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TCR β Combinatorial Immunoreceptor Expression by Neutrophils Correlates with Parasite Burden and Enhanced Phagocytosis during a *Plasmodium berghei* ANKA Malaria Infection

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ABSTRACT Recent studies have demonstrated that a subpopulation of neutrophils express the TCR $\alpha\beta$ combinatorial immunoreceptor in humans and mice. Here, we report that a *Plasmodium berghei* ANKA murine malaria infection induces expansion of TCR β expressing CD11b⁺ Ly6G⁺ neutrophils in the spleen during the early phase of infection. Measurement of TCR β transcript and protein levels of neutrophils in wild-type versus nude and *Rag1* knockout mice establishes that the observed expression is not a consequence of nonspecific antibody staining or passive receptor expression due to phagocytosis or trogocytosis of peripheral T cells. Remarkably, on day 3 postinfection, we observed a highly significant correlation between the proportion of neutrophils that express TCR β and peripheral blood parasite burden. In addition, TCR β ⁺ neutrophils phagocytose parasitized erythrocytes with 4-fold greater efficiency than TCR β ⁻ neutrophils. Together these results signify that TCR expression by the neutrophil plays an important role in the regulation of parasite burden by enhancing the phagocytic capacity of the neutrophil.

KEYWORDS *Plasmodium berghei* ANKA, neutrophil, TCR β

Neutrophils are considered the first line of defense against invading pathogens and injury. Neutrophils perform their protective function against pathogens through phagocytic clearance, release of reactive oxygen species and proteases, and formation of extracellular traps (1). More recent studies depict neutrophils as part of a sophisticated innate surveillance system that influences the downstream immune response by modulating components of the innate and adaptive immune systems (2).

The roles of neutrophils in malaria immunity and pathogenesis are under extensive investigation. In a study of *Plasmodium falciparum*-infected patients in Thailand, neutrophil granulocyte counts were significantly higher in patients with high parasitemias than in patients with low and moderate parasitemias (3). *In vitro* studies have demonstrated that the addition of activated neutrophils to *P. falciparum* cultures suppresses the growth of parasites (4). However, in one study, early depletion of neutrophils with the RB6-8C4 monoclonal antibody during a *P. berghei* ANKA infection of CBA/NSIc mice did not modify parasitemia, suggesting that neutrophils do not affect parasite growth (5).

In contrast to the unresolved role of neutrophils in regulating parasite density, several studies have established that neutrophils can be pathogenic during a malaria infection. An association of activated neutrophils with parasitized erythrocyte seques-

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tration during pediatric cerebral malaria was recently identified in Malawian children (6), and studies in the strain ANKA murine model have shown that Fc ϵ RI⁺ neutrophils are essential for the pathogenesis of experimental cerebral malaria (ECM) (5, 7). In addition, hemolysis during a malaria infection can result in heme oxygenase-1-dependent neutrophil dysfunction, which impairs the ability of developing neutrophils to mount a competent oxidative burst and consequently causes increased susceptibility to coinfection with nontyphoidal *Salmonella* (8, 9). Lastly, a recent study suggests that neutrophil activation by type I interferon mediates liver pathology during *P. vivax* and *P. chabaudi* malaria (10).

Neutrophils have been generally considered a homogenous population of leukocytes that recognize their targets through a set of invariant receptors. However, recent research has suggested a vast degree of heterogeneity and plasticity in neutrophil populations that are governed by the cellular environment and biological functions being performed (11). Puellmann et al. have identified a subpopulation (5 to 8%) of mammalian neutrophils that express the TCR $\alpha\beta$ combinatorial receptor (12). In this study, it was shown that TCR $\alpha\beta$ expression in neutrophils is modulated by granulocyte colony-stimulating factor, and stimulation of TCR $\alpha\beta$ protects neutrophils from apoptosis and results in increased interleukin-8 (IL-8) secretion. A case study reporting that 80% of circulating neutrophils expressed membrane-bound TCR $\alpha\beta$ in a patient diagnosed with autoimmune hemolytic anemia who also displayed extensive neutrophil erythrophagocytosis suggests that TCR expression by the neutrophil can have important consequences in the immunopathogenesis of disease (13).

In this study, we report that early strain ANKA infection induces the expression of combinatorial TCR β on murine CD11b⁺ Ly6G⁺ neutrophils in the spleen. We demonstrate through extensive experimentation that expression of TCR β by the splenic neutrophil is not an artifact of passive receptor expression or anti-TCR β binding to a cross-reactive epitope or Fc receptor on the neutrophil. Remarkably, we identified a highly significant correlation in the proportion of splenic neutrophils that express TCR β and parasite burden on day 3 postinfection. Furthermore, we show that TCR β expression also associates with enhanced neutrophil phagocytosis of strain ANKA-infected erythrocytes. Our results have improved our knowledge of the biological diversity of neutrophil populations and demonstrate that neutrophil TCR β may regulate parasite burden by facilitating phagocytosis of parasitized erythrocytes during the early phase of a strain ANKA infection.

RESULTS

Effect of strain ANKA infection on the neutrophil population in the brain and spleen. We first measured the effect of a *P. berghei* ANKA infection (Fig. 1A) on the neutrophil population in the spleen in C57BL/6 mice, which are susceptible to ECM, and BALB/c mice, which are resistant to ECM (Fig. 1B). Although the symptoms displayed and pathogenesis exhibited differ greatly between ECM-susceptible C57BL/6 mice and ECM-resistant BALB/c mice, there was no significant difference in parasitemia between the two strains of mice (Fig. 1A). In naive C57BL/6 mice ($n = 5$), approximately 2.7% (1.6×10^6) of live lymphocyte singlets are CD11b⁺ Ly6G⁺ neutrophils (Fig. 1C). Enumeration of neutrophils on days 3 and 6 postinfection indicates that strain ANKA infection of C57BL/6 mice does not result in expansion of the neutrophil compartment of the spleen. In contrast, the splenic neutrophil compartment of BALB/c mice expanded over the course of a strain ANKA infection; a 1.8-fold expansion of splenic neutrophils from day 0 (1.6×10^6) to day 6 (2.9×10^6) postinfection was observed (Fig. 1D).

We also quantitated the number of sequestered neutrophils in perfused brain tissue (Fig. 1E to G) of ECM-susceptible C57BL/6 mice on days 0, 3, and 6 postinfection and ECM-resistant BALB/c mice on day 6 postinfection. In naive C57BL/6 mice, 8.1% ($1.4 \times 10^4 \pm 0.2 \times 10^4$) of live brain-sequestered leukocytes (BSLs) were CD11b⁺ Ly6G⁺ neutrophils. On day 3 postinfection, 7.1% ($1.1 \times 10^4 \pm 0.1 \times 10^4$) of live BSLs were neutrophils. By day 6 postinfection, when ECM-susceptible C57BL/6 mice are moribund,

