INNATE LYMPHOCYTES IN YOUNG MICE PROMOTE PROTECTION AGAINST *PLASMODIUM CHABAUDI* INFECTION

A Thesis
by
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Abstract

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Understanding the immune response to malaria, especially in children under five, is limited due to the lack of a reliable animal model to study the pathogenesis of the disease. By utilizing a newly developed young rodent model in our lab, we have observed that both splenocytes and purified $CD4_+$ T cells from 8-week adult mice proliferate faster than day 15 old young mice (pups) when stimulated with a plate bound anti- CD3 and CD28 *in vitro*. Using adoptive transfer technique, we show that both pup and adult cells protect immunocompromised RAG1 knockout (RAG1KO) mice from death. To better understand the responsive and protective cell populations in the splenocytes from the young mice, we adoptively transferred splenocytes from both pups and adult mice into RAG1KO mice and infected the recipients with *P. chabaudi* to determine both innate and adaptive immune cell populations. We observed higher numbers and proportions of innate lymphocytes including gamma delta ($\gamma\delta$) T cells and natural killer (NK) cells in the pup splenocyte recipients when compared to adult counterparts on day 9 post-infection. In contrast, there were significantly higher proportions of macrophages in the adult splenocyte recipients. Taken together, our findings suggest that pup splenocytes are enriched with innate lymphocytes that may promote protection against *P. chabaudi* infection.

Acknowledgments

I would like to express my sincerest gratitude to my faculty mentor Dr. Michael Opata for his incredible patience and guidance, and for making me a better scientist and person. Special thanks to my committee members Drs. Maryam Ahmed and Darren Seals for guidance, advice and encouragement through my thesis project. I would also like to acknowledge Dr. Nicholas Shaw for his unwavering support and for always saving me a place at his dinner table. Special appreciation and gratitude to my colleagues in both chemistry and biology, for I would not have been able to complete this without your hard work and dedication to pursuing great science. I would like to give all my love and thanks to the future Dr. Jennifer Pilotos Mcbride, who not only grew alongside me as a scientist, but who also grew to be a dear friend and sister. All the tears and late nights together were always worth it, and we will both do such great things. I would also like to give thanks to Monique Eckard, whom we could never have accomplished our work without her great care and attention to our mice. A final thanks to my family for all of their love and continuous encouragement throughout the years of me pursuing science, and for always pushing me to be the best I could be. I thank you all, for I would not be who I am today without your presence in my life.

Dedication

To those who inspired it but will never read it: my mother, father, and cat.

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List of Abbreviations

iRBCs: infected red blood cells

NK: Natural killer cells

IFN-γ: Interferon gamma

IL-2: Interleukin 2

IL-10: Interleukin 2

DCs: Dendritic cells

TNF-α: Tumor necrosis factor alpha

APCs: Antigen presenting cells

MHCII: Major histocompatibility complex II

RTS, S: *Plasmodium falciparum* vaccine

VLP: Virus-like particle

CSP: Circumsporozoite protein

FACS: Fluorescence-activated cell sorting

RAG1KO: Rag-1 knockout mice

Pups: Young mice

NC: No cell controls

SEM: Standard error of the mean

Chapter 1

INTRODUCTION

Throughout history, malaria has proved to be a significant threat to human health, and 300–500 million people have a malarial illness annually (1). Despite extensive strides towards reducing the progress of infection, malaria continues to inflict extensive morbidity and mortality in resource-limited countries, particularly in sub-Saharan Africa.

Malaria is caused by protozoan parasites of the genus *Plasmodium*. There are five parasite species known to infect humans including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* (2). However, the vast majority of malaria related deaths are caused by *P. falciparum* (3). Transmission of *Plasmodium* parasites to the mammalian host begins with the deposition of the infectious, motile sporozoites into the human skin by a bite from an infected female *Anopheles* mosquito as it takes a blood meal. The sporozoites traverse through the skin to capillaries, where they gain access to the bloodstream and get transported to the liver (4, 5). In the liver, each individual sporozoite infects a healthy hepatocyte (liver cell), then transforms and undergoes growth, and genome replication (4). In both humans and rodents, merozoites are released from the liver into the bloodstream 7–10 days after initial transmission. The liver stage is predominately asymptomatic, and the host is generally unaware of the fact that they are already infected with malaria. As merozoites are released from the liver, they infect red blood cells (iRBCs), replicate within, and are released again to undergo continuous cycles of infection and replication. This continuous release cycles allow parasite numbers to reach billions within a week (6, 7).

The clinical manifestations of malaria, and severity of clinical attack depends on the species and strain of the infecting *Plasmodium* parasite, as well as the person's age, immune status, malaria specific immunity, nutritional status, and the mode of transmission (8). The first clinical symptoms and awareness of malaria infection are observed during the rupture of the schizont, which initiates the symptomatic blood-stage of malaria infection. Upon merozoite infection of the red blood cells, the merozoites form the trophocyte (ring form), which later fully develop into late blood-stage schizonts. The rupturing of the blood-stage schizonts inside the infected red blood cells releases on average of 15 to 32 merozoite progeny that infect surrounding red blood cells (9, 10). The most well-known clinical manifestations in human infection are anemia fever, impaired consciousness, seizures, vomiting, and respiratory distress. In children, features of severe *P. falciparum* can include severe sepsis and cerebral malaria [8].

Children between six months and five years of age are at highest risk of malaria disease due to loss of maternal immunity. At this age, children have not yet developed specific immunity to malaria, and therefore rely heavily on a rapid, robust, and non-specific immune response in order to quickly counter the progression of malaria pathology and infection. The initial inflammatory response by the innate immune cells is critical in quickly combating malaria infection. It does this by (*i*) acting rapidly as a first line of defense in which it clears the parasite early, thus inhibiting its progressive growth, and (*ii*) initiating the adaptive immune response and subsequently leading to the control of re-infection. The innate immune response in *P. falciparum* infection is orchestrated by macrophages, innate lymphocytes (natural killer cells), and innate-like lymphocytes (gamma delta T cells), due to their ability to directly sense the presence of the parasite through specific innate immune mechanisms (11).

Natural killer (NK) cells contribute to providing a first line of defense against pathogens through their ability to respond to a pathogenic challenge prior to antigen presentation and recognition (12). This is particularly important in children, due to their naive and still developing immune system. Despite being relatively non-specific, NK cells have an array of innate cell receptors to "sense" their environment (13). NK cells control infection through the robust production of pro-inflammatory cytokines and the direct destructions of damaged, dysfunctional, or infected host cells through cytotoxic activity (12). In malaria-infected children, elevated NK cell counts, and increased NK cell cytotoxicity have been correlated with lower parasitemia. This is due to the observed ability of NK cells to directly lyse *Plasmodium*-infected red blood cells, and are also one of the earliest sources of IFN-γ following *P. falciparum* infection (14). In order for this to take place, NK cells require activation signals from three sources: (*i*) priming by IL-2 or IL-15, which is produced and released by T cells or dendritic cells (DCs) respectively, (*ii*) IL-12 or IL-15 from macrophages, or (*iii*) from direct cell-to-cell contact with macrophages (11).

Gamma delta (γδ) T cells are an unconventional T cell subset, which only constitute 2-5% of the total circulating lymphocytes, γδ T cells play an integral role in both the innate and adaptive arms of the immune system. γδ T cells are the first T cells to arise during fetal development and provide a protective function even prior to birth (15). However, the percentage of this population declines after birth and only constitutes 0.5% of all mature T cells in adults (15). These cells have long been known to rapidly proliferate in both humans and mice following primary malaria infection (16, 17), with many of their functions including: robust production of inflammatory, cytotoxic killing, and immune cell recruitment (16-18). γδ T cells cytotoxic killing heavily contributes toward antiparasitic activity through the secretion of cytotoxic molecules perforin, granzyme A and B, and Fas-Ligand/CD95L expression (18, 19), as well as

inflammatory cytokines (IFN-γ, TNF-α and TNF-β/LT). All of which work in concert with other mechanisms in order to directly eliminate parasite, mediating protection against recrudescence and/or reinfection, particularly in the context of malaria (20), and lead to the recruitment of other immune cells (18, 19).

The prevalence of IFN-γ production by immune cells has been associated with a greater likelihood of uncomplicated malaria (8), as well as reduced severe malarial anemia (9). Importantly, experiments in mice have demonstrated that IFN-γ production by both NK and γδ T cells play a major role in the direct control of *Plasmodium* infection through the recruitment of other key players in the immune response to malaria disease. The IFN-γ is specifically involved in the activation of macrophages, which control parasite through phagocytosis and the production of other inflammatory mediators and reactive oxygen species (ROS).

Macrophages play an indispensable role during malaria infection through antigen recognition, processing, and presentation (21). Interactions between these cell subsets and *Plasmodium* parasites occur at every stage of infection (22, 23). *P. falciparum* is predominately found in the blood-stage and provides many opportunities for antigen presenting cells (APCs) to interact with the *Plasmodium* parasites, therefore leading to the internalization (phagocytosis) of the merozoites and whole parasitized erythrocytes in both an opsonization-dependent and opsonization-independent manner (11, 24). This allows for antigen processing and presentation to T cells via the major histocompatibility complex II (MHCII) (23, 25).

