INVESTIGATION OF NEUTROPHIL QUANTITY AND FUNCTION IN TISSUE-REGENERATING MURIDS

by

JENNIFER LYNN CYR

(Under the Direction of Vanessa Ezenwa)

ABSTRACT

Regeneration is rare in mammals, but mammalian species such spiny mice (*Acomys spp.*) can undergo this process. Evidence suggests that regeneration-competent organisms exhibit reduced inflammatory responses, such as lower numbers of inflammatory cells in circulation. This thesis explores a potential inflammation-regeneration trade-off by comparing the quantity and functionality of neutrophils in regeneration-competent (*Acomys spp.*) and -incompetent (*Mus musculus*) murids from varying environments. Results from this work suggest that regeneration-competent murids have nuanced differences in neutrophil quantity and function. Specifically, *Acomys* exhibit decreased neutrophil proportions in circulation and decreased proportions of mature neutrophil reserves in the bone marrow compared to *Mus*. Additionally, *Acomys* neutrophils can ingest more material than *Mus* neutrophils without altering bacteria-killing functions of neutrophils. However, bacterial killing by the serum alone was strongly enhanced in *Acomys* compared to *Mus.* Overall, this work suggests that *Acomys* utilize immunologic strategies that decrease inflammation without compromising pathogen defense.

INDEX WORDS: *Acomys*, inflammation, mammals, neutrophils, tissue regeneration

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW: WHY SOME ANIMALS REGENERATE WHILE OTHERS DO NOT: THE RELATIONSHIP BETWEEN INFLAMMATION AND REGENERATION

Introduction

Fibrosis, the formation of scars during wound healing, can be detrimental to health because scar formation can cause the loss of tissue function, decreased mobility, and other negative physiological outcomes. In contrast, regeneration, the ability to heal wounds without forming scar tissue, can preserve tissue structure and function and restore mobility following wounding (Ferguson and O'Kane, 2004). Regeneration is common in some animals, including several vertebrate species. Fish, tadpoles, and salamanders can all regenerate following tissue injury (Godwin and Brockes, 2006; King and Newmark, 2012). In addition, during fetal development, mammals may undergo regeneration (Colwell et al., 2005; Mescher and Neff, 2005); however, this type of healing is not generally observed in adult mammals (Seifert et al., 2012b). Given the benefits of regeneration (preservation of tissue structure and function, restoration of mobility), one intriguing biological question is why adult mammals do not undergo this type of wound healing.

Across vertebrate taxa, the ability to regenerate appears to have been lost as increasingly pro-inflammatory immunity was acquired. Thus, vertebrates with more complex immune systems and more potent mechanisms of inflammation (e.g. increased subsets of specialized inflammatory

cells, pathogen specificity, and memory), such as mammals, generally do not regenerate (Mescher and Neff, 2005; Godwin and Brockes, 2006;King et al., 2012; Mescher et al., 2013). This immune comparison across vertebrate taxa prompted the hypothesis that regeneration may trade off with inflammation (Harty et al., 2003; Godwin and Brockes, 2006; Eming et al., 2009; Eming et al., 2017).

In mammals, the immune response to injury typically involves the infiltration of the injured area by inflammatory cells, such as neutrophils (Kolaczkowska and Kubes, 2013). This inflammation is important to ensure that the wound does not become infected, but it can also exacerbate tissue damage, which is eventually repaired via fibrosis (Clark, 1988; Bergamini et al., 2004; Dreifke et al., 2015). For this reason, there is a growing body of evidence supporting the idea that regeneration may trade-off with high levels of inflammation (King et al., 2012; Eming et al., 2017; Mescher et al., 2017), possibly explaining why many mammals do not commonly undergo this process. Investigating this potential trade-off would increase our understanding of the potential costs and benefits associated with different wound healing strategies.

Evidence for and against an inflammation-regeneration trade-off

Evidence in support of the regeneration-inflammation trade-off hypothesis comes from recent studies of (i) regeneration-competent non-mammalian vertebrates, (ii) regeneration-competent mammalian fetuses, and (iii) rare, regeneration-competent adult mammals. First, evidence from non-mammalian regeneration-competent vertebrates suggests the capacity to regenerate may trade off with high levels of inflammation (reviewed in Eming et al., 2009). For example, axolotl salamanders that can regenerate complex structures decrease the numbers of

white blood cells recruited to wounds during injury (Schotté and Sicard, 1982; Sicard, 1983). Neutrophil recruitment, in particular, is significantly reduced during regeneration in axolotls (Lévesque et al., 2010; Seifert et al., 2012c). The salamander eye, capable of full regeneration, contains low levels of neutrophils and pro-inflammatory cytokines (Eming et al., 2009). Furthermore, regeneration is halted in axolotls when high levels of inflammation are present; for example, when salamanders are treated with the light metal Beryllium (Be^{2+}), which induces inflammation, regenerative processes stop (Thornton, 1949). Like salamanders, the frog species *Xenopus laevis* can regenerate. However, regeneration only occurs in these frogs at a precise larval life stage pre-metamorphosis; post-metamorphosis, frogs are unable to regenerate. *X. laevis* larvae and adults possess drastically different immune phenotypes, with the regeneration-competent larval stage containing few pro-inflammatory mechanisms and the regeneration-incompetent adult stage having an immune system more closely resembling those of mammals (Robert and Cohen, 1998). Transcriptome analysis in *X. laevis* has revealed distinct immunological mRNA profiles during the regeneration-competent and -incompetent life stages. Regeneration-incompetent stages are marked by increased inflammatory markers, whereas regeneration-competent phases are marked by increased inflammation resolution markers (Grow et al., 2006; King et al., 2012; Mescher et al., 2013). Thus, in non-mammalian vertebrates, high levels of some inflammatory cells and molecules appear to inhibit regeneration.

Second, most mammals, including mice, primates, and humans, can undergo regeneration during fetal development (e.g. Sullivan et al., 1995; Florence et al., 1996; Han et al., 2003), and mammalian fetal environments generally have lower levels of inflammation compared to adult mammals (Sullivan et al., 1995; Mescher and Neff, 2005). For example, the fetal environment in mammals is largely devoid of pro-inflammatory cytokines and innate immune cells such as

neutrophils and macrophages. Instead, it is characterized by increased levels of anti-inflammatory and regulatory cytokines (e.g. IL-10) as well as the increased prevalence of humoral immune factors such as serum immunoglobulin and antimicrobial peptides (Singer and Clark, 1999; Harty et al., 2003). Furthermore, in murine fetuses, knocking out the regulatory cytokine IL-10 increases inflammation and suppresses regeneration in fetuses. When wounded, these knockout fetuses produce scars and recruit significant numbers of leukocytes, particularly neutrophils, to the wound (Liechty et al., 1999). Therefore, in mammalian species that can regenerate during development, high levels of inflammation also appear to be incompatible with regeneration.

Third, while regeneration is uncommon in adult mammals, some mammalian species are capable of undergoing regeneration during adulthood. For example, several species of deer can regenerate antler tissue (Li et al., 2004; Kierdorf et al., 2009), and rabbits and pika can fully regenerate ear holes (Williams-Boyce and Daniel, 1980). Both models provide some support for the idea that inflammation is inhibitory to regeneration. For instance, deer antlers are characterized by high levels of anti-inflammatory, pro-repair cytokines such as TGF-β (Faucheux et al., 2004; Price et al., 2005). The immune environment of the rabbit ear has not been as extensively studied. However, one study suggests that when inflammation is increased in the rabbit ear following the introduction of a pathogen (in this case, *Trypanosoma brucei*), excess blood vessel damage occurs, and regeneration is slowed (Goodwin, 1971).

Perhaps the most useful model of adult mammalian tissue regeneration is the African spiny mouse (Muridae: *Acomys spp.*, Figure 1.1), which is closely related to laboratory mice, making comparative studies of wound healing possible (Seifert et al., 2012a;Seifert and Maden, 2014;Gawriluk et al., 2016). Spiny mice are among the few mammalian species that can regenerate during adulthood (Seifert et al., 2012a; Figure 1.1). Recently, three species of *Acomys* (*A.*

cahirinus, *A. percivali*, and *A. kempi*) have been identified as regeneration-competent (Seifert et al., 2012a; Brant et al., 2016; Gawriluk et al., 2016). Recent studies suggest that during regeneration, fewer neutrophils are recruited to *A. cahirinus* wounds when compared to wounds of laboratory *Mus musculus*, a regeneration-incompetent murid (Simkin et al., 2017). At baseline, *Acomys* have also been shown to have fewer neutrophils in circulation when compared to *Mus* (CD-1, Brant et al., 2016). Finally, regeneration in *Acomys* is characterized by lower levels of the enzyme myeloperoxidase (MPO), a neutrophil-specific enzyme that produces reactive oxygen species (ROS), when compared to wound healing in *Mus* (Swiss webster; Simkin et al., 2017). Thus, the *Acomys* immune system appears to be characterized by lower levels of inflammation, particularly regarding neutrophil quantity and function, than the immune system of *Mus*, a regeneration-incompetent murid. Collectively, evidence from studies of regeneration-competent non-mammalian vertebrates, mammalian fetuses, and regeneration-competent adult mammals suggest that regeneration trades off with inflammation, at least to some degree.

Despite a growing body of evidence in support of a trade-off between inflammation and regeneration, it is important to note that not all aspects of inflammation appear to hinder regeneration. While low numbers of leukocytes are commonly seen in the wounds of regeneration-competent species, the complete absence of leukocytes halts regeneration (Schotté and Sicard, 1982). Some inflammatory cell types, such as macrophages, are necessary for regeneration to occur (Godwin et al., 2013; Simkin et al., 2017). Additionally, reactive oxygen species (ROS), molecules produced by inflammatory cells, are required for the initiation of regeneration in some species (Gauron et al., 2013; Love et al., 2013; Simkin et al., 2017). For example, ROS production is heightened in *Acomys* during regenerative healing compared to *Mus* during fibrotic healing (Simkin et al., 2017). In *Xenopus* tadpoles, ROS promote regeneration by increasing cell

proliferation and initiating signaling cascades that are important to the regenerative process. When ROS levels are dampened in *Xenopus,* tadpoles can no longer regenerate (Love et al., 2013). ROS have also been implicated in regeneration in zebrafish (Gauron et al., 2013). Therefore, while regeneration may trade off with some aspects of inflammation, other aspects seem to be required for regeneration to proceed.