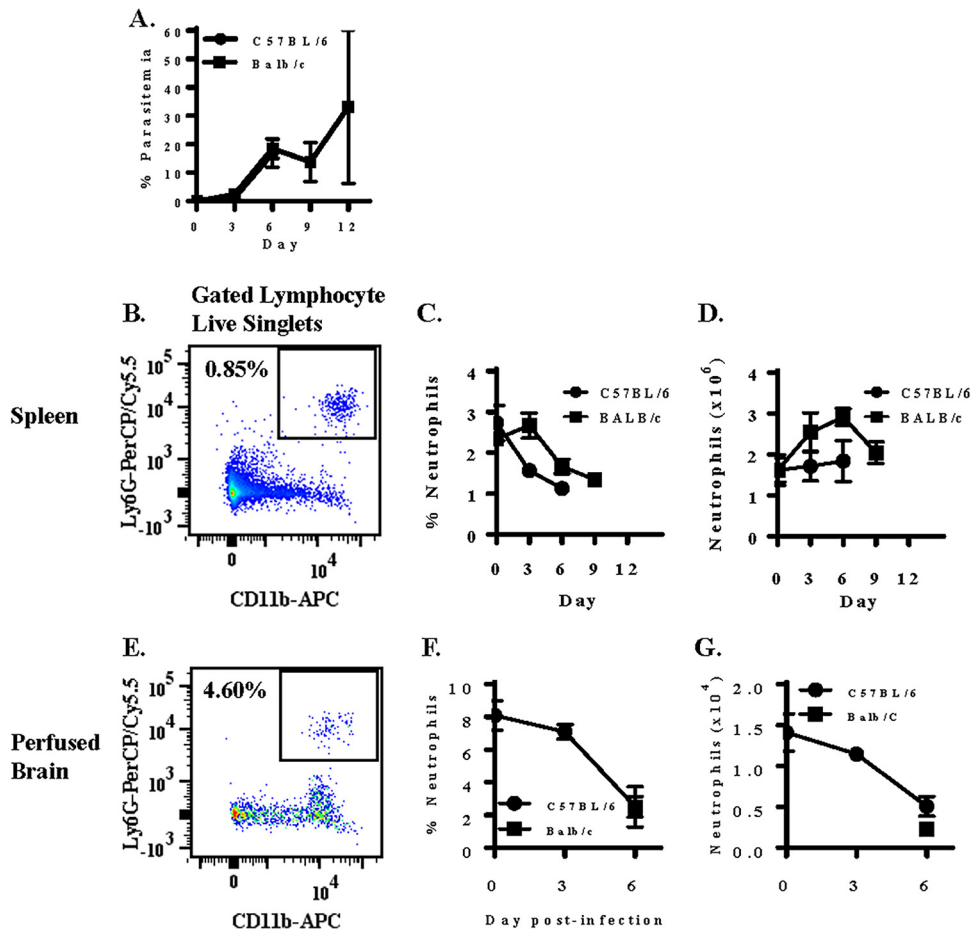


FIG 1 Measurement of neutrophils during a *Plasmodium berghei* ANKA infection in ECM-susceptible C57BL/6 and -resistant BALB/c mice. Mice were infected with 10^6 *Plasmodium berghei* ANKA (*Pb-A*) parasites. (A to D) Parasitemia was enumerated in the two strains of mice (A), and neutrophils were quantitated by measuring the expression of CD11b and Ly6G on live lymphocyte singlets in spleen tissue (B) and plotted as proportion (C) and absolute number (D) over the course of a strain ANKA infection. (E to G) Neutrophils were also quantitated in perfused brain tissue (E), and the percentage (F) and absolute number (G) of brain-sequestered leukocytes that were CD11b⁺ Ly6G⁺ neutrophils was also determined in perfused brain tissue of susceptible C57BL/6 mice on days 0, 3, and 6 postinfection and resistant BALB/c mice on day 6 postinfection. $n = 5$ (spleen) and $n = 4$ (brain) for each time point for each strain of mouse, and results presented are representative of three independent experiments.

only 2.5% ($0.5 \times 10^4 \pm 0.1 \times 10^4$) of live BSLs were CD11b⁺ Ly6G⁺ neutrophils. A 2.8-fold decrease in brain-sequestered neutrophils in naive versus moribund C57BL/6 mice was unexpected and is contrary to the observed 14.3-fold increase in brain-sequestered CD8⁺ T cells (data not shown). However, a paucity of brain-sequestered neutrophils during the cerebral phase is consistent with previous findings demonstrating that neutrophils are pathogenic during the early phase of ECM (5).

Strain ANKA infection induces expansion of a novel population of TCR β -expressing neutrophils. Previous studies report that a small subset of neutrophils expresses TCR $\alpha\beta$ in humans and mice. We measured the effect of strain ANKA infection on the expression of TCR β on neutrophils in spleen tissue of C57BL/6 and BALB/c mice. In response to strain ANKA infection, we observed expansion of a subset of neutrophils that expressed TCR β but was negative for the T lymphocyte marker CD3 ϵ (Fig. 2A). This population of TCR β^+ CD3 ϵ^- neutrophils was also CD4⁻ CD8⁻ and F4/80⁻ (data not shown). In C57BL/6 mice, only 1.6% (2.5×10^4) of neutrophils were TCR β^+ and CD3 ϵ^- in naive mice. However, 4.2% (7.6×10^4) and 4.1% (8.7×10^4) of neutrophils were TCR β^+ CD3 ϵ^- on days 3 and 6 postinfection with strain ANKA, respectively (Fig. 2B). Therefore, early but not late infection of C57BL/6 mice with strain ANKA caused a

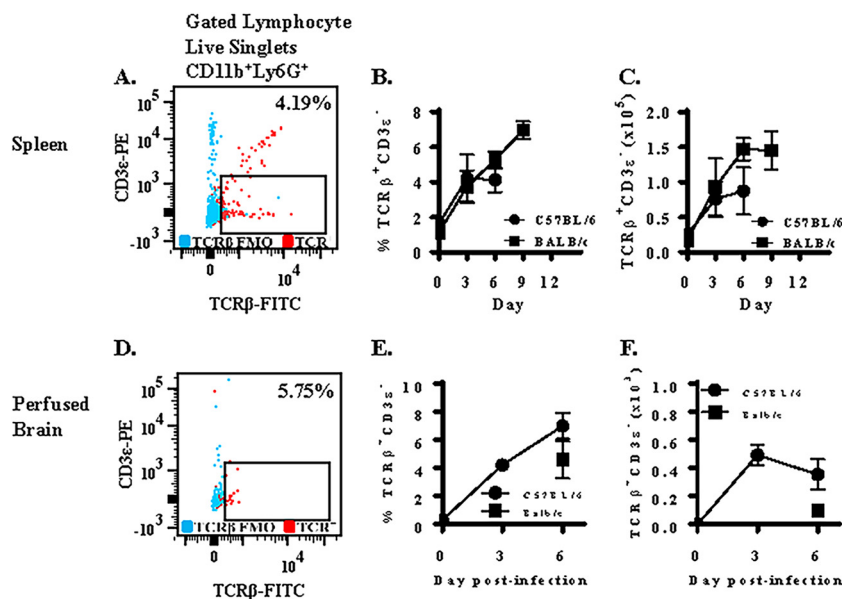


FIG 2 *Plasmodium berghei* ANKA induces expression of TCR β on neutrophils in C57BL/6 and BALB/c mice. Mice were infected with 10^6 *Plasmodium berghei* ANKA (*Pb-A*) parasites. The proportion of neutrophils that were TCR β^+ CD3 ϵ^- (A) was then determined and graphed as proportion (B) and absolute number (C) during a strain ANKA infection in C57BL/6 and BALB/c mice. (D) TCR β and CD3 ϵ expression was also measured on brain-sequestered neutrophils of susceptible C57BL/6 mice on days 0, 3, and 6 postinfection and resistant BALB/c mice on day 6 postinfection. Findings are presented as proportion (E) and absolute number (F). A fluorescence-minus-one control for TCR β was used for gating purposes. $n = 5$ (spleen) and $n = 4$ (brain) for each time point for each strain of mouse, and results presented are representative of three independent experiments.

significant increase (3.5-fold) in the absolute number of TCR β^+ CD3 ϵ^- neutrophils (Fig. 2C). Importantly, this novel population of TCR β -expressing neutrophils selectively expanded in the neutrophil compartment of the spleen that otherwise remained unaltered in size in response to parasitemia (Fig. 1D). In BALB/c mice, there was a 5.5-fold increase in the number of TCR β^+ CD3 ϵ^- neutrophils from day 0 to day 3 and a 1.6-fold increase from day 3 to day 6 in BALB/c spleen tissue (Fig. 2C), indicating that TCR β^+ CD3 ϵ^- neutrophils expanded significantly from day 0 to day 6 postinfection in spleen tissue.

