopulation and bone marrow cell numbers after combined injury. Wildtype C57BL/6 or Nlrp12−/− mice were subjected to sham or combined radiation and burn injury (RCI). Lungs were harvested 14 days post injury and the total number of (A) CD45+ cells, (B) neutrophils (CD11b+ Ly6Cint Ly6G+ F4/80−) and immature monocytes (iMOs; CD11b+ Ly6C+ Ly6Ghi F4/80hi) were quantified by flow cytometry analysis. Wildtype C57BL/6 or Nlrp12−/− mice were subjected to sham or combined radiation and burn injury (RCI). Bone marrow from femurs and tibias and blood from a cheek bleed were harvested 3, 7 and 14 days post injury and the total number of (C) bone marrow cells, immature monocytes (iMOs; CD11b+ Ly6C+ Ly6Ghi F4/80hi), neutrophils (CD11b+ Ly6Cint Ly6Ghi F4/80hi) from the bone marrow and platelets (CD62P+TER119−), monocyte and neutrophils from blood were quantified by flow cytometry analysis. Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test with n=6 mice per group, with experiments performed in triplicate.
Figure 2.4. NLRP12 deficiency does not result in macrophage, B or T cell changes after injury. Wildtype C57BL/6 or Nlrp12-/- mice were subjected to sham, burn, radiation, or combined radiation and burn injury (RCI). Spleens were harvested at 14 days post injury and the total number of (A) splenic and (B) pulmonary macrophages (CD11b+ Ly6C+ Ly6G F4/80\textsuperscript{hi}), and (C) pulmonary B and (D) T cells were quantified by flow cytometry analysis. Data represented as mean ± SEM, with statistical significance defined as *, p<0.05 and **, p<0.005 by Student’s t test with n=4 mice per group, with experiments performed in triplicate.
Figure 2.5. Defects in myelopoiesis following RCI are not observed in inflammasome-deficient animals. Caspase1/11−/−, adaptor Asc−/−, or receptor Il1r−/− mouse strains were subjected to sham or combined radiation and burn injury (RCI). After 14 days we quantified the (A) serum levels of IL-1β by ELISA total number of (B) splenocytes, (C) pulmonary CD45+ cells and (D) total bone marrow cells by flow cytometry (E) survival of mice after injury.
Figure 2.6. Nlrp12−/− animals have increased serum cytokine and bone marrow receptor expression following combined injury. Wildtype C57BL/6 or Nlrp12−/− mice were subjected to sham or combined radiation and burn injury (RCI). The concentration of (A) TNF was quantified using ELISA in serum 3, 7, and 14 days post injury. We also analyzed mean fluorescent intensity of (B) TNFR, CD40, and RANK on bone marrow cells harvested at 14 days post injury using flow cytometry. (D) The percentage of TNF producing iMos was determined using intracellular staining and flow cytometry. (E) The level of phospho-IκBα, phospho-IKKα/β, phosphor-p65, and phospho-p38 was quantified using intracellular staining and flow cytometry. Data represented as mean ± SEM, with statistical significance defined as ** p<0.005 by Student’s t test with n=5 mice per group, with experiments performed in triplicate.
Figure 2.7. NLRP12 regulates serum cytokines. Wildtype C57BL/6 or Nlrp12−/− mice were subjected to sham or combined radiation and burn injury (RCI). (A) Serum was collected at days 3, 7, 14 and IFNα, IFNγ, IL-6 and IL-12p40 were quantified by ELISA. (B) Serum was collected at day 14 and IL-4, IL-10, and GM-CSF were quantified by ELISA. Data represented as mean ± SEM, with statistical significance defined as *, p<0.05 and **, p<0.005 by Student’s t test with n=6 mice per group, with experiments performed in triplicate.
Figure 2.8 *Nlrp12/-* animals have increased granulocyte/monocyte progenitor apoptosis after combined injury. Wildtype C57BL/6 or *Nlrp12/-* mice were subjected to sham or combined radiation and burn injury (RCI). Bone marrow was collected from wild type and *Nlrp12/-* mice at 3, 7, and 14 days post RCI or sham treatment (n=6/group). Using flow cytometric analysis, (A) the total number of bone marrow Granulocyte/Monocyte Progenitors (GMP, Lin- IL7R- Sca1- ckit+ FcγRhi CD34+) and (B) the percentage of GMP cells undergoing apoptosis was determined by positive Annexin V staining in the absence of 7-AAD-staining cells; representative flow staining from *Nlrp12/-* mice is shown in (C). Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test with n=5 mice per group. In separate experiments, wildtype C57BL/6 or *Nlrp12/-* mice were subjected to sham, irradiation, burn or RCI. Femurs were collected 14 days post injury and H&E staining performed for histological analysis. (D) shows representative sections from each group, with areas of cell death marked by white arrow (white bar represents 25um); (E) quantification of cell death area was performed using ImageJ, with experiments performed in triplicate.
Figure 2.9. NLRP12 deficiency results in no changes in lymphoid progenitors. Wildtype C57BL/6 or Nlrp12-/- mice were subjected to sham or combined radiation and burn injury (RCI). Bone marrow was harvested at day 14 and (A) common lymphoid progenitors (Flk2+Lin- IL7R+ ckit+ Sca1+) were quantified by flow cytometric analysis. Data represented as mean ± SEM, with n=5 mice per group, with experiments performed in triplicate.
Figure 2.10. Anti-TNF antibody administration prevents NLRP12-associated GMP apoptosis after combined injury. Wild type and Nlrp12-/- C57/BL6 mice received a single administration of anti-TNF or isotype control antibody immediately following combined radiation and burn injury (RCI). We harvested bone marrow and lung from these mice 14 days after injury. We quantified (A) the total number of bone marrow Granulocyte/Monocyte Progenitors (GMP, Lin- IL7R- Sca1- ckit+ FcγRhi CD34+) and (B) the percentage of GMP cells undergoing apoptosis by 7-AAD- Annexin V+ staining by flow cytometry. We measured (C) the total number of pulmonary CD45+ cells and immature monocytes (iMOS; CD11b+ Ly6C+ Ly6Ghi F4/80hi) by flow cytometry analysis. Data represented as mean ± SEM, with statistical significance defined as *, p<0.05 by Student’s t test with n=5 mice per group, with experiments performed in triplicate.
Figure 2.11 Nlrp12−/− mice lack control of pulmonary infection following combined injury. Wildtype C57BL/6 or Nlrp12−/− mice were subjected to sham or combined radiation and burn injury (RCI). Mice were inoculated 14 days post-injury intratracheally with 1x10⁶ CFU of Pseudomonas aeruginosa (PAK). We quantified (A) survival, (B) bacterial load within lungs and liver by culture, and (C) number of splenic CD45+ cells, neutrophils (CD11b+ Ly6C<sup>−</sup> Ly6G<sup>+</sup> F4/80<sup>−</sup>) and immature monocytes (iMOs; CD11b+ Ly6C<sup>+</sup> Ly6G<sup>+</sup>F4/80<sup>hi</sup>) harvested two days after inoculation. Data represented as mean ± SEM, with statistical significance defined as *, p<0.05; **, p<0.05 by Student’s t test with n=6 mice per group (3 for burn and radiation only), with experiments performed in triplicate.
CHAPTER 3: DELETION OF NLRP12 IMPARES INNATE IMMUNE RESPONSES DURING RADIATION- THERMAL COMBINED INJURY AND SHOCK

3.1 Summary

During a traumatic event that induces emergency myelopoiesis, innate immune signaling helps to control how inflammatory cells respond and what new immune cells the hematopoietic system will produce. The severe immune impairment after a radiation thermal combined injury is exacerbated in the absence of NLRP12 as NLRP12 has been shown to play a role in limiting TNF-induced apoptosis of progenitors. Using a bone marrow chimera, a mixture of wild type and Nlrp12/- cells, we show that NLRP12 acts intrinsically within the bone marrow progenitor population to directly limit induction of TNF and to limit TNF- and inflammation-induced apoptosis of progenitor cells after a radiation-thermal combined injury. Additionally, that excessive TNF signaling leads to further defects in innate immune signaling, by altering TLR signaling. Furthermore, TNF administration without further injury is sufficient to induce progenitor apoptosis. Taken together, these data show the NLRP12 acts as an intrinsic regulator of innate inflammation with progenitors during emergency hematopoiesis.

3.2 Introduction

The innate immune system is the primary responder and major source of dysfunction after injury (Manz & Boettcher, 2014; Mendoza et al., 2012; Vieira-de-Abreu et al., 2012). The absence of NLRP12 leads increased production of TNF and a subsequent increased in TNF-induced apoptosis of bone marrow progenitors, specifically granulocyte monocyte progenitor cells, the cells responsible for the production of the cell type the predominates the immune
system after RCI – as shown in Chapter 1. This increased TNF and reduction of peripheral innate immune cells leads to an increased susceptibility to infection. Several open questions remain regarding NLRP12: Is NLRP12 acting within the hematopoietic compartment to limit inflammation, or acting with peripheral immune cells to reduce TNF production? Is NLRP12 impacting other inflammatory pathways? Can the increased apoptosis of progenitors be seen in other models of shock?

Use of bone marrow chimeras has been an integral part of studying basic immunology using mouse models(Kuida et al., 1995; Onoe, Fernandes, & Good, 1980). This allows scientist to differentiate between affects non-hematologic cells play and those from the bone marrow compartment. More importantly, chimeras allow to determine the affect an immune environment plays on two different cells (i.e. Wt and Nlrp12-/-progenitors) after injury.

TNFR1 ligation of TNF signals through p65/NFkB and induces production of pro-inflammatory cytokines like IL-6, pro-IL1β, chemoattractants, and further promotes cell survival(Rothe, Pan, Henzel, Ayres, & Goeddel, 1995; Wicovsky et al., 2009). Contrastingly, the TNFR2 variant signals through the Fas-associated protein with a death domain (FADD) adaptor protein to engage Caspases 3 and 8 and causes the cell to undergo apoptosis(Rothe et al., 1995; F. Wang et al., 2006).

TLRs are responsible for conducting and initiating inflammatory responses as pattern recognition receptors to respond to bacterial, fungal, and viral ligands(B. A. Cairns et al., 2008). These signaling prime and activate innate immune cells to create an anti-microbial environment and cellular phenotype that will leads to the eventual clearance of the pathogen. However, increased signaling can lead to immune exhaustion, tissue immunopathology, and that inability to fight an infection.
Given that NLRP12 suppresses apoptosis of GMPs during emergency hematopoiesis, we sought to determine NLRP12 impact on innate immune signaling and reconstitution using RCI and other models of shock. We demonstrate that NLRP12 works intrinsically within the bone marrow compartment and works to limit pro-inflammatory responses during an infection and that a model of TNF-shock can replicate the apoptosis phenotype seen in Nlrp12−/− animals after RCI.