Trade-offs between neutrophil quantity and regeneration

Given the apparent complexities of the relationship between inflammation and regeneration, additional studies assessing this relationship are needed. Assessing different aspects of pro-inflammatory immunity separately may help to elucidate which aspects of inflammation are detrimental to regeneration and which are not. One aspect of inflammation that appears to consistently trade off with regeneration is neutrophil quantity. Lower neutrophil numbers have been documented across multiple regeneration-competent model systems, including mammalian fetuses (Liechty et al., 1999; Singer and Clark, 1999; Harty et al., 2003), axolotls (Lévesque et al., 2010), and *Acomys cahirinus* (Brant et al., 2016).

Why might large numbers of neutrophils hinder regeneration? During wound healing in mammals, neutrophils are required to kill pathogens, ensuring the wound does not become infected. Neutrophils primarily destroy pathogens by internalizing them via phagocytosis and using chemicals such as ROS to break down microbes internally (Segal, 2005). During this process, tissue damage can occur, especially when large numbers of neutrophils are present, because ROS can leak into the extracellular environment (Weissmann et al., 1980; Weiss, 1989). For most mammals this is not especially problematic because the subsequent stages of healing

clear excess inflammation and quickly repair tissue via fibrosis (Clark, 1988). Thus, excess inflammation might bias the wound healing response toward fibrosis and away from inflammation (Mescher and Neff, 2005; Eming et al., 2009).

If regeneration-competent animals have fewer neutrophils recruited to wounds, they could potentially be more susceptible to infection during wound healing. Therefore, a related question that remains to be addressed is that if regeneration-competent animals reduce neutrophil-mediated pathogen killing, do they compensate for this using alternative immune strategies? If so, what would these alternative strategies consist of? There is some evidence that regeneration-competent animals rely more heavily on blood serum than on inflammatory cells for pathogen defense. For example, *Xenopus* tadpoles have lower numbers of inflammatory cells and higher levels of serum peptides such as heat shock proteins when compared to the regeneration-incompetent adult stage (Robert and Cohen, 1998; King et al., 2012). The reliance of regenerators on humoral immune mechanisms is conceivable for two reasons. First, as mentioned, fibrosis is largely driven by tissue damage resulting from cell-mediated immunity and inflammation (Clark, 1988; Calve et al., 2010; Dreifke et al., 2015). Second, not only do defenses that occur via serum peptides prevent the extensive tissue damage associated with cell-mediated responses; recent studies have shown that these factors can promote mitosis and angiogenesis, two processes crucial to regeneration (Koczulla and Bals, 2003; Sørensen, 2016). Therefore, even though regeneration-competent animals may have lower numbers of inflammatory cells, these animals might protect themselves from infection by increasing defensive measures in the blood serum.

Unanswered questions

While the immune mechanisms associated with regeneration are not completely understood, it appears that some aspects of inflammation may trade off with the ability to regenerate. In particular, high neutrophil numbers (such as those of neutrophils recruited to wounds in most mammals) appear to be incompatible with regeneration. However, one question that arises from prior regeneration studies is whether the neutrophils that are present during wound healing function similarly between regeneration-competent and regeneration-competent species. Another unanswered question is that if regeneration-competent animals indeed have fewer neutrophils in circulation, are these animals more susceptible to infection during wounding or do other immune mechanisms make up for this deficiency? Finally, what are the health and fitness costs and benefits associated with regeneration in *Acomys*?

Specific aims

The goal of this thesis project was to further explore the hypothesis that regeneration trades off with inflammation. To do so, the following specific aims were addressed:

Specific Aim 1: To explore the potential trade-off between neutrophil quantity and the regeneration phenotype in uninjured animals.

Neutrophil quantity was compared among uninjured adult, regeneration-competent murids (*Acomys spp.*) and uninjured adult, regeneration-incompetent murids (*Mus musculus*).

Because immune parameters can vary within murid species due to differences in environmental origins (Beura et al., 2016; Abolins et al., 2017), multiple species and strains of mice sourced from both captive and wild environments were utilized in this study to distinguish the potential effects of environment from the potential effects of having a regenerative phenotype.

Specific Aim 2: To examine relationships between neutrophil function and the regeneration phenotype in uninjured animals.

Three distinct neutrophil functions (i) migration ability, (ii) reactive oxygen species production, and (iii) phagocytosis capacity were compared among uninjured adult, regeneration-competent murids (*Acomys cahirinus*) and uninjured adult, regeneration-incompetent murids (*Mus musculus:* Swiss webster and wild-caught *M. musculus*). Animals derived from both captive and wild environments were compared to distinguish the potential effects of environment from the potential effects of having a regenerative phenotype.

Specific Aim 3: To investigate potential compensating immune strategies used by *Acomys.*

The abilities of whole blood, serum, and neutrophils from uninjured, adult regeneration-competent (*Acomys cahirinus*) and -incompetent (*Mus musculus*) murids to kill bacteria *in vitro* were compared. This comparison was used to investigate (i) the bacteria-killing capacity of neutrophils and (ii) the relative killing potential of different blood components between *Acomys* and *Mus.*

Figures

Figure 1.1

The African spiny mouse, *Acomys percivali*. Several species of African spiny mice (Muridae: *Acomys spp.*) can successfully regenerate dermis and cartilage following tissue injury.

CHAPTER 2

COMPARISON OF NEUTROPHILS FROM REGENERATION-COMPETENT AND -INCOMPETENT MURIDS REVEALS SUBTLE DIFFERENCES IN CELL QUANTITY AND FUNCTION [1]

[1] Cyr, J.L., Gawriluk, T.G., Kimani, J.M., Rada, B., Watford, W.T., Kiama, S.G., Seifert, A.W., and Ezenwa, V.O. Submitted to *Scientific Reports*, 06/08/2018.

Abstract

Regeneration is rare in mammals, but spiny mice (*Acomys spp*.) naturally regenerate skin and ear holes. Inflammation is thought to inhibit regeneration during wound healing, but aspects of inflammation contribute to both regeneration and pathogen defense. We investigated the relationship between regeneration and inflammation by comparing neutrophil traits among uninjured regeneration-competent (*Acomys*: *A. cahirinus, A. kempi, A. percivali*) and -incompetent (*Mus musculus:* Swiss webster, wild-caught strains) murids. Neutrophil quantity differed significantly among species. In blood, *Acomys* had lower percentages of circulating neutrophils than *Mus*; and in bone marrow, *Acomys* had higher proportions of less mature band neutrophils and lower proportions of more mature segmented neutrophils. Functionally, *Acomys* and *Mus* neutrophils did not differ in their ability to migrate or produce reactive oxygen species, but *Acomys* neutrophils phagocytosed more material. Despite enhanced phagocytosis activity, *Acomys* neutrophils were not more effective than *Mus* neutrophils at killing *Escherichia coli*. Moreover, whole blood bacteria killing was dominated by serum in *Acomys* versus neutrophils only or neutrophils and serum in *Mus*, suggesting that *Acomys* primarily rely on serum to kill bacteria whereas *Mus* do not. These subtle differences in neutrophil traits may allow regeneration-competent species to offset damaging effects of inflammation without compromising pathogen defense.

Introduction

Most adult mammals lack the ability to regenerate damaged tissue and instead repair wounds by forming scar tissue. Wound healing in mammals is a multi-step process that involves inflammation, cell migration, new tissue formation, and tissue remodeling (Clark, 1988; Grose and Werner, 2004; Gurtner et al., 2008; Guo and DiPietro, 2010). Typically, tissue damage elicits the production of pro-inflammatory cytokines and the immediate recruitment of neutrophils to the wound site (Gillitzer and Goebeler, 2001; Werner and Grose, 2003; Grose and Werner, 2004). This early wave of inflammation is required to ensure the wound does not become infected after barrier defenses are compromised. Later in the wound-healing process, excess neutrophils are ingested by macrophages as anti-inflammatory cytokines help to resolve the acute inflammatory state and promote new tissue formation (Martin, 1997; Stramer et al., 2007; Guo and DiPietro, 2010). Although this entire process is efficient at closing wounds rapidly and stimulating fibrotic repair, the initial inflammatory phase can exacerbate tissue damage and promote excess scarring (Clark, 1988; Bergamini et al., 2004; Stramer et al., 2007; Dreifke et al., 2015). Thus, the tendency of the mammalian immune system to mount a strong inflammatory response to injury may directly impede tissue regeneration (Mescher and Neff, 2005; Eming et al., 2009), contributing to the rarity of this phenomenon in mammals.

African spiny mice (Muridae: *Acomys spp.*) are among the few mammals known to regenerate complex structures in response to tissue injury. At least three species in this genus, *A. kempi*, *A. percivali*, and *A. cahirinus*, have been shown to successfully regenerate full-thickness skin including hair follicles, and are capable of re-growing the complex tissue assemblage of the ear pinna (Seifert et al., 2012a; Brant et al., 2016; Gawriluk et al., 2016; Matias Santos et al.,

2016). The cellular mechanisms that allow spiny mice to regenerate in response to injury remain largely unknown. However, recent studies suggest that the inflammatory response of *Acomys cahirinus* is somewhat distinct from the *Mus musculus* inflammatory response, characterized, for example, by delayed neutrophil infiltration at the wound site and differences in reactive oxygen species (ROS) production (Simkin et al., 2017). Non-mammalian species with regenerative abilities also show anti-inflammatory immune biases associated with wound healing (Godwin et al., 2013). For example, while limb amputation in salamanders elicits a strong pro-inflammatory cytokine signature, anti-inflammatory cytokines are simultaneously produced at the wound site (Godwin et al., 2013). Likewise, neutrophil recruitment to salamander skin wounds is significantly reduced compared to that of mammals during wound healing (Lévesque et al., 2010; Seifert et al., 2012c). Furthermore, comparisons between pre-metamorphic *Xenopus laevis* tadpoles, which are capable of regeneration, and juveniles, which are not, show that genes associated with inflammatory responses and immune cell recruitment are downregulated in tadpoles (Grow et al., 2006). Altogether, these observations support the hypothesis that regeneration trades off with at least some aspects of inflammation.

Building on these studies, we used spiny mice as a model to better understand potential regeneration-associated biases in immune cell function by focusing on neutrophils, central players in the acute inflammatory response to tissue injury. Following an injury, neutrophils increase dramatically in circulation (Grose and Werner, 2004), and these cells infiltrate the wound site in large numbers where their main function is to kill pathogens (Brinkmann et al., 2004; Martin and Leibovich, 2005). Pathogen-killing is accomplished by phagocytosis, degranulation, or the use of neutrophil extracellular traps (Brinkmann et al., 2004; Mayadas et al., 2014). These activities destroy pathogens using chemicals, such as ROS, that are hazardous

to microbes but can also damage tissue if they leak into the extracellular environment (Weissmann et al., 1980; Weiss, 1989; Smith, 1994). Given the tissue-damaging side effects of neutrophil-mediated pathogen killing, we hypothesized that these functions might be constitutively dampened in *Acomys* compared to regeneration-incompetent murids. However, inflammation is a key adaptive response to noxious stimuli and an effective inflammatory response is required to eliminate pathogenic threats during injury (Tracey, 2002). Moreover, some aspects of the inflammatory response, such as ROS production, are essential for the initiation of regeneration (Gauron et al., 2013; Love et al., 2013; Simkin et al., 2017). Given this, we expected that any differences in neutrophil behavior observed in *Acomys* might be nuanced, balancing the negative effects of the inflammatory response (tissue damage) against its positive effects (pathogen defense, polarization of regeneration).

