



SYMPOSIUM

Regeneration-Competent and -Incompetent Murids Differ in Neutrophil Quantity and Function

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Synopsis 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 compared neutrophil traits among uninjured, regeneration-competent (*Acomys*: *A. cahirinus*, *A. kempi*, *A. percivali*) and -incompetent (*Mus musculus*: Swiss Webster, wild-caught strains) murids to test for constitutive differences in neutrophil quantity and function between these groups. Neutrophil quantity differed significantly among species. In blood, *Acomys* had lower percentages of circulating neutrophils than *Mus*; and in bone marrow, *Acomys* had higher percentages of band neutrophils and lower percentages of 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 fungal zymosan. Despite this enhanced phagocytosis activity, *Acomys* neutrophils were not more effective than *Mus* neutrophils at killing *Escherichia coli*. Interestingly, 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 tissue and instead repair wounds by forming scars. Wound healing in mammals is a multi-step process involving inflammation, cell migration, and new tissue formation and remodeling (Grose and Werner 2004; Gurtner et al. 2008; Guo and DiPietro 2010). Typically, tissue damage promotes pro-inflammatory cytokine production and recruitment of neutrophils to the wound (Gillitzer and Goebeler 2001; Werner and Grose 2003; Grose and Werner 2004). At the wound site, the main function of neutrophils 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 kill pathogens using chemicals, such as reactive oxygen species (ROS), that are hazardous to microbes but can also damage tissue (Weissmann et al. 1980; Weiss 1989; Smith 1994). This early wave of inflammation is required to prevent infection after barrier defenses are compromised. Later in the wound-healing process, excess neutrophils are ingested by macrophages as anti-inflammatory cytokines help to resolve inflammation (Martin 1997; Stramer et al. 2007; Guo and DiPietro 2010). Although this process is efficient at closing wounds rapidly and stimulating fibrotic repair, the

initial inflammatory phase can exacerbate tissue damage and promote scarring (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 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 injury. At least three species in this genus, *Acomys kempfi* (AK), *Acomys percivali* (AP), and *Acomys cahirinus* (AC), have been shown to successfully regenerate full-thickness skin, including hair follicles, and can re-grow 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 *Acomys* to regenerate remain largely unknown, although recent studies suggest that the inflammatory response of *A. cahirinus* is somewhat distinct from the *Mus musculus* (MM) inflammatory response and that some aspects of inflammation appear to inhibit regeneration. For example, a study by Simkin et al. (2017) examining inflammatory cell activation during regeneration found that *Acomys* wounds are characterized by delayed neutrophil infiltration and differences in ROS production, including decreased granulocyte-specific ROS production. However, whether differences in neutrophil function exist between *Acomys* and regeneration-incompetent murids outside the context of wound healing remains an open question.

Evidence of *Acomys* having a dampened inflammatory response during wound healing is further supported by non-mammalian species with regenerative abilities, many of which show that the resolution of inflammation is generally important for regeneration (reviewed in Eming et al. [2009] and Mescher et al. [2017]). For example, neutrophil recruitment to salamander skin wounds is significantly reduced compared with that of mammals during wound healing (Lévesque et al. 2010; Seifert et al. 2012b). Furthermore, comparisons between pre-metamorphic *Xenopus laevis* tadpoles, which are capable of regeneration, and juveniles, which are not, show that genes associated with inflammation are downregulated in tadpoles (Grow et al. 2006). Taken together, these observations support the overall hypothesis that regeneration trades off with at least some aspects of inflammation.

Building on these studies, we used spiny mice as a model to understand potential regeneration-associated biases in immune cell function by

focusing on neutrophils, central players in the acute inflammatory response to tissue injury. Given the tissue-damaging side effects of neutrophil-mediated pathogen killing, we hypothesized that these functions might be constitutively dampened in *Acomys* compared with regeneration-incompetent murids. However, inflammation is a key adaptive response to noxious stimuli and an effective inflammatory response is required to eliminate pathogens during injury (Tracey 2002). Moreover, aspects of the inflammatory response, such as ROS production, are essential for the initiation of regeneration in some species (Gauron et al. 2013; Godwin et al. 2013; Love et al. 2013; Simkin et al. 2017). Given this, we expected that any constitutive differences in neutrophil behavior observed in *Acomys* might be nuanced, balancing the negative effects of inflammation (tissue damage) against its positive effects (pathogen defense, initiation of regeneration).