3.3 Methods and Materials

Mice and Combined Irradiation and Burn Injury procedure

The Wt and Nlrp12+/− C57/B6 mouse strains have been described (Arthur et al., 2010; Honda et al., 2005; Kaisho & Akira, 2001). All experiments were conducted with female mice housed under SPF conditions that were age-matched and backcrossed for at least nine generations onto the C57BL/6 background. All studies were conducted in accordance with the IACUC guidelines of the University of North Carolina at Chapel Hill and NIH Guidelines for the Care and Use of Laboratory Animals. Our model of RCI has been previously described; briefly, mice received a subcutaneous injection of morphine (3mg/kg body weight) for pain control immediately before burn injury. A full-thickness contact burn of 20% total body surface area (TBSA) was produced and within 1 hour, mice received a 5Gy (dose rate of 0.98 Gy/min) whole-body dose of ionizing radiation and were maintained on oral morphine for the duration of the experiment. Sham controls with 0% TBSA underwent all described interventions except for the burn and γ-irradiation exposure.

Murine Chimeras

4-6 week old, Wt C57/B6.SJL mice (B6 CD45.1, Pep Boy), a congenic strain of mice who express a differential Ptprca pan leukocyte marker known as CD45.1. These mice differ
from C57/B6 mice as they express the canonical CD45.1 marker (Mercier, Sykes, & Scadden, 2016). Wt CD45.1 and Nlrp12/- CD45.2 mice were given a lethal dose of γ-irradiation from a Cs137 source (Stanford and Associate, CA, USA) as previously described. Mice were then maintained for 24 hours after irradiation and then given an adoptive transfer of Wt CD45.1 and Nlrp12/- CD45.2 bone marrow (10^7 cells, intravenously via tail vein). Mice were allowed to recover from radiation and bone marrow transplant for 4-6 weeks and then underwent burn and radiation procedures as described above.

**Pseudomonas aeruginosa infection**

A wildtype strain (PAK) of *P. aeruginosa* was obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC). 10^6 bacteria were then aerosolized intratracheally as described previously.

**Serum Collection and Cytokine ELISA**

Animals underwent a submandibular bleed and systemic cytokines were measured by single-plex ELISA (Biolegend, CA, USA) according to the manufacturer’s instructions.

**Flow Cytometry**

All fluorescence-conjugated FACS antibodies were purchased from BD Biosciences or Biolegend. Antibody panel used to identify neutrophils and macrophages are described in the figures. The antibody panel for monocyte and neutrophil analysis was comprised of CD11c-PerPCy5.5, CD11b-PECy7, Ly6G-APC, Ly6G-PE, and F4/80-FITC. The antibody panel for progenitor analysis was comprised of CD3, CD8, NK1.1, CD19, CD45RA, TER-119 (Ly-76) as a lineage negative gate with all antibodies conjugated to FITC, CD127-PE/CF594, Sca1-APC, cKit- BVU395, FcγR-BV605, CD34-Alexa647, and Annexin V-Pacific Blue. CD45.2-BV630 was used to distinguish between CD45.2 and CD45.1 transplanted cells. TLR2-PE, TLR4-PE,
TLR5-PE, and TLR9-PE were used to determine TLR expression on monocytes. In each case, a million cells per organ were used for flow cytometric analysis.

**Intracellular Staining**

Intracellular staining was performed using a BD Bioscience Cytofix/Cytoperm kit. Antibodies used were TNF-PE (BD Biosciences). In each case, a million cells per organ were used for flow cytometric analysis.

**TNF-Induced Shock**

Wt and Nlrp12/- C57/B6 mice were given 50 or 100ug of murine recombinant-TNF (R&D System, CA, USA) intravenously via tail vein injection suspended in PBS. Mice were monitored for weight and temperature changes after induction of shock.

**LPS- and PolyIC-induced shock**

Wt and Nlrp12/- C57/B6 mice were given 50ug of LPS or 100ug of PolyIC (R&D systems, CA, USA) intravenously via tail vein suspended in PBS. Animals were monitored for signs of disease after LPS or PolyIC administration.

**Statistical Analysis**

Analysis was carried out with Prism 7.0 for Windows. All data are presented as the mean +/- standard error of the mean (SEM). Complex data sets were analyzed by analysis of variance (ANOVA) with a Tukey-Kramer post-test HSD for multiple comparisons. Single data points were assessed by the Student’s two-tailed t test. The product limit method of Kaplan-Meier was utilized for generating the survival curves, which were compared using the log rank test. A p value less than 0.05 was considered statistically significant for all data sets.
3.4 Results

Adoptive transfer of chimera bone marrow (Wt:Nlrp12-/−) reveals intrinsic defect in Nlrp12-/− GMP apoptosis signaling

Following a radiation-thermal combined injury, Nlrp12-/− deficient animals show decreased peripheral innate immune cell repopulation that is attributed to dysregulation of TNF, resulting in increased TNF-induced apoptosis of myeloid progenitor cells. This data, however, does not address whether NLRP12 is acting within the remaining peripheral immune cells in which more TNF is produced or whether Nlrp12-/− myeloid cells are more susceptible to the basal level of TNF to undergo apoptosis. In order to investigate this, Wild type and Nlrp12-/− mice were irradiated to destroy their native bone marrow, and transplanted with a 1:1 mixture of CD45.2 marker Wild type bone marrow cells and CD45.1 marked Nlrp12-/− bone marrow cells resulting in a Wt:Nlrp12-/− bone marrow chimera. After allowing transplant recipient animals immune systems to reach homeostasis, animals either underwent sham or RCI procedures. No changes in TNF production in Wt or Nlrp12-/− recipient animals was observed (Figure 3.1A). This supports previous evidence that NLRP12 does not act from epithelial cells, but rather from myeloid cells – of which the two recipients are identical after transplant. However, when comparing the ratio of Wt:Nlrp12-/− granulocyte monocyte progenitors undergoing apoptosis (competitive index, normalized to the input ratio), we observed that nearly two-fold more Nlrp12-/− GMPs were actively undergoing programmed cell death than Wild type GMPs (Figure 3.1B). Additionally, we observed no significant increase in the total number of GMPs from Wt to Nlrp12-/− recipients (Figure 3.1C), again suggesting that defects due to NLRP12 deficiency is arising within the bone marrow compartment. Moreover, when comparing the ratio of Wt GMPs to Nlrp12-/− GMPs, we see that by two weeks after injury, Wt GMPs outnumber Nlrp12-/− GMPs two-fold (Figure 3.1D). Taken together, these data suggest that NLRP12 works intrinsically, on a
single cell basis, from the bone marrow compartment to limit TNF-mediated inflammation and apoptosis.

**NLRP12 deficiency leads to higher TNFR1 but not TNFR2 on GMPs after RCI**

In Chapter 2, we showed that TNFR expression was increased on bone marrow cells in *Nlrp12/-* animals after injury. However, the antibody used recognized both variants of the TNF-receptor (TNFR1 and TNFR2). We therefore examined whether there were differences in expression of TNFR1 and TNFR2 on GMPs following RCI. We see that after 14 days post injury, GMPs express high levels of the pro-apoptotic CD120b (TNFR2) compared to Wild type, RCI animals (Figure 3.2A); whereas, there are no differences from *Nlrp12/-* and Wild type expression of the pro-inflammatory CD120a (TNFR1) after RCI (Figure 3.2B). This data furthers the assertion that TNF is species responsible for the increased apoptosis of bone marrow progenitor after RCI, specifically through induction of TNFR2.

**RCI-NLRP12 Have Greater M1 Balance and More Polarization following Infection AND NLRP12 Controls TLR Expression following RCI on iMos**

The ratio of the anti-inflammatory cytokine IL-10 compared to the pro-inflammatory IL-12 can be used as a clinical indicator of injury severity and gives an impression of the M1/M2 axis of macrophage polarization after injury. We therefor sought to determine whether NLRP12-deficiency would alter pulmonary macrophages responses when trying to fight a pulmonary infection. RCI animals were infected with *P. aeruginosa* two weeks after injury via aerosolization. Whereas Wt animals had a reduction in the cells actively producing either IL-10 and IL-12 after infection, displaying a reduction in responses to infection to limit immunopathology, *Nlrp12/-* animals showed higher M1 skewing after injury alone and an increase in both IL-10 producing M2s and IL-12 producing M1s (Figure 3.3A). Moreover, *Nlrp12/-* macrophages expressed higher levels off all TLRs examined, TLR2, TLR4, TLR5, and
TLR9 when compared to Wild type-infected macrophages (Figure 3.3B). This data leads us to conclude that NLRP12’s role in limiting inflammation may be more widespread than just TNF-induction of apoptosis.

**Wild type animals are less susceptible to TNF-shock compared to Nlrp12-/- animals**

Following RCI, we observe an initial spike of TNF which wains within days following injury. However, in Nlrp12-/- animals this spike persists and wreaks havoc on the hematopoietic system. Despite our attributing TNF as responsible for the increased apoptosis seen in the bone marrow compartment of Nlrp12-/- animals, burn and radiation injuries are complex injuries, resulting in numerous physiological and immunological impairments that make studying more difficult, but nonetheless pressing for study. We therefore chose to examine whether the decreased resistance to apoptosis could be replicated in a model of TNF-shock. No appreciable differences in mortality were observed in Wt and Nlrp12-/- animals given 50ug of recombinant-TNF (Figure 3.4A); however, Nlrp12-/- mice given 100ug of TNF exhibited higher mortality than Wt animals with all animals succumbing to shock by 15 hours post injection (Figure 3.4A) and Nlrp12-/- mice lost more weight than Wt mice (Figure 3.4B). This increase in mortality was correlated with a significant decrease in the number of GMPs present in the bone marrow (Figure 3.4C) when bone marrow aspirates were collected at 8 hours post injection. Moreover, at this time, Nlrp12-/- TNF-treated animals showed an increase in both IL-6 and IL-12, in addition to an increase in the anti-inflammatory IL-10 (Figure 3.4D). Taken together, this data shows the clinical relevance of RCI to study basic immunology as well as supporting NLRP12 control of TNF-induced apoptosis within the hematopoietic compartment during a traumatic event.
**LPS, but not PolyIC, induces granulocyte monocyte progenitor expansion**

Administration of LPS to Wild type animals induces expansion of the GMP population, however this increase is not seen in Nlrp12/- animals (3.5A). However, when further staining the cells with Annexin V and 7-AAD to examine their apoptotic status, there were no appreciable differences between Wild type and Nlrp12/- levels of apoptosis (3.5B). Administration of PolyIC resulted in expansion of GMPs in both Wt and Nlrp12/- animals (Figure 3.5C), but no significant increase in apoptosis (Figure 3.5D). From this, we conclude that the LPS induction of inflammation is sufficient to cause differences in the GMP populations in Wt and Nlrp12/- animals. However, we observed no elevation in the level of apoptosis after LPS administration, indicating that the inflammatory response may not be strong enough from LPS alone.