To test these ideas, we compared neutrophil quantity and function in regeneration-competent *Acomys* and regeneration-incompetent *Mus musculus.* First, we measured neutrophil quantity by comparing neutrophil proportions in the peripheral blood and bone marrow of uninjured *Acomys* and *Mus*. Second, we assessed neutrophil function by examining the ability of bone marrow neutrophils from both genera to perform key pathogen-killing functions, including migration, phagocytosis, ROS production, and bacterial killing. Third, because within-species differences in immune function are common (e.g. Lochmiller et al., 1994; Christe et al., 2000; Harms et al., 2010), including among *Mus musculus* populations originating from different environments (Beura et al., 2016; Abolins et al., 2017), we used comparisons of wild and captive animals to help distinguish between effects that might be due to environmental variation versus the trait of interest (i.e., regenerative ability). Specifically, we compared captive *Acomys* (*A. cahirinus*, AC) to wild-caught (MM) and captive (Swiss webster, SW) *Mus musculus*. For

analyses involving peripheral blood cell comparisons we included an additional comparison of wild-caught *Acomys* (*A. kempi,* AK and *A. percivali,* AP) to captive (SW) *Mus.*

Results

Acomys have lower percentages of neutrophils in peripheral blood compared to *Mus*

To assess differences in neutrophil quantity between *Acomys* and *Mus,* we performed white blood cell differential counts on whole blood from a combination of captive and wild-caught animals housed at either the University of Georgia (UGA) or University of Nairobi (UON). UGA species included regeneration-competent, captive-bred *A. cahirinus* (ac-uga) and regeneration-incompetent captive-bred (sw-uga) and wild-caught (mm-uga) *Mus musculus* (Table 1). UON species included regeneration-competent, wild-caught *A. kempi* (ak-uon) and *A. percivali* (ap-uon) and regeneration-incompetent, captive-bred *M. musculus* (sw-uon) (Table 2.1).

For the UGA species, we found significant among-species differences in neutrophil (Figure 2.1a) percentages in the blood. Ac-uga had significantly fewer circulating neutrophils than both laboratory sw-uga and wild mm-uga (Figure 2.1b; Table 2.2). The lower neutrophil percentages in ac-uga were also accompanied by significantly higher percentages of lymphocytes and lower percentages of monocytes when compared to sw-uga and mm-uga (Table 2.2), suggesting that lower proportions of phagocytic cells in *Acomys* are counterbalanced by increased proportions of lymphocytes. The blood cell differences observed in the UGA species were perfectly recapitulated in the UON species. Both ap-uon and ak-uon had significantly fewer

neutrophils than did sw-uon (Figure 2.1c; Table 2.3). Both ap-uon and ak-uon also had significantly fewer lymphocytes and more monocytes compared to sw-uon (Table 2.3).

Acomys and *Mus* do not differ in the percentage of total neutrophils in the bone marrow, but *Acomys* have fewer segmented neutrophils

To determine whether neutrophil percentages in the bone marrow differed between *Acomys* and *Mus*, we performed differential white blood cell counts on bone marrow homogenates from the UGA species (ac-uga, mm-uga, and sw-uga), focusing on differences in the proportions of morphologically distinct neutrophil stages (Figure 2.2a). There was no difference among species in the percentage of total neutrophils present in the bone marrow (Figure 2.2b; Table 2.4). However, percentages of less mature band neutrophils were significantly higher in ac-uga compared to laboratory sw-uga and wild mm-uga (Figure 2.2c; Table 2.4), whereas percentages of more mature segmented neutrophils showed the reverse pattern (Figure 2.2d; Table 2.4). No differences were observed among species in any other cell type except basophils, for which laboratory sw-uga differed from wild mm-uga only (Table 2.4).

Migratory ability does not differ between *Acomys* and *Mus* bone marrow neutrophils

To examine neutrophil functionality in the UGA species (ac-uga, sw-uga, mm-uga), we tested for differences in the ability of bone marrow cells to migrate across a semi-permeable membrane toward migration-inducing stimuli. Neutrophils were either treated with the stimulant phorbol myristate acetate (PMA) or left untreated, and treatment status significantly affected

migratory ability, whereas species did not (LMM, n = 47; treatment: df = 1, F = 16.3, p < 0.0001; species: df = 2, F = 0.510, p = 0.6025, Figure 2.3).

ROS pathway components do not differ consistently between *Acomys* and *Mus*

We used two approaches to compare the functionality of reactive oxygen species (ROS) and ROS-producing enzymes in bone marrow neutrophils isolated from the UGA species (ac-uga, sw-uga, mm-uga). First, we compared the ability of untreated and PMA-treated neutrophils to produce the ROS, superoxide anion. For untreated samples, superoxide production differed among species (LMM, n = 47; species: df = 2, F = 5.11, p = 0.0106), but post-hoc analysis showed no consistent difference between *Acomys* and *Mus* (Figure 2.4a). Neutrophils from ac-uga produced more superoxide than neutrophils from laboratory sw-uga (Tukey's test: p = 0.0128), but neutrophils from wild mm-uga did not differ from either ac-uga (p = 0.0589) or sw-uga (p = 0.9332). For PMA-treated samples, superoxide production did not differ between species (LMM, n = 47; df = 2, F = 2.74, p = 0.0767; Figure 2.4b). Altogether, these data suggest that variation in superoxide production is not a function of regenerative ability.

Second, we quantified the activity of myeloperoxidase (MPO), an enzyme that converts hydrogen peroxide to hypochlorous acid downstream of superoxide anion production, in PMA-treated and untreated neutrophil supernatants. MPO activity differed significantly by treatment and among species (LMM, n = 47; treatment: df = 1, F = 8.23, p = 0.0062; species: df = 2, F = 26.4, p < 0.0001). However, once again there was no consistent difference between *Acomys* and *Mus* (Figure 2.4c). Neutrophils from laboratory sw-uga showed higher MPO activity than neutrophils from either wild mm-uga (Tukey's test: p < 0.0001) or ac-uga (p < 0.0001), whereas

neutrophils from ac-uga and mm-uga were not different from one another ($p = 0.9069$). Thus, as with superoxide production, MPO activity did not appear to be associated with regenerative capacity.

Acomys bone marrow neutrophils show enhanced phagocytic ability compared to *Mus*

To determine whether differences in phagocytic capacity existed between *Acomys* and *Mus* neutrophils, we investigated the ability of bone marrow cells from the UGA species (ac-uga, sw-uga, and mm-uga) to consume the immunogenic fungal glucan and toll-like receptor 2 (TLR2) activator, zymosan. PMA treatment did not affect phagocytic capacity, but phagocytic ability differed significantly among species (LMM, $n = 47$; treatment: $df = 1$, $F = 1.43$, $p = 0.23$; species: $df = 2$, $F = 13.6$, $p < 0.0001$; Figure 2.5). Ac-uga neutrophils consumed more zymosan than neutrophils from both sw-uga (Tukey's test: $p = 0.0015$) and mm-uga ($p < 0.0001$), whereas sw-uga and mm-uga neutrophils did not differ from one other in phagocytic capacity ($p = 0.2029$).

Acomys and *Mus* neutrophils do not differ in *E. coli*-killing ability, but killing activity in the two groups is driven by different blood components

Finally, to understand the relative role of neutrophils in performing pathogen killing functions in the blood of *Acomys* and *Mus*, we compared the ability of different blood components (neutrophils, serum, and whole blood) isolated from the UGA species (ac-uga, sw-uga, mm-uga) to kill *Escherichia coli* in vitro. Analyses of killing ability by blood component

revealed that while there was no difference among species in the ability of neutrophils to kill bacteria (LMM, n = 48; species: df = 2, F = 0.508, p = 0.6056; Figure 2.6a), killing ability did vary among species for serum (LMM, n = 48; species: df = 2, F = 262, p < 0.0001; Figure 2.6b) and whole blood (LMM, n = 48; species: df = 2, F = 72.4, p < 0.0001; Figure 2.6c). Both serum and whole blood from ac-uga killed significantly more bacteria than serum and whole blood from mm-uga (Tukey's test: serum, p < 0.0001; whole blood, p < 0.0001) and sw-uga (serum, p < 0.0001; whole blood, p < 0.0001).

When we examined killing responses within species and across blood components (whole blood, serum, and neutrophils), we found that bacteria killing in *Mus* appeared to be driven either by a combination of serum and neutrophils (sw-uga: LMM, n = 48; tissue: df = 2, F = 5.24, p = 0.0097; Tukey's test; whole blood vs. serum: p = 0.0051, whole blood vs. neutrophils: p = 0.4162, serum vs. neutrophils: p = 0.1099; Figure 2.6d, left panel), or primarily by neutrophils (mm-uga: LMM, n = 48; tissue: df = 2, F = 8.43, p = 0.0008; Tukey's test; whole blood vs. serum: p = 0.0094, whole blood vs. neutrophils: p = 0.6865, serum vs. neutrophils: p = 0.0009; Figure 2.6d, center panel). In contrast, whole blood bacteria-killing in ac-uga appeared to be driven entirely by serum, with serum killing an equivalent amount of bacteria as whole blood, and neutrophils killing significantly less bacteria than either whole blood or serum (LMM, n = 48; tissue: df = 2, F = 83.0, p < 0.0001; Tukey's test; whole blood vs. serum: p = 0.7595, whole blood vs. neutrophils: p < 0.0001, serum vs. neutrophils: p < 0.0001; Figure 2.6d, right panel). These data suggest that microbial killing in *Acomys*, but not *Mus*, is dominated by non-cellular blood components.