To test this hypothesis, we compared neutrophil traits among regeneration-competent *Acomys* and regeneration-incompetent *Mus*. 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 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 MM populations originating from different environments (Beura et al. 2016; Abolins et al. 2017), we used comparisons of wild and captive animals to distinguish between effects that might be due to environmental variation versus the trait of interest (i.e., regenerative ability).

Materials and methods

Animals and sampling

Six species/strains of murids (Rodentia: Muridae) were used in this study and assays were performed at two different locations: the University of Georgia, USA (UGA) and the University of Nairobi, Kenya (UON). Specific details on animals, sample sizes, and assays performed on each species are summarized in Table 1. The regeneration-competent study species were: captive-bred AC, wild-caught AK, and wild-caught AP. The regeneration-incompetent study species were: captive-bred MM—Swiss Webster (SW) and wild-caught MM. AC were obtained from a breeding colony at the University of

Table 1 Animal, sample size, and assay information

	Species/strain	Abbreviation	Study site	Approximate age	Mass range (g)	Obtained from	Captivity status	n	Assays
Regeneration-incompetent murids	<i>Mus musculus</i> /Swiss Webster	SW	UGA	6 months/adult	31–41	Envigo Corp.	Captive-bred	16	WBC differentials, functional neutrophil assays
	<i>Mus musculus</i> /Swiss Webster	SW	UON	6 months/adult	14–19	Local breeders in Nairobi	Captive-bred	6	WBC differentials
	<i>Mus musculus</i> (wild-caught)	MM	UGA	Unknown/adult	16–25	UGA dairy and swine farm satellite units	Wild	16	WBC differentials, functional, neutrophil assays
Regeneration-competent murids	<i>Acomys cahirinus</i>	AC	UGA	6–18 months/adult	41–54	UK breeding colony	Captive-bred	16	WBC differentials, functional, neutrophil assays
	<i>Acomys kempi</i>	AK	UON	Unknown/adult	23–34	Mpala Research Centre	Wild	7	WBC differentials
	<i>Acomys percivali</i>	AP	UON	Unknown/adult	25–37	Mpala Research Centre	Wild	6	WBC differentials

Kentucky, Lexington, KY, USA and husbandry for this species followed established protocols (Haughton et al. 2016). AK and AP were live-caught at the Mpala Research Centre, Laikipia, Kenya (0°17'N, 37°52'E). SW were used due to their outbred status and obtained from either a commercial vendor (SW: Hsd: ND4, Envigo Corp., Huntingdon, UK) for assays conducted at UGA or from local breeders in Nairobi for assays conducted at UON. MM were live-caught at the UGA swine and dairy satellite units (33°55'N, 83°15'W, and 33°54'N, 83°14'W). Experimental procedures were approved by the Institutional Animal Care and Use Committees of the University of Georgia, University of Nairobi, and University of Kentucky. Research in Kenya was approved by the Kenyan National Council for Science and Technology (NACOSTI).

Bone marrow and peripheral blood neutrophil isolation

Bone marrow neutrophils were collected for use in migration, ROS, and phagocytosis assays. Peripheral blood neutrophils, serum, and whole blood were collected for use in bacteria-killing assays. To isolate bone marrow neutrophils, bone marrow was flushed from femurs and tibias using 3 mL of RPMI 1640 plus 1% penicillin/streptomycin. Collected marrow was passed through a cell strainer and centrifuged at 300 g for 10 min, after which 2 mL of ammonium–chloride–potassium (ACK) buffer (ThermoFisher Scientific, Waltham, MA, USA) was added to the resulting pellet followed by 10 mL of sterile phosphate buffered saline (PBS). Cells were centrifuged at 300 g for 10 min and resuspended in 1× PBS. Neutrophils were isolated using the Histopaque gradient method as previously described for *Mus* (Swamydas and Lionakis 2013; Swamydas et al. 2015) and then resuspended in RPMI supplemented with 0.5% glucose and 1.0% donor serum for 1–24 h until use. Cardiac blood neutrophils were isolated according to the same protocol. Following isolation, cell yield and viability were assessed using a hemocytometer and Trypan blue exclusion staining.