**3.5 Conclusions**

This study demonstrates that wide impact that NLRP12 deficiency plays in the innate immune response to RCI, infection, and during shock. In Chapter 2, we showed that NLRP12 deficiency results in a global immunocompromised state that results from increased serum levels of TNF, causing TNF-dependent apoptosis of myeloid progenitor cells after a radiation-thermal combined injury. However, these studies did not address in which compartment (i.e. myeloid or progenitor) NLRP12 works under homeostatic conditions. We therefor utilized Wt:Nlrp12/- chimeras in both Wt and Nlrp12/- recipients. When Wt and NLRP12-deficient animals’ immune systems were reconstituted with equal parts Wt:Nlrp12/- cells, we observed a noted specific increase of apoptosis only those progenitors lacking Nlrp12, whereas Wt progenitors were able to survive and proliferate support that NLRP12 activity is intrinsic to hematopoietic cells after injury.
Previous work has shown IL-6, IL-12, and IL-10 to be use prognostic tools to examine patient outcomes after burn and trauma (Jeannin, Duluc, & Delneste, 2011; S. W. Jones et al., 2013; Steensberg, Fischer, Keller, Møller, & Pedersen, 2003; Steinhauser et al., 1999). Moreover, after burn injury the ratio of IL-10 compared to IL-12 is a useful tool to determine a patient’s pro-bacterial responses compared to anti-inflammatory and wound healing responses as shown in bronchoscopy-derived correlates after burn and radiation injuries (S. W. Jones et al., 2013).

Furthermore, we show that NLRP12 is vital to prime resident macrophages to limit immunopathology as seen by the increase in IL-10 and IL-12 polarization and TLR surface expression. These studies would be valuable as a basis to further study the role NLRP12 plays during injuries that induce complex, robust inflammatory responses resulting in wide spread immunocompromised states.

Utilizing a model of a model of radiation-thermal combined injury may not be warranted in the future. Primarily, the model induces numerous immunological and hematological dysfunction, whose ramifications are not fully understood. Using a high dose of TNF, animals showed rapid development of morbidity in both Wt and Nlrp1/- animals, but Nlrrp12/- animals succumb to shock significantly more quickly than their Wt counterpoints. Additionally, Nlrp12/- animals showed significant increases in granulocyte monocyte progenitor apoptosis, correlated with a decrease in total GMP numbers. Conversely, treatment with PolyIC, the ligand for TLR9, showed neither an increase in apoptosis nor a decrease in GMP numbers. Moreover, treatment with TLR4, the ligand for TLR4 and NLRC4, showed a significant reduction in GMP numbers, but no differences between apoptosis. These studies provide support that investigating NLRP12 may better be done utilizing a model of shock using TNF-administration alone, but not LPS or PolyIC.
Figure 3.1 NLRP12 acts intrinsically to granulocyte monocyte progenitor apoptosis signaling in bone marrow chimeras. Wild type B6.SJL CD45.2 and Nlrp12/- CD45.1 mice were subjected to 9Gy of lethal irradiation and received a bone marrow transplant 24 hours after injury of a 1:1 mixture of Wt:Nlrp12/- bone marrow. After 4 weeks of recovery, animals were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI). (A) serum levels of TNF were quantified using an ELISA at 14 days post injury. (B) The ratio of Wt:Nlrp12/- granulocyte monocyte undergoing apoptosis was determined using flow cytometry and CD45.1/CD45.2 to distinguish between the two donor types, at 14 days post injury. (C) The total number of GMPs and (D) the competitive index of Wt:Nlrp12/- GMPs were quantified using flow cytometry. Data represented as mean ± SEM with statistical significance compared to sham defined as *, p<0.05 by Student’s t test.
Figure 3.2 TNFR2 expression, but not TNFR1, is increased on GMPs after injury. Wild type and Nlrp12-/- C57/B6 animals were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI). (A) TNFR2 (CD120b) and (B) TNFR1 (CD120a) expression was quantified using flow cytometry. Data represented as mean ± SEM with statistical significance compared to sham defined as *, p<0.05, ***P<0.001 by Student’s t test.
**Figure 3.3 Nlrp12--/- show greater monocyte inflammation after injury.** Wild type and Nlrp12--/- C57/B6 animals were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI). (A) Splenocytes were collect at 14 days post injury and intracellular flow cytometry staining was used to quantify expression of IL-10 and IL-12 directly within splenic monocytes. (B) TLR2, TLR4, TLR5, and TLR9 expression was quantified on monocytes using flow cytometry. Data represented as mean ± SEM with statistical significance compared to sham defined as *, p<0.05, **, p<0.001 by Student’s t test.
Figure 3.4 A model of TNF-shock replicates granulocyte monocyte progenitor apoptosis after RCI. Wild type and Nlrp12/- mice were administered either 50 or 100ug of recombinant murine TNF and followed for (A) survival, data represented as Kaplan-Meier log-rank test with data represented as *, p<0.05. Wild type and Nlrp12/- mice were given 100ug of murine TNF and their (B) body temperature was quantified. 8 hours after TNF treatment, bone marrow aspirates were collected and used to determine the (C) total numbers of GMPs, (D) serum IL-12p40, IL-6, and IL-10 cytokines were measured using ELISA. Data represented as mean ± SEM with statistical significance compared to sham defined as *, p<0.05, **, p<0.005 by Student’s t test or two-way ANOVA.
Figure 3.5 LPS treatment, but not PolyIC, reduces granulocyte monocyte progenitor numbers. Wt and Nlrp12-/-mice were given either 50ug of LPS or 100ug of PolyIC. Twelve hours after injection, bone marrow aspirates were collected and stained using flow cytometry for (A) granulocyte monocyte numbers after LPS administration, (B) quantification of GMP apoptosis after LPS administration, (C) GMP numbers after PolyIC administration, and (D) quantification of GMP apoptosis after PolyIC administration. Data represented as mean ± SEM with statistical significance compared to sham defined as *, p<0.05, by Student’s t test.
CHAPTER 4: GM-CSF TREATMENT FOLLOWING RADIATION-THERMAL COMBINED INJURY IMPROVES PLATELETS AND MONOCYTE RECOVERY AND FUNCTION

4.1 Summary

With increased concerns for a terrorist attack, countermeasures are needed to adequately improve patient outcomes following a combined radiation exposure and thermal injury (RCI). Radiation destruction of bone marrow progenitors in concert with the immune consequences of a thermal injury result in severe leukopenia and platelet dysfunction. Under homeostatic condition, granulocyte-monocyte colony stimulating factor is tightly controlled to regulate innate immune cell and platelet production. We hypothesized that using our animal model of RCI, GM-CSF administration would result in increases leukocyte and platelet production as well as improving cellular functions in response to sterile injury and an infectious challenge. In GM-CSF-treated animals, we observed a significant increase in numbers of granulocyte-monocyte and megakaryocyte-erythrocyte progenitors, but no increase in lymphoid progenitors. This heightened number of progenitors correlated with a decrease in progenitor apoptosis, as well as an increase of circulating platelets and monocytes as well as their function. Additionally, when challenged with a systemic infection of *Pseudomonas aeruginosa*, GM-CSF treated animals showed a nearly 10-fold improved ability to control infection systemically and at target organs compared to vehicle treated and injury animals. Our studies suggest that GM-CSF administration can improve hematopoietic production of new cells after a radiation-thermal combined injury and may be a useful therapeutic to treat this unique form of trauma.

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2 This chapter is currently under review as an article at *Shock, injury, Inflammation, and Sepsis.*
4.2 Introduction

The hematopoietic system can rapidly increase myelopoiesis in response to tissue damage and plays a vital role in cellular responses for wound healing and infection clearance (T. D. Jones et al., 1993; Manz & Boettcher, 2014). Emergency myelopoiesis is a tightly coupled process governed by the inflammatory cytokines TNF, IL-1β, and IL-6 through NFκB activation as well as specific growth factors, namely granulocyte-monocyte colony stimulating factor (GM-CSF) (T. Chen et al., 2007; Kopf et al., 1995; Stein & Baldwin, 2013). TNF and IL-6, produced through toll-like receptor signaling, can act to promote the expansion of granulocyte/monocyte progenitors (GMP), resulting in systemic myeloid expansion (Buechler et al., 2013). GM-CSF signaling can limit apoptosis and promote survival and differentiation of progenitor cells within the bone marrow niche; specifically, by promoting progenitor amplification, megakaryocyte production, and differentiation of progenitors into innate cell subsets, including monocytes, eosinophils, neutrophils, and dendritic cells (Egea et al., 2013; Reeves, 2014).

After severe burn and radiation injury, many complications may arise that affect the patient’s blood supply and circulation. Vessel damage can result in excessive fluid loss and hypovolemia; tissue damage results in excessive platelet degranulation and inflammatory activation and binding of platelets resulting in increased tissue damage; and depletion of coagulation factors after the initial injury leads to the inability to properly clot after a burn injury (Heslet et al., 2012). Prolonged hospitalization, mechanical ventilation, and immunological perturbations from burn and radiation injuries lead to an increased susceptibility to lung and system infections within survivors (S. W. Jones et al., 2013). This is a pressing clinical problem in the face of nuclear accidents and possible incorporation of nuclear materials within explosives.
This susceptibility has been attributed to a loss of inflammatory regulation, incomplete immune restoration and a systemic anti-inflammatory response following sepsis and shock (S. W. Jones et al., 2013; Neely et al., 2014; Neely et al., 2011). Following a radiation-thermal combined injury (RCI), an immature monocyte population (iMo) rapidly expands and predominates the periphery (Mendoza et al., 2012). This weakly inflammatory population is necessary to fight infection, but lacks a fully mature phenotype needed for phagocytic and immune regulatory functions.

Using our murine model of radiation and thermal combined injury (RCI), we tested whether repeated administration of GMSF would improve progenitor differentiation of immature immune cells and improve responses to injury and challenge to infection. We observed that GM-CSF treated, RCI mice had greater survival and peripheral cellularity compared to vehicle treated controls. Additionally, these animals displayed a reduction in serum inflammatory cytokines and platelet activation. When challenged with a systemic infection of Pseudomonas aeruginosa, GM-CSF treated injured animals were able to better control bacterial numbers than animals treated with vehicle alone. These results suggest that GM-CSF administration after a traumatic injury may be a suitable treatment to reverse leukopenia and immunosuppression.