Discussion

The degree to which inflammatory cells and their products impact the regenerative response to tissue injury, as well as potential consequences for pathogen defense, are poorly understood. Using a comparative approach, we investigated neutrophil behavior in three species of spiny mice (*Acomys spp.,* regeneration-competent murids) and two strains of house mice (*Mus musculus,* regeneration-incompetent murids) originating from both captive (ac-uga, sw-uga, sw-uon) and wild (ak-uon, ap-uon, mm-uga) populations. We tested whether uninjured *Acomys* showed constitutive differences in blood and bone marrow neutrophil quantity and function compared to *Mus*. We found that *Acomys* had significantly lower percentages of neutrophils in the blood and higher percentages of less mature band neutrophils in the bone marrow when compared to *Mus*. However, functionally, bone marrow neutrophils from *Acomys* did not differ from *Mus* neutrophils in their ability to migrate toward stimuli. There were also no consistent differences between the two genera in reactive oxygen species (ROS) production or ROS-producing enzymatic activity of their neutrophils. Despite similarities in neutrophil functions including migration ability and ROS production between *Acomys* and *Mus*, *Acomys* bone marrow neutrophils did differ from *Mus* neutrophils in phagocytic ability, showing increased consumption of fungal particles compared to *Mus* cells. Yet despite this difference, peripheral blood neutrophils from the two genera did not differ in their ability to kill *E. coli in vitro*. Moreover, *Acomys* serum exhibited a significantly stronger bacteria-killing ability than *Mus* serum. Intriguingly, whole blood microbial killing appeared to be dominated by the serum component in *Acomys,* whereas inflammatory cells seemed to play a more important role in *Mus*. Together, these results suggest that subtle differences in neutrophil development, mobilization,

and phagocytic function in *Acomys* may help to maintain a balance between the tissue damaging and pro-regenerative roles of inflammation.

The difference we observed in the percentage of circulating neutrophils in peripheral blood of *Acomys* and *Mus* were consistent across wild and captive animals. For the UGA species, captive *Acomys* (AC) had lower percentages of neutrophils than both captive (SW) and wild (MM) *Mus*. Similarly, for the UON species, wild *Acomys* (AK, AP) had lower neutrophil percentages than captive (SW) *Mus*. The lower neutrophil proportions we observed in *Acomys* support a previous observation by Brant *et al.* (Brant et al., 2016), who documented a marked difference in the proportion of circulating neutrophils in *Acomys* (AC) when compared to an outbred CD-1 strain of *Mus*. Interestingly, a recent study by Simkin *et al*. (Simkin et al., 2017), described equal numbers of neutrophils in the peripheral blood of AC and SW. However, Simkin *et al.* visualized neutrophils using a different approach from Brant *et al.* and the current study, possibly accounting for this disparity.

In addition to the difference in neutrophils, we also found that all three species of *Acomys* had proportionately fewer circulating monocytes and more lymphocytes than did the two *Mus* strains. Like neutrophils, monocytes are also involved in the early inflammatory response. During wound healing, monocytes differentiate into macrophages, which play a key role in phagocytosis at the wound site, including ingesting excess neutrophils (Martin and Leibovich, 2005;Mosser and Edwards, 2008;Koh and DiPietro, 2011;Kibe et al., 2017). Monocytes are also involved in regeneration in some species, including *Acomys cahirinus* (e.g. Godwin et al., 2013;Simkin et al., 2017). In combination, our comparison of the peripheral blood cell profiles between *Acomys* and *Mus* suggest a robust pattern in which *Acomys* have proportionately lower numbers of circulating inflammatory cell types (neutrophils and monocytes) than do *Mus*.

We also found subtle differences in the composition of bone marrow neutrophils between *Acomys* (AC) and *Mus* (SW and MM) that may help explain the difference in circulating neutrophil proportions we observed. In murine bone marrow, neutrophil development progresses from promyelocytes to myelocytes and then metamyelocytes, which develop into band neutrophils and finally mature into segmented neutrophils (Pillay et al., 2013). Interestingly, although AC did not differ from SW and MM in the total percentage of neutrophils in the bone marrow, SW and MM had significantly higher percentages of segmented neutrophils, whereas AC had significantly higher percentages of band neutrophils. Since band neutrophils are developmentally less mature than segmented neutrophils (da Silva et al., 1994; Pillay et al., 2013), this pattern suggests that *Acomys* may have a smaller reservoir of fully mature neutrophils in the bone marrow compared to *Mus*. In laboratory *Mus* (C57BL6), bone marrow has been shown to contain large numbers of mature, functionally competent neutrophils that can be released during infection (Boxio et al., 2004). Thus, a smaller reservoir of segmented neutrophils in *Acomys* bone marrow could explain the lower numbers of mature neutrophils in circulation. Another possible explanation that is compatible with the pattern of variation in blood and bone marrow cells we observed is that *Acomys* neutrophils may not be released from the bone marrow at the same frequency as *Mus* neutrophils. This idea is supported by a previous study which found that *Acomys* (AC) skin wounds appear to be deficient in granulocyte colony stimulating factor (G-CSF) compared to *Mus* (CD-1) wounds (Brant et al., 2016). Importantly, G-CSF signaling controls the trafficking of neutrophils from bone marrow to blood (Semerad et al., 2002); administration of G-CSF has been shown to increase circulating neutrophil numbers in both mice and humans (Lord et al., 1991; Furze and Rankin, 2008); mice lacking G-CSF exhibit profound neutropenia (Lieschke et al., 1994); and transgenic mice with truncated murine G-CSF

receptors have been shown to exhibit impaired neutrophil maturation (Mitsui et al., 2003). Thus, if the reduced levels of G-CSF reported in *Acomys* wounds reflect levels in the blood of uninjured animals, it is possible that impaired neutrophil maturation and a slower release of neutrophils from *Acomys* bone marrow, driven by low constitutive amounts of G-CSF, can explain both the smaller reservoir of segmented neutrophils in *Acomys* bone marrow and the maintenance of lower levels of circulating neutrophils in *Acomys* blood. Indeed, a slower release of neutrophils from *Acomys* bone marrow would explain the recent finding that neutrophil accumulation at the site of injury is delayed in AC wounds compared to SW wounds (Simkin et al., 2017).

In addition to exploring differences in neutrophil quantity between *Acomys* and *Mus*, we also examined differences in neutrophil function. Neutrophils perform multiple functions that contribute to pathogen killing during wound healing (reviewed in Kolaczkowska and Kubes, 2013), and some of these functions can exacerbate tissue damage (Smith, 1994;Bergamini et al., 2004). For example, destructive chemicals, such as ROS, are released into the extracellular space by neutrophils during microbial killing, contributing to tissue damage (Weissmann et al., 1980; Weiss, 1989; De Groot, 1994; Groot and Rauen, 1998; Mittal et al., 2014). Given that ROS are integral to the tissue-damaging effects of neutrophils, we expected differences in ROS production between *Acomys* and *Mus* to be more pronounced than any differences observed for other, less tissue-damaging neutrophil functions. Interestingly, we found no evidence of consistent differences in either superoxide production or MPO activity between *Acomys* and *Mus*. In the case of superoxide, untreated AC neutrophils produced more superoxide than untreated SW neutrophils, but neither species differed from MM, suggesting that some factor other than regeneration competence explains variation in baseline neutrophil superoxide

production among the species. Likewise, in the case of MPO activity, SW neutrophils had significantly higher MPO activity than AC neutrophils, but MPO activity was not different between AC and MM. Thus, the differences in ROS and ROS-producing enzymes we observed among species in this study were not associated with the ability to regenerate. The recent observation that ROS play an important role in the regenerative process may help explain the lack of a consistent difference in ROS production and activity we observed between *Acomys* and *Mus* neutrophils. Indeed, ROS production by NADPH oxidase, measured during the inflammatory phase of wound healing, was significantly higher in AC during regeneration than in SW during scarring (Simkin et al., 2017). Moreover, in other regeneration-competent species, ROS have been implicated in the initiation of regeneration (Gauron et al., 2013; Love et al., 2013). For instance, in *Xenopus* tadpoles, tail amputation prompts an increase in ROS levels that in turn facilitate key regenerative processes such as cell proliferation (Love et al., 2013). Overall, these observations suggest that ROS production is an unlikely axis of variation in which *Acomys* neutrophil function should differ from that of *Mus*.

Phagocytosis is another key function of neutrophils that is used to kill pathogens (Hampton et al., 1998; Segal, 2005; Mayadas et al., 2014). However, because ROS can leak into the extracellular environment and damage tissues during phagocytosis (Weissmann et al., 1980; Weiss, 1989; Smith, 1994), this function also has potentially tissue-damaging side effects. As such, we predicted that *Acomys* might have dampened phagocytosis capabilities compared to *Mus*. Counter to our expectations, we found that AC bone marrow neutrophils were better able to consume the fungal glucan zymosan than SW or MM neutrophils. Since neutrophil phagocytosis is also thought to play a role in wound debridement and resolving inflammation (Simpson and Ross, 1972; Park and Barbul, 2004; Fournier and Parkos, 2012; Kolaczkowska and Kubes,

2013), it is possible that these functions counterbalance any damaging effects. Indeed, if neutrophil phagocytosis plays an important role in resolving inflammation in *Acomys,* this may explain the heightened phagocytosis capabilities we observed in this species compared to *Mus*.

If *Acomys* and *Mus* neutrophils differed in key pathogen-killing functions, we expected these differences to translate directly into variation in microbial killing ability. To quantify differences in microbial killing, we compared the *in vitro E. coli* killing capacities of whole blood, neutrophils, and serum both among and within the three UGA species. Intriguingly, even though *Acomys* neutrophils were better able to phagocytose immunogenic particles, we found no difference between *Acomys* (AC) and *Mus* (MM, SW) in the ability of peripheral blood neutrophils to kill *E. coli*. This result suggests that the increased phagocytic ability of *Acomys* neutrophils may be used for functions other than bacteria-killing. With respect to whole blood and serum, we found that both AC whole blood and serum killed significantly more bacteria than did SW or MM blood and serum. Moreover, when we compared bacterial killing within each species by blood component, we found that in MM and SW, neutrophils at least partly dominated the killing effect of whole blood. In contrast, AC neutrophils killed significantly less bacteria than serum or whole blood, and *Acomys* appeared to rely more heavily on serum than cells for bacterial killing. This strategy could allow *Acomys* to reduce or delay the number of inflammatory cells recruited to wounds without increasing susceptibility to infection.

Environmental conditions play a key role in driving immune variation in animals (Maizels and Nussey, 2013; Beura et al., 2016; Abolins et al., 2017; Graham, 2017; Masopust et al., 2017). For example, within a species, the conditions in which an animal lives can shape immune responses, as has been shown in *Mus*, where wild mice have been shown to have more activated or "primed" immune systems compared to laboratory mice. Wild mice have even been

shown to possess an activated myeloid cell type not present in laboratory mice (Abolins et al., 2017). To account for these types of environmental effects, we compared multiple species/strains of *Acomys* and *Mus* sourced from both captive (AC, SW) and wild (AK, AP, MM) environments.