White blood cell counts in blood and bone marrow

Smears were prepared from blood or from bone marrow homogenates treated with ACK buffer. Smears were fixed in 100% methanol and stained 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 cells 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 counts were expressed as a percentage of the total cells counted.

Migration assay

Migration ability of bone marrow neutrophils was quantified using a Boyden chamber assay (EZCell[®] Cell Migration/Chemotaxis Assay, BioVision, Milpitas, CA, USA). Two replicates of 50,000 neutrophils per animal were added to the top of the chamber. A Control Migration Inducer, supplied by the manufacturer, was added to the bottom. Cells were incubated for 2.5 h at 37°C and permitted to migrate across the membrane between chamber components. Cells in the bottom component were isolated and counted using a hemocytometer.

ROS assays

Two assays were used to determine the functionality of ROS and ROS-producing enzymes in bone marrow neutrophils. First, the amount of superoxide produced was quantified using a chemiluminescence detection assay (Diogenes Superoxide Chemiluminescence Kit, National Diagnostics, Atlanta, GA, USA). Neutrophils were resuspended in 1 mL Hank's Balanced Salt Solution (HBSS) and two replicates of 50,000 cells per animal were placed in a 96-well white microplate with an equivalent volume of Diogenes Reagent. Total chemiluminescence per well was detected every 30 s for 30 min. The total superoxide generated, in relative luminescence units, was calculated as the area under the resulting curve.

Second, the activity of myeloperoxidase (MPO), an enzyme that converts hydrogen peroxide to hypochlorous acid downstream of superoxide production, was quantified using an Amplex Red Hydrogen Peroxide assay (ThermoFisher Scientific). Two replicates of 100,000 neutrophils per animal were incubated for 30 min at 37°C and centrifuged at 300 g for 3 min. Supernatants were collected and transferred in duplicate to a 96-well microplate alongside serially diluted (1:1 to 1:64) standards of horseradish peroxidase. 10-Acetyl-3, 7-dihydroxyphenoxazine and hydrogen peroxide were added to standards and samples. The plate was incubated for 30 min at room temperature. Absorbance was measured at 560 nm. Results were expressed in ng/mL peroxidase equivalent.

Phagocytosis assays

Phagocytosis ability of bone marrow neutrophils was quantified using zymosan, an immunogenic glucan prepared from *Saccharomyces cerevisiae* cell wall (EZCell[®] Phagocytosis Assay Kit, Red Zymosan, BioVision). Two replicates of 50,000 neutrophils per animal were added to a 96-well clear microplate, and 5 μ L zymosan was added per well. Samples were incubated for 2 h, then centrifuged at 500 g for 5 min before cells were discarded. Supernatants were transferred to a black microplate alongside a zymosan standard curve. Phagocytosis ability was calculated as the inverse of remaining zymosan at Ex/Em 540/570 nm.

Bacteria-killing assays

The ability of circulating neutrophils to effectively kill *Escherichia coli* was evaluated using bacteria-killing assays (Rada et al. 2004; Matson et al. 2006) and compared with the *E. coli*-killing abilities of serum and whole blood. For these assays, *E. coli* pellets (ATCC #8739: EPower Microorganisms, Microbiologics, St. Cloud, MN, USA) were reconstituted in PBS following the manufacturer's instructions to create bacterial stock solutions. For neutrophil-only assays, peripheral blood neutrophils were added to *E. coli* in a 1:10 cell:bacteria ratio (50,000–100,000 neutrophils: 5.0–10.0 $\times 10^5$ colony forming units [CFU] *E. coli*). This mixture was incubated at 37°C for 1 h, then centrifuged at 300 g for 5 min before supernatants were collected. The supernatants were diluted 100-fold, and 50 μ L 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 $\times 10^4$ CFU were prepared each day assays were run. For serum-only assays, clotted whole blood was centrifuged at 5000 g for 10 min to harvest serum, and 5 μ L of serum was added to 95 μ L PBS and 20 μ L of *E. coli* working solution to achieve a 1:20 dilution. For whole blood assays, blood was collected into heparinized tubes and individual hematocrit values were measured during blood collection to calculate serum:whole blood ratios. These ratios were used to calculate the volume of blood from each animal containing ~ 5 μ L serum (the volume of serum used in the serum-only assays), and this volume of blood was added to PBS and 20 μ L *E. coli* to achieve a 1:20 dilution. Serum and whole blood assays were incubated at 37°C for 30 min and 50 μ L of each sample was plated in duplicate on TSA plates. For all killing assays, TSA plates inoculated with appropriately