4.3 Materials and Methods

*Mice and combined irradiation and burn injury procedure*

All experiments were conducted with C57BL/6 female mice housed under SPF conditions that were age-matched. All studies were conducted in accordance with the IACUC guidelines of the University of North Carolina at Chapel Hill and NIH Guidelines for the Care and Use of Laboratory Animals. Our model of RCI has been previously described; briefly, following a subcutaneous injection of morphine, a full-thickness contact burn of 20% total body
surface area (TBSA) was produced and within 1 hour, mice received a 5Gy (dose rate of 0.98 Gy/min) whole-body dose of ionizing radiation. Sham controls with 0% TBSA underwent all described interventions except for the burn and γ-irradiation exposure. Mice were weighed and had body temperatures taken rectally through the duration of experiments.

**GM-CSF administration**

GM-CSF (2.7mg/kg in PBS) (Life Technologies, Carlsbad, CA) or vehicle control were administered intravenously immediately following injury and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-injuries.

**Pseudomonas aeruginosa infection**

A wildtype strain (PA01) of *P. aeruginosa* was obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC). 10⁴ bacteria were then injected intravenously as described previously.

**Serum collection and cytokine quantification**

Animals underwent a submandibular bleed, blood was collected in EDTA coated collection tubes, and systemic cytokines were measured by LEGENDplex Mouse Cytokine Panel 2 bead-based immunoassay (Biolegend, CA, USA) according to the manufacturer’s instructions on a BDFortessa at the UNC Flow Cytometry Core Facility.

**Flow cytometry**

All fluorescence-conjugated FACS antibodies were purchased from Biolegend or BD Biosciences (CA, USA). Antibody panel used to identify neutrophils and macrophages are CD11b-PE/Cy7, CD11c-PerCP/Cy5.5, CD45-Pacific Blue, Ly6G-APC, Ly6C-PE, F4/80-FITC. The antibody panel for progenitor analysis was comprised of CD3, CD8, NK1.1, CD19, CD45RA, TER-119 (Ly-76) as a lineage negative gate with all antibodies conjugated to FITC,
CD127-PE/CF594, Sca1-APC, cKit-BUV395, FcγR-BV605, CD34-Alexa647, and Annexin V-Pacific Blue. Whole blood samples were stained using TER-119-FITC to discriminate against red blood cells, platelets were then identified using CD62P-APC, CD107a-BV421, CD63-PerCPCy5.5, CD62P-PECy7, and CD42b-BV605. In each case, a million cells per sample were used for flow cytometric analysis.

**Statistical analysis**

Statistical analysis was carried out with Prism 7.0 for Windows and flow cytometry results were analyzed using FlowJoVX for Windows. All data are presented as the mean +/- standard error of the mean (SEM). Complex data sets were analyzed by analysis of variance (ANOVA) with a Tukey-Kramer post-test HSD for multiple comparisons. Single data sets were assessed by the Student’s two-tailed t test. The product limit method of Kaplan-Meier was utilized for generating the survival curves, which were compared using the log rank test. A p value less than 0.05 was considered statistically significant for all data sets.

**4.4 Results**

**GM-CSF increases survival and weight following RCI**

GM-CSF under homeostatic conditions is a cytokine that maintains survival and a low level of differentiation of hematopoietic stem cells and myeloid progenitor cells. Moreover, following a tissue ablative injury such as a total body radiation exposure, GM-CSF production is increased by macrophages, T and NK cells, fibroblasts, and endothelial cells. However, dual exposure to radiation and burn results in not only bone marrow ablation, but also a severe immunocompromised state. This weakens the ability of these cells to respond appropriately to the injury, namely increased inflammatory cytokine production and delayed production of GM-CSF.
and G-CSF. We therefore investigated whether repeated administration of GM-CSF to radiation-thermal combined injured animals would improve survival and immune responses.

No differences in mortality were observed in sham treated animals, however GM-CSF treated, injured animals showed no instances of mortality compared to the 20 percent mortality observed in vehicle treated animals (Figure 4.1A). GM-CSF treated injured animals increased body weight and temperature more quickly than vehicle control animals (Figure 4.1B-C). These data suggest that repeated GM-CSF administration was able to reduce mortality and improve body temperature and weight recovery after a radiation-thermal combined injury.

**GM-CSF increases granulocyte-monocyte progenitor numbers following RCI**

Following a radiation-thermal combined injury, we have shown highly granular, immature monocyte predominate the peripheral immune system (Mendoza et al., 2012). These cells were immediately produced through GM-CSF induced differentiation of granulocyte-monocyte progenitor cells (GMPs) within the bone marrow. We therefore investigated the affect GM-CSF administration plays on these and other progenitor cells following RCI. GM-CSF treated, RCI animals had significantly increased numbers of GMPs at one and two weeks after injury compared to vehicle animals (Figure 4.2A). Additionally, megakaryocyte-erythrocyte progenitors (MEPs), that give rise to megakaryocytes and erythrocytes, also showed greater signs of expansion compared to vehicle treated, injured animals (Figure 4.2B). In contrast, the common B and T cell lymphoid progenitor, and has not been reported to be affected by GM-CSF, showed no significant expansion (Figure 4.2C). These data implicated that GM-CSF specifically improves myeloid progenitor survival and proliferation after RCI.
**Progenitor increase is attributed to decreased apoptosis of progenitor cells**

The inflammatory cytokine milieu present after RCI is unique in that it results in a sustained increase in pro-inflammatory and anti-inflammatory cytokines long after the initial injury (Mendoza et al., 2012). Prolonged exposure to elevated pro-inflammatory cytokines, namely TNF, can induce program cell death instead of initiating survival and proliferation (Micheau & Tschopp, 2003). We therefore investigated whether GM-CSF treatment would impact the level of apoptosis that occurs in progenitor cells after injury. Both the GMP and MEK populations showed significantly lower levels of apoptosis as detected by Annexin-V/7-AAD staining (Figure 4.3A-B), while no reduction was seen in CLPs (Figure 4.3C). These data suggest that GM-CSF treatment improves progenitor survival by reducing their susceptibility to inflammation-mediated apoptosis.

**Peripheral innate immune cells and platelets expand following GM-CSF treatment after RCI**

Following RCI, we have previously observed that despite radiation-ablation of much of the bone marrow and peripheral immune system including peripheral blood mononuclear cells (PBMCs) and platelets, we observed the expansion of a radio-resistant, immature myeloid population. We then sought to determine if GM-CSF administration improves expansion of monocytes and platelets after injury. We found that GM-CSF significantly increases the numbers of platelets (Figure 4.4A) after one week of GM-CSF treatment compared to untreated controls, while neutrophils (Figure 4.4B) and monocytes (Figure 4.4C) had a weak, yet significant expansion at 7 and 14 days, respectively. These data indicated that repeated GM-CSF administration improves platelet and PBMC expansion following RCI.

**Platelets display normalized phenotype following GM-CSF treatment and RCI**

Following a burn or radiation injury, platelets rapidly respond to clot off any open wounds and to initiate a wound healing response. However, this initial injury depletes many
clotting factors and circulating platelets (Levin & Egorihina, 2010; Takashima, 1997). Following the immunosuppression that follows, many new platelets are not able to function appropriately leading to defects in clotting, increased levels of degranulation and release of inflammatory mediators, and increased adhesion to circulating monocyte and neutrophils, leading to further aberrant immune activation. We sought to determine the impact GM-CSF has on platelet function after injury.

To examine the coagulation ability of platelets, we utilized flow cytometry staining of CD42b (glycoprotein 1b), whose expression is directly correlated with platelets ability to initiate the coagulation cascade. GM-CSF treated, injured animals showed higher platelet expression of CD42b (Figure 5A) indicating there are better prepared clot should the need arise. Additionally, GM-CSF treated animal’s platelets displayed decreased surface expression of CD62P (P-selectin) (Figure 4.5B) indicating a decreased activation and decreased potential to effectively bind circulating monocyte and neutrophils compared to untreated RCI mice.

As previously reported, platelets after RCI show an increased degranulation of both alpha and dense granules compared to uninjured controls (Bergmann et al., 2016; Vieira-de-Abreu et al., 2012). Alpha granules, which contain insulin-like growth factor-1, platelet-derived growth factor, TFGβ, and numerous clotting proteins, also express CD107a (LAMP-1) on the vesicle wall. Upon alpha granule release, platelets display increased surface expression of CD107a compared to untreated RCI mice (Figure 4.5C). GM-CSF administration reduces as early as three days post injury compared to untreated RCI mice, the expression of CD107a indicating a reduction in degranulation. Additionally, dense granules, which contain ADP, ATP, ionized calcium, and histamine and serotonin, can be traced by surface expression of CD63 (LAMP-3). Platelets from GM-CSF treated, injury animals showed a reduction of CD63 expression as early
as 3 days compared to untreated RCI mice. These data show that GM-CSF treatment is not only able to increase platelet numbers, but also to return them to a more homeostatic state after RCI.

**GM-CSF treated animals show a reduction in serum IL-6 and an increase in IL-10**

Following a burn and radiation injury, heightened levels of IL-6 or reduced levels of IL-12 following trauma have been shown to be the major predictor of poor outcome (bacterial infection) following a traumatic injury (Gebhard et al., 2000). We observed a reduction in IL-6 after one week of GM-CSF treatment (Figure 4.6A) and reduction of IL-12 after two weeks (Figure 4.6B) compared to untreated RCI mice. Increased TNF levels are beneficial to promote bacterial clearance, while prolonged heightened levels induce immunopathology of sensitive organs, namely the lung, after a burn or radiation injury. We observe no difference in TNF after GM-CSF treatment (Figure 4.6C). The anti-inflammatory cytokine IL-10 promotes a wound healing response (Edwards & Harding, 2004; Werner & Grose, 2003). We observe an increase in serum IL-10 at 3 days compared to untreated RCI controls, but see no significant differences at one and two weeks after injury following GM-CSF treatment (Figure 4.6D). These data suggest that GM-CSF treatment reduces the inflammatory responses to injury, while promoting a wound healing environment.

**GM-CSF treatment improves systemic infection responses to Pseudomonas aeruginosa**

Following a prolonged hospital stay, patients that survive the initial shock from a burn or radiation-thermal combined injury succumb to systemic or pulmonary infections (Chitkara & Feierabend, 1980). We sought to determine the effect GM-CSF has on the ability of animals to control a systemic infectious challenge. Animals underwent the radiation-thermal combined injury as described before, mice were then sustained for two weeks in individual housing wherein they were infected intravenously with $1 \times 10^4$ CFU of *Pseudomonas aeruginosa* (PA01).
Spleen and lungs from infected animals that survived until 48 hours post infection were collected and plated to enumerate bacterial load systemically. We observed nearly a 5-fold reduction in bacterial load in the spleen (Figure 4.7A) and a 10-fold reduction in the lung load (Figure 4.7B) in GM-CSF treated animals compared to vehicle treated RCI animals. These data suggest that GM-CSF can significantly improve the ability of animals to effectively control an infectious challenge after a radiation-thermal combined injury.