Importantly, this element of our study design revealed key instances in which immune parameters, such as superoxide production and MPO activity, differed between captive *Acomys* (AC) and captive *Mus* (SW), but not between captive *Acomys* (AC) and wild *Mus* (MM). The fact that AC and MM resembled each other in both instances when AC and SW did not could be explained by differences in the number of generations in which AC has been in captivity compared to SW (Gonet et al., 1966; Pictet et al., 1967; Lynch, 1969; Rice and O'Brien, 1980; Chia et al., 2005). This is because differences in generation time in captivity may translate into differences in overall levels of genetic variation (Earnhardt et al., 2001; Wang and Ryman, 2001; Woodworth et al., 2002), another key factor that can affect immune function (Acevedo-Whitehouse et al., 2003; Hawley et al., 2005). Although we specifically chose an outbred line of captive *Mus* (SW) in our study so that our captive *Mus* would resemble free-living *Mus* as closely as possible, SW populations are still less genetically variable than wild *Mus* populations (Hayakawa et al., 1980;Rice and O'Brien, 1980;Cui et al., 1993). Thus, differences in the levels of genetic variation in laboratory *Mus* populations compared to wild *Mus* populations may make laboratory *Mus* a less optimal choice for comparative studies seeking to compare *Mus* immune phenotypes to those of natural or recently captive populations of other species. Indeed, our results emphasize the importance of considering environmental and genetic context in comparative immunological studies.

Collectively, our results suggest that *Acomys* may use several subtle strategies to reduce neutrophil-associated inflammation and tissue damage without compromising pathogen defense.

First, *Acomys* have fewer neutrophils in circulation, perhaps as a result of the delayed maturation of neutrophil precursors and/or slower release of cells from the bone marrow. Crucially, having fewer neutrophils in circulation and smaller reserves of mature neutrophils in the bone marrow may reduce, or at least delay, the number of neutrophils recruited to wounds, minimizing inflammation and tissue damage during healing. Second, *Acomys* bone marrow neutrophils phagocytose more pathogenic material than do *Mus* neutrophils without differing from *Mus* cells in bacteria-killing ability. This increased phagocytosis may help facilitate neutrophil-mediated activities associated with tissue clean-up and wound repair without altering pathogen killing ability. Third, *Acomys* appear to compensate for the lower numbers of neutrophils they have in circulation with a highly effective non-cellular response to pathogen killing. This work contributes to our growing understanding of basic immune characteristics that may differentiate regeneration-competent and -incompetent mammals.

Materials and methods

Animals and sampling

Six species/strains of mice were studied at the University of Georgia, Athens, GA, USA (UGA) and the University of Nairobi, Nairobi, Kenya (UON). Ac-uga were obtained from the University of Kentucky, Lexington, KY. Outbred sw-uga (SW Hsd: ND4) were obtained from Envigo Corp., Huntington, UK. Mm-uga were live-caught using Sherman traps at the University of Georgia swine and dairy farm satellite units (33º55'N, 83º15'W and 33º54'N, 83º14'W). Ak-uon and ap-uon were live-caught using Sherman traps at the Mpala Research Centre, Laikipia,

Kenya (0°17'N, 37°52'E). Captive-bred sw-uon were obtained from local breeders in Nairobi. Husbandry for all *Acomys* species followed previously described protocols (Haughton et al., 2016). Animal species/strains, sample sizes, and assays performed on each species/strain are summarized in Table 1.

Blood and bone marrow samples were collected for use in a series of assays described below. Bone marrow neutrophils were used for migration, ROS, and phagocytosis assays. Peripheral blood neutrophils, serum, and whole blood were used for bacteria killing assays. All experimental procedures were approved by and carried out in accordance with the Animal Care and Use Protocol Guidelines established by the Institutional Animal Care and Use Committees of the University of Georgia, University of Nairobi, and University of Kentucky.

Bone marrow and peripheral blood neutrophil isolation

Bone marrow neutrophils were isolated from femurs and tibias. Bones were placed in RPMI 1640 plus 1% penicillin/streptomycin following dissection. Bones were rinsed in sterile phosphate buffered saline (PBS) followed by RPMI 1640 and then dipped in 70% ethanol before cell isolation. Bones were cut at both ends and marrow was flushed into a conical tube containing sterile RPMI 1640 using a 3mL syringe and 25-gauge needle. Collected marrow in RPMI was passed through a cell strainer and centrifuged at 300*g* for 10 minutes. After the supernatant was discarded, 2 mL of ammonium-chloride-potassium (ACK) lysis buffer was added to the resulting pellet and 10mL PBS was added after 30 seconds. Cells were centrifuged at 300*g* for 10 minutes and resuspended in 1mL ice-cold, sterile PBS. Neutrophils were isolated using the Histopaque density gradient method previously described for use in *Mus* (Swamydas

and Lionakis, 2013; Swamydas et al., 2015). Briefly, cells resuspended in PBS were carefully placed to overlay 3mL of Histopaque 1077 (Sigma Aldrich, St. Louis, MO, USA, Ref # 10771) and 3mL Histopaque 1119 (Sigma Aldrich, St. Louis, MO, USA, Ref # 11191). This gradient was centrifuged at 870*g* for 30 minutes and neutrophils were collected from the 1077-1119 interface, washed using sterile PBS, and resuspended in fresh RPMI supplemented with 0.5% glucose and 1.0% donor serum for 1 to 24 hours until use in assays. Peripheral blood neutrophils were isolated from cardiac blood according to the same protocol. Following isolation, cell yield and viability were assessed using hemocytometer counts and Trypan blue exclusion staining. The percentage of neutrophils isolated compared to all white blood cell types was assessed by staining isolates with Wright-Giemsa, counting 200 leukocytes, and calculating total neutrophil percentages. This technique, when used in *Acomys*, resulted in the isolation of 92.8 ± 3.3% neutrophils.

White blood cell counts in blood and bone marrow

Smears were prepared from whole blood or from bone marrow homogenates treated with ACK buffer to lyse red blood cells (prior to neutrophil isolation). Smears were fixed in 100% methanol and stained for 15 minutes with Wright-Giemsa. A differential count of 200 (blood) or 300 (bone marrow) white blood cells per slide was performed and two slides were counted per animal. For blood smears, neutrophils, lymphocytes, monocytes, eosinophils, and basophils were distinguished based on morphology. For bone marrow smears, the following cell types were distinguished based on morphology: neutrophils of various maturity stages (promyelocytes, myelocytes, metamyelocytes, band neutrophils, segmented neutrophils), lymphocytes,

monocytes, eosinophils, basophils, and other (precursor cells, blasts, and plasma cells). All cell counts were expressed as a percentage of the total number of cells counted.

Migration assays

Migration ability of bone marrow neutrophils was quantified using a Boyden chamber assay (EZCell© Cell Migration/ Chemotaxis Assay, 96-well, 3μm, BioVision, Milpitas, CA, USA, Ref# K907-100). Two replicates of 50,000 neutrophils were treated with 100nM phorbol myristate acetate (PMA) and placed into the top component of a 3μm Boyden chamber; an additional two replicates of 50,000 neutrophils were left untreated and added to the top component. A Control Migration Inducer, supplied by the manufacturer, was added to the bottom component. Cells were incubated for 2.5 hours at 37ºC and permitted to migrate across the membrane between components. Cells in the bottom component were then isolated and counted using a hemocytometer.

Reactive oxygen species assays

The amount of superoxide produced by bone marrow neutrophils was quantified using a chemiluminescence detection assay (Diogenes Superoxide Chemiluminescence Kit, National Diagnostics, Atlanta, GA, USA, Ref# CL-202). Neutrophils were resuspended in 1mL Hank's Balanced Salt Solution (HBSS), and for each animal, 50,000 cells were used per well in a 96-well white microplate. A 1:1 ratio of Diogenes reagent was added to two of these wells to obtain a reading for superoxide production in untreated neutrophils, and a 1:1 ratio of Diogenes reagent

plus 100nM PMA was added to two wells to assess superoxide production following stimulation. Total chemiluminescence per well was detected every 30 seconds for 30 minutes. The total superoxide generated was calculated as the area under the resulting curve.

MPO activity in bone marrow neutrophil supernatants was quantified using an Amplex Red Hydrogen Peroxide assay (ThermoFisher Scientific, Waltham, MA, USA, Ref# A22188) as described previously (Yoo et al., 2014; Sil et al., 2016). Briefly, 200,000 neutrophils per animal were isolated and placed into two microcentrifuge tubes, with 100,000 cells per tube. The first set of tubes was left untreated and the second set of tubes was treated with 100nM PMA. Cells were incubated for 30 minutes at 37°C and then centrifuged at 300*g* for 3 minutes. Supernatants were collected and transferred in duplicate to a 96-well microplate alongside serially diluted (1:1 to 1:64) standard samples of horseradish peroxidase (HRP). 10-acetyl-3,7-dihydroxyphenoxazine and hydrogen peroxide were added to standards and samples. The plate was incubated for 30 minutes at room temperature. Absorbance was measured at 560nm. Results were expressed in ng/ml peroxidase equivalent as described previously (Yoo et al., 2014).

Phagocytosis assays

The ability of bone marrow neutrophils to phagocytose particles was quantified using zymosan (EZCell© Phagocytosis Assay Kit, Red Zymosan, BioVision, Milpitas, CA, USA, Ref # K398-100). Two replicates of 50,000 neutrophils per animal were treated with 100nM PMA while an additional two replicates of 50,000 cells were left untreated in a 96-well clear microplate. 5uL zymosan was added to each well and cells were incubated for 2h. Samples were centrifuged at 500g for 5 minutes and cells were removed and discarded. Supernatants were

transferred to a sterile black microplate alongside a zymosan standard curve. Concentrations of zymosan were calculated using the standard curve. Phagocytosis ability was reported as the inverse of remaining zymosan at Ex/Em 540/570nm.