Table 2 Differences in white blood cell percentages in peripheral blood of regeneration-competent (*Acomys*: AC) and -incompetent (*Mus*: SW, MM) murid species sampled at the University of Georgia (UGA) and University of Nairobi (UON)

		Regeneration-incompetent		Regeneration-competent	Statistics		
		SW (n = 16)	MM (n = 16)	AC (n = 16)	F-ratio	P-value	Relationship
UGA	[§] Neutrophils	15.8 (8.5–23.0)	13.4 (3.8–30.5)	7.4 (2.5–9.5)	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	7.5 (3.8–13.3)	6.6 (2.3–15.0)	4.8 (2.0–7.8)	7.6288	0.0014	sw, mm>ac
	[§] Eosinophils	1.0 (0.3–2.5)	2.3 (0.0–4.0)	0.9 (0.0–3.5)	1.1181	0.3358	
	[§] Basophils	0.3 (0.0–1.5)	0.3 (0.0–2.3)	0.3 (0.0–3.0)	0.2215	0.8022	
		Regeneration-incompetent		Regeneration-competent		Statistics	
		SW (n=6)	AK (n=7)	AP (n=6)	F-ratio	P-value	Relationship
UON	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	

Counts are reported as the mean percent of total cells counted (\pm standard error) for cell types that were not transformed for analysis, and as the median (lower range value–upper range value) for cell types that were subject to transformation prior to analysis (§ denotes cell types that were arcsine square root transformed). Significant effects are highlighted in bold and the direction of these effects, as determined by *post hoc* Tukey tests, are summarized in the “Relationship” column.

diluted *E. coli* alone served as positive controls and plates inoculated with PBS alone served as negative controls. Plates were incubated at 37°C for 24 h. Bacteria-killing ability was calculated as $[1 - ((\text{mean CFU}_{\text{sample}}) / (\text{mean CFU}_{\text{control}}))] * 100\%$.

Statistical analyses

White blood cell counts were analyzed separately by assay location (UGA or UON), tissue (blood or bone marrow), and cell type using analysis of variance (ANOVA). In cases where cell counts were not normally distributed, data were arcsine square root transformed to normalize residuals (see Tables 2 and 3).

Data from the migration, superoxide, MPO, and phagocytosis assays were analyzed using linear mixed effects models (LMMs). This analytical approach was used to account for variation arising from the experimental design in which study replicates were run across seven different days. Thus, for each model, species was included as a fixed effect and experiment date was included as a random effect. To normalize model residuals, migration data were log transformed and superoxide and MPO data were box-cox transformed.

Finally, analysis of the bacteria-killing assay data was performed in two ways. First, to test for among-species differences in killing by blood component (neutrophils, serum, or whole blood), the killing ability of each component served as the response

variable in separate LMMs with species included as a fixed effect and date as a random effect. Once again, we used an LMM approach to account for variation arising from an experimental design that involved running assays across multiple days. Second, to test for within-species differences in killing ability across blood components, the killing ability of all blood components for a single species served as the response variable in separate LMMs with blood component included as a fixed effect and date as a random effect. Data for these models were arcsine square root transformed to normalize residuals. For all analyses, significance was accepted at $P < 0.05$ and Tukey’s HSD *post hoc* tests were used to assess pairwise relationships between species.