4.5 Discussion

Our study demonstrates that repeated GM-CSF administration improves survival after a radiation-thermal combined injury. Moreover, GM-CSF improves progenitor cell survival and differentiation, resulting in an increase in total peripheral and tissue leukocyte numbers compared to injured animals alone. This increased cellularity and immune responses lead to an improved ability to control and infectious challenge.

Hematopoietic stem cell expansion and differentiation have been shown to be important factors in the immune suppression that follows a traumatic injury (Manz & Boettcher, 2014; J. G. Noel et al., 2002). Previous studies have shown GM-CSF stimulation can improve survival, and enhance proliferation and differentiation of bone marrow progenitors (Reeves, 2014). With little study of the biology of radiation combined with a thermal injury, there have been few therapeutics developed to combat this unique and devastating injury. We propose that repeated GM-CSF administration following a traumatic injury that causes emergency hematopoiesis will improve peripheral immune reconstitution and survival.

Under homeostatic conditions, bone marrow progenitor cells undergo low levels of differentiation and are resistant to many pro-apoptotic signaling pathways (Wilson & Trumpp, 2006). GM-CSF has been shown to be important to activate, Mcl1, a member of the anti-
apoptotic BCL2 family (Derouet, Thomas, Cross, Moots, & Edwards, 2004; Opferman et al., 2005). Additionally, others have shown that GM-CSF reverses monocyte anergy that occurs during sepsis (Williams, Withington, Newland, & Kelsey, 1998). GM-CSF is a pluripotent cytokine whose role as a therapeutic following RCI requires more investigation.

However, after RCI, we observed a limited repopulation of the peripheral immune system with an immature monocyte population in addition to an increase in apoptosis of granulocyte-monocyte progenitor and megakaryocyte-monocyte progenitor cells.

Because of its anti-apoptotic and stimulatory effects, we sought to determine the impact GM-CSF would have on immune reconstitution and cell function following RCI. GM-CSF treatment not only abrogates the progenitor apoptosis and survival defects observed after injury. Additionally, GM-CSF treatment improves platelet numbers and function from the improves megakaryocyte function. This increased function of platelets and improved repopulation after GM-CSF treatment improves systemic responses to an infectious challenge. Taken together, our studies suggest that GM-CSF could be utilized as a treatment to improve immune outcomes and responses following a traumatic injury.
Figure 4.1. GM-CSF treatment increases temperature, survival and weight following RCI C57BL/6 mice were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI); mice were then either given 2.7mg/kg GM-CSF or vehicle control. (A) survival, (B) weight, and (C) temperature was measured at 1, 3, 5, 7, 11, and 16 days post injury. Survival data was analyzed using Kaplan-Meier log rank test. Data represented as mean ± SEM, with statistical significance compared to sham defined as *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test, with experiments performed in duplicate (n=5 per group).
Figure 4.2. GM-CSF administration increases progenitor cell numbers following RCI

Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI) and GM-CSF treatment. Bone marrow was aspirated at 3, 7 and 16 days post injury and the total number of (A) common lymphoid progenitors, (B) granulocyte-monocyte progenitors, and (C) megakaryocyte-erythrocyte progenitors were quantified by flow cytometry. Data represented as mean ± SEM, with statistical significance defined as *, p<0.05 and **, p<0.005 by Student’s t test with n=5 mice per group, with experiments performed in duplicate.
Figure 4.3. GM-CSF reduces progenitor apoptosis following RCI Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI) and GM-CSF treatment. Bone marrow was aspirated at 3, 7, and 16 days post injury and the percentage of (A) granulocyte-monocyte progenitors, (B) megakaryocyte-erythrocyte progenitors, and (C) common lymphoid progenitors cells undergoing apoptosis was determined by positive Annexin V staining in the absence of 7-AAD-staining cells. Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test with n=5 mice per group, with experiments performed in duplicate.
Figure 4.4. GM-CSF increases circulating platelet, neutrophil, and monocyte numbers after RCI
Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI) and GM-CSF treatment. Cheek bleeds were performed at 3, 7 and 16 days post injury and the number of (A) platelets, (B) neutrophils, and (C) monocytes were quantified by flow cytometry. Data represented as mean ± SEM with n=5 mice per group, with experiments performed in duplicate.
Figure 4.5. GM-CSF increases platelet function after RCI Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI) along with GM-CSF treatment or vehicle alone. Cheek bleeds were performed at 3, 7, and 16 days post injury and (A) CD42b, (B) CD62P, (C) CD107a and (D) CD63 mean fluorescent intensity were determined using flow cytometry. Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test with n=5 mice per group with experiments performed in duplicate.
Figure 4.6. GM-CSF treatment reduces IL-6 and increases IL-10 after RCI Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI) along with GM-CSF treatment or vehicle alone. Cheek bleeds were performed at 3, 7, and 16 days post injury and cytokine concentrations of (A) IL6, (B) IL12, (C) TNF, and (D) IL10 were determined using LEGENDplex bead-based assay. Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student’s t test with n=5 mice per group with experiments performed in duplicate.
Figure 4.7. GM-CSF mice show greater bacterial clearance than vehicle treated Wildtype C57BL/6 mice were subjected to sham or combined radiation and burn injury (RCI). Mice were injected 14 days post-injury intravenously with $1 \times 10^4$ CFU of *Pseudomonas aeruginosa* (PA01). We quantified (A) bacterial load within spleen and (B) lungs liver by culture. Data represented as mean ± SEM, with statistical significance defined as *, $p<0.05$; **, $p<0.05$ by Student’s t test with n=5 mice per group, with experiments performed in duplicate.
CHAPTER 5: FLAGELLIN TREATMENT PREVENTS INCREASED SUSCEPTIBILITY TO SYSTEMIC BACTERIAL INFECTION AFTER INJURY BY INHIBITING IL-10+ IL-12- NEUTROPHIL POLARIZATION

5.1 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 innate immune modulations observed during sepsis also contribute to increased bacterial susceptibility after severe trauma. A well-established murine model of burn injury, used to replicate infection following trauma, showed that wound inoculation with P. aeruginosa quickly spreads systemically. The systemic IL-10/IL-12 axis was 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 (ΔCyaB) 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. This suggests that neutrophil polarization influences bacterial clearance after burn injury. Administration of a TLR5 agonist, flagellin, after burn injury restored the neutrophil response towards a N1 phenotype resulting in an increased clearance of wildtype P. aeruginosa after wound inoculation. This study details specific alterations in innate cell populations after burn injury that contribute to increased

3 This chapter was previously appeared as an article in Plos One. The original citation is as follows: Neely, Crystal J., Kartchner, L, Mendoza, A, Linz, B, Wolfgang M, Frehlinger J, Maile R, Cairns B. "Flagellin treatment prevents increased susceptibility to systemic bacterial infection after injury by inhibiting anti-inflammatory IL-10+ IL-12- neutrophil polarization." PloS one 9.1 (2014): e85623.
susceptibility to bacterial infection. In addition, for the first time, it identifies neutrophil polarization as a therapeutic target for the reversal of bacterial susceptibility after injury.

5.2 Introduction

Each year traumatic injury accounts for over 40 million emergency room visits and 2 million hospital admissions across the United States (Santaniello et al., 2004). Severe trauma predisposes patients to infection with rates as high as 37% of patients (Papia et al., 1999). Infectious complications, such as sepsis and pneumonia, increase the length of hospitalization and cost of treatment (Glance, Stone, Mukamel, & Dick, 2011; Niven, Fick, Kirkpatrick, Grant, & Laupland, 2010). Furthermore, infection increases a traumatically injured patient's mortality rate by 5-fold (Patel, Mollitt, & Tepas, 2000).

It is clear that severe burn-injury results in a complex interaction of both innate and adaptive immunity that leads to immune dysfunction, infection and often sepsis. Much work has been focused on defining alterations in the adaptive immune system, with T cell apoptosis (Fukuzuka, Edwards III, et al., 2000; Pellegrini et al., 2000), lymphopenia (Maile et al., 2006), T cell cytokine polarization (B. A. Cairns et al., 2001; Guo et al., 2003; Kovacs, Duffner, & Plackett, 2004; Zedler, Bone, Baue, Donnersmarck, & Faist, 1999) and upregulation of regulatory T cell (Treg) suppressive function (Choileain et al., 2006; MacConmara et al., 2006; Scumpia et al., 2006) being key players. However, in healthy individuals, the innate immune system is sufficient for clearing 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, release of granule proteins, and generation of neutrophil extracellular traps (NETs) (Borregaard, 1997; Brinkmann et al., 2004; Nordenfelt & Tapper, 2011). Macrophages and dendritic cells are also phagocytic, and antigen presentation and pro-
inflammatory cytokine secretion (such as TNF-α and IL-12) by these cells induce and shape the adaptive immune response (Fujiwara & Kobayashi, 2005; Savina & Amigorena, 2007). Toll-like receptors (TLRs), which recognize conserved microbial products, are vital for detection of invading pathogens. TLR signaling leads to the induction or suppression of hundreds of inflammatory genes that further influence an immune response (Lien & Ingalls, 2002; van Putten, Bouwman, & de Zoete, 2010). Collectively, these innate immune responses lead to clearance of invading bacteria.

During sepsis, defective bacterial clearance has been linked to alterations in the innate immune response. TLR expression and signaling is often altered leading to hypo- or hyper-responsiveness (Harter, Mica, & Stocker, 2004; Martins et al., 2008). In addition, macrophages and neutrophils, which are extremely plastic, tend to be polarized into an anti-inflammatory phenotype due to TLR-signaling by danger-associated molecular patterns (DAMPS) released from damaged tissue (Biswas, Chittezhath, Shalova, & Lim, 2012; Hotchkiss, Coopersmith, McDunn, & Ferguson, 2009; Kasten, Muenzer, & Caldwell, 2010; Qin et al., 2012; X. Zhang & Mosser, 2008). These polarized macrophage (M2) and neutrophil (N2) cells secrete high amounts of IL-10, a potent anti-inflammatory cytokine and have been implicated in burn injury (B. A. Cairns et al., 2008; M. J. Cohen et al., 2007; Greg Noel et al., 2011; Schwacha, Chaudry, & Alexander, 2003). IL-10 can limit tissue damage by dampening the exaggerated production of pro-inflammatory cytokines observed during sepsis and induce tissue healing (Kahlke et al., 2002; Kalechman et al., 2002). However, excessive IL-10 has been shown to be detrimental for bacterial clearance by attenuating protective pro-inflammatory cytokines, such as IL-12 (Steinhauser et al., 1999; L. Sun et al., 2009; Yamaguchi et al., 2000) and correlates with worse outcome after burn injury (S. W. Jones et al., 2013). Additionally, in various models of trauma a
Ly6g+ CD11b+ myeloid cell population has been shown to arise (Cuenca et al., 2011; John G Noel et al., 2007) which are thought to be analogous to the Myeloid-derived Suppressor Cells (MDSC) that mediate immune suppression in the tumor microenvironment although their role in injury is controversial (Cuenca et al., 2011; Cuenca & Moldawer, 2012). We hypothesized that these innate immune modulations observed during sepsis also contribute to increased bacterial susceptibility after severe trauma.