We also quantitated the proportion and number of neutrophils that express TCR β in perfused brain tissue of ECM-susceptible C57BL/6 mice on days 0, 3, and 6 postinfection and ECM-resistant BALB/c mice on day 6 postinfection (Fig. 2D to F). In C57BL/6 mice, TCR β expression on brain-sequestered neutrophils increased over the course of infection and was similar to TCR β expression on splenic neutrophils. We observed no significant difference in the proportion of neutrophils that express TCR β in the brain (6.0%) versus the spleen (4.1%), indicating that TCR β -expressing neutrophils do not preferentially migrate to the brain. However, on day 6 postinfection, we observed a 4.4-fold difference in brain-sequestered TCR β^+ neutrophils between moribund C57BL/6 and resistant BALB/c mice that approached significance (P value of 0.057 by Mann-Whitney test).

Measurement of TCR β on neutrophils isolated from nude and *rag1* knockout mice. To confirm that observed TCR β expression by the neutrophil was not due to phagocytosis or trogocytosis of peripheral T cells, we compared expression of TCR β on the surface of splenic neutrophils in C57BL/6 wild-type (WT) versus nude (which lack T cells) and *rag1* knockout (KO; which lack T and B cells) mice on the C57BL/6 background (Fig. 3A to F).

Previous studies have shown that nude (14) and *rag1* KO (15) mice are resistant to ECM. Consistent with these studies, in our hands, following strain ANKA infection, nude and *rag1* KO mice did not exhibit symptoms of ECM, whereas 100% of WT mice were

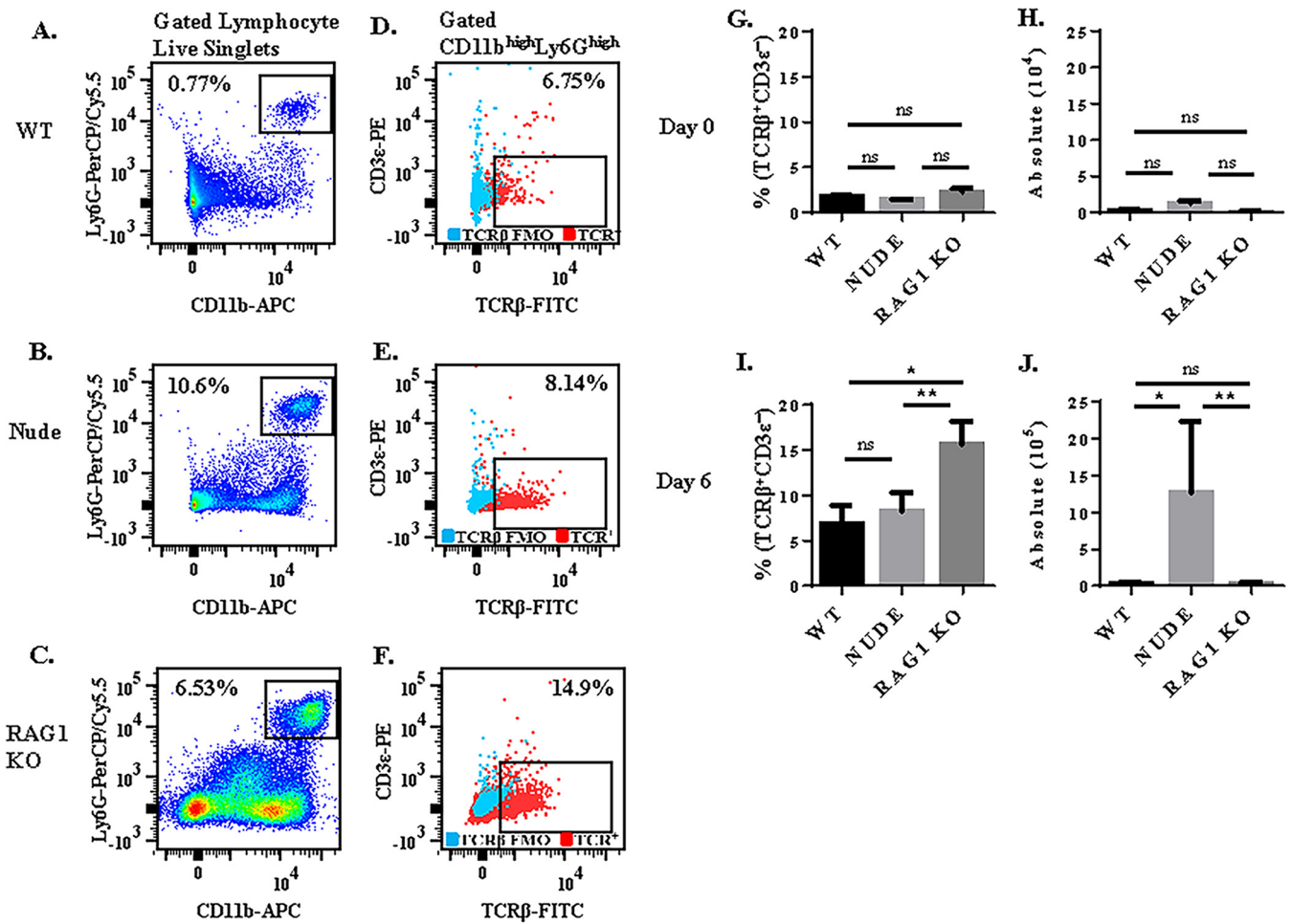


FIG 3 Comparison of TCRβ expression on neutrophils in C57BL/6 wild-type versus nude and *rag1* knockout mice. Mice were infected with 10⁶ *Plasmodium berghei* ANKA (*Pb-A*) parasites, and TCRβ expression was compared in gated CD11b⁺ Ly6G⁺ neutrophils isolated from wild-type (A and D) versus nude (B and E) and *rag1* knockout (C and F) spleen tissue. The proportion of neutrophils that express TCRβ and the absolute number of TCRβ-expressing neutrophils was compared in naive mice (G and H) and in strain ANKA-infected mice on day 6 postinfection (I and J). Absence of peripheral T cells in nude and *Rag1* knockout mice did not reduce TCRβ expression by neutrophils. A fluorescence-minus-one control was used to select the gate for TCRβ. *n* = 5 for each time point for each group of mice, and results presented are representative of two independent experiments. ns, not significant; *, *P* < 0.05; **, *P* < 0.01 (*P* values calculated using the Mann-Whitney *U* test).

moribund. In naive mice, we observed no significant difference in the proportion (Fig. 3G) of splenic neutrophils that expressed TCRβ or the absolute number (Fig. 3H) of TCRβ-expressing neutrophils in WT versus nude and *rag1* KO mice (Fig. 3G). On day 6 postinfection, a significantly higher proportion of lymphocyte live singlets were CD11b⁺ Ly6G⁺ neutrophils in nude (10.6%) and *rag1* KO (6.53%) mice compared to WT controls (0.77%). While a similar proportion of neutrophils expressed TCRβ in nude (8.7%) versus WT (6.8%) mice (*P* value 0.35 by Mann-Whitney test), a significantly higher proportion (14.9%) of neutrophils expressed TCRβ in *rag1* KO mice than in WT mice (*P* value of 0.018 by Mann-Whitney test) (Fig. 3I). However, because *rag1* KO mice had very small spleens, when normalized for cell volume, there was no significant difference between the absolute number of TCRβ-expressing neutrophils in WT (3.0 × 10⁴) and *rag1* KO (2.8 × 10⁴) mice. However, due to an abundance of splenic neutrophils likely caused by immune compensatory mechanisms (10.6% of lymphocyte live singlets were neutrophils in nude mice compared to 0.77% in WT mice), nude mice had 42-fold more TCRβ-expressing neutrophils than WT mice (Fig. 3J). In summary, these results demonstrate that the proportion of neutrophils that express TCRβ is not increased by the presence of peripheral T cells in WT mice.