Another function of macrophages is their ability to kill parasite through secretory products. After erythrocyte invasion by merozoites, major transformations are induced on the surface of iRBCs, which can directly interact with receptors on the surface of macrophages (21, 26). In response to infection caused by *Plasmodium* parasites, macrophages release antimicrobial

reactive oxygen species (ROS) and proinflammatory cytokines and chemokines (TNF-α, IL-1β, and IL-6), which are highly damaging and lead to efficient killing of iRBCs (12, 27). This generation of ROS causes an imbalance with antioxidant species, that triggers oxidative stress, which is an important response to infections (28-30). In the case of malaria, parasite control is caused by oxidative damage to both the parasite as well as parasitized erythrocytes, which takes place once ROS are able to diffuse through the membrane of infected red blood cells (28, 29).

The innate immune response provides a critical mechanism for the rapid sensing, elimination, and control of the chronic pathology associated with *P. falciparum* infection. However, the adaptive immune response provides a more slowly developed, more specific, and fine-tuned repertoire of recognition for both self- and non-self-antigens (31, 32). Adaptive immunity involves a highly controlled and regulated communication between APCs, T cells, and B cells. All working in tandem in order to orchestrate and facilitate pathogen-specific pathways, generation of memory, and regulation of the host immune response (31). It is important to note that sterile immunity to malaria is never achieved by the host (3). But after repeated exposures to the infection, individuals eventually develop effective partial immunity that controls parasitemia and prevents severe and life-threatening complications (31).

Despite considerable efforts to control malaria over decades, there has yet to be a highly effective vaccine for any of the five plasmodial species which cause human malaria. This is predominately due to the plasmodial life cycle being incredibly complex, and thus the task of designing an effective vaccine has been difficult (4). In addition to the insufficiently understood parasite biology, malaria infection is currently undergoing a worldwide resurgence because of the spread of drug resistant parasite strains and increasing resistance of mosquitoes to insecticides. Several molecules from the sporozoite at the hepatocyte or asexual erythrocyte, and

gametocyte stages have been identified and studied as potential vaccine candidates with the aim of eliciting protection against infection, clinical illness, and/or transmission of malaria, but none has so far been highly effective (33). Thus, the lack of an effective vaccine necessitates intensive research on human malaria parasites that can inform the development of new intervention tools (34).

There are currently three developed strategies implemented by the World Health Organization (WHO) for protection and development of acquired protective immunity to *P. falciparum* infection in young African children: (*i*) insecticide-treated bed nets, (*ii*) preventive therapy, and (*iii*) anti-sporozoite vaccines [35]. Resistance has now been detected in malaria vectors to the four classes of public health insecticides used in malaria vector control (pyrethroids, organochlorines, organophosphates and carbamates), which are components in insecticide-treated bed nets.

Most current vaccine candidates target a single stage of the parasite's life cycle and vaccines against the early pre-erythrocytic stages have shown most success (36). Vaccines based on the pre-erythrocytic stages usually aim to completely prevent infection, while blood stage vaccines aim to reduce (and preferably eliminate) the parasite load once a person has been infected (37). Gametocyte vaccines would prevent the parasite being transmitted to others through mosquitoes. Overall, an ideal vaccine would be effective against all parasite stages. The ultimate goal of developing a vaccine for malaria is to ensure complete cure, through the rapid and full elimination of the *Plasmodium* parasite from the patient's blood, in order to prevent progression of uncomplicated malaria to severe disease, as well as prevent the chronic infection that leads to malaria-related anemia as well as death.

The most current and promising vaccine design is the RTS, S, which was recently approved for use in some African countries (38). It is a malaria recombinant vaccine, that is structured on the foundation of the hepatitis B surface antigen virus-like particle (VLP or HBsAg), and is genetically-engineered to include the carboxy terminus (amino acids 207-395) of the circumsporozite antigen (CSP) (33, 38). CSP is a malaria parasite sequence that is species-specific, but highly conserved for isolates from each of the five species of *Plasmodium* (39).

The RTS, S vaccine accomplishes limited protection against the *Plasmodium* circumsporocyte, and its ability to illicit the production of anti-CSP antibodies by plasma cells. It also leads to activation of antigen specific $CD4^+$ T cells which can provide immunogenicity with or without boosters (40). RTS, S induces very high immunoglobulin G (IgG) concentrations and moderate to high $CD4^+$ T cell responses in vaccinated humans (33). Protection by the RTS, S vaccine varies from 12 to 68% efficacy in the first year but has been shown to degrade to only a 2.5% efficacy after four years (41). The limited efficacy of this vaccine may be due to the selective and minimal immune responses that it induces, which consists mostly of antibodies against the repeat domain of the CSP and are known to neutralize and halt the progression of sporozoites in the liver (33, 42). An additional limitation of the RTS, S vaccine is that it does not induce $CD8^+$ T cell responses, as these cells are known to be effective and have efficient anti-parasite mechanisms that eliminates malaria during the liver stages (33, 42). The development of a vaccine capable of inducing $CD8^+$ T cell responses in addition to antibodies may require major changes in the design of the vaccine construct of the RTS, S in order for it to fully illicit an effective protective immune response against *P. falciparum* (33, 42).

Studying the protective host responses against the *Plasmodium* parasite has emerged as a key factor in reducing morbidity and mortality associated with severe infections. This is

particularly important early in the infection as well as for susceptible groups who are most likely to succumb to infection. This study aligns with this goal in that understanding malaria responsive cells early in life can inform better vaccine designs that are protective to the vulnerable population. Therefore, we use an adoptive transfer technique to better understand *Plasmodium* responsive cells in young and adult mice. We hypothesize that young mice have more innate like lymphocytes (γδ T and NK cells) that promote protection against malaria in *P. chabaudi* infection.

Chapter 2

MATERIALS AND METHODS

Mice and Parasite

Adult C57BL/6 mice were obtained from Harlan labs, and Recombinant Activating Gene knockout mice (RAG1KO) that are immunocompromised, were obtained from The Jackson laboratories. All mice were maintained at Appalachian State University as breeding pairs and kept on a 12:12 light/dark cycle. We utilized the rodent strain of malaria, *Plasmodium chabaudi* (*P. chabaudi*), which manifests disease symptoms that are similar to that caused by the human parasite strain *Plasmodium falciparum* (*P. falciparum*), such as a synchronized life cycle, pathology and long-term chronic infection. The parasite was a gift from Dr. Robin Stephens from the University of Texas Medical Branch at Galveston with permission from Dr. Jean Langhorne (Francis Creek Institute, UK). Parasites were counted by light microscopy after Diff Quick staining.

In vitro cultures

Splenocytes were collected from days 10, 15 and adult 8-week old mice that were not infected. Single cell suspensions were obtained by homogenization of the spleens through a cell strainer, and red blood cells were lysed using red blood cell lysis buffer (Tonbo Biosciences, San Diego, CA). Cells were washed with PBS and resuspended in complete ISCOVES culture media supplemented with 2 mM L-glutamine, 5mM sodium pyruvate, non-essential amino acids (MEM NEAA), 10 mM HEPES, 100 U/ml Penicillin, 100 U/ml Streptomycin and 25 μM 2-Mercaptoethanol. Cells were counted using a hemocytometer and trypan exclusion. After counting aliquots for culture were labeled with CFSE according to the manufacturer's instructions (Tonbo Bioscience) and then cultured in anti-CD3/CD28 bound 24 well plates at a

concentration of $5x10_5$ cells/well. Proliferation was determined at 2- and 4-days post-culture by determining CFSE intensity.

Age Determination

The average lifespan of laboratory mice is about 24 months, whereas the life expectancy of humans globally is about 80 years. Therefore, considering both lifespans, the ages of mice were calculated as follows utilizing a murine to human age equivalency calculation determined by Dutta and Sengupta [43]:

$$\frac{(80 \times 365)}{(2 \times 365)} = 40\ human\ days = 1\ mouse\ day$$

$$\frac{365}{40} = 9.125\ mouse\ days = 1\ human\ year$$

Therefore, when utilizing these calculations 1 human year is approximately equivalent to 9 mouse days when considering entire lifespan of both species. We used C57BL/6 10 and 15-day-old pups and 8-week-old adult mice.

Adoptive Transfers

Both C57BL/6 15-day-old pups and 8-week-old adult mice were euthanized, and spleens were harvested. Single cell suspensions were obtained, and RBCs were lysed as described above. After counting, splenocytes were transferred at a concentration of $2x10_7$ intraperitonially into immunocompromised RAG1KO mice. The recipient mice of either pup or adult cells were inoculated with $1x10_5$ *Plasmodium* infected red blood cells (iRBCs) one day post-transfer. On

day 9 post-infection, tail blood smears were collected for parasite determination in the recipient RAG1KO mice before they were sacrificed to determine immune cells in the spleens as shown in the schematic in Figure 1 below.