Results

Acomys and *Mus* show differences in blood and bone marrow neutrophil numbers

For white blood cell comparisons performed on species sampled at both UGA (AC, SW, MM) and UON (AK, AP, SW), there were significant among-species differences in neutrophil percentages in the blood. For the species sampled at UGA, AC had significantly fewer circulating neutrophils than both SW and MM (Table 2). The lower neutrophil percentages in AC were also accompanied by significantly higher percentages of lymphocytes and lower percentages of monocytes when compared with SW and

Table 3 Differences in white blood cell percentages in bone marrow of regeneration-competent (*Acomys*: AC) and -incompetent (*Mus*: SW, MM) murid species sampled at UGA

	Regeneration-incompetent		Regeneration-competent AC (n = 14)	Statistics		
	SW (n = 15)	MM (n = 16)		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 (4.0–9.8)	7.8 (5.2–12.3)	17.8 (11.7–21.8)	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	

Counts are reported as the mean percent of the total cells counted (\pm standard error) for cell types that were not transformed for analysis, and as the median (lower range value–upper range value) percent of the total cells counted for cell types that were subject to transformation prior to analysis (§ denotes cell types that were arcsine square root transformed). Significant effects are highlighted in bold and the direction of these effects, based on *post hoc* Tukey tests, are summarized in the “Relationship” column.

MM (Table 2), suggesting that lower proportions of phagocytic cells in *Acomys* are counterbalanced by increased proportions of lymphocytes. The differences observed in the species sampled at UGA were perfectly recapitulated in the species sampled at UON. Both AP and AK had significantly fewer neutrophils than did SW (Table 2). Both AP and AK also had significantly fewer lymphocytes and more monocytes compared with SW (Table 2).

In the bone marrow, there was no difference among the species sampled at UGA (AC, SW, MM) in the percentage of total neutrophils (Fig. 1A, B and Table 3). However, percentages of less mature band neutrophils were significantly higher in AC compared with SW and MM (Fig. 1C and Table 3), whereas percentages of more mature segmented neutrophils showed the reverse pattern (Fig. 1D and Table 3). No differences were observed among species in other cell types, except basophils, for which SW differed from MM (Table 3).

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

There was no significant difference among species sampled at UGA (AC, SW, MM) in the ability of bone marrow neutrophils to migrate in response to stimuli (LMM, $n=47$; species: $df=2$, $F=0.387$, $P=0.6817$). In contrast, superoxide production

differed among species (LMM, $n=47$; species: $df=2$, $F=5.11$, $P=0.0106$); however, *post hoc* analysis showed no consistent difference between *Acomys* and *Mus*. Neutrophils from AC produced more superoxide than neutrophils from SW (Tukey’s test: $P=0.0128$), but neutrophils from MM did not differ from either AC ($P=0.0589$) or SW ($P=0.9332$). MPO activity also differed significantly among species (LMM, $n=47$; species: $df=2$, $F=20.9$, $P<0.0001$). However, once again there was no consistent difference between *Acomys* and *Mus*. Neutrophils from SW showed higher MPO activity than neutrophils from either MM or AC (Tukey’s test: $P<0.0001$), whereas neutrophils from AC and MM were not different from one another ($P=0.9807$).

Acomys bone marrow neutrophils show enhanced phagocytosis compared with *Mus*, but blood neutrophils show no difference in *E. coli*-killing ability

The phagocytosis ability of bone marrow neutrophils differed significantly among the species sampled at UGA (AC, SW, MM; LMM, $n=47$; species: $df=2$, $F=10.2$, $P=0.0002$; Fig. 2A). AC neutrophils consumed more zymosan than neutrophils from both SW (Tukey’s test: $P=0.0213$) and MM ($P<0.0001$), whereas SW and MM neutrophils did not differ from one other in phagocytic capacity ($P=0.1099$). However, this did not translate to among-species differences in *E. coli*-killing ability