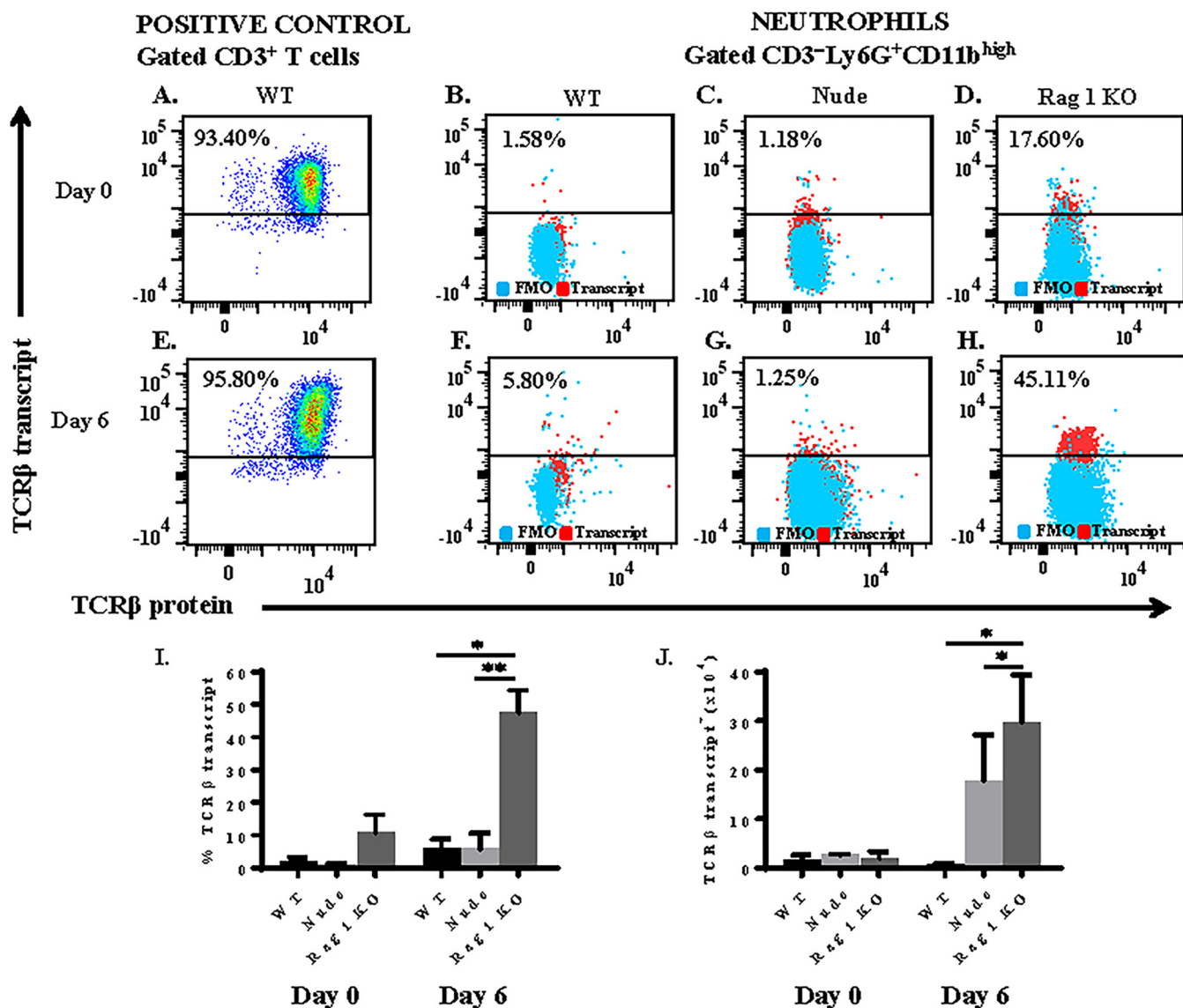


FIG 4 *Plasmodium berghei* ANKA infection induces expression of TCRβ mRNA by neutrophils. Mice were infected with 10⁶ strain ANKA parasites, and TCRβ transcription was assessed by the PrimeFlow RNA assay. (A and E) Abundant levels of TCRβ mRNA were observed in positive-control CD3⁺ T cells isolated from spleen tissue of naive and strain ANKA wild-type mice. TCRβ mRNA was also quantitated in gated CD3⁻ CD11b⁺ Ly6G⁺ neutrophils isolated from naive (day 0) and strain ANKA-infected (day 6) C57BL/6 wild-type (B and F), nude (C and G), and *rag1* knockout (D and H) spleen tissue. Results are presented as proportion of neutrophils that express TCRβ transcript (I) and absolute number of TCRβ transcript-expressing neutrophils (J). A fluorescence-minus-one (FMO) control was used to select the gate for TCRβ transcript, and values plotted were calculated by subtracting the background in each FMO control from its corresponding sample. Results shown are representative of two independent experiments. *, *P* < 0.05; **, *P* < 0.01 (*P* values calculated using the Mann-Whitney *U* test).

Neutrophils express comparable levels of TCRβ transcript and protein. To verify that TCRβ expression by the neutrophil was not a consequence of anti-TCRβ binding to an Fc receptor or a cross-reactive epitope on the neutrophil, we measured intracellular transcript levels of mouse T cell receptor beta, constant region 1 (TRBC1), using a flow cytometry-based PrimeFlow RNA assay (Fig. 4A to H). This assay detects RNA using a set of 40 short oligonucleotide probes specific for a target gene. Due to the small size of each oligonucleotide probe, a single-nucleotide difference in TRBC1 transcript will prevent hybridization of the probe to TRBC1 RNA. In WT mice, the proportion of splenic neutrophils that express TCRβ transcript increased from 2.1% to 5.8% from day 0 to day 6 postinfection with strain ANKA (Fig. 4I). Importantly, the proportion of neutrophils that express TCRβ transcript was similar to the proportion of neutrophils that express TCRβ protein in both naive and strain ANKA-infected mice (Fig. 2B and 3G and I). Similar