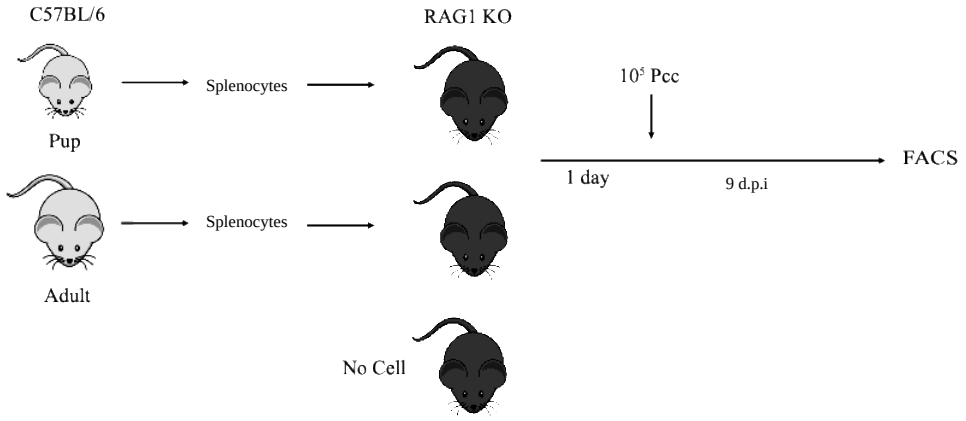

FIGURE 1. Schematic for RAG1 KO adoptive transfer and timeframe. RAG1 KO mice received purified splenocytes from either pup (15- day- old) or adult (8- week- old) C57BL/6 mice at a concentration of $2x10_7$ cell/mouse. One day post transfer the RAG1 KO were inoculated with a parasite dosage of $1x10_5$. Mice were then sacrificed 9 days post-infection (d.p.i).Pcc = *Plasmodium chabaudi*

Determining Parasitemia

Parasite burden was determined using thin blood smears that were obtained through tail snips of infected mice at day 9 post-infection. The slides were stained with Diff-Quick and parasites were counted under a light microscope in 10 to 50 different fields, depending on the level of anemia/parasite load. To determine percent parasitemia, the number of infected red blood cells were divided by the total number of red blood cells (both infected and un-infected) in all counted fields. The outcome was then multiplied by 100 to get a percentage value as shown in the formula below:

$$\%\ Parasitemia = \frac{iRBC}{Total\ RBC} \times 100$$

Flow cytometry

a) Surface staining

Aliquots were taken from the single cell suspension after processing from the spleens as described above. The cells were counted with the use of a hemocytometer and an aliquot of $3x10_6$ cells/mL was transferred into polystyrene round-bottom tubes and washed in cold FACS buffer (PBS, 2% FBS, and 0.1% NaN3 sodium azide). The cells were then incubated with Fc receptor blocking antibody for 20 minutes. After this incubation, florescent antibody stain master mix was added directly to the tubes and incubated for an additional 40 minutes for surface molecule staining. The master mix was made with the following fluorescently conjugated antibodies: $CD4_+$ T cell panel (CD4, CD44, CD62L, CDlla), Gamma Delta T cell panel (CD3, γδ), Macrophage panel (CDllb, F4/80, MHCII, MHCI, CD86), and Natural Killer cells (NK) panel (CD49, NK1.1). Stained cells were then washed and resuspended in FACS buffer for flow cytometry analysis after filtration with nanomesh screens.

b) Intracellular staining

After preparation of spleen cells as described above, splenocytes were incubated in complete Iscoves media (described in the *in vitro* section). A stimulation cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin and Brefeldin A (Tonbo Biosciences) was added to each well, except for an unstimulated control. Treatment with PMA and ionomycin is sufficient to induce activation of many cell types to produce cytokines. After 4 hours incubation, cells were harvested and washed with FACS buffer. Cells were Fc blocked and then immediately stained

for surface markers. The cell membrane was stabilized with 2% paraformaldehyde (PFA) and permeabilized with a perm wash buffer (Tonbo Biosciences). The cells were then stained with anti-IFN-γ, TNFα, IL-2 and IL-10, or perforin, and granzyme B, then incubated for 30 minutes. Stained cells were then washed three times with perm wash buffer to remove excess unbound antibodies and filtered as mentioned above for flow analysis.

Data Analysis

All flow cytometry data were collected on an FC500 Beckman Coulter flow cytometer (Indianapolis, IN). Cytometry data was analyzed with FlowJo8 software (Ashland, OR) and data calculations were done using Microsoft excel. Graphs and statistical evaluations were done using the Student's *t* test to compare distribution of pairs of groups in Prism GraphPad (San Diego, CA). In some experiments that include four-group comparisons, a non-parametric One-way ANOVA was used followed by comparison among individual groups. A p value of <0.5 was considered significant.

Chapter 3

RESULTS

Previous results from our laboratory showed that when splenocytes from young mice are transferred into immunocompromised RAG1KO knockout mice (RAG1KO), the immunocompromised mice are protected from death and these mice regain weight better than adult cell recipients after the peak of infection (Smith, *et al*., unpublished data). This suggested to us that the splenocytes from young mice contained cell populations that could confer protection against malaria infection. In order to investigate this further, we determined the proliferation capacity of the young versus adult cells. We harvested spleen cells from 10, and 15-days old young or adult 8-week old mice, then cultured them *in vitro* on plate bound anti-CD3/CD28, to selectively stimulate T cell proliferation and expansion.

We observed that both the young and adult splenocytes proliferated as measured by CFSE dilution. As expected, the adult splenocytes proliferated faster on days 2 and 4 than the cells from both 10 and 15-days young mice. Comparing only the pup cells, 15-days old splenocytes proliferated faster than the 10-day pup cells **(Fig. 2 A & B)**. Based on the proliferation pattern of these splenocytes, we concluded that as the mice grow, their cells have the potential to proliferate faster.

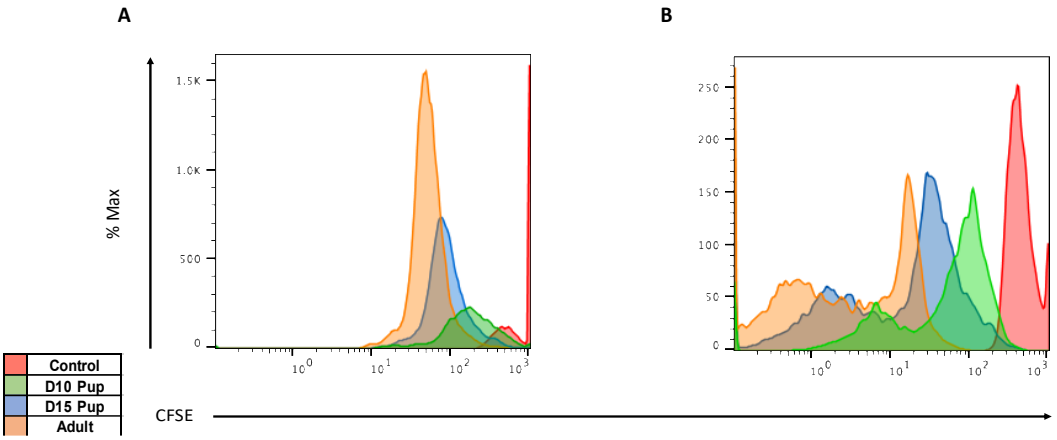

FIGURE 2. Adult splenocytes proliferate faster than young mice splenocytes. Splenocytes from young 10 days old (green), or 15 days old (blue), and 8-week old adult (orange) C57BL/6 mice were labeled with CFSE and cultured *in vitro*. Cells were harvested on **(A)** day 2 and **(B)** day 4 post stimulation with plate bound anti-CD3/CD28 to determine proliferation by CFSE dilution. (Red) indicate CFSE labeled control cells that were not cultured.

To better understand the responsive immune cells in young mice infected with malaria, we infected 15 days old pups or 8 week old adult mice with $1x10^5$ *P. chabaudi* and determined activation of $CD4_+$ T cells and presence of γδ T cells, which are known to be highly present in young mice, as well as children in human studies [44]. There was no significant difference in the percentage of activated $CD4_+$ T cells between infected pups and adult mice, but due to larger spleen sizes, cell numbers were higher in adult mice **(Fig. 3A & B)**. We also observed significantly higher proportions and numbers of γδ T cells in the adult than young mice **(Fig. 4A & B)**. Taken together, these results suggest that in the physiological environment, there is higher numbers of both activated $CD4_+$ T cells as well as γδ T cells in the adult mice.

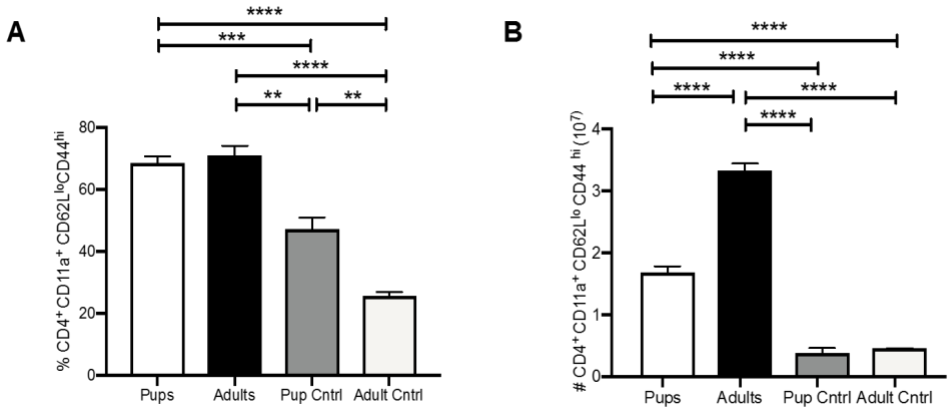

FIGURE 3. Numbers but not proportions of activated CD4 T cells are higher in adult mice. C57BL/6 15-day old pups and 8-week-old adult mice were infected with $1x10_5$ *P. chabaudi* and sacrificed on day 8 post-infection. Activation of $CD4_+$ T cells was determined using surface markers CDlla, CD62L, CD44 antibodies. **(A)** Percent and **(B)** number of activated $CD4_+$ T cells was determined with flow cytometry. These data represent 3-7 mice per group from 2 independent experiments. Error bars represent SEM and significance was determined using One-way Anova. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