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. 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-10+ IL-12− N2 anti-inflammatory phenotype. To confirm if neutrophil polarization played a role in bacterial clearance after burn injury, mice were then infected with attenuated *P. aeruginosa* strain (ΔCyaB). We found that better clearance of ΔCyaB 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 that 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 and reveal neutrophil polarization as a therapeutic target for the reversal of bacterial susceptibility after injury.
5.3 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 (3 mg/kg body weight; Baxter Healthcare) was given prior to burn injury for pain control, and an intraperitoneal injection of lactated Ringer's solution (0.1 mL/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

A wildtype strain (PAK) and a mutant strain (ΔCyaB) of *P. aeruginosa* were obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC) (Wolfgang, Lee, Gilmore, & Lory, 2003). 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 or until mid-log phase. The cultures were centrifuged at 13,000 rpm for 30 seconds, and the pellet washed with 1 mL 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 600 nm. After diluting the bacteria to obtain the desired concentration, the inoculum was verified by plating serial 10-fold dilutions on LB agar plates containing 25 µg/L irgasan (Sigma-Aldrich, St. Louis, MO).

Animal infections

Twenty-four hours following burn or sham injury, mice were anesthetized by intraperitoneal injection of Avertin (0.475 mg/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 *S. typhimurium* (InvivoGen; San Diego, CA) was given intraperitoneally at a concentration of 0.125 ng/100 µl per mouse.

Determination of bacterial infection

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

**CD11b+ cell enrichment**

Cells suspensions were prepared from spleens of mice. CD11b+ cells were positively selected using the BD Imag Mouse CD11b MagneticParticles 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 10⁶ cells/mL. Cells were plated in a 48 well plate and cultured for 6 hours with 0.1 ng/mL of LPS (Sigma-Aldrich) at 37°C at 5% CO₂. During the last 4 hours of the stimulation, 3.0 µl/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 1 µg 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-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). Data were collected on a Dako CyAN and analyzed using Summit software (Dako; Carpinteria, CA).

**Serum cytokine analysis**

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; two-way ANOVA with Tukey post-test was used to test differences in TLR expression GraphPad Prism version 5 was used for the analyses. Statistical significance was defined as $p \leq 0.05$ unless indicated otherwise.

**5.4 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 a wildtype strain of *P. aeruginosa*, PAK. At 24 hours following burn or sham procedure, mice were anesthetized and given a subcutaneous injection of bacteria ($2 \times 10^3$, $2 \times 10^4$, or $2 \times 10^5$ CFU/100 μl) at the mid-dorsum. There was 100% survival of sham mice, even with the highest dose of $2 \times 10^5$ CFU (Figure 5.1A). Burn mice, however, exhibited mortality that was dose dependent (Figure 5.1A). Mortality of infected burn mice began as early as 1 day after inoculation. To evaluate bacterial clearance in burn and sham mice various tissues were harvested 48 hours following infection ($2 \times 10^4$ CFU/100ul), which was before significant
mortality occurred. As shown in Figure 5.1B, sham mice had no bacteria recovered from skin biopsies of the injection site while all burn mice had bacteria detected. Furthermore, the number 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 were actively replicating in the skin. Distal organs were also analyzed to examine bacterial dissemination. The liver, lungs, wound-draining lymph nodes and spleen of sham mice had no detectable bacteria, whereas the organs of burn mice had a high bacterial load (Figure 5.1C–1F). These data show that burn mice develop a systemic infection by 48 hours following wound inoculation with a wildtype strain of *P. aeruginosa* (PAK).

**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 (Bruce A Cairns, Carie M Barnes, Stefan Mlot, Anthony A Meyer, & Robert Maile, 2008; Maung et al., 2005) and protein (B. A. Cairns et al., 2008) levels changes after burn injury. Since TLR2, TLR4, and TLR5 are involved in control of *P. aeruginosa* infection by recognizing outer membrane lipoproteins, LPS, and flagellin, respectively (Balloy et al., 2007; Feldman et al., 1998; Hajjar, Ernst, Tsai, Wilson, & Miller, 2002), we hypothesized that decreased bacterial clearance after burn injury was due to reduced expression of these TLRs on innate immune cells. 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 to differentiate between innate cell populations (Neutrophils, Gr1+, Ly6C+, Ly6G+, CD11b+, CD11c+, F4/80-; macrophages, Gr1+, Ly6C+, Ly6G-, CD11b+, CD11c-, F4/80+; dendritic cells, Gr1-, Ly6C-, Ly6G-, CD11b+, CD11c-, F4/80- and Gr1+ myeloid MDSC, Gr1+, Ly6C+, Ly6G+, CD11b+, CD11c-, F4/80-). The absolute number of these innate populations were similar in all treatment groups (data not
shown). Adaptive cell populations (T and B cell) were also largely unchanged (data not shown), with a significant decrease in CD8 T cell number only upon injury, as we have documented before (Maile et al., 2006). 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 5.2A and 5.2B). 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 5.2C). These data demonstrate that on specific innate cell populations there are acute alterations in TLR expression in response to bacterial infection after burn injury.

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

Many studies have shown that IL-10 is deleterious whereas IL-12 is beneficial for clearance of *P. aeruginosa* (Steinhauser et al., 1999; L. Sun et al., 2009; Yamaguchi et al., 2000). 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 5.3A). 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 5.3B). 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**

Macrophages and neutrophils can be polarized into pro- (M1/N1) and anti-inflammatory states (M2/N2) (Biswas et al., 2012; Hotchkiss et al., 2009; Kasten et al., 2010; Qin et al., 2012)
after TLR stimulation, particularly in the context of injury where there is release of tissue DAMPs (X. Zhang & Mosser, 2008). Infected burn mice had a systemic anti-inflammatory response following infection, which was marked by elevated serum IL-10 levels; therefore, we hypothesized 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 5.4A). Due to these data along with previous reports about IL-10 production by innate cells following burn injury (Greg Noel et al., 2011; Schwacha, 2003), we focused our subsequent studies on these cell types. Splenocytes were harvested at 48 following infection, then underwent CD11b enrichment by magnetic selection. CD11b⁺ cells were cultured in the presence of LPS and brefeldin-A to measure intracellular accumulation of IL-10 and IL-12. Cell surface, intracellular staining and side/forward scatter indicated that neutrophils (Gr1+, Ly6C+, Ly6G+, CD11b+, CD11c+, F4/80⁻), but not macrophages (Gr1+, Ly6C+, Ly6G⁻, CD11b+, CD11c⁻, F4/80⁺), 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 5.4B). 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 5.4C–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 (ΔCyaB) P. aeruginosa correlated with reduced N2 polarization of neutrophils

ΔCyaB is a mutant strain of P. aeruginosa that has been previously reported to be attenuated in an adult mouse model of acute pneumonia (Smith, Wolfgang, & Lory, 2004). We predicted that burn mice could control infection with ΔCyaB better than wildtype PAK. Also, we hypothesized that any differences in the innate immune response between ΔCyaB and PAK 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 of wildtype PAK or ΔCyaB mutant (2×10⁴ CFU/100 µl). 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 PAK or ΔCyaB following burn injury resulted in a similar bacterial load at the injection site (data not shown). In contrast, burn mice infected with ΔCyaB had significantly less bacterial recovery in the distal organs than burn mice infected with PAK (Figure 5.5A and 5.5B). These data indicate that burn mice are more resistant to developing systemic infection with an attenuated strain of P. aeruginosa than with wildtype PAK. 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 5.5C). In contrast to PAK, infection with ΔCyaB following burn injury resulted in a significant increase in serum IL-12 levels (Figure 5.5D). 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 ΔCyaB and those infected
with PAK (Figure 5.5E). ΔCyaB infection also resulted in a higher percentage of IL-12+ neutrophils within the spleen (Figure 5.5F). Hence, infection with ΔCyaB following burn injury results in a higher percentage of IL-12+ cells within the spleen and an increase in serum IL-12. TLR2, TLR4, and TLR5 expression on the various immune cells was comparable between PAK and ΔCyaB infected burn mice (data not shown). These data suggest that the reduced susceptibility to ΔCyaB 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 (Vicente-Suarez et al., 2007). Therefore, we hypothesized that flagellin could improve clearance of wildtype *P. aeruginosa* (PAK) in burn mice by increasing the protective IL-12 response. Burn mice were resuscitated after burn injury and received an intraperitoneal injection of flagellin (0.125 ng/100 µl) twenty-hours later. Twenty-four hours after burn they were then infected subcutaneously with PAK. Forty-eight hours following infection with or without treatment 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 5.6A and 5.6B). 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 5.6C and 5.6D). The absolute number of innate and adaptive cells were unchanged between flagellin and flagellin-untreated burn mice. 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.

**5.5 Discussion**

Severe trauma results in a period of immune impairment that predisposes the patient 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), bacteria replicate to a high titer and spread to distal organs resulting in bacterial sepsis. Neutrophils and Ly6g$^+$ CD11b$^+$ myeloid cells have decreased TLR expression. In addition, the 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 strain of *P. aeruginosa* (ΔCyaB). We found that burn mice have greater control of ΔCyaB 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$^-$) is responsible for improved clearance of ΔCyaB 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 (Vicente-Suarez et al., 2007). We found that
treatment with flagellin after burn injury enhances clearance of wildtype *P. aeruginosa* (PAK) 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, which is a very pressing clinical need. 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 (F4/80− Gr1+ (Ly6C+ Ly6G+) CD11b+ CD11c+), macrophages (F4/80+ Gr1+ (Ly6C+ Ly6G−) CD11b+ CD11c−), a Ly6g+ CD11b+ myeloid population (F4/80+ Gr1+ (Ly6C+ Ly6G+) CD11b+CD11c−) and dendritic cells (F4/80− CD11b+ Gr1− CD11c−). 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.