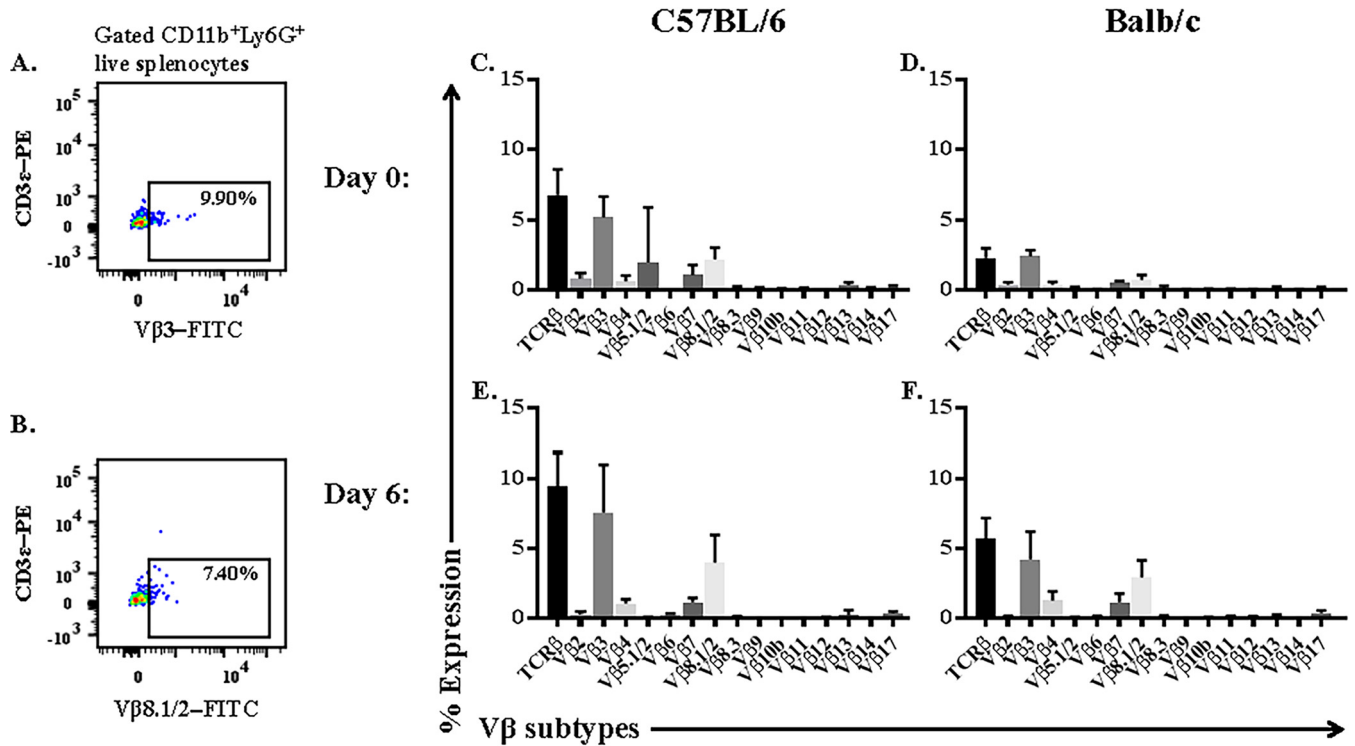


FIG 5 Analysis of the TCR $V\beta$ repertoire of splenic neutrophils during *Plasmodium berghei* ANKA (*Pb-A*) infection of C57BL/6 and BALB/c mice. Mice were infected with strain ANKA, and expression of 15 $V\beta$ TCR subtypes was then assessed by flow cytometry. (A and B) $V\beta$ subtype expression was measured on gated CD11b⁺ Ly6G⁺ splenic neutrophils in naive (C and D) and strain ANKA-infected (E and F) C57BL/6 and BALB/c mice. An FITC channel fluorescence-minus-one control was used to select the most appropriate gate that was applied to all $V\beta$ subtypes. $n = 5$ for each time point for each strain of mice, and results presented are representative of two independent experiments.

results were observed in nude mice: the percentage of splenic neutrophils that express TCR β transcript increased from 1.1% (day 0) to 6.5% (day 6). In *rag1* KO mice, 10.9% of neutrophils expressed TCR β transcript in naive mice, and this proportion increased to 47.6% by day 6 postinfection. Therefore, strain ANKA infection induced TCR β transcription in neutrophils in all three types of mice. Consistent with protein results, a significantly higher proportion of neutrophils expressed TCR β transcript in *rag1* KO compared to WT and nude mice (Fig. 4).

Neutrophils preferentially use $V\beta 3$ and $V\beta 8.1/8.2$ TCR subtypes. We measured the expression of 15 $V\beta$ TCR subtypes on the splenic neutrophil flow cytometry to assess the diversity of and preference for $V\beta$ subtypes (Fig. 5A and B). In this experiment, CD11b⁺ Ly6G⁺ neutrophils expressed an array ($V\beta 2$, $V\beta 3$, $V\beta 4$, $V\beta 5.1/2$, $V\beta 7$, and $V\beta 8.1/8.2$) of subtypes (Fig. 5C to F). However, neutrophils isolated from both C57BL/6 and BALB/c mice preferentially expressed the $V\beta 3$ subtype and, to a lesser degree, the $V\beta 8.1/2$ subtype. For example, on day 6 postinfection, when $9.4\% \pm 1.1\%$ of neutrophils in C57BL/6 mice expressed TCR β , the majority of these neutrophils expressed the $V\beta 3$ subtype. Importantly, $V\beta 3$ was the dominant subtype on neutrophils in both naive and strain ANKA-infected mice, and we note that although strain ANKA infection increased the proportion of neutrophils that express TCR β , it did not dramatically influence the diversity of and preference for $V\beta$ subtypes.

Neutrophil TCR β undergoes productive gene rearrangements. We next sequenced the complementarity-determining region 3 (CDR3) of TCR β transcripts purified from approximately 1,000 TCR β ⁺ CD3 ϵ ⁻ CD11b⁺ Ly6G⁺ neutrophils sorted from splenocytes isolated from four individual C57BL/6 mice exhibiting symptoms of ECM on day 6 postinfection with strain ANKA using an established protocol (16) (Fig. 6). Using this approach, we found that neutrophil TCR β was capable of undergoing productive gene rearrangements. We also used this molecular method to measure the diversity of