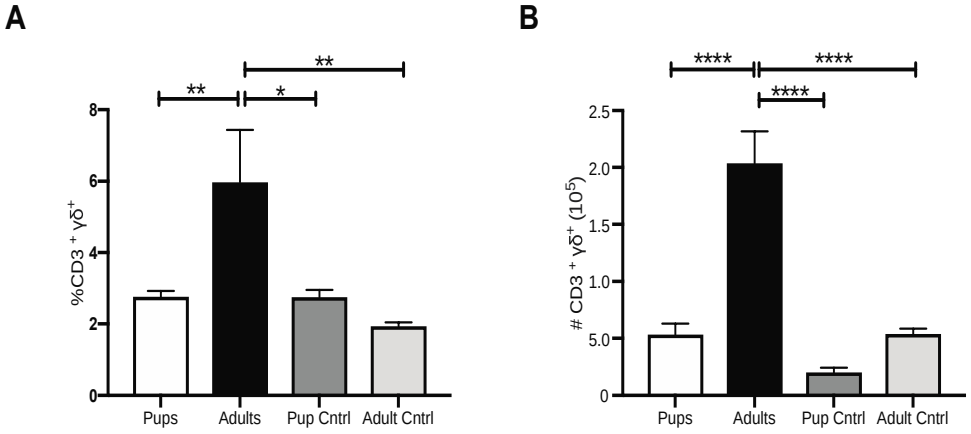

FIGURE 4. Proportions and numbers of γδ T cells are higher in adult mice. C57BL/6 15-day old pups and 8-week-old adult mice were infected with $1x10_5$ *P. chabaudi* and sacrificed day 8 post-infection. The γδ T cells were determined using anti- CD3 and γδ antibodies. **(A)** Percent and **(B)** number of γδ T cells was determined by flow cytometry. Data represent 3-7 mice per group from 3 independent experiments. Error bars represent SEM and significance was determined by One-way Anova. *$p < 0.05$, ** $p < 0.01$, *** p <, **** $p < 0.0001$.

Pup splenocytes reduce parasitemia in immunocompromised mice infected with malaria.

Since young mice have a naive immune system that is still developing, the results from the infection of the pups and adult mice in **Figures 3 and 4** above may be biased in favor of the adult mice due to a fully developed immune system and bigger spleens. To address this concern, we performed an adoptive transfer of the splenocytes from both pups and adult mice into RAG1 KO recipient mice, which do not produce mature T or B cells, and therefore lack an adaptive immune system [45, 46]. But it is important to note that RAG1KO still retain their own innate immune cell populations prior to an adoptive transfer.

The recipient mice were infected with $1x10^5$ *P. chabaudi* one day after cell transfer and sacrificed 9 days post-infection. When looking at the demonstrable presence of parasite in the blood (parasitemia) at the peak of infection (day 9), we observed that RAG1 KO mice that had received splenocytes from either adult or young mice had significantly lower percent parasitemia when compared to the no cell controls, but both pups and adult cell recipients had similar reduction in parasitemia (**Fig. 5A).** Recipients of both pup and adult spleenocytes also retained their weight when compared to no cell control mice which lost significantly more weight between 7-9 days post infection.

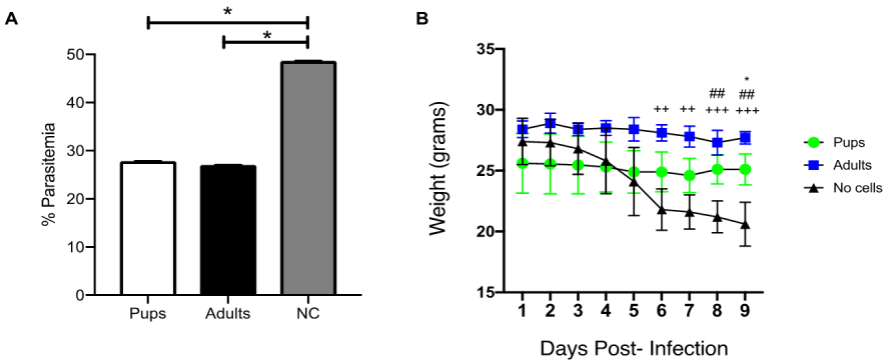

Figure 5. Both adult and pup cells reduce parasitemia and protect from weight loss in immunocompromised RAG1KO mice. $2x10_7$ splenocyte cells were adoptively transferred into adult RAG1KO mice infected with $1x10_5$ *P. chabaudi*. (**A**) Parasitemia in pup recipients, adult recipients, and no cell controls (NC) was determined by microscopy from blood smears on day 8 p.i. (**B**) Mouse weight in grams was recorded over 9-day infection period. Data represent 3-4 mice per group from 2 independent experiments. Error bars represent SEM and significance was determined by two-tailed t-test *p < 0.05.

Based on the reduction in parasite load in recipient RAG1KO mice of both pup and adult cells, we next determined the adoptively transferred immune cell populations that could have contributed to parasite control in the immunocompromised mice. Therefore, we measured the presence of both $CD4_+$ T and γδ T cells as was performed in physiological studies (**Fig. 3 & 4**). As expected, we observed higher proportions and number of $CD4_+$ T cells in RAG1KO mice that received adult splenocytes (**Fig. 6A & B**), which was reflective of what was observed in physiological studies (**Fig. 3A & B**) above. $CD4_+$ T cells from adult donor mice also produced more IFN-γ as determined by proportion and numbers, when compared to pup donor cells **(Fig. 6C & D)**. This is consistent with other investigators reporting that classical $CD4_+$ T cells are the primary producers of malaria- induced IFN-γ [24].

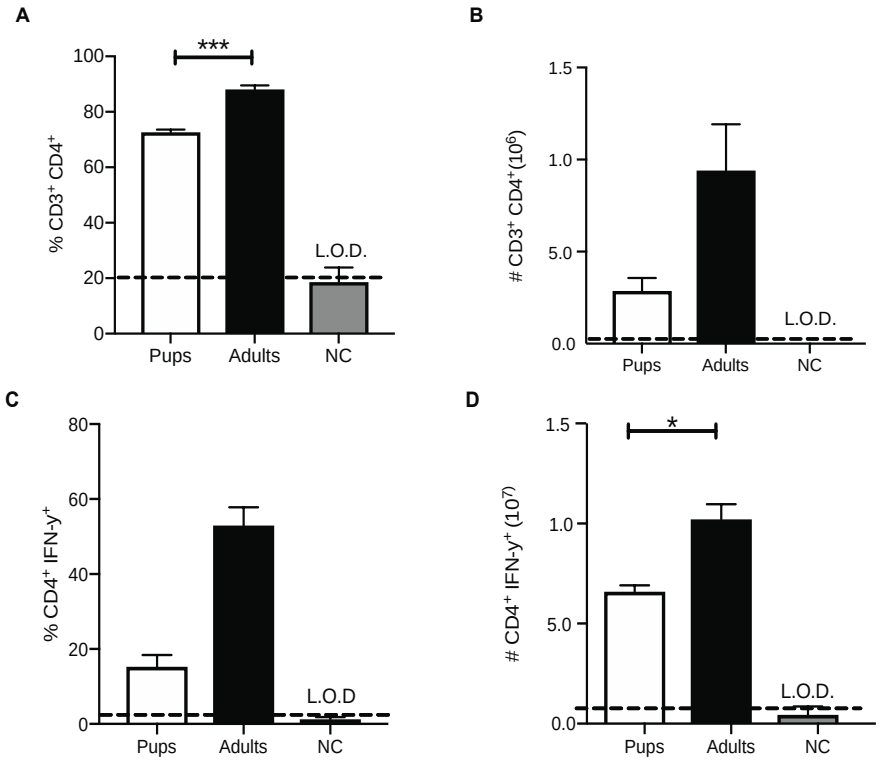

FIGURE 6. Adult CD4+ T cells produce more IFN-y after malaria infection. 2x10$_7$ splenocytes were transferred into RAG1KO mice infected with 1x10$_5$ *P. chabaudi.* (**A**) proportion and (**B**) number of CD4+ T cells were identified using CD3 and CD4 surface markers. **(C)** proportions and **(D)** numbers of CD4+ IFN-γ T cells in pup recipients, adult recipients, and no cell controls (NC). Data represents 3 mice per group from 2 independent experiments. Error bars represent SEM and significance was determined by two-tailed t-test. *p < 0.05, *** p < 0.001. L.O.D. = Limit of detection

Pup splenocytes have increased percent and number of γδ T cells in malaria infection.

It has been reported that there are higher amounts of CD4+ T cells in infected adult mice, and γδ T cells are higher in young mice [15, 17]. Since we did not observe this initially when directly infecting pup and adult mice, due to bigger spleens in the adult mice as mentioned above (**Fig. 3 and 4A & B**), we sought to determine the γδ T cells in the adoptive transfer RAG1KO mouse model. We observed significantly higher proportions and numbers of γδ T cells in the pup cell recipients, than what was observed in the physiological infection between the two groups (**Fig. 7A & B**). Therefore, taken together, these results indicate that pup cell recipients may be

protected by the γδ T cells while the adult cell recipient mice may be protected by conventional CD4+ T cells, as well as their production and secretion of IFN-γ.