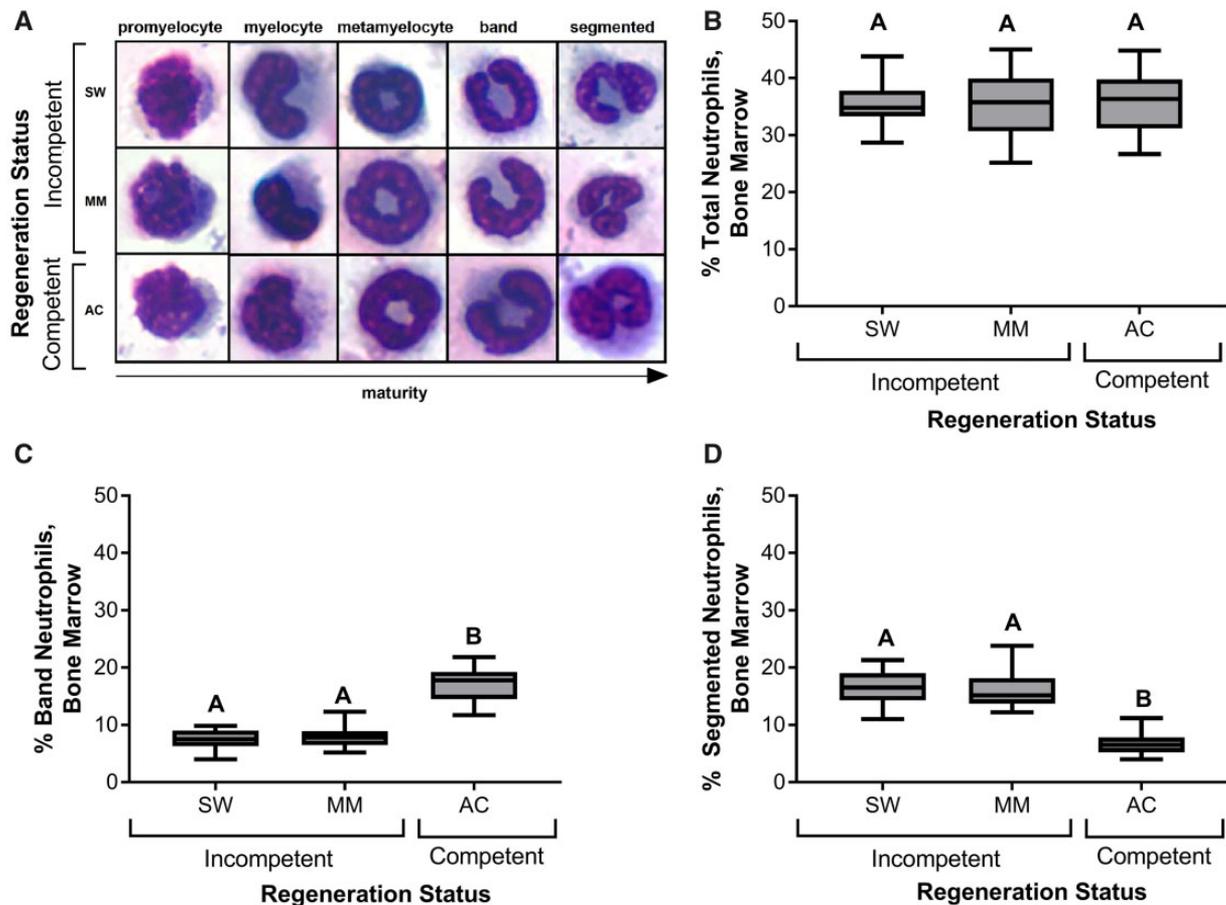


Fig. 1 (A) Bone marrow neutrophils of different maturity stages in regeneration-competent (*Acomys*: AC) and -incompetent (*Mus*: SW, MM) murid species. Comparison of bone marrow (B) total neutrophils, (C) band neutrophils, and (D) segmented neutrophils among *Acomys* and *Mus* shown as the median and interquartile range of the percent of cells counted. Different letters indicate significant differences based on *post hoc* Tukey tests.

by blood neutrophils (LMM, $n=48$; species: $df=2$, $F=0.508$, $P=0.6056$; Fig. 2B). In contrast, the killing ability of serum (LMM, $n=48$; species: $df=2$, $F=262$, $P<0.0001$) and whole blood (LMM, $n=48$; species: $df=2$, $F=72.4$, $P<0.0001$) did differ, with AC showing consistently higher killing than both MM and SW (Tukey's test: $P<0.0001$). The median killing ability of AC serum (83.9%) was 26 times higher than the median killing ability of SW serum (3.15%) and more than 80 times higher than that of MM serum (0%).