Bacteria-killing assays

In vitro E. coli-killing abilities of blood neutrophils, serum, and whole blood were performed according to previously described protocols with modification (Rada et al., 2004; Matson et al., 2006). For all assays, lyophilized *E. coli* pellets (EPower Microorganisms, Microbiologics, St. Cloud, MN, USA, Ref #0483E7) were reconstituted in PBS following the manufacturer's instructions to create stock solutions of bacteria. For neutrophil-only assays, cells isolated from cardiac blood and re-suspended in sterile PBS were added to *E. coli* in a 1:10 cell: bacteria ratio (50,000 to 100,000 neutrophils were added to 5.0-10.0 x 10^5 CFU *E. coli*). This mixture was incubated at 37°C for 1 hour and then centrifuged at 300*g* for five minutes before supernatants were collected and neutrophils were discarded. The *E. coli*-containing supernatants were diluted 100-fold, and 50uL of the mixture was plated in duplicate on tryptic soy agar (TSA) plates. For serum-only and whole blood assays, working solutions of bacteria at concentrations of 4.0-4.8 x 10^4 CFU were prepared each day assays were run. For serum-only assays, clotted whole blood was centrifuged at 5,000rpm for 10 minutes to harvest serum, and 5uL of fresh serum was added to 95uL PBS and 20uL of *E. coli* working solution to achieve a 1:20 dilution. For whole blood assays, fresh whole blood was collected into heparinized tubes. Individual hematocrit values were measured during blood collection to calculate serum: whole blood ratios. These ratios were used to calculate the volume of whole blood from each animal containing

~5uL serum (the volume of serum used in the serum-only assays), and the estimated volume of whole blood was added to PBS and 20uL *E. coli* to achieve a 1:20 dilution. Serum and whole blood mixtures were incubated at 37ºC for 30 minutes and 50uL of each mixture was plated in duplicate on TSA plates. For all killing assays, TSA plates inoculated with appropriately diluted *E. coli* in lieu of cells, serum, or whole blood served as positive controls and plates inoculated with PBS only served as negative controls. Plates were incubated at 37°C for 24 hours before colony forming units were quantified. Bacteria-killing ability was calculated as [1 - [(mean CFU_{sample})/(mean $CFU_{control}$)]].

Statistical Analyses

White blood cell counts were analyzed separately by study site (UGA or UON), tissue (blood or bone marrow), and cell type using analysis of variance (ANOVA). Where necessary, data were arcsine square root transformed to normalize model residuals. For the neutrophil migration ability, MPO activity, and phagocytosis assays, differences among species and by treatment (untreated or PMA-treated) were evaluated using linear mixed effects models (LMMs). Species and treatment status were included as fixed effects in each model, and date was included as a random effect since experimental replicates (n = 7) were run on different days. To normalize model residuals, migration data were log transformed and MPO data were box-cox transformed. Data from the superoxide production assays were also analyzed using LMMs, but in this case, box-cox transformed values were modeled separately by treatment status (untreated or PMA-treated) given the bimodal distribution of the data. Each model included species as a fixed effect and date as a random effect. Analysis of the bacteria-killing data was done in two ways to

evaluate: (i) among-species differences in killing ability for a given blood component (neutrophils, serum, or whole blood), and (ii) within-species differences in killing ability across blood components. For (i), killing ability for each blood component served as the response variable in separate LMMs, species was a fixed predictor variable, and date was a random effect. For (ii), killing ability across all tissues of a single species was the response variable, blood component was a fixed effect, and date was a random effect. For both (i) and (ii), the data were arcsine square root transformed to normalize residuals. Finally, for all analyses, significance was accepted at $p < 0.05$ and Tukey's HSD post-hoc tests were used to assess the significance of pairwise relationships between species. All datasets used for analyses will be publicly available on Dryad upon acceptance of this thesis.

Figures

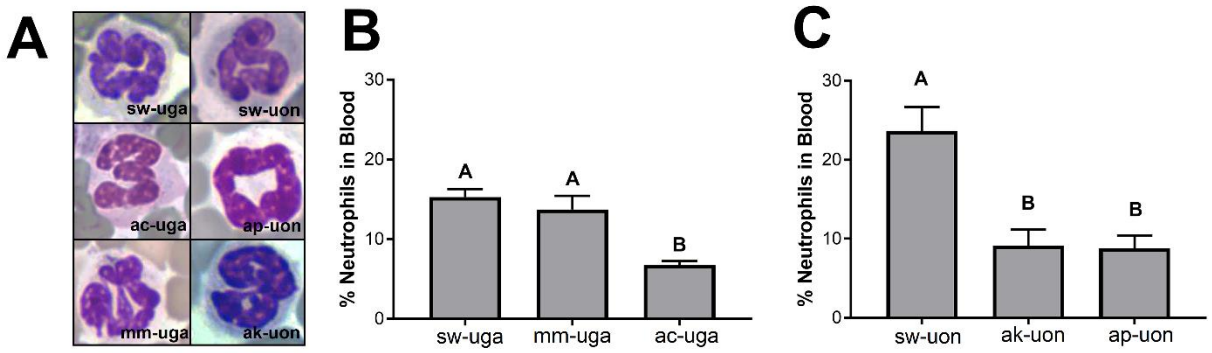

Figure 2.1

The percentage of circulating neutrophils was lower in *Acomys* than in *Mus.* Blood smears were stained with Wright-Giemsa and white blood cell differential counts were performed. **(A)** Neutrophils were identified based on morphology. **(B-C)** Neutrophil numbers are shown as mean percentages of total white blood cells +/- s.e.m. in the blood of UGA mice (sw-uga, n = 16; mm-uga, n = 16; ac-uga, n = 16) and UON mice (sw-uon, n = 6; ak-uon, n = 7; ap-uon, n = 6). Letters indicate significant differences based on post-hoc Tukey HSD tests.

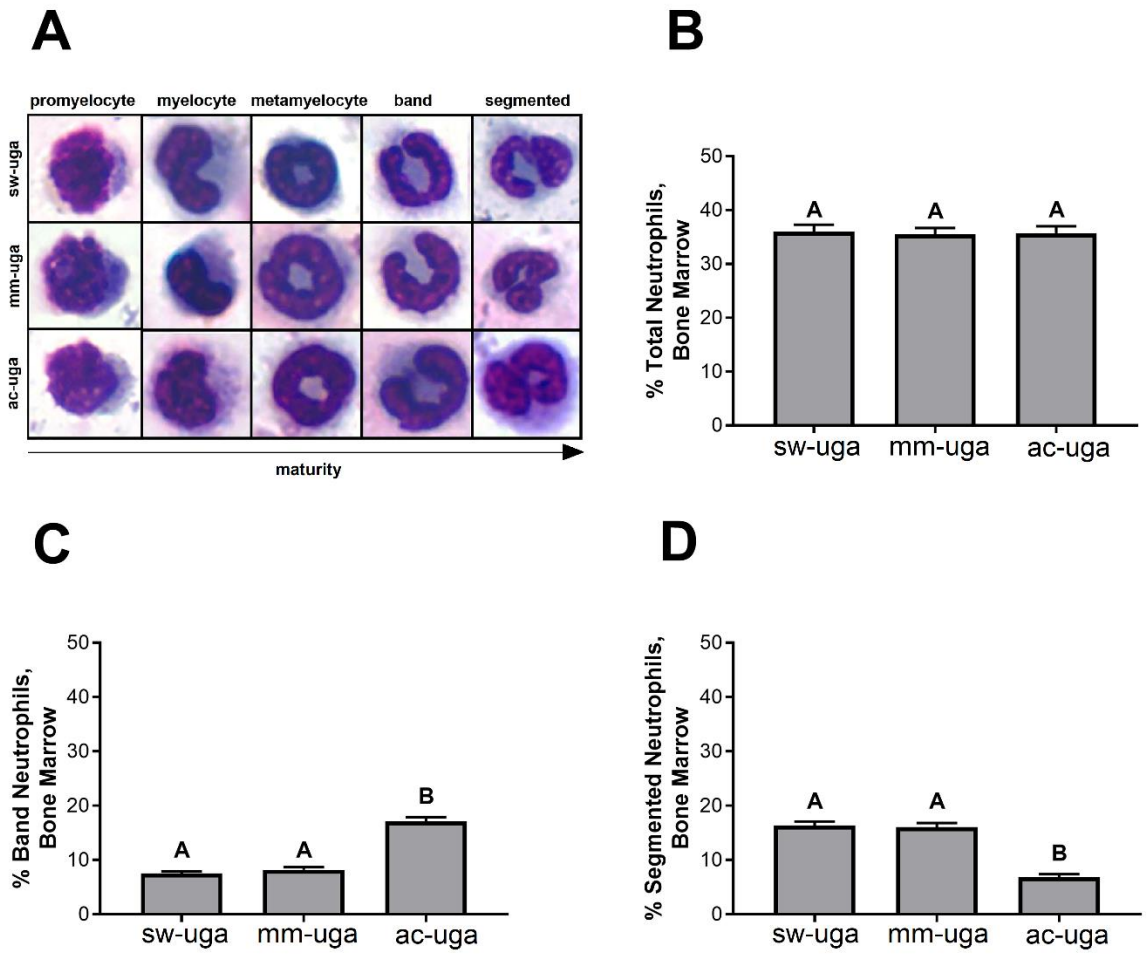

Figure 2.2

Total neutrophil percentages do not differ between *Mus* and *Acomys* in the bone marrow, but *Mus* bone marrow contains proportionately more segmented neutrophils whereas *Acomys* bone marrow contains proportionately more band neutrophils. Bone marrow smears from sw-uga (n=15), mm-uga (n=16), and ac-uga (n=14) were stained with Wright-Giemsa and white blood cell differential counts were performed. **(A)** Proportions of neutrophils in various maturity stages were identified based on morphology. **(B-D)** Total neutrophil, band neutrophil, and segmented neutrophil values are expressed as mean percentages of total white blood cells +/- s.e.m. Letters indicate significant differences based on post-hoc Tukey HSD tests.

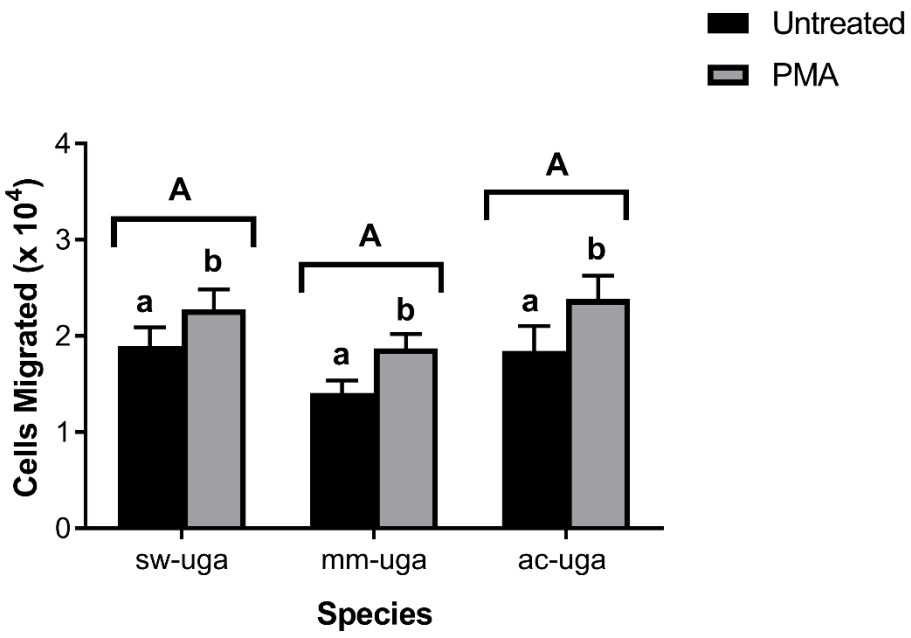

Figure 2.3

***Acomys* and *Mus* do not differ in the ability of bone marrow neutrophils to migrate toward stimuli.** PMA treatment increased the migratory ability of neutrophils in all species. However, neutrophils from sw-uga (n=16), mm-uga (n=16), and ac-uga (n=15) did not differ in their ability to migrate toward stimuli. Migration ability is expressed as the mean number of cells migrated +/- s.e.m. Lowercase letters indicate significant differences between treatment groups, and uppercase letters indicate significant differences among species.