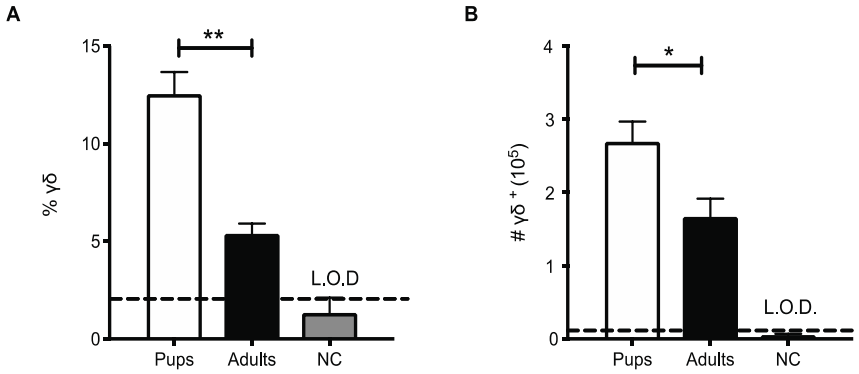

FIGURE 7. Proportions and number of γδ T cells are higher in pup splenocyte recipients after malaria infection. 2x10$_7$ cells were transferred into RAG1KO mice which were then infected with 1x10$_5$ *P. chabaudi.* (**A**) proportion and (**B**) number of γδ T cells in pup recipients, adult recipients, and no cell controls (NC). γδ T cells were identified using anti-mouse CD3 and γδ antibodies. This data represents 3 mice per group from 2 independent experiments. Error bars represent SEM and significance was determined by two-tailed t test, *p < 0.05, ** p < 0.01. L.O.D. = Limit of detection

Pup splenocytes increased the natural killer cells in immunocompromised mice in malaria infection.

It has been reported that NK cells significantly contribute to protection against malaria (12). Similarly, previous studies have shown that NK cells are increased during early infection with experimental malaria (47). We therefore determined if NK cells would also be contributing to the protection in pup recipient hosts. As expected, we observed significantly higher proportions and numbers of NK cells in the recipients of young splenocytes compared to adult splenocytes (**Fig. 8A & B**). Surprisingly, the no cell control mice also had higher proportions of the NK cell population. This data suggests that the immunocompromised mice have higher proportions of NK cells due to RAG1KO lacking an adaptive immune response, but it is

important to note that they retain their innate immune cell populations (48), therefore the cell recipients have their own NK cells prior to the adoptive transfer of splenocytes. The observed NK cell-based immunity and amount of NK cells is either maintained or enhanced when cells from young mice are transferred but is subsequently decreased upon transfer of adult splenocytes.

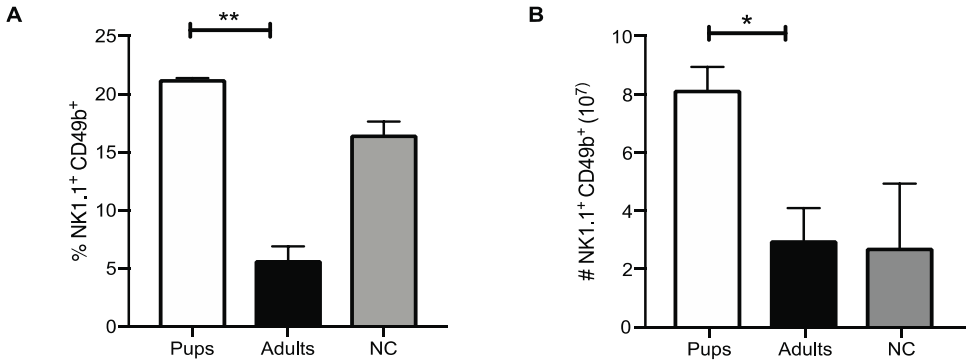

FIGURE 8. Proportions and numbers of NK cells are higher in pup splenocyte recipients in malaria infection. $2x10^7$ cells were transferred into RAG1KO mice, which were infected with $1x10^5$ *P. chabaudi.* (**A**) proportion and (**B**) number of NK cells in pup recipients, adult recipients, and no cell controls (NC). The NK populations were identified using anti-mouse CD49b and NK1.1 antibodies. These graphs represent 3 mice per group from 3 experiments. Error bars represent SEM and significance was determined by two-tailed T test. *$p < 0.05$, ** $p < 0.01$.

There is no difference in the ability of natural killer cells to respond to IL-2 in both young and adult splenocyte recipients.

Natural killer cells expand in response to IL-2, since we observed higher proportions of NK cells in both the pup cell recipients and no cell recipients, (**Fig. 8A & B**), we wondered if they had similar response to Interleukin 2 (IL-2). To test this, we determined the expression of the high-affinity IL-2 receptor alpha chain (CD25) on NK cells in all the groups after malaria infection. CD25 has been shown to be upregulated upon NK cell activation, leading to local or systemic inflammation, through production of inflammatory cytokines (12, 49). We observed lower proportion of CD25 expression in the no cell control which did not reach significant

difference, but both pup and adult cell recipients had similar numbers of CD25 expressing NK cells.

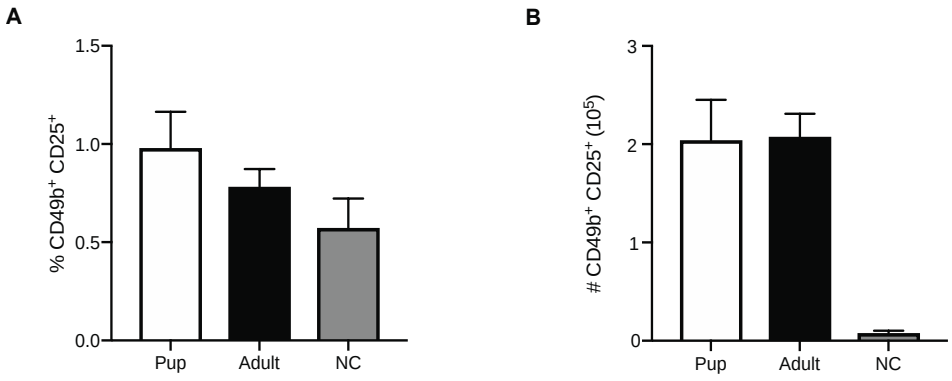

FIGURE 9. There is no difference in CD25 expression in NK cells in both pup and adult splenocyte recipients. $2x10_7$ cells were transferred into RAG1KO mice, which were infected with $1x10_5$ *P. chabaudi.* (**a**) proportion and (**b**) number of NK cells expressing CD25 in pup recipients, adult recipients, and no cell controls (NC). The NK populations were identified using antibody markers for CD49b and CD25. These graphs represent 3 mice per group from 2 experiments. Error bars represent SEM and significance was determined by two-tailed t test. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

There is no difference in the secretion of IFN-γ and IL-10 by natural killer cells in splenocyte recipients.

Several studies have demonstrated a crucial role for NK cells in the production of cytokines during early murine malaria infections (44, 49, 50). We next tested the functionality of NK cells by determining cytokine secretion and cytotoxic capacity. IFN-γ is significantly important during early stages of malaria infection and subsequent parasite clearance (22). Given that we observed both higher numbers and proportions of NK cells in pup cell recipients, we used intracellular cytokine staining technique to determine IFN-γ production by the NK cells (24, 47, 51). We found that despite observing significantly higher proportions and number of NK cells in the recipients of young mice splenocytes (**Fig. 8A & B**), these cells produced similar percentages and number of IFN-γ to that produced by the adult cells (**Fig. 10A & B)**.

The rapid and robust production of pro-inflammatory cytokines like IFN-γ early in malaria infections correlate with more severe clinical symptoms, but better parasite clearance (24). In malaria, IFN-γ secreting cells have been reported to co-produce the anti-inflammatory cytokine IL-10 to reduce any potential damage due to inflammation (52, 53). We thus explored the secretion of IL-10 by NK cells (54). Similar with what we observed for IFN-γ, there was no difference in the proportion or number of IL-10 secretion by NK cells between the two splenocyte recipient groups (**Fig. 10C & D**). Taken together, these data suggest that NK cells in pups may significantly contribute to protection from malaria by secreting both IFN-γ or IL-10.