***Escherichia coli*-killing ability in *Acomys* and *Mus* is driven by different blood components**

Further examination of the bacteria killing responses of the species sampled at UGA (AC, SW, MM) showed that whole blood bacteria killing in *Mus* appeared to be driven either by a combination of serum and neutrophils (SW: 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: $P=0.1099$; Fig. 2C), or primarily by neutrophils (MM: 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: $P=0.0009$; Fig. 2C). In contrast, whole blood bacteria killing in *Acomys* appeared to be driven entirely by serum, with AC serum killing an equivalent amount of bacteria as whole blood, and neutrophils killing significantly fewer 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: $P<0.0001$; Fig. 2C). 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

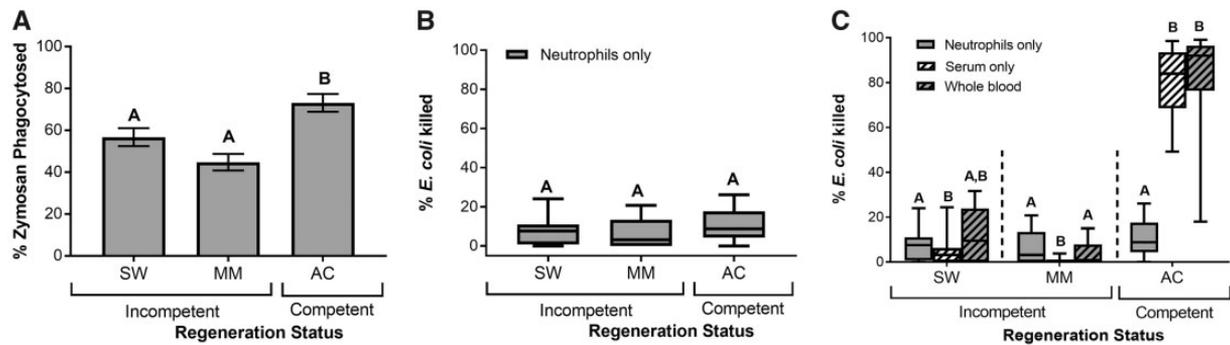


Fig. 2 (A) Phagocytosis ability of bone marrow neutrophils from regeneration-competent (*Acomys*: AC) and -incompetent (*Mus*: SW, MM) murid species. Phagocytosis ability is expressed as the mean percent of zymosan remaining with standard error. (B) Among-species comparison of *E. coli*-killing ability of blood neutrophils shown as the median and interquartile range of the percent of bacteria killed. (C) Within-species comparison of *E. coli*-killing ability across blood components (neutrophils, serum, whole blood) shown as the median and interquartile range of the percent of bacteria killed. Different letters indicate significant differences based on *post hoc* Tukey tests.

injury, as well as potential consequences for pathogen defense, are poorly understood. Using a comparative approach, we tested whether uninjured, regeneration-competent *Acomys* showed constitutive differences in blood and bone marrow neutrophil quantity and function compared with regeneration-incompetent *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 with *Mus*. However, functionally, bone marrow neutrophils from *Acomys* did not differ from *Mus* neutrophils in their ability to migrate. There were also no consistent differences between the two genera in ROS production or ROS-producing enzymatic activity of neutrophils. Interestingly, *Acomys* bone marrow neutrophils differed from *Mus* neutrophils in phagocytic ability, showing increased consumption of zymosan compared with *Mus* cells, yet peripheral blood neutrophils from the two genera did not differ in their ability to kill *E. coli in vitro*. Intriguingly, whole blood *E. coli* killing was significantly higher in *Acomys* compared with *Mus*, and this 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 constitutive 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 differences we observed in circulating neutrophils in *Acomys* and *Mus* were consistent across wild and captive animals. For species sampled at UGA, captive *Acomys* (AC) had lower percentages of neutrophils in blood than both captive (SW) and wild

(MM) *Mus*. Similarly, for species sampled at UON, 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. (2016), who documented a marked difference in the proportion of circulating neutrophils in AC when compared with an outbred CD-1 strain of *Mus*. Interestingly, though, Simkin et al. (2017) found no difference in neutrophil numbers between AC and SW. The contradictory pattern observed for the peripheral blood neutrophil comparisons between our study and that of Simkin et al. (2017) may relate to the fact that Simkin and colleagues used a Sudan Black-modified Wright Giemsa stain to morphologically identify neutrophils. Sudan Black stains leukocyte granules (Sheehan and Storey 1947) and can differentiate neutrophils in various stages of development (Ackerman 1964); therefore, it is possible that the neutrophil populations quantified by Simkin et al. (2017) were different than those quantified in our study in terms of representation of less mature, non-segmented cells. Thus, our results may reflect predominantly mature neutrophil populations, while those of Simkin et al. reflect both mature and immature populations. Interestingly, these contrasting results align with our observation from bone marrow cells showing that differences in neutrophil numbers between the two species may depend on cell maturity.