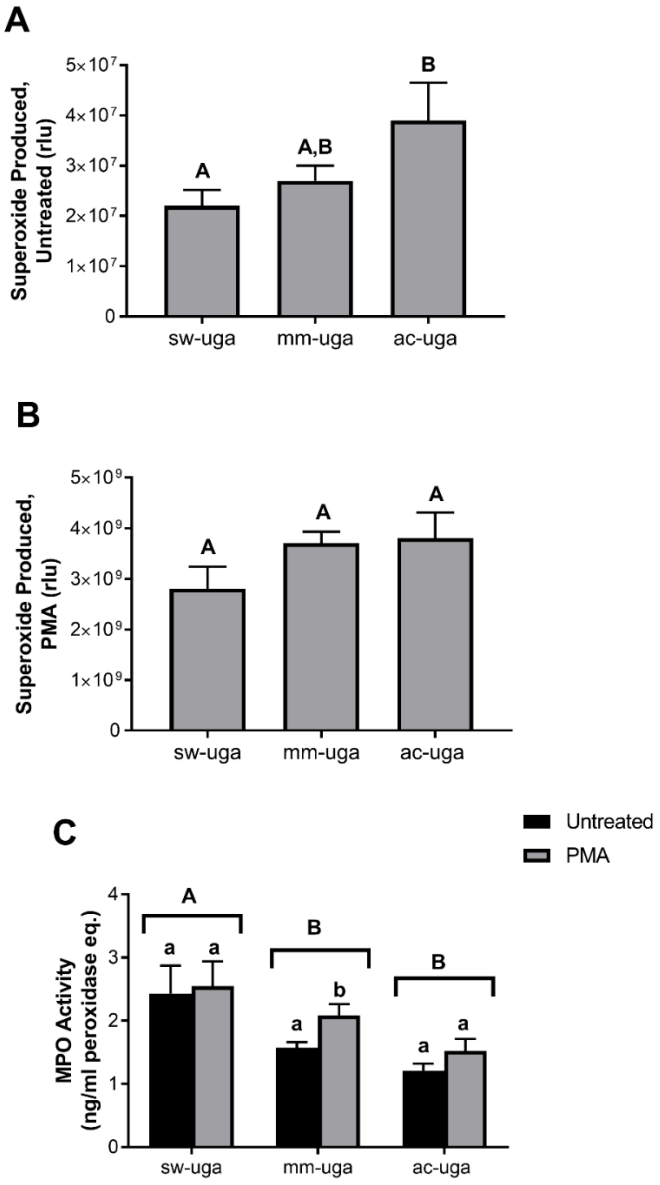

Figure 2.4

***Acomys* and *Mus* bone marrow neutrophils do not differ consistently in the functionality of ROS and ROS-producing enzymes. (A)** Superoxide anion production was significantly different between ac-uga (n=15) and sw-uga (n=16) untreated neutrophils, but mm-uga (n=16) neutrophils did not differ from sw-uga or ac-uga neutrophils. **(B)**There was no difference among species in the ability of PMA-treated neutrophils to produce superoxide. Superoxide production

is expressed in mean relative luminescence units (rlu) +/- s.e.m. and different uppercase letters indicate significant differences among species based on post-hoc Tukey HSD tests. **(C)** MPO activity was lower in mm-uga and ac-uga neutrophils compared to sw-uga neutrophils. MPO activity is expressed as the mean concentration (ng/ml) of peroxidase equivalent +/- s.e.m. Lowercase letters indicate significant differences between treatment groups, and uppercase letters indicate significant differences among species.

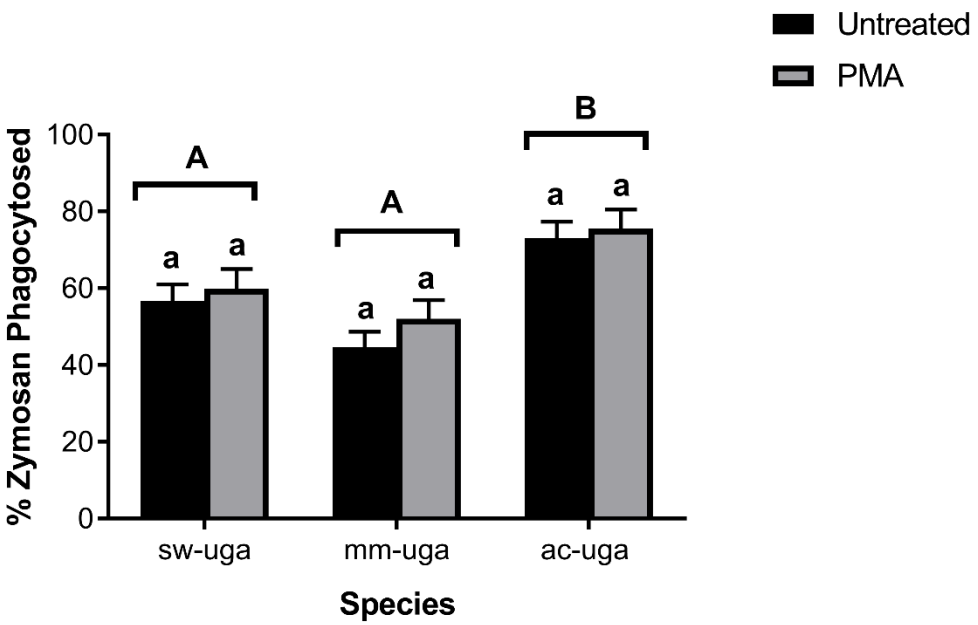

Figure 2.5

Bone marrow neutrophils from *Acomys* show increased phagocytosis compared to neutrophils from *Mus*. PMA treatment had no effect on phagocytosis within species, but ac-uga neutrophils consumed significantly more zymosan than neutrophils from sw-uga and mm-uga. Phagocytosis ability is expressed as the mean percent of zymosan consumed +/- s.e.m. Lowercase letters indicate significant differences between treatment groups and uppercase letters indicate significant differences among species.

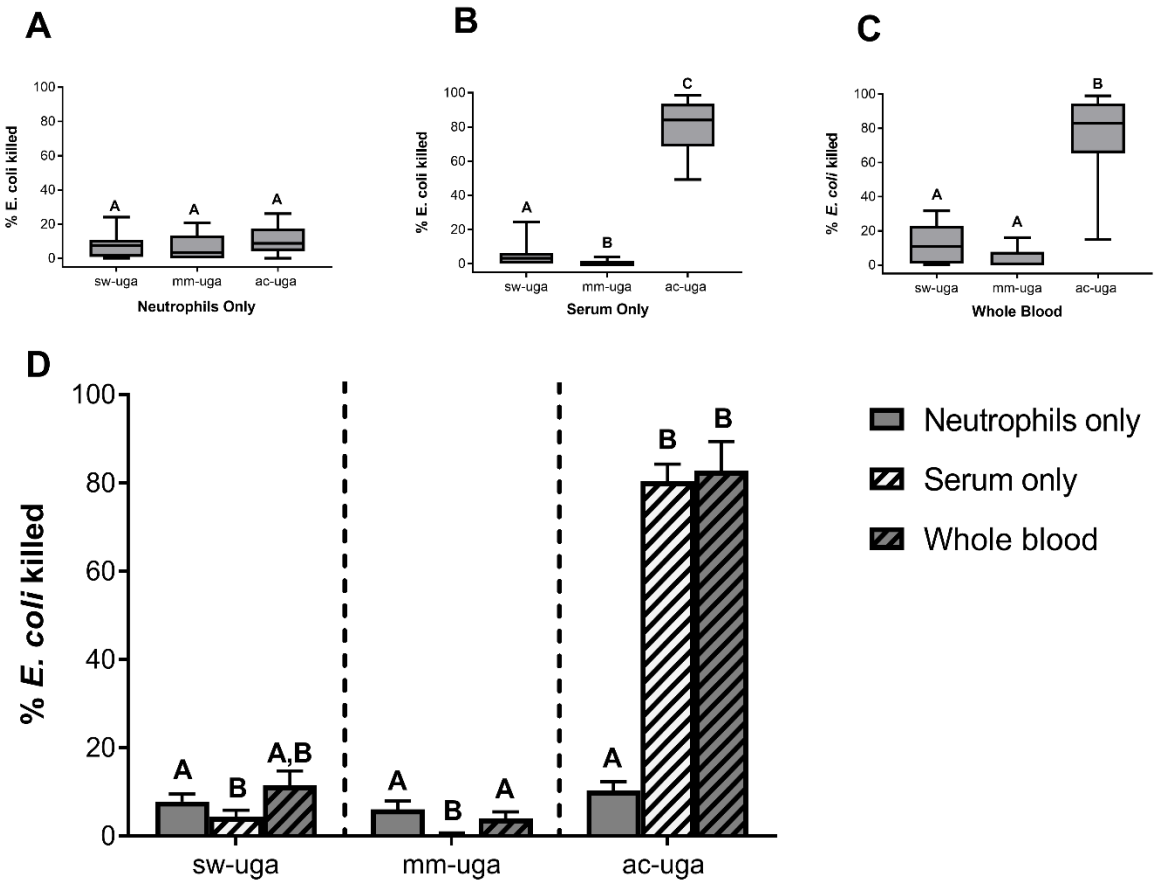

Figure 2.6

Summary of the bacteria killing abilities of *Acomys* and *Mus* whole blood, serum, and neutrophils. (A-C) Among-species analyses of bacteria-killing ability by blood component revealed that the ability of peripheral blood neutrophils to kill *E. coli* did not differ between *Mus* and *Acomys*, while serum and whole blood bacteria-killing abilities did differ significantly among species. Ac-uga (n=16) serum and whole blood killed significantly more bacteria than serum and whole blood from sw-uga (n=16) and mm-uga (n=16). **(D)** Within-species analyses across blood components revealed that *Acomys* use primarily serum to kill bacteria, whereas *Mus* rely on either neutrophils alone (mm-uga) or a combination of neutrophils and serum (sw-uga). In sw-uga (n=16) and mm-uga (n=16), cellular killing was not different from whole blood

killing, whereas in ac-uga (n=16), serum killing was not different from whole blood killing. Bacteria-killing ability is expressed as the mean percent of *E. coli* killed +/- s.e.m., and uppercase letters indicate significant differences.