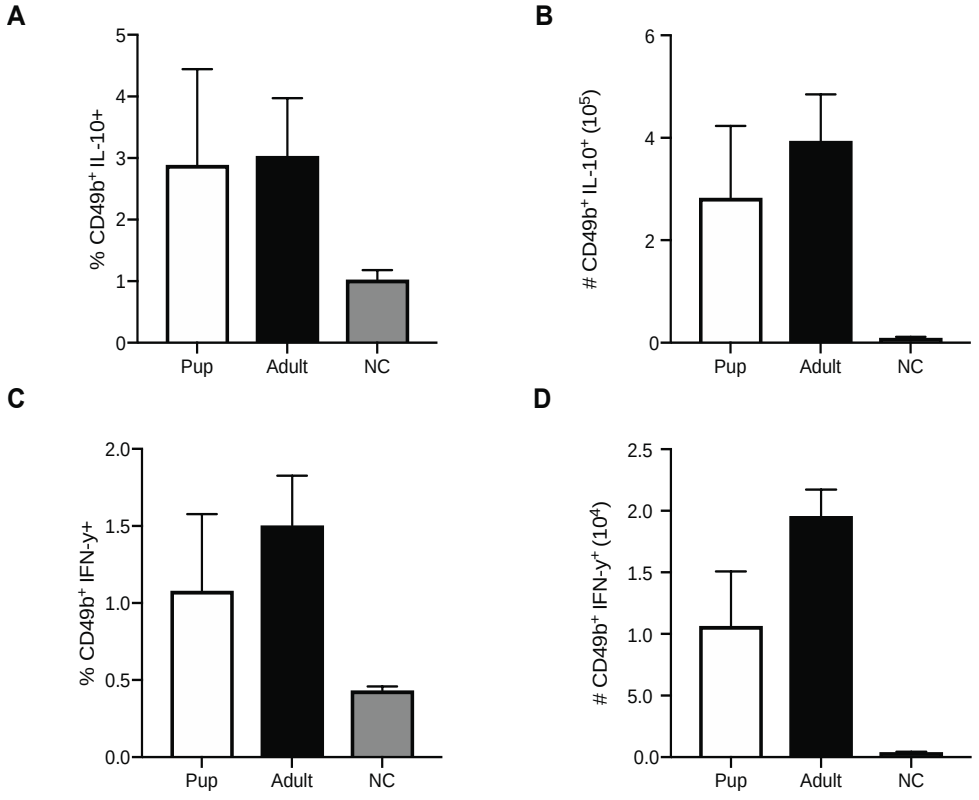

FIGURE 10. There is no defect in NK cell production of IFN-γ or IL-10 in pup cell recipients. $2x10^7$ splenocytes were adoptively transferred into adult RAG1KO mice, infected with $1x10^5$ *P. chabaudi*. (**A**) Proportion and (**B**) number of NK cells producing IL-10 in pup recipients, adult recipients, and no cell controls (NC). NK cells were identified with markers for CD49b, and cytokines were identified with IFN-γ and IL-10 antibodies. Data represent 3 mice per group from 2 experiments. Error bars represent SEM and significance was determined by two-tailed t test.

Pup splenocytes promote higher numbers of granzyme B production during malaria infection.

In addition to the well-investigated role for NK cells in cytokine production during malaria infection, there is limited but growing evidence to suggest that NK cells may also be capable of directly killing *Plasmodium*-infected cells through cytotoxic activity (11). Therefore, we determined the cytotoxic potential of the NK cells through their production of granzyme B and Perforin. Pup cells produced significantly more granzyme B compared to adult cells, but there was no difference in perforin between the two groups.

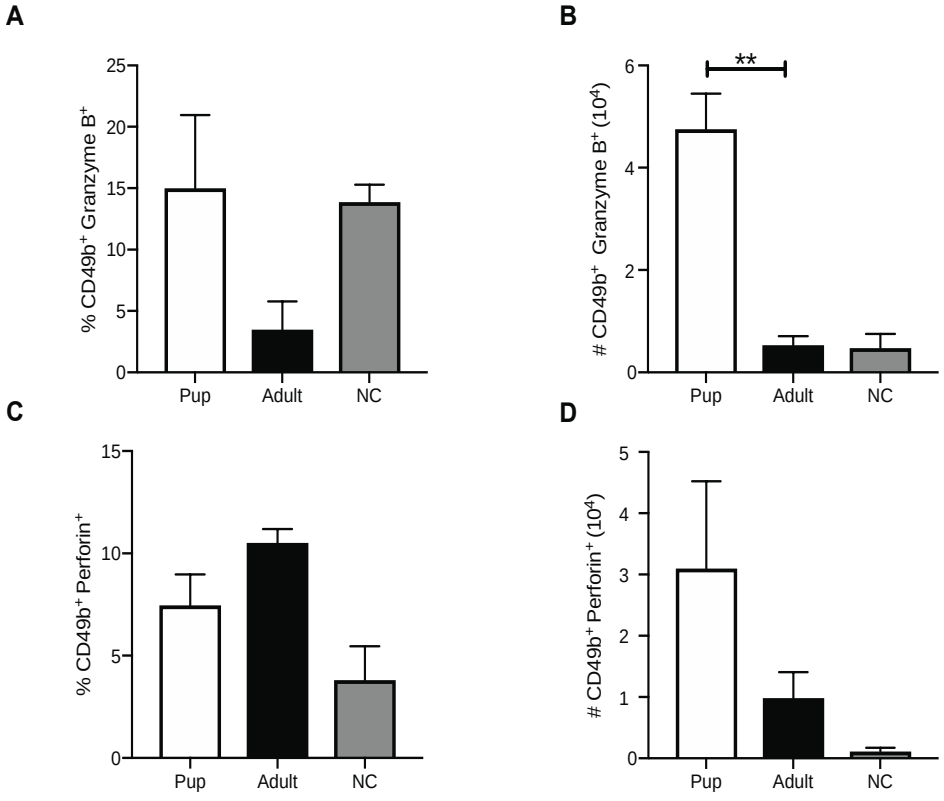

FIGURE 11. Pup cells promote increased granzyme B section by the NK cells in RAG1 KO mice. $2x10^7$ splenocytes were adoptively transferred into adult RAG1KO mice, infected with $1x10^5$ *P. chabaudi*. (**A**) proportion and (**B**) number of granzyme B producing NK cells pup recipients, adult recipients, and no cell controls (NC). (**C**) Proportion and (**D**) number of perforin producing NK cells were identified by markers for CD49b and cytotoxic killing was determined by anti-granzyme B and perforin antibodies. Data represent 3 mice per group. Error bars represent SEM and significance was determined by two-tailed t test. ** $p < 0.01$.

Adult splenocytes promote production of granzyme B and perforin in macrophages during malaria infection.

Since pup cell recipients had higher numbers of the innate lymphocytes (**Fig. 8A & B**), we wondered if normal innate immune cells like macrophages were also contributing to protection in pup cells as seen with NK cells above. Therefore, we determined the number ($CD11b^{+}F4/80^{+}$) and activation status ($CD11b^{+}MHCII^{+}CD86^{+}$) of the macrophages as they are important for phagocytosis, clearance of parasite, and disposal of debris from dead infected erythrocytes (21). There was no difference in the proportions or number of activated macrophages (**Fig. 12A & B**).

Similar to NK cells, macrophages also have cytotoxic potential and function through the production and secretion of perforin and granzyme B (55). Macrophages in adult donor cells produced more granzyme B and perforin than the pup donor cells (**Figure 13A & B**). Taken together, these data suggest that pup cells may functionally depend on NK cells for their cytotoxic killing, while adult cells depend on macrophages.

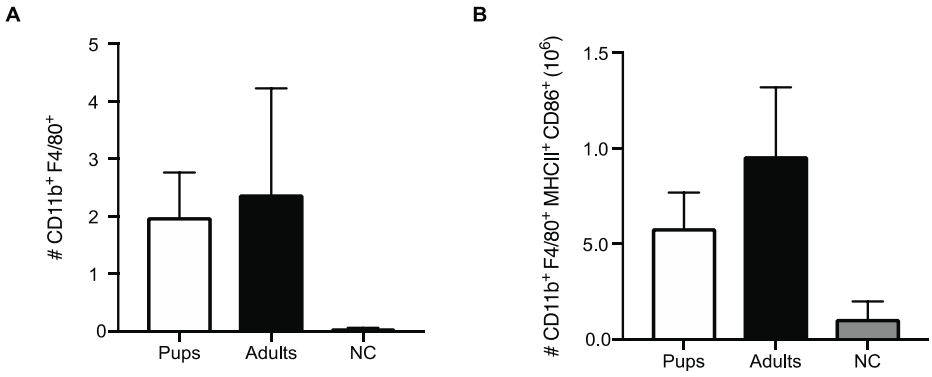

FIGURE 12. Adult and pup cells have similar proportions and number of macrophages. $2x10^7$ splenocytes were adoptively transferred into adult RAG1KO mice, infected with $1x10^5$ *P. chabaudi*. (**A**) Number of macrophages (**B**) number of activated macrophages in pup recipients, adult recipients, and no cell controls (NC). Macrophages were identified by markers for CD11b, F4/80, and activation was determined by markers for MHCII and CD86. Data represent 3 mice per group from 2 experiments. Error bars represent SEM and significance was determined by two-tailed t test.

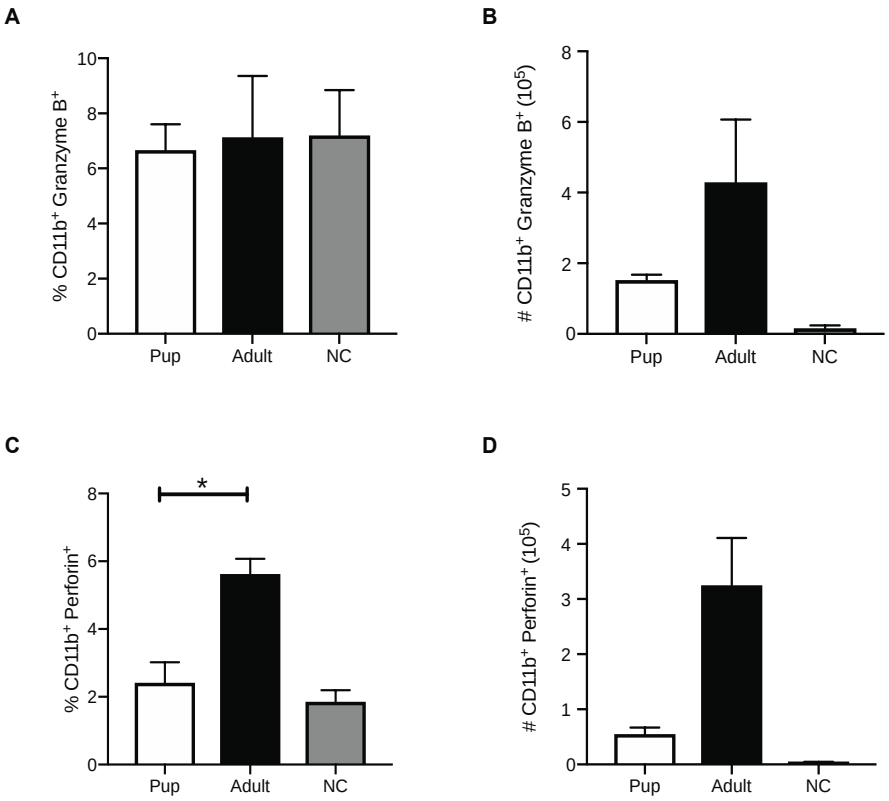

FIGURE 13. Macrophages in adult cell recipients express higher numbers of granzyme B and perforin. 2x10$_7$ splenocytes were adoptively transferred into adult RAG1KO mice, infected with 1x10$_5$ *P. chabaudi*. (**A**) proportion and (**B**) number of macrophages producing granzyme B. **(C)** proportion and (**D**) number of macrophages producing perforin in pup recipients, adult recipients, and no cell controls (NC). Macrophages were identified by markers for CD11b, and cytotoxic killing was determined by granzyme B and perforin antibodies. Data represent 3 mice per group from 2 experiments. Error bars represent SEM and significance was determined by two-tailed t test. * $p < 0.05$.