In the bone marrow, we saw subtle differences in neutrophils between *Acomys* (AC) and *Mus* (SW and MM) that help explain the difference in circulating neutrophil proportions. 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). While AC did not differ from SW and MM in the total percentage of neutrophils in 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 with *Mus*. In C57BL6 mice, bone marrow contains 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 data showing that AC skin wounds appear to be deficient in granulocyte colony stimulating factor (G-CSF) compared with *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 increases circulating neutrophil numbers in mice and humans (Lord et al. 1991; Furze and Rankin 2008); and G-CSF impairment negatively affects neutrophils numbers (Lieschke et al. 1994) and maturation (Mitsui et al. 2003) in mice. 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 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 with 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, such as the ingestion of microbes via phagocytosis and the destruction of pathogens using chemicals like ROS (reviewed in Kolaczowska and Kubes 2013). Some of these

functions can exacerbate tissue damage (Smith 1994; Bergamini et al. 2004). Because 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 differences observed for other, less tissue-damaging functions. However, we found no evidence of consistent differences in ROS functionality between *Acomys* and *Mus*. In the case of superoxide, AC neutrophils produced more superoxide than SW neutrophils, but neither species differed from MM. Likewise, 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 observed among species in this study cannot be linked to regeneration competence. The recent observation that some ROS play a role in the regenerative process in AC (Simkin et al. 2017) may help explain the lack of a consistent difference in ROS production and activity between *Acomys* and *Mus* neutrophils from unwounded animals. Moreover, ROS have been implicated in the initiation of regeneration in other regeneration-competent species (Gauron et al. 2013; Love et al. 2013), suggesting 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 combat 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 (Weiss 1989; Weissmann et al. 1980; Smith 1994), this function also has potentially tissue-damaging effects. As such, we predicted that *Acomys* might have dampened phagocytosis capabilities compared with *Mus*. Counter to our expectations, 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; Kolaczowska and Kubes 2013), it is possible that these functions counterbalance any damaging effects.

If *Acomys* and *Mus* neutrophils differed in key pathogen-killing functions, we expected these differences to translate directly into variation in microbial killing abilities. However, even though *Acomys* neutrophils were better able to phagocytose zymosan, 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. Interestingly, AC whole blood and serum killed significantly more bacteria than did SW or MM blood and serum. Moreover, in MM and SW, neutrophils at least partly dominated the killing effect of whole blood, whereas serum dominated the killing effect in AC. This serum biased killing strategy could allow *Acomys* to reduce or delay the number of inflammatory cells recruited to wounds without increasing susceptibility to infection.

Finally, the substantial difference in serum bacteria-killing ability we documented between *Acomys* and *Mus* is intriguing. Killing of the *E. coli* strain we used (ATCC #8739) is largely complement-dependent (Demas et al. 2011; Scotti et al. 2015), and complement plays a critical role in inflammation (Carroll 1998; Dunkelberger and Song 2010). Considering our initial hypothesis that aspects of inflammation can hinder regeneration, we would not expect high levels of complement in *Acomys* serum. Thus, it is possible that other effector molecules may instead be responsible for the high serum bacteria-killing capacity we observed in *Acomys*. For instance, lysozyme, a molecule found in serum, destroys bacteria and elicits anti-inflammatory responses (Ogundele 1998). Certain antimicrobial peptides, such as defensins, also exhibit anti-inflammatory functions (Lillard et al. 1999). It is possible that differences in concentrations of effectors such as these allow *Acomys* to combat bacterial pathogens in a manner that regulates excessive inflammation. However, future studies are needed to evaluate the degree to which complement or other molecules may account for the strong serum bacteria-killing ability of *Acomys*. Collectively, though, our results suggest that *Acomys* may use several subtle strategies to reduce neutrophil-associated inflammation and tissue damage without compromising pathogen defense. This study contributes to our growing understanding of constitutive immune characteristics that may differentiate regeneration-competent and -incompetent mammals.

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