Tables

Table 2.1

Animal, Sample Size, and Assay Use Information.

Abbreviation	Species/ Strain	Location	Captivity Status	Regeneration Status	*n*	Assays
sw-uga	*Mus musculus*/ Swiss webster (SW)	UGA	captive-bred	incompetent	16	WBC differentials (blood, bone marrow), functional neutrophil assays
mm-uga	*Mus musculus* (MM)	UGA	wild	incompetent	16	WBC differentials (blood, bone marrow), functional neutrophil assays
ac-uga	*Acomys cahirinus* (AC)	UGA	captive-bred	competent	16	WBC differentials (blood, bone marrow), functional neutrophil assays
sw-uon	SW	UON	captive-bred	incompetent	6	WBC differentials (blood only)
ak-uon	*Acomys kempi* (AK)	UON	wild	competent	7	WBC differentials (blood only)
ap-uon	*Acomys percivali* (AP)	UON	wild	competent	6	WBC differentials (blood only)

Table 2.2

Differences in white blood cell percentages in peripheral blood of the UGA species.

Differential counts are expressed as mean % total cells counted ± s.e.m. and were compared using ANOVA. ANOVA p-values are indicated in bold, and pairwise relationships based on post-hoc tests are summarized under 'relationship'. Data for all cell types were arcsine square root transformed for analysis. Abbreviations = Mus *musculus* (Swiss webster): sw-uga, wild-caught *Mus musculus*: mm-uga, *Acomys cahirinus:* ac-uga

Cell type	**Mean ± SEM**			**Statistics**		
	sw-uga (n = 16)	*mm-uga (n = 16)*	*ac-uga (n = 16)*	*F Ratio*	*p-value*	*Relationship*
Neutrophils	15.3 ± 1.0	13.7 ± 1.7	6.7 ± 0.5	16.6035	< **0.0001**	sw, mm > ac
Lymphocytes	75.2 ± 1.2	76.8 ± 2.7	86.9 ± 0.7	14.0138	< **0.0001**	sw, mm < ac
Monocytes	8.1 ± 0.7	7.1 ± 0.9	4.5 ± 0.4	7.6288	**0.0014**	sw, mm > ac
Eosinophils	1.1 ± 0.1	1.8 ± 0.3	1.2 ± 0.3	1.1181	0.3358	
Basophils	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.2215	0.8022	

Table 2.3

Differences in white blood cell percentages in peripheral blood of the UON species.

Differential counts are expressed as mean % total cells counted ± s.e.m. and were compared using ANOVA. Significant ANOVA p-values are indicated in bold, and significant pairwise relationships based on post-hoc tests are summarized under 'relationship'. Abbreviations = *Mus musculus* (Swiss webster): sw-uon, *Acomys kempi*: ak-uon, *Acomys percivali*: ap-uon

Cell type	**Mean ± SEM**			**Statistics**		
	sw-uon (n = 6)	*ak-uon (n = 7)*	*ap-uon (n = 6)*	*F Ratio*	*p-value*	*relationship*
Neutrophils	23.7 ± 3.0	9.1 ± 2.0	8.8 ± 1.6	13.2945	**0.0004**	sw > ak, ap
Lymphocytes	64.8 ± 3.0	82.0 ± 3.3	80.8 ± 2.2	10.6672	**0.0011**	sw < ak, ap
Monocytes	8.8 ± 0.7	4.8 ± 0.8	5.3 ± 0.6	8.6838	**0.0028**	sw > ak, ap
Eosinophils	2.3 ± 0.5	2.5 ± 0.8	4.4 ± 0.5	3.2660	0.0647	
Basophils	0.5 ± 0.1	1.5 ± 0.7	0.6 ± 0.2	1.5300	0.2466	

Table 2.4

Differences in white blood cell percentages in bone marrow of the UGA species. Differential white blood cells counts were used to assess relative percentages of neutrophils (including promyelocytes, myelocytes, metamyelocytes, band neutrophils, and segmented neutrophils), lymphocytes, monocytes, eosinophils, basophils, and other cell types (precursors, blasts, and plasma cells) in bone marrow. Counts are expressed as mean % total cells counted ± s.e.m. and were compared using ANOVA. Significant ANOVA p-values are indicated in bold, and significant pairwise relationships based on post-hoc tests are summarized under 'relationship'. Band neutrophil data were arcsin square root transformed for analysis. Abbreviations = Mus *musculus* (Swiss webster): sw-uga, wild-caught *Mus musculus*: mm-uga, *Acomys cahirinus:* ac-uga

Cell type	**Mean ± SEM**			**Statistics**		
	sw-uga (n = 15)	*mm-uga (n=16)*	*ac-uga (n=14)*	*F Ratio*	*p-value*	*relationship*
Neutrophils	36.0 ± 1.3	35.5 ± 1.2	35.7 ± 1.3	0.0257	0.9747	
Promyelocytes	5.1 ± 0.6	5.3 ± 0.5	4.8 ± 0.5	0.2391	0.7884	
Myelocytes	2.6 ± 0.4	3.2 ± 0.4	2.7 ± 0.4	0.7835	0.4633	
Metamyelocytes	4.4 ± 0.6	2.9 ± 0.4	4.3 ± 0.6	2.7170	0.0777	
Band neutrophils	7.5 ± 0.4	8.2 ± 0.5	17.1 ± 0.8	72.5230	**< 0.0001**	sw, mm < ac
Segmented neutrophils	16.3 ± 0.8	16.0 ± 0.8	6.8 ± 0.6	54.8633	**< 0.0001**	sw, mm > ac
Lymphocytes	41.6 ± 1.1	39.1 ± 1.1	42.8 ± 1.1	2.9978	0.0607	
Monocytes	5.9 ± 0.8	5.5 ± 0.5	5.9 ± 0.6	0.1413	0.8687	
Eosinophils	2.1 ± 0.4	2.5 ± 0.4	1.8 ± 0.4	0.6656	0.5193	
Basophils	0.6 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	3.4526	**0.0409**	sw>mm, mm= ac, sw = ac
Other	13.9 ± 1.6	17.2 ± 1.5	13.5 ± 1.7	1.6667	0.2011	

CHAPTER 3

CONCLUSIONS

This thesis addressed the overarching hypothesis that regeneration trades off with inflammation. Results from this study suggest that regeneration-competent murids (*Acomys spp.*) utilize subtly different immune mechanisms compared to regeneration-incompetent murids (*Mus musculus*). In particular, *Acomys* have fewer neutrophils in circulation and fewer mature neutrophils in the bone marrow, possibly due to differences in neutrophil maturation or release processes. In addition, while many neutrophil functions are consistent between *Acomys* and *Mus*, one key disparity revealed in this study is that *Acomys* neutrophils appear to have enhanced phagocytic capabilities. Surprisingly, despite this increased phagocytosis, *Acomys* neutrophils are not more effective at destroying *E. coli*, suggesting that this increased phagocytosis is used by *Acomys* neutrophils for functions other than pathogen defense. However, *Acomys* serum was more effective than *Mus* serum at killing *E. coli*. Therefore, *Acomys* might compensate for reduced neutrophil numbers by relying more heavily on serum for defense against pathogens in the blood.

The results of this work have implications for several fields of science, including immunology and medicine. With respect to immunology, this work adds additional support to the hypothesis that regeneration trades off with inflammation. Study results show that neutrophil quantities robustly differ across multiple species/strains of *Acomys* and *Mus*, strongly suggesting that the regenerative phenotype may underlie this difference. Prior to this study, bone marrow

neutrophil proportions had not previously been explored in comparative studies of regeneration. The finding that *Acomys* bone marrow contained fewer mature neutrophils than *Mus* bone marrow, in conjunction with a previous study which documented low levels of the neutrophil maturation-associated cytokine G-CSF (Brant et al., 2016), calls for future work investigating whether delayed neutrophil maturation does in fact impact neutrophil quantity in *Acomys*.

Second, and perhaps more importantly, this work supports the recent idea that not all inflammatory mechanisms are detrimental to regenerative healing. In fact, some key inflammatory measures did not differ between regeneration-competent and -incompetent animals in this study. For example, ROS production and migration ability of neutrophils did not differ consistently between *Acomys* and *Mus.* Previous studies suggest that both macrophages and ROS are necessary for regeneration to occur in some contexts (Gauron et al., 2013; Godwin et al., 2013; Love et al., 2013; Simkin et al., 2017). Thus, this work highlights the value of investigating different aspects of inflammation separately to generate a broader view of specific aspects of inflammation that may either help or hinder the regeneration process.

Third, this work highlights the importance of using multiple species and strains of animals in comparative immunological studies. In this study, ROS production did not consistently differ between *Acomys* and *Mus.* However, both superoxide production and MPO activity did differ between laboratory-raised (Swiss webster) and wild-caught *Mus musculus.* This finding supports previous studies which show that within a species, immune function can vary due to environmental origin (Beura et al., 2016; Abolins et al., 2017; Masopust et al., 2017). For example, wild *Mus musculus* have been documented to have more primed immune systems than laboratory mice of the same species. Wild mice even have an entire unique T cell subset that laboratory mice do not (Abolins et al., 2017). Similarly, mice raised in “dirtier” environments

such as pet stores have more active immune systems than their laboratory counterparts, raised in sterile conditions (Beura et al., 2016). Differences in immune function within a species such as these suggest that accounting for environmental context is essential in comparative immunological studies, particularly if laboratory or captive strains of animals are being compared to animals of wild origin.

Finally, this research also provides preliminary evidence of the strong bacteria-killing capacity of *Acomys* serum, which has implications for the field of medicine. Currently, one of the most pressing public health concerns is that many bacterial pathogens are becoming increasingly resistant to conventional antibiotics. In fact, the Centers for Disease Control and Prevention reports that approximately two million people become infected with antibiotic-resistant bacteria each year (CDC, 2018). Therefore, the discovery of new antimicrobial drugs is needed. The finding that *Acomys* serum is significantly more effective at destroying Gram-negative bacteria (*E. coli*) than *Mus* serum, so much so that whole blood killing in *Acomys* is driven entirely by serum, was an interesting addition to this study. Future investigations should test the efficacy of this serum on a more comprehensive panel of microbes, including Gram-positive bacteria and fungi, to determine whether molecules found in *Acomys* serum might be of interest for pharmacological purposes. This method has been effective for identifying potential drug candidate molecules from the serum of other wildlife species, such as alligators and spiders (Silva et al., 2000; Merchant et al., 2003).

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