Chapter 4

DISCUSSION

Although the increased efforts toward prevention of malaria spread have resulted in a significant decline in disease, most of the progress has focused on reducing further transmission of the infection. There is still a considerable lack of knowledge on immune development during malaria disease, especially in children. Due to the insufficiently understood parasite biology and emergence of drug resistance, there is a growing need for intensive research to better understand immunity in order to contribute toward the development of new intervention tools (34). To enable progress in these studies, our laboratory established a young rodent model to malaria using the *P. chabaudi* parasite, a well-established rodent parasite strain that has been extensively utilized in laboratory settings to study the pathogenesis of malaria (2).

Although various *Plasmodium* species differ in their life-cycle characteristics, there is significant conservation of both genetic and phenotypic traits between the human malaria parasites and their rodent counterparts, therefore making them useful models for studying human malaria (2). Due to this, there are several reasons to favor the use of animal models to study malaria: (*i*) animal models allow scientists to investigate the progress of the disease, which is more difficult to study in humans, as well as (*ii*) permit studies of organs to which the parasite sequesters, such as the spleen, lungs and brain, and (*iii*) it is not ethical, practical or financially viable to use human volunteers (5, 56). Overall, there are many parallels between data on *P. chabaudi* experimental malaria and human malaria, which suggests that the use of this species in laboratory experiments will continue to yield further important contributions to our understanding of the immunobiology of malaria for years to come. With this in mind, our

research has utilized a mouse model to investigate differences in immune response between cells from young and adult mice that are exposed to a *P. chabaudi* infection.

These spleen cells (splenocytes) are comprised of many immune cell populations which include all lymphocytes, innate immune cells, and inflammatory mediators. Therefore, it was evident that young splenocytes contain cell populations that are capable of transferring protection to immunocompromised hosts, based off our previous studies. In this study, we sought to investigate the cell populations in the splenocytes of young mice that promote protection against malaria infection in comparison to adult cells.

We first looked at $CD4_+$ T cells, which have been shown to play a central role in the immune control of infection with *Plasmodium* parasites (57). Cell mediated immunity is largely T cell-driven, which have been supported in experimental studies that $CD4_+$ T cells rescue immunodeficient animals from lethal blood-stage *P. chabaudi* infection and may also modulate susceptibility to re-infection in humans (58, 59). $CD4_+$ T cells control *Plasmodium* infection in multiple ways: (*i*) cytotoxic responses in the liver, (*ii*) driving antibody production in the spleen and bone marrow through interacting with B cells, and (*iii*) secreting several cytokines during blood-stage infection (58, 60, 61). Our studies indeed show that there is an increased percent and number of activated $CD4_+$ T cells in the spleens of 8-week-old adult mice at the peak of malaria infection (9 d.p.i). Similar observations were seen with the RAG1 knockout model as well (**Fig. 2 and 5**).

There has been a considerable body of research assessing the role of cytokines in protection or risk of malaria, particularly the inflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), as well as the regulatory cytokines IL-10, IL-27, and TGF-β (61-63). With great importance being placed on IFN-γ, vaccine developers now regard

this cytokine production to be the hallmark of effector T cell function for combating malaria infection due to its ability to induce innate immune cell function, such as macrophage activation (63, 63). Both *P. chabaudi* and *P. falciparum* infection generate malaria-specific T cells with the ability to make IFN-γ which has been shown to correlate with protection against *P. falciparum* malaria (61).

Importantly, there is evidence that children with severe *P. falciparum* infection have lower concentrations of $CD4^+$ T cells secreting IFN-γ than children with uncomplicated malaria, as well as infected adults (60, 65). Interferon is essential for the resolution of primary infection by limiting the initial phase of parasite replication, but when left unchecked could heavily contribute to the acute symptoms such as fever, nausea, and headache (60, 62), and in extreme cases, cerebral malaria (66). In our study there were significantly greater proportion and number of IFN-γ producing $CD4^+$ T cells in adult cell recipients when compared to the pup counterparts (**Fig. 5**). This suggests that older mice not only had more IFN-γ producing $CD4^+$ T cells when compared to younger mice in malaria infection, but that this population may be responsible for protecting the adult RAG1KO mice in early malaria infection. But since parasite load was similar in both pup and adult cell recipients, we further investigated the cell populations that may confer protection to the recipient RAG1KO mice.

In contrast to conventional T lymphocytes which carry an αβ T-cell receptor, there are gamma delta T cells that carry the γδ T-cell receptor. Both of these T cell populations differ in their activation, function, and arm of the immune system (being either the innate or adaptive) (17, 67). Many of γδ T-cell functions include robust production of inflammatory cytokines, cytotoxic killing, as well as immune cell recruitment (16-18). As γδ T cells are seen in high numbers in children than adults (15, 17), it is highly certain that these cells play a role in

controlling malaria infection in pup splenocyte recipients due to their ability to rapidly respond to foreign invaders prior to antigen presentation (17).

Despite literature suggesting that there is a greater dependence on γδ T cells in young mice in innate infection (15, 17), this was not observed in our current study when we directly infected young mice with *P. chabaudi* (**Fig. 3**). But upon adoptive transfer of young and adult splenocytes into RAG1KO recipients, we observed significantly greater proportions and numbers of γδ T cells in the pup cell recipients that we did not see in previous studies (**Fig. 6**). Therefore, suggesting that there is an enrichment of γδ T cells in the splenocytes of young mice that may contribute to reduction in parasitemia and weight loss during early infection as previously observed in our lab.

Similar to γδ T cells, NK cells are also beneficial during the early phase of *Plasmodium* infection as they act as first responders to infection. They act as an effective rapid fire force through (*i*) rapid response prior to the activation and expansion of antigen specific $CD4_+$ T cells, which is crucial towards the early control of parasite pathology (12), and (*ii*) in a similar manner to γδ T cells, NK cells are relatively promiscuous in their targets (13, 68). Recently published studies have also indicated that NK cells are frequently the first cells to directly respond after *in vitro* exposure of human *P. falciparum*-infected erythrocytes (69). In malaria infections, NK cells control pathology through cytolysis and rapid cytokine secretion which activate macrophages to eliminate the parasites faster.

With this in mind, we wanted to look at the presence of NK cells in the recipients of our splenocyte cells. Upon adoptive transfer we observed that there were higher proportions and numbers of NK cells in recipients of pup splenocytes (**Fig. 7**). Which is reflective of the idea that this population may be playing a protective role in early *Plasmodium* infection. But surprisingly,

we observed that the adult cell recipients had significantly lower percentage and numbers of NK cells. Which can be observed when comparing them to the no cell recipients.

The NK cells expand due to their ability to respond to IL-2, an important cytokine for immune cell proliferation. As IL-2 can only function upon upregulation of the high-affinity IL-2 receptor α chain (CD25), which has been well-documented to be upregulated upon activation, allowing NK cells to expand as a result of local or systemic inflammation (55). In a study performed by Korbel it was observed that NK cells upregulated the expression of CD25 upon stimulation with iRBCs, which resulted in the rapid production of IFN-γ as well as the cytolytic products perforin and granzyme B (70). This is important because the expression of these molecules by NK cells not only indicate cell activation, but their ability to produce important mediators that are crucial towards the control of malaria pathology. In our research we demonstrated that there was no difference in the expression of CD25 by the NK cells between the groups suggesting proper functionality (**Fig. 8**).

Cytokines play an important role in immune cell response as described by various investigators (24, 54, 55). The cytokines amplify the immune response during the crucial early timeframe of infection and ultimately determine pathology as well as disease outcome. In rodent malaria, the difference between lethal and non-lethal infections depends on the ability of the mouse to mount an IL-12, IFN-y, or TNF-α response early in infection. In the presence of *Plasmodium*, observed in both mouse and human studies, NK cell production of IFN-γ has been associated with control of parasitemia as well as delayed re-infection (11, 71). In our studies, we observed that there was no difference in IFN-γ secretion by NK cells between splenocyte recipient groups (**Fig. 9**). This was interesting due to our observation of a significant decrease in NK cells upon adoptive transfer of adult cells in RAG1KO mice, but despite this reduction in

amount, their functionality was retained. With this in mind, there is still much debate on whether the NK cells are more harmful than helpful due to their ability to rapidly overproduce inflammatory cytokines in response to infection, which can also contribute to immunopathology (47, 52, 53).