Controversy exists as to whether the Ly6g+ CD11b+ cells that arise after trauma are analogous to the Myeloid-derived Suppressor Cells (MDSC) that mediate T cell suppression in the tumor microenvironment (Cuenca et al., 2011). Our laboratory has recently described that burn-induced Ly6g+ CD11b+ cells suppress T cell proliferation (Mendoza et al., 2012) and polarize T cells towards a Th2-anti-inflammatory response (B. Cairns, Maile, Barnes, Frelinger, & Meyer, 2006) suggesting they mimic aspects of MDSC function. Ly6g+ CD11b+ cells employ various mechanisms, such as arginase, IL-10, and nitric oxide production, to inhibit T cell
proliferation and activation (Movahedi et al., 2008; Youn, Nagaraj, Collazo, & Gabrilovich, 2008). 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 time points after burn injury (Mendoza et al., 2012) due to continuous myelopoiesis. IL-10 itself has many effects on other immune cells, including upregulation of T_{reg} suppression, decreased effector T cell function. IL-10 can also downregulate innate cell function including inhibition of reactive oxygen species vital for killing of bacteria. The innate system is thought to drive the resultant adaptive response. 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 other 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 (Mosmann & Sad, 1996). 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 mainly focuses on macrophage polarization (Sica et al., 2008). 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-gamma (Jeannin et al., 2011). 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-10⁺ IL-12⁻) (Ambarus et al.,
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 to local tissue since the robust pro-inflammatory cytokine production by the macrophages can exacerbate tissue damage (Qin et al., 2012). Also, an overt M2 response is believed to deleterious by significantly impairing bacterial clearance (Shigematsu, Asai, Kobayashi, Herndon, & Suzuki, 2009). 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 (Csontos et al., 2010; Steinhauser et al., 1999; L. Sun et al., 2009). 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 5.1. Burn mice, but not sham mice, exhibit dose-dependent mortality and develop a systemic infection following a *P. aeruginosa* wound inoculation. Wildtype *P. aeruginosa* (PAK) was administered subcutaneously at 24 hours after burn or sham treatment. A) Various doses of bacteria (2×10³, 2×10⁴, or 2×10⁵ CFU/100 µl) were given and survival was monitored for 120 hours post infection (hpi). B–F) Using a dose of 2×10⁴ CFU, bacterial load at the injection site and distal organs was assessed at 48 hpi in sham (open circles) and burn (closed circles) mice. (n=4–9 per group) *, p≤0.05. **, p≤0.005. These experiments were repeated three times with similar results.
Figure 5.2. 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+ cells, and B) macrophages at 3 days post burn (solid) or sham (open) 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 by two-way ANOVA with Tukey posttest. These experiments were repeated three times with similar results.
Figure 5.3. Burn mice, but not sham mice, mount a robust serum IL-10 response after *P. aeruginosa* wound inoculation. Twenty-four hours after sham (open) or burn (solid) treatment, mice were given a subcutaneous injection of wild-type *P. aeruginosa* PAK. Forty-hours following infection, serum was collected to determine circulating levels of A) IL-10 and B) IL-12p40 by cytometric bead array. Data expressed as mean ± SEM. (n=10–15) *, p≤0.05.
Figure 5.4. 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 collected 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. Percentage of B) IL-10⁺ neutrophils, as well as IL-12⁺ C) neutrophils, D) dendritic cells, and E) macrophages were measured for infected sham (open) and burn (solid) mice. Data expressed as mean ± SEM. (n=6, 7) **, p≤0.005. ***, p≤0.0005. ****, p<0.0001. These experiments were repeated three times with similar results.
Figure 5.5. Reduced bacterial load at distal organs following wound inoculation with an attenuated *P. aeruginosa* strain (∆CyaB) is associated with an increased serum IL-12 and pro-inflammatory neutrophil (N1; IL-10⁺IL-12⁺) response in burn mice. Forty-eight hours following wildtype PAK (circles/solid bars) or ∆CyaB (triangles/checkered bars) wound infection, various organs were harvested from burn mice. Bacterial load in A) liver and B) lung samples was determined by colony forming unit (CFU) assay. 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 5.6. Administration of flagellin at burn resuscitation and 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. Burn mice were given an intraperitoneal injection of flagellin (circles/solid bars) or left untreated (squared/striped bars) twenty-two hours after burn injury. Twenty-four hours after burn injury, mice were challenged with subcutaneous wound infection with PAK. Forty-eight hours following the bacterial challenge, various organs were harvested. Bacterial load in A) liver and B) lung samples was determined by colony forming unit (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≤0.05. **, p≤0.005. These experiments were repeated three times with similar results.
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 A clinical need

Currently there are 444 nuclear reactors operating in 30 countries. Despite wavering public opinion of nuclear energy, there are 63 new nuclear plants that are under construction in 15 countries (Budnitz, 2016). With increasing concern for a terrorist attack and the potential for state-sanctioned nuclear attacks, research concerning the medical consequences of a blast is necessary to properly treat any potential victims. Radiation accidents or attacks are not isolated exposures, but are often coupled with initial blasts, secondary gas explosions, as well as other various forms of physical trauma (Bui et al., 2014). From the documented nuclear accidents, ranging from the Three Mile Island plant partial meltdown, the Chernobyl nuclear meltdown in 1986, as well as the more recent Fukushima-Diachi that occurred after a tsunami hit mainland Japan, those that were exposed to radiation, between 20-60% also experienced a secondary form of trauma (Fushiki, 2013).

Both burn and radiation injuries induces profound immune system dysfunction that renders patients to be highly susceptible to infection (Church et al., 2006; Gordon, Ruml, Hahne, & Miller, 1955). The immune cell ablation of the radiation injury, combined in the context of a full-thickness skin burn distorts the immune systems response even further (Cherry et al., 2013; Mendoza et al., 2012). Despite progress made in reducing instances of wound infection after a burn through wound debridement and the aggressive use of antibiotics, wound and pulmonary infections remain a major cause of mortality (Church et al., 2006). Therefore, to improve patient outcome, it is necessary to elucidate the immune responses to a radiation-thermal combined
injury, and how the immune system responses to an infection after such injury. Additionally, due to the highly dynamic responses of the immune system after a burn injury, it is important to also study the impact that burn alone has on the ability of the immune system to control and infection.

6.2 Cellular mechanisms of immune dysfunction following radiation-thermal combined injury in the absence of NLRP12

Immediately following a thermal injury, the resident macrophages and T cells respond by rapidly elevating numerous pro-inflammatory cytokines (Bergmann et al., 2016; Greg Noel et al., 2011; J. G. Noel et al., 2005). These circulate systemically and reach the bone marrow compartment. Hematopoiesis is a tightly controlled, homeostatic process that involves hematopoietic stem cells and progenitor cells expanding and producing new, naïve immune cells. Under natural, homeostatic conditions the hematopoietic compartment works to slowly produce new lymphoid (e.g. B and T cells), that leave the tissue for maturation in the thymus, spleen, lymph nodes, or other target tissues (Ansel & Cyster, 2001; Kincade, Lee, Pietrangeli, Hayashi, & Gimble, 1989). Myeloid lineage cells are also produced within the bone marrow and undergo minimal amount of maturation and priming (T. D. Jones et al., 1993). Upon stimulation with through a chemotactic gradient, myeloid cells will leave the bone marrow niche. Macrophages, monocyte, and neutrophils have a variety of half-lives ranging from several days to weeks for macrophages, and hours to days for neutrophils. Naturally, these are the most predominant species of cell present in the hematopoietic compartment (J. G. Noel et al., 2002). Upon recognition of a damage or pathogen associated molecular pattern, these myeloid cells will receive chemotactic signals and follow this gradient to the site of injury (J. G. Noel et al., 2002).

In immune cells, NFkB acts as a transcription factor to initiate production of pro-inflammatory cytokines, chemokines, and changes to the cell cycle. Moreover, canonical NFkB, or p65/RelA, works to maintain HSC renewal and proliferation during homeostatic conditions.
and in response insults (Stein & Baldwin, 2013). Previous studies have shown NLRP12 to act as negative regulator of NFkB through association with NIK (I. C. Allen et al., 2012). Increased activity of NLRP12 leads to a reduction in the production of NFkB-dependent chemokines and pro-inflammatory cytokines. Canonical NFkB signaling through STAT3 and the type II TNF-receptor promotes pro-inflammatory environment, improves survival, but under prolonged exposure to TNF, expression of the type I TNF-receptor and signaling through TRADD/FADD can initiate inflammation-induced, programmed cell death – apoptosis (Croft et al., 2013; Hayden & Ghosh, 2014).

The NLR family of proteins has been most widely studied in the context of the inflammasome, a catalytic oligomer that processes IL-1β and IL-18 into the bioactive forms, and support secretion from the cytosol. However, some members of the NLR family have been shown to be direct regulators of innate immune signaling and the NFkB pathway (Irving C Allen et al., 2011; Vladimer et al., 2012). NLRP12 has been shown to act as an inflammasome in the lungs of animals infected with Yersinia pestis and acting specifically to cleave pro-IL-18 to activate and recruit Natural Killer (NK cells) (Vladimer et al., 2012). NLRP12 has also been shown to process IL-18 in the cytosol of malaria parasite infected monocyte (Ataide et al., 2014).

Using our animal model of radiation-thermal combined injury, we investigated the contribution NLRP12 plays in the immune and hematopoietic response following injury. We observed relative increases in Nlrp12 transcript in lung, spleen, and bone marrow tissues of wild type mice after RCI, but not after burn or radiation alone. Nlrp12-/- deficient animals also displayed a significant increase in morbidity and mortality after RCI compared to wild type animal. This decrease in survival also correlated with decrease myeloid cellularity in the spleen, lungs, bone marrow, and blood. Contrastingly, animals deficient in other components of the
inflammasome complex (Caspases 1 and 11, and ASC) or IL1ra did not display a myeloid cell defect indicating that NLRP12 is not acting as an inflammasome within our model.

In addition to decreased myeloid cellularity in the bone marrow compartment, Nlrp12-/- animals have a significant reduction in the numbers of myeloid progenitors as early as three days post injury. This decrease was correlated with significantly high levels of TNF in Nlrp12-/- RCI animals, but not in wild type RCI animals. These high TNF levels were then shown to be responsible for the progenitor cell apoptosis by neutralizing circulating TNF immediately after injury. In addition to higher levels of progenitor cells actively undergoing apoptosis, Nlrp12-/- RCI animals showed higher levels of canonical NFkB signaling through phosphorylation of IkBa and p65, but not IKK or p38. Animals were then subjected to a systemic infection of Pseudomonas aeruginosa, with all NLPR12 animals eventually succumbing to infection. Taken together, these data show that NLRP12 acts to control the inflammatory responses during an emergency hematopoietic event by limiting TNF production and responses to excessive inflammatory signaling.

6.3 Future directions: Characterization of NLRP12 impact on canonical NFkB induction of apoptosis

Using our model of radiation thermal combined injury and mice deficient in Nlrp12, we showed that excessive levels of TNF in NLRP12-deficient animals results in increased levels of TNF-induced apoptosis of progenitor cells. Within the cells, we observed increase activation of the canonical NFkB signaling indicated by increased phosphorylation of p65/RelA and the inhibitor IkBa with no changes observed in non-canonical pathway proteins. Further investigation should elucidate the signaling abnormalities in Nlrp12-/- animals after injury. More specifically if NLRP12 is working to limit canonical signaling of NFkB and limiting Caspase 3 and Caspase 8 activity to suppress TNF-induced apoptosis, or if it suppressing RIP1/3 kinases
induction of necroptosis, another form of programmed cell death. Staining of progenitor cells for the phosphorylated epitopes of these proteins would help to examine this. Additionally, co-immunoprecipitations of progenitor cells would allow for the study of the proteins NLRP12 is interacting with in progenitor cells and monocyte.