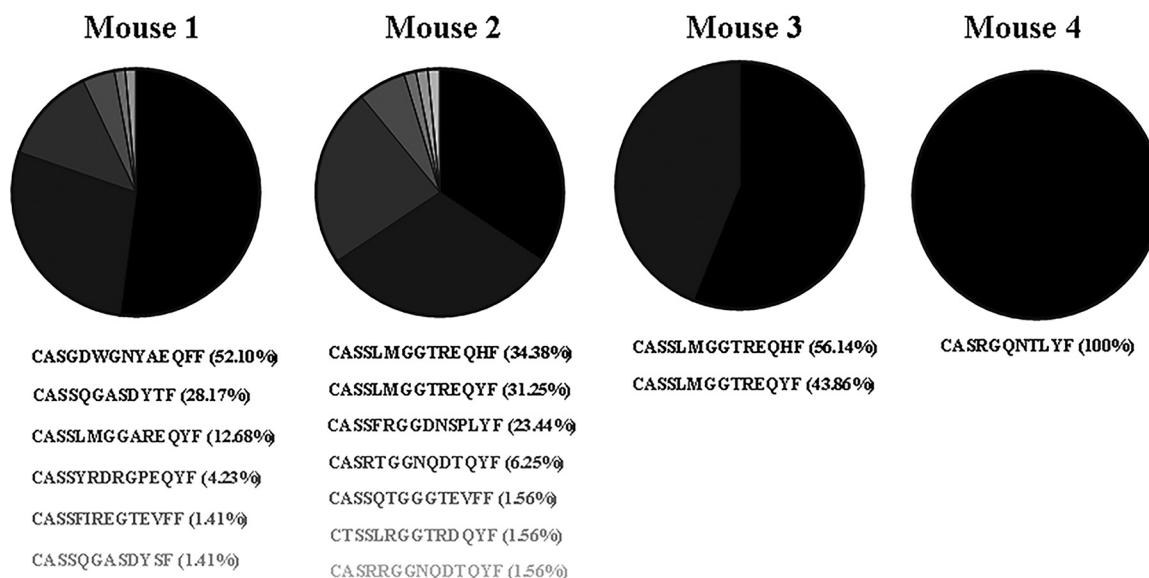


FIG 6 Repertoire of productive TCRβ gene rearrangements. Mice were infected with strain ANKA, and unbiased molecular analysis of T cell receptor expression was performed on TCRβ⁺ CD3ε⁻ CD11b⁺ Ly6G⁺ neutrophils sorted from spleen tissue of four moribund mice. Although neutrophil TCRβ undergoes productive gene rearrangements, neutrophil TCRβ has a restricted repertoire and displays preferred usage.

the TCRβ repertoire in the neutrophil population. We analyzed a minimum of 50 productive TCRβ rearranged sequences per mouse and found that the repertoire of TCRβ was limited (mice expressed only 1 to 7 types of TCRβ), and each mouse expressed a dominant TCRβ CDR3 region, indicating preferred usage.

TCRβ expression on CD11b⁺ Ly6G⁺ neutrophils correlates with parasite burden on day 3 after strain ANKA infection. It is well recognized that neutrophils are critical during the early immune response to acute infections. Remarkably, on day 3 postinfection, we observed a highly significant positive correlation (*P* value of ≤0.01, Pearson *r* correlation *R*² value of 0.92) between the proportion of splenic neutrophils that were TCRβ⁺ CD3ε⁻ and peripheral parasitemia in individual C57BL/6 mice (Fig. 7); a smaller percentage of neutrophils were TCRβ⁺ CD3ε⁻ in mice with low parasitemia than in mice with high parasitemia. A similar but less significant correlation was also observed in BALB/c mice (data not shown). However, on day 6 postinfection, there was no significant correlation between the proportion of neutrophils that were TCRβ⁺ CD3ε⁻ and peripheral parasitemia. These results suggest that rising parasite burden is driving the expansion of TCRβ-expressing neutrophils. In turn, this could play an important role in regulating parasite burden during the early phase of a strain ANKA infection, which fits the prescribed role of neutrophils in early host defense.

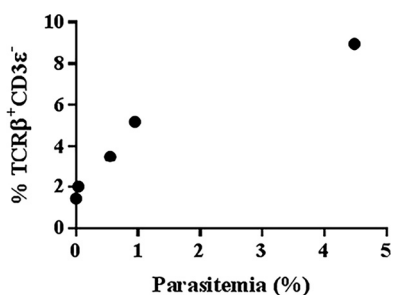


FIG 7 TCRβ expression by the neutrophil correlates with parasite burden on day 3 postinfection with *Plasmodium berghei* ANKA. On day 3 postinfection, the percentage of live singlet CD11b⁺ Ly6G⁺ neutrophils in the spleen that are TCRβ⁺ CD3ε⁻ was correlated with peripheral parasitemia (parasitized erythrocytes/total erythrocytes × 100) in five individual mice. Results presented are representative of four independent experiments. *P* ≤ 0.01; Pearson *r* correlation *R*² value of 0.92.

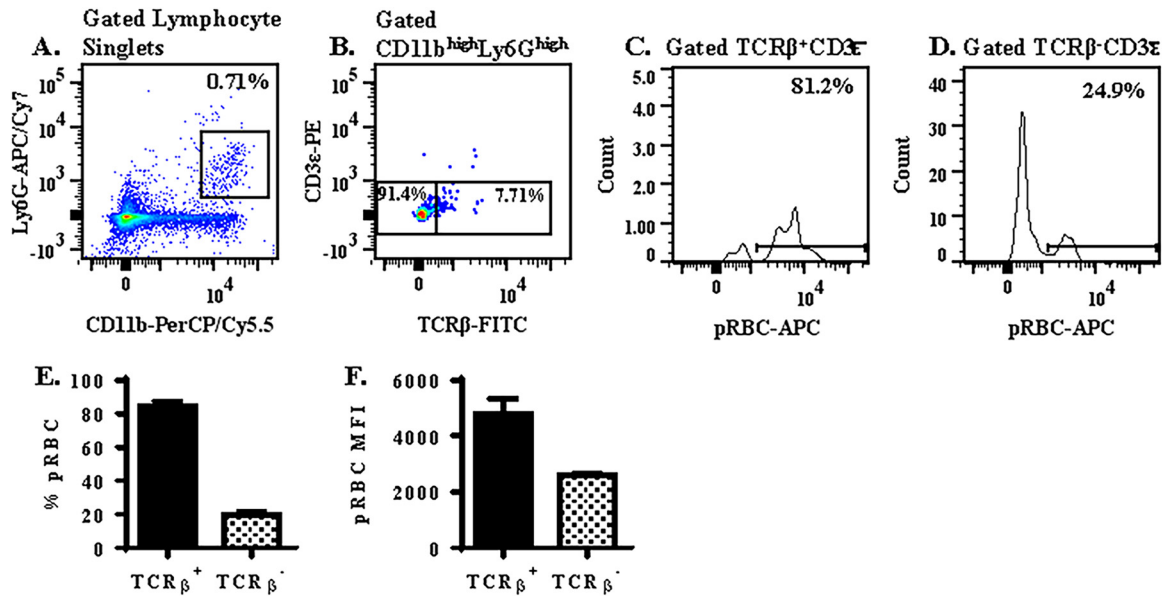


FIG 8 TCR β^+ neutrophils display enhanced phagocytosis of pRBC compared to TCR β^- neutrophils. (A to D) The ability of CD11b⁺ Ly6G⁺ neutrophils (A) that are TCR β^+ versus TCR β^- (B) to phagocytose pRBC (C and D) after 90 min was analyzed in an *in vitro* phagocytosis assay consisting of a 1:1 ratio of pRBC (labeled with CellTrace Far Red), and splenocytes (stained with neutrophil and T cell markers) isolated from C57BL/6 mice on day 3 postinfection with strain ANKA. (E) The percentage of neutrophils that phagocytosed pRBC was compared in TCR β^+ versus TCR β^- neutrophils. (F) In addition, the pRBC MFI was compared in TCR β^+ versus TCR β^- neutrophils that had undergone phagocytosis to determine if TCR β expression influenced the quantity of pRBC phagocytosed by a single neutrophil. A fluorescence-minus-one control was used to select the gate for TCR β . $n = 3$ for each phagocytosis assay, and results presented are a replicate from three independent experiments.