NK cells also participate by controlling malaria infection through cytotoxic killing. When inhibitory signals are reduced, the result is the cytotoxic activation of NK cells and their direct interaction with the *Plasmodium* parasite as well as iRBCs (13). Previous studies conducted by Orago *et al.*, provided the initial evidence that there is a direct NK cell cytotoxic response to *P. falciparum* iRBC*s* in malaria infection (70). Release of perforin and granzymes reflect the activation of cytotoxicity in NK cells. Cytolytic products and a number of pro-inflammatory cytokines were shown to be elevated in response to *P. falciparum* infections in African children with clinical malaria (72). Here we observed that NK cells from pups produced slightly more perforin and granzyme B, compared to the adult cells (**Fig. 10**).

Excess circulating inflammatory cytokines can lead to complications and further progression of pathology. Therefore, there is a crucial need for control and balance of that inflammation. In order to temper this, NK cells are well-documented in their production of the immunoregulatory cytokine, IL-10 (53). Similar to IFN-γ production from NK cells, there was no significant difference between the two groups in the production of IL-10 by NK cells (**Fig. 9)**. While we observed higher numbers of NK cells in the pup cell recipients, there was no difference in their functionality in terms of cytokine secretion. This indicates that the NK cells in the pup splenocytes may promote protection through direct killing by secreting granzymes, but not cytokine production. NK cells could also function through other mechanisms that have not

been established yet. One such mechanism could be induction of cell death in the infected cells through Fas mediated apoptosis, even though we did not test this possibility.

All together we observed, in regard to NK cells and their functionality, that in early malaria infection, pup splenocytes had more NK cells early in the infection, and that these cells had greater cytotoxic potential than the cells from the adult mice. We also observed that there was no difference in the expression of CD25 by the NK cells, nor their ability to produce IL-10 or IFN-y between the two groups. Some potential possibilities for this observation could be that there may be differences in the NK cells activation status and expression of natural cytotoxicity triggering receptor 3 (NKp30) or natural-killer receptor group 2, member D (NKG2D) in response to early malaria infection. While both receptors have been shown to increase during NK cell activation in direct response to iRBCs (73), much has yet to be explored.

It has previously been demonstrated using *in vitro* studies that both NKp30 and NKG2D bind to the *P. falciparum* protein *Pf* EMP1, leading to NK cell activation (73). In particular Artavanis-Tsakonas et al. demonstrated that the engagement of NKG2D stimulates the production of cytokines, as well as cytotoxic molecules (73) Traditionally, this receptor is viewed as a molecule that mediates direct responses against cellular threats, such as *plasmodium*. Once activated, NKG2D results in (*i*) signaling for adhesion to the target, (*ii*) granule polarization, (*iii*) degranularization, and ultimately (*iv*) the apoptosis of the target cell (74, 75). Applying this to our study, future studies will investigate if NKp30 and/ or NKG2D is downregulated in the RAG1KO adult cell recipient mice and upregulated in pup cell recipients

The activation of NK cells is dictated by a balance between positive signals promoted by a variety of activating receptors, such as NKG2A, and negative signals provided by inhibitory receptors upon interaction with molecules on the target cell. It has been observed that NK cell

inhibitory receptors maintain an inactive state through the recognition of constitutively expressed “self-molecules” on potential target cells (13). These molecules are used to identify non target cells: killer immunoglobulin receptors (KIRs), NKG2A, Ly49 and Siglecs, as well as MHCI (76). In particular, major histocompatibility complex (MHC) class I acts as an inhibitory signal to NK cells as it signifies that the cells with MHCI are “self”. When cells do not have MHCI on their surface, it signifies to NK cells that the cell in question has been compromised, resulting in the activation of the NK cells (13, 74). The MHC class I binding of inhibitory NK cell receptors may be another potential explanation as to why we observed a reduction of NK cells in the adult cell recipients.

The crosstalk between immune cells is critical for the innate immune system to combat the early attacks from pathogenic challenge, particularly the interaction between NK cells and macrophages. The macrophage-NK interaction is a major first-line defense against pathogenic challenge. Similar to NK cells, macrophages, play an indispensable role during early malaria infection through antigen recognition, processing, and presentation (21). Interactions between these cell subsets and *Plasmodium* parasites occur at every stage of the life cycle within the host, but most importantly the blood and spleen, where the majority of *P. chabaudi* pathology occurs (22, 23). Blood-stage parasitemia provides many opportunities for antigen presenting cells (APCs), such as macrophages, to interact with *Plasmodium* parasites (4, 30). The direct binding of immunoglobulin domains to target cells and the parasite itself are important to initiate immunological defenses against pathogens including antigen presentation, phagocytosis, cytotoxicity, induction of inflammatory processes and modulation of immune responses (77). With emphasis on phagocytosis which results in the internalization of the parasite, and the processing and presentation to adaptive immune cells (23, 25). In contrast to pup immune cells

which were mostly comprised of NK cells, adult splenocytes were enriched for macrophages (**Fig. 11**).

Along with NK cells, macrophages are also known to destroy antibody-coated targets via antibody-dependent cellular cytotoxicity (ADCC). ADCC is a cell-mediated immune defense mechanism in which an immune cell actively lyses a target, whose membrane-surface antigens have been bound by specific antibodies. Once contact is made there is the release of cytotoxic factors (perforin and granzyme) that cause the death of the target cell. In malaria infection, the target of ADCC is the surface of iRBCs as well as whole parasite (21, 30). However, the precise mechanisms by which they carry out this function remains elusive (21). Cytolytic products, perforin and granzymes, are critical effectors of the cytolytic immune responses. Particularly, granzyme B expression has been shown to be enhanced by agonists of toll-like receptor 8 (TLR8), which is documented to directly interact with RNA from *P. falciparum* infected iRBCs (78, 79). In our research we observed that macrophages from adult cell recipients produced more perforin and granzyme B, than pup splenocyte recipients (**Fig. 12**). Therefore, it can be concluded that despite similar numbers of macrophages in pup and adult splenocyte recipients, pup macrophages may be slightly defective in their cytolytic ability.

Our research predominately focused on the innate immune response in malaria infection, but it is important to note that for sterile immunity to be achieved, it will be dependent on the adaptive arm of the immune response. And although immune mechanisms of protection against malaria have been extensively studied in both rodent models and humans, they are still not completely defined. Protective humoral response against *Plasmodium* infection can be acquired after repeated infections of malaria (3, 59). However, it does not persist over long periods of time, is generally incomplete, and requires both exposure to multiple antigenic variants of

malaria parasites, and the maturation of the immune system over time (2, 13). And like many other infectious diseases, malaria is an inflammatory response-driven disease, and positive outcomes to infection depend on finely tuned regulation of the immune responses that leads to the effective clearance and control of the parasite over time.

All these data suggest that pup splenocytes are enriched in γδ T cells and NK cells. However, adult splenocytes were not only enriched in $CD4_+$ T cell but also expressed higher proportions of cytotoxic macrophages in the adult spleen cell recipients. Taken together, our findings suggest that pup cells are enriched with innate lymphocytes that may promote protection against *P. chabaudi* infection, through reducing parasite burden as well as weight loss. This information could be used towards further understanding the childhood innate immune response to malaria. Such information would be essential for vaccine design that can help reduce mortality rates caused by malaria infection in the most susceptible group.

Chapter 5

CONCLUSIONS

Today, children under 5 years remain the most susceptible group that succumb to malaria infections and with the threat of antimalarial resistance, there is an increasing need for new methods of counteracting malarial progression. Our data suggests that in a mouse model, pup splenocytes are enriched in γδ T cells and NK cells. We propose that innate lymphocyte populations found in pup splenocytes can transfer protection to immunocompromised RAG1KO mice, through the observed reduction of parasitemia as well as weight loss in malaria infection. In contrast we observed that RAG1KO mice that received adult splenocytes had higher numbers of macrophages as well as $CD4^+$ T cells, which were also protective in RAG1KO recipient mice. Overall, we propose that despite the differences in the populations of immune cells present in pup and adult splenocytes, they are both protective in malaria infection. Further research will need to be conducted in order to investigate the functionality of γδ T cells in malaria infection.

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Vita

Lyndsay Richard was born in small-town Gastonia, North Carolina in 1994. From a very early age she was fascinated by the world around her and always knew that she wanted to pursue a career in science. She attended Appalachian State University where she received a bachelor's degree in cell and molecular biology, with a minor in chemistry. This passion for science led her to join the Shaw Research Group (SRG) and the Opata lab, where she was able become a seasoned researcher in both chemistry and immunology. Her next research endeavor will take place at East Carolina University where she will take on her role as a research specialist. Based on her years of research experience and love for her work in science, she plans on pursing a PhD in biomedical science with a concentration in immunology.

Due to her diligent work and dedication to research, she was able not only discover a new methodology for synthesizing peptides, but she is a co-inventor for a provisional patent and multiple forthcoming utility patents for her work. She was also able to present and engage at a variety of conferences including the American Chemical Society (ACS) 2018 meeting, North Carolina branch of American Society for Microbiology (NC-ASM) 2018 & 2019, and Immunology 2018 & 2019 hosted by the American Association of Immunologists (AAI). She has also received awards which include the Student Faculty and Excellence (SAFE) Fund award and the Office of Student Research travel and research awards. Her work and research culminated in the pending manuscript titled "Organic Synthesis in Nano-Reactors: Development and Application" to the *American Chemical Societ*y journal.

Outside of academics she can be found in the blue-ridge mountains with her camera or with her cat, Pascal.