6.4 Rescue of platelet and myeloid populations after radiation-thermal combined injury

Platelets are one of the first cells to respond to a burn injury. They initiate coagulation to close any open wound and degranulate, releasing chemokines, chemoattractants, and cytokines to recruit activated monocytes, neutrophils, and T cells that begin the wound healing process and countervene any infectious insult (Levin & Egorihina, 2010; Takashima, 1997). However, after the initial injury, many soluble clotting factors and the systemic platelet compartment is severely diminished (Takashima, 1997). Additionally, the platelets that remain are hyper-reactive, displaying more degranulation for several days after the initial injury (Takashima, 1997). GM-CSF is a glycoprotein growth factor that stimulates proliferation and survival of myeloid lineage progenitor cells and myeloid cells (Reeves, 2014). Previous studies have shown the beneficial impact that GM-CSF plays after a radiation injury alone with limit success in restoring myelopoiesis and peripheral restoration.

Following two week of continuous treatment with GM-CSF, injured animals displayed increased peripheral innate populations of monocyte and neutrophils, as well as platelets. Moreover, these platelets showed higher ability to initiate coagulation, in addition to a reduction in degranulation. This increased cellularity correlated not only with an increase in myeloid cell progenitor expansion in the GM-CSF treated, injured animals compared to vehicle-treated, injured animals, but also with a decrease in the number of progenitors undergoing inflammation-
induced apoptosis. Consequently, when challenged with a systemic infection, GM-CSF-treated, injured animals were better able to control spread of bacteria.

6.5 Future directions: Investigate contribution of platelets to radiation-thermal combined injury

Using our model of radiation-thermal combined injury, regular administration of granulocyte-monocyte colony stimulating factor (GM-CSF) after RCI improves innate immune cell and platelet function compared to vehicle treated controls. Further understanding of the role GM-CSF plays as a cellular therapeutic after injury may help to provide for better care and patient outcomes. The impact GM-CSF is playing on specific progenitor populations, specifically on their proliferation, inflammatory-induction of apoptosis, and their ability to resist exhaustion through prolonged exposure to stimulatory cytokine could be examined by using a methylcellulose-based colony forming unit assay. Additionally, higher doses administered as a bolus or in a contracted regime may further improve cellular and infection outcomes.

Further studies are necessary to elucidate platelet phenotypic improvements following GM-CSF including megakaryocyte phenotyping, platelet clotting and activity assays through aggregrometry. Moreover, the role GM-CSF plays in improving innate immune cell responses against pathogens requires further study. Other bacteria, fungal or viral challenges such as the Graham + Staphylococcus aureus, Aspergillus fumigatis, and Human/Murine Cytomegalovirus are all common within burn units and patients, and the use of GM-CSF may improve outcomes to these agents following a burn and radiation injury.

6.6 Cellular mechanisms of increased susceptibility to early wound infection after thermal injury

The major cause of mortality of both burn and radiation-thermal combined injury patients is a wound or lung infection often acquired in their long-term hospital stay and attributed to their highly immunocompromised state (Roth & Hughes, 2015). Immediately following a burn injury,
the body enters burn shock – a highly specific type of shock in which hypovolemic shock, septic shock, and emotional shock – in which many organ systems are directly impact (Angus & Van der Poll, 2013; Roth & Hughes, 2015). In addition to the obvious dermatologic, cardiologic, and gastrointestinal complications that occur, the immune system initial enters into a profound pro-inflammatory cytokine storm (Church et al., 2006).

Despite this pro-inflammatory, which can be beneficial in response to an infectious insult, the destructive damage caused by incidental immune cell activation, however, elicits a poor response to infectious challenge, especially at the damaged wound bed (Church et al., 2006). This initially pro-inflammatory response dampens in the days after injury, but the immunocompromised state remains (Chastre & Fagon, 2007; Shorr et al., 2005). The wound healing process involves a tight coordination of growth factors, anti-inflammatory cytokines, and cell migration toward the injured site. In the first weeks after a burn, the overwhelming anti-inflammatory, wound healing state hinders the innate and adaptive immune systems in mounting appropriate reactions to bacterial, fungal, and viral challenges (S. W. Jones et al., 2013). This is all in spite of CD4+ and CD8+ T cells showing enhanced allograft and xenograft rejection (B. Cairns et al., 2006).

Utilizing our murine model of thermal injury, the initial innate immune responses to a wound infection were investigated. The most common Graham negative bacteria that burn patients, let alone hospital patients acquire taken all together, is the pathogen Pseudomonas aeruginosa. Twenty-four hours following the burn or sham procedures, mice were challenged with P. aeruginosa at the site of injury. Within 48 hours of infection, the bacteria had replicated to a high titer and spread resulting in a bacterial sepsis. Dendritic cells and CD8+ T cells after infection are necessary regulatory cells to promote an anti-bacterial response (D’Arpa et al.,
2009), however these cells in burned mice exhibit hire levels of apoptosis, a form of programmed cell death, in addition to alterations in TLR expression also exhibited by other innate immune cells.

As previously mentioned, the body response to traumatic injuries initially with a pro-inflammatory response, burn shock. The hallmark cytokines that have been used as biomarkers to predict patients are IL-6 and IL-12. IL-6 is a pleiotropic cytokine/myokine that is vital for bactericidal activity and to lessen TNF and IL1 family responses (Pedersen & Fischer, 2007; Steensberg et al., 2003). IL-12 is produced by various immune cells – dendritic cells, neutrophils, macrophages, and B cells – that alters the transcriptional responses of immune cells to increase their production of TNF and IFNγ to in turn activate Natural Killer (NK) cells and T cells (T. Chen et al., 2007). In a wound healing response, the anti-inflammatory cytokine IL-10 regulates production of growth factors for angiogenesis and tissue repair, but lessens the bactericidal capacity of many innate immune cells. Consequently, the balance of these two cytokines is vital to patient outcomes and responses to infection (S. W. Jones et al., 2013). The systemic IL-10/IL-12 axis was skewed in burn animals after infection, with elevated levels of IL-10 and a concordant polarization of neutrophils into an anti-inflammatory phenotype (N2; IL-10+ IL-12).

The adenylate cyclase gene cyaB is a bacterial enzyme of P. aeruginosa that converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) (Topal et al., 2012). cAMP acts as a secondary messenger to initiate an acute virulence program including downregulation of flagellin production, increased virulence factor production, and decreased biofilm formation. Infection with an attenuated isogenic mutant strain of P. aeruginosa, lacking the adenylate cyclase gene cyaB, (ΔcyAB) 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. Moreover, administration of bacterial flagellin increases bacterial clearance of wild type PAK and neutrophils skewing toward an N1 phenotype.

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

Utilization of our model of thermal injury has shown that neutrophil polarity with regards to IL-12 and IL-10 is directly correlated with bacterial clearance. Further understanding of the molecular processes that govern neutrophil polarization is needed. Delineating the phagocytic capacity, oxidative burst potential, and innate immune signaling cascades of neutrophils after a burn injury could help to improve patient outcomes by providing interventions or treatments to directly modulate these changes.

We find that neutrophils, not macrophages, are the primary innate immune cell population that is polarized early after a thermal injury. Neutrophil activation during infection or following trauma can be regulated by the pattern recognition receptors (TLRs). TLR2, TLR4, and TLR5 ligation with lipoproteins, LPS, and bacterial flagellin, respectively, can rapidly induce a neutrophil burst to produce radical oxygen and nitrogen species (Ayala et al., 2002; Maung et al., 2005; Sabroe et al., 2003; Shen, de Almeida, Kang, Yao, & Chan, 2012). We find that early after a thermal injury and subsequent wound infection with WT P. aeruginosa, that neutrophils display decreased expression of TLR2, TLR4, and TLR5 compared to infected, sham controls. Moreover, following infection neutrophils shift toward an anti-inflammatory (N2: IL-10+ IL-12+) phenotype that is detrimental to bacterial clearance. This decrease in TLR expression and resulting diminishment of bactericidal activity or hypo-responsiveness should be investigated further.
Similarly, as within the tumor microenvironment, the burn wound has high levels of TNF locally and drives a systemic increase as well. This TGFβ increase drives anti-inflammatory neutrophil responses. TGFβ drives naïve CD4+ T cells to differentiate in Th17 cells within the wound draining lymph nodes, it is therefore possible that TGFβ-receptors on neutrophils have alterations in p38 and ERK1/2 signal transduction (Amento & Beck, 2008). Use of sTβRII, a soluble TGFβ type II receptor, and a TGFβ broadly neutralizing antibody, could be used to determine TGFβ’s involvement in neutrophil polarization. Glucocorticoid release from the wound bed induces an anti-inflammatory macrophage phenotype that is beneficial to wound healing and induces lymphocyte apoptosis (Fukuzuka, Edwards, et al., 2000; Lang, Silvis, Nystrom, & Frost, 2001). It is therefore conceivable that burn-dependent glucocorticoids are driving an anti-inflammatory response within the neutrophil compartment. A possible means to test this would be by blocking the glucocorticoid receptor 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. It is currently unclear which immune sensor is mediating the polarization of neutrophils towards a N1 response. Lastly, it is not known if flagellin is interactive directly with neutrophils or another cell type. In order to use flagellin as a viable therapeutic, further investigation is required to delineate how flagellin impacts neutrophil functions – i.e. phagocytosis, NET formation, degranulation – after burn injury and infection.

6.8 Closing remarks

Collectively, these studies demonstrate the dynamic responses that the immune system has following a radiation-thermal combined injury or a thermal injury alone. The havoc that a prolonged immunocompromised state response wreaks on the body following a trauma is not
well understood. Moreover, the understanding of the complex interplay the immune system and hematopoietic system play in trying to regulate an appropriate balance between pro-inflammatory, bacterial response and that of an anti-inflammatory, wound healing response is poorly understood.

Taken together, these studies describe that cytokines that act to activate and drive responses peripherally can be detrimental to stable hematopoiesis. NLRP12 seems to act as a repressor of inflammation in this instance, but reducing the TNF-dependent apoptosis of hematopoietic progenitor cells. TLR signaling promotes an altered neutrophil inflammatory phenotype, resulting in poorer bactericidal activity. GM-CSF, which under hemostatic conditions induces survival and proliferation of progenitors, can be used to improve poor cellular recovery due to radio-ablation of immune cells. Additionally, flagellin administration can help to limit neutrophil anti-inflammatory polarization to help neutrophils mount an appropriate response to fight bacterial challenges. The work presented in this dissertation furthers our knowledge of the role innate immune cells in the responses to traumatic events that induce emergency myelopoiesis and increased susceptibilities to infection.
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