TCR β^+ neutrophils display enhanced phagocytosis of pRBC compared to TCR β^- neutrophils. To better understand whether neutrophil TCR β plays a role in regulating parasite burden during a strain ANKA infection, we measured the effect of TCR β expression on the ability of neutrophils to phagocytose parasitized erythrocytes in an *in vitro* assay. In this assay, splenocytes isolated from day 3 strain ANKA-infected C57BL/6 mice were labeled with neutrophil (Ly6G and CD11b) and T cell (TCR β and CD3 ϵ) markers and incubated for 90 min at 37°C with CellTrace-labeled parasitized red blood cells (pRBC) also isolated from day 3 strain ANKA-infected C57BL/6 mice. Phagocytosis of pRBC by gated TCR β^+ versus TCR β^- CD11b⁺ Ly6G⁺ neutrophils was then compared by flow cytometry (Fig. 8A to D). Remarkably, expression of TCR β was associated with enhanced phagocytosis of pRBC by the neutrophil; 84.3% \pm 3.2% of TCR β^+ versus 19.9% \pm 2.7% TCR β^- samples were CellTrace-allophycocyanin (APC) positive by flow cytometric analysis (Fig. 8E). Furthermore, we also compared the CellTrace-APC median fluorescence intensity (MFI) of CellTrace-positive TCR β^+ versus TCR β^- neutrophils. The MFI of TCR β^+ neutrophils (4,784 \pm 521) was significantly higher (1.8-fold) than that of TCR β^- neutrophils (2,601 \pm 103) (Fig. 8F), suggesting that each TCR β^+ neutrophil had phagocytosed a greater number or different stage of pRBC than each TCR β^- neutrophil. Together, these results demonstrate a strong positive association between TCR β expression and neutrophil phagocytosis of pRBC.

DISCUSSION

Since the discovery of TCR in 1984 (17, 18), it is widely accepted that expression of this molecule is limited to T lymphocytes. Combinatorial rearrangement permits T cells to express antigen-specific TCR combinations that enable recognition of potentially inordinate numbers of existing and emerging pathogens that a host may encounter over a lifetime. On the other hand, nonlymphoid cells are known to express invariant receptors for nonspecific phagocytic clearance of invading pathogens as a part of early host defense (19). However, in recent years, a few studies have reported TCR expression by nonlymphoid cells (20), including neutrophils, eosinophils, and

monocytes/macrophages. In 2006, Puellmann et al. reported that a subpopulation (5 to 8%) of human neutrophils expressed TCR $\alpha\beta$, and engagement of the neutrophil TCR $\alpha\beta$ complex inhibited apoptosis and increased IL-8 secretion (12). A case report documenting that 80% of circulating neutrophils expressed TCR in a patient diagnosed with autoimmune hemolytic anemia who also displayed abundant neutrophil erythrophagocytosis suggests that neutrophil TCR plays a biologically relevant role in the immunopathogenesis of disease (13). Likewise, macrophages have also been shown to express TCR, and high numbers of TCR $\alpha\beta$ -bearing macrophages have been reported in tuberculosis granulomas (21), atherosclerotic lesions (22), and the tumor microenvironment (23).

In this study, we report that early infection with *P. berghei* ANKA malaria results in significant expansion of TCR β -expressing CD11b⁺ Ly6G⁺ neutrophils in the splenic compartment of C57BL/6 and BALB/c mice. Importantly, we demonstrate experimentally that the observed malaria-induced expression of TCR β by the neutrophil is not simply an artifact of (i) passive receptor expression via phagocytosis or trogocytosis of peripheral T cells or (ii) nonspecific anti-TCR β binding to a cross-reactive epitope or Fc receptor on the neutrophil surface. Strain ANKA infection induces expression of both TCR β transcript and protein in neutrophils isolated from nude (which lack T cells) and *rag1* knockout (which lack T and B cells) mice, eliminating the likelihood that peripheral T cells are the source of neutrophil TCR β . In addition, measurement of TCR β RNA in neutrophils by PrimeFlow, molecular analysis of the TCR β repertoire by template switch PCR of neutrophil RNA, and demonstration of preferential usage of V β by neutrophils using a flow cytometry-based V β TCR screening panel confirm that expression of TCR by the neutrophil is not a consequence of nonspecific binding of the H57-597 clone of anti-TCR β to a non-TCR β epitope on the neutrophil.

An unexpected result of our study was that TCR β -expressing neutrophils were CD3 ϵ ⁻. On classical T cells, TCR forms a complex with CD3. This complex consists of two TCR chains (usually $\alpha\beta$) and six CD3 chains ($\delta\epsilon$, $\gamma\epsilon$, and $\zeta\zeta$). While it is difficult to conceive how TCR can operate in the absence of CD3 ϵ , previous studies of TCR-expressing neutrophils (12) and macrophages (21) also reported undetectable levels of CD3 ϵ . Detection of high levels of TCR β transcript and protein on neutrophils in *rag1* KO mice in our study and the Puellmann et al. study support the notion that the requirements for TCR expression differ between lymphoid and nonlymphoid cells. Future studies in our laboratory will be directed at defining the TCR machinery in myeloid cells of the immune system.

A signature finding of our study was that there was a highly significant correlation between the proportion of splenic neutrophils that express TCR β and parasitemia in the peripheral blood of individual C57BL/6 mice on day 3 postinfection. This finding suggests that parasite burden influences the recruitment of TCR β -expressing neutrophils from the bone marrow (the site of neutrophil production) to the spleen (the site of parasite clearance) during the early phase of a strain ANKA infection.

An important function of the neutrophil is the phagocytosis of invading pathogens. In an *in vitro* phagocytosis assay, we found that TCR β ⁺ neutrophils phagocytosed pRBC with considerably higher efficiency than TCR β ⁻ neutrophils; 84.3% of TCR β ⁺ versus 19.9% of TCR β ⁻ neutrophils had phagocytosed pRBC in this *in vitro* phagocytosis assay, indicating that TCR β expression by neutrophils contributes toward regulation of parasite density prior to acquisition of adaptive immunity. This strong association between TCR β expression by the neutrophil and erythrophagocytosis of pRBC is reminiscent of the case report documenting massive induction of TCR expression by neutrophils in a patient diagnosed with autoimmune hemolytic anemia who also displayed extensive neutrophil erythrophagocytosis (13).

In summary, our findings demonstrate a highly significant correlation between TCR β expression and (i) parasite burden and (ii) neutrophil phagocytic capacity, warranting further investigation of a novel immune mechanism whereby induction of TCR β expression by the neutrophil regulates parasite burden by enhancing phagocytosis of parasitized erythrocytes during the innate immune response to a strain ANKA infection.

MATERIALS AND METHODS

Mice and parasite infections. Six- to 10-week-old female C57BL/6, BALB/c, B6.129S7-*Rag1*^{tm1Mom/J}, and B6.Cg-*Foxn1*^{nu/J} (nude) mice purchased from The Jackson Laboratory (Bar Harbor, ME) were maintained at the Food and Drug Administration animal care facility and treated in accordance with the guidelines of the Animal Care and Use Committee. For infections, a donor mouse was first injected with a thawed vial of an uncloned line of strain ANKA parasites. When peripheral parasitemia reached approximately 5%, blood was collected from the donor mouse and diluted in phosphate-buffered saline (PBS) to 10⁷ parasites/ml. Experimental mice were then infected by intraperitoneal injection of 10⁶ parasites in a 100- μ l volume. Infected mice were monitored for clinical symptoms of ECM as previously described (24–26), and thin blood films were prepared to assess parasite burden. Parasite burden in peripheral blood was enumerated by calculating the percent parasitemia (parasitized erythrocytes/total erythrocytes \times 100) of Giemsa-stained thin blood films.

Flow cytometry. Expression of TCR β and the V β subtypes on the neutrophil was assessed by flow cytometry, as previously described (26, 27), in C57BL/6, BALB/c, nude, and *rag1* KO mice. Expression of TCR β was measured on splenic and brain-sequestered neutrophils. Briefly, a single-cell suspension was prepared from spleens of naive and strain ANKA-infected mice harvested on days 3, 6, and 9 postinfection. This single-cell suspension was subjected to ACK (Gibco by Life Technologies) lysis for 4 min to eliminate erythrocytes, and lymphocytes were then stained with eFluor 506 viability dye (eBiosciences, Santa Clara, CA) for 30 min, washed three times, and then seeded at 10⁶ cells/100 μ l of staining buffer (Hanks' balanced salt solution [HBSS] containing 1% bovine serum albumin [BSA]). A single-cell suspension of brain-sequestered leukocytes isolated from perfused brain tissue of C57BL/6 and BALB/c mice was prepared by treatment with DNase (3 U/ml) and collagenase (0.5 mg/ml) (Roche Applied Sciences, Indianapolis, IN) for 1 h at room temperature, followed by purification by centrifugation at 515 \times *g* for 30 min at 21°C on 33% Percoll (Sigma-Aldrich, Saint Louis, MO). Lymphocytes were then blocked with TruStain FcX (anti-mouse CD16/32) (BioLegend, San Diego, CA) for 15 min, stained with APC anti-mouse/human CD11b, peridinin chlorophyll protein (PerCP)/Cy5.5 anti-mouse Ly-6G, fluorescein isothiocyanate (FITC) anti-mouse TCR β chain (clone H57-597), phycoerythrin (PE) anti-mouse CD3 ϵ , Alexa Fluor 700 anti-mouse CD4, and APC/Cy7 anti-mouse CD8a antibodies, purchased from BioLegend (San Diego, CA) for 30 min, and washed three times with wash buffer (HBSS containing 0.1% BSA and 0.05% sodium azide). All incubations were performed at 4°C and were protected from light. Unfixed cells were resuspended in 150 to 200 μ l of wash buffer and then acquired on an LSR Fortessa X-20 (BD Biosciences, San Jose, CA). All data analysis was performed using FlowJo software. Importantly, a fluorescence-minus-one control was used to determine the appropriate gate for TCR β . Expression of V β 2, V β 3, V β 4, V β 5.1, 5.2, V β 6, V β 7, V β 8.1,8.2, V β 8.3, V β 9, V β 10^b, V β 11, V β 12, V β 13, V β 14, and V β 17^a TCR was also measured on splenic neutrophils isolated from C57BL/6 and BALB/c mice on days 0 and 6 postinfection with strain ANKA using the mouse V β screening panel (BD Biosciences, San Jose, CA).

PrimeFlow RNA assay. The PrimeFlow RNA assay (eBiosciences, Santa Clara, CA) was used to compare transcript levels of the mouse T cell receptor beta, constant region 1 (TRBC1), gene in naive and infected (day 6) wild-type, nude, and *rag1* KO mice on the C57BL/6 background. Splenocytes isolated from strain ANKA-infected mice were treated with ACK buffer (Gibco by Life Technologies) to remove erythrocytes, stained with eFluor 506 viability dye (eBiosciences), incubated with TruStain FcX (anti-mouse CD16/32) (BioLegend, San Diego, CA), and then stained with antibodies specific for extracellular neutrophil markers (CD11b and Ly-6G) and T cell markers (TCR β and CD3 ϵ). To prepare cells for target probe hybridization, cells were fixed with fixation buffer 1 for 30 min at 4°C, washed 3 times with permeabilization buffer containing RNase inhibitors, fixed again with fixation buffer 2 for 60 min at room temperature, and then washed 3 times. Cells were then incubated with a target probe specific for the *trbc1* gene for 2 h at 40°C in a hybridization oven. Amplification of signal was then performed in three sequential hybridization steps; briefly, cells were first incubated with preamplifier DNA (which hybridizes to the target probe) for 1.5 h at 40°C, incubated with amplifier DNA (which hybridizes to the preamplifier molecules) for 1.5 h at 40°C, and lastly incubated with label probe oligonucleotide (which hybridizes to the amplifier molecules) conjugated to the Alexa Fluor 647 fluorescent dye for 1 h at 40°C. When possible, a minimum of 200,000 lymphocyte events were acquired on an LSR Fortessa X-20 (BD Biosciences, San Jose, CA).

Unbiased molecular analysis of TCR expression by neutrophils. Unbiased quantification and characterization of all expressed TCR gene products within CD11b⁺ Ly6G⁺ neutrophils was performed using an established protocol (16). Approximately 1,000 CD11b⁺ Ly6G⁺ TCR β ⁺ CD3 ϵ ⁻ neutrophils sorted from spleen tissue of moribund C57BL/6 mice on day 6 postinfection with strain ANKA were snap frozen in RNAlater and stored at -80°C until use. mRNA was extracted from TCR β -expressing neutrophils using the Oligotex direct mRNA minikit (Qiagen, Valencia, CA), and cDNA was then synthesized using the SMARTer PCR cDNA synthesis kit (Clontech, Mountain View, CA). The MuMBC primer (TGGCTCAAACAA GGAGACCT), specific for mouse TRBC (Eurofins, Huntsville, AL), permits sequencing across the CDR3 region of the TCR gene and was used for PCR amplification of rearranged TCR products. After amplification, TCR gene products were run on a 1% agarose gel, and amplicons that were appropriate in size (500 to 700 bp) were extracted from the gel and purified using the NucleoSpin extract II kit (Clontech, Mountain View, CA). Each TCR gene product was then ligated into the pGEM-T Easy vector and transformed into competent *Escherichia coli* cells. After transformation, a minimum of 50 colonies were picked for amplification of plasmid inserts using the commercially obtained M13F (TTTCCAGTCACGAC) and M13R (CAGGAAACAGCTATGAC) primers (Eurofins, Huntsville, AL). Amplified products were then run on a 1% agarose gel to confirm sizes of products prior to sequencing using MuMBC primer. Alignment of the sequences was performed using the IMG/QUEST alignment tool for TCR nucleotide sequences.

Phagocytosis assay. The effect of TCR β on the ability of neutrophils to phagocytose parasitized red blood cells (pRBC) during a strain ANKA infection was assessed in an *in vitro* assay. This *in vitro* phagocytosis assay was performed using a 1:1 ratio of pRBC (labeled with CellTrace Far Red [Invitrogen, Carlsbad, CA]) and splenocytes (labeled with neutrophil and T cell markers) isolated from C57BL/6 mice at postinfection day 3. A single-cell suspension of harvested spleen was prepared and subjected to ACK (Gibco by Life Technologies) lysis to eliminate RBC. A total of 10^6 lymphocytes were then blocked with TruStain FcX (anti-mouse CD16/32) (BioLegend, San Diego, CA) and stained with neutrophil (PerCP/Cy5.5 anti-mouse/human CD11b and APC/Cy7 anti-mouse Ly-6G) and T cell (FITC anti-mouse TCR β chain and PE anti-mouse CD3 ϵ) markers. A total of 10^5 CellTrace-labeled pRBC and 10^5 antibody-labeled splenocytes were then mixed together and incubated at 37°C. After 90 min of incubation, samples were then washed once with PBS prior to acquisition on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Phagocytosis of CellTrace Far Red pRBC by TCR β^+ versus TCR β^- neutrophils was compared using FlowJo software. A fluorescence-minus-one control was used to select an appropriate gate to distinguish TCR β^+ from TCR β^- neutrophils.

Statistics. Differences in cell proportions and counts were determined using the Mann-Whitney *U* test. Correlation between the proportion of neutrophils that were TCR β^+ CD3 ϵ^- and peripheral parasitemia was determined using the Pearson correlation coefficient.

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