A Proposed Mouse Model for Childhood Malaria and Immunity

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A thesis

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DEDICATION

To the people that have influenced me throughout my life.

Acknowledgements

I would like to thank my mentor Dr. Opata for your amazing support and patience with me. Thank you for allowing me to be your first undergraduate in the lab and encouraging me no matter what. I would also like to thank Dr. Dempsey and Dr. Bouldin for their help with editing and preparation of my thesis. Thank you to Coach Pinnix for being my mentor and talking me off the ledge when I am overwhelmed. Thank you to Mrs. Lobree, who assigned me the project back in 3rd grade that introduced me to Epidemiology. I am forever grateful to you for helping me find my passion early on in life. No words can describe my gratitude to my family and how much I appreciate your guidance and support. Thank you especially to my father for all the papers you have read and will read in the future.

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Abstract

CD4+ T cells are imperative in the immune response to infections, but the immune response to malaria, especially in young children, is poorly understood, due to a lack of a young rodent animal model to study the pathogenesis of the disease. In this thesis, we focused on developing, a young mouse (pup) malaria model to help understand the immune response and development of protective CD4+ T cells. Using day 14-17 old pups, we are able to replicate childhood malaria in mice. We demonstrate that pups suffer severe malaria that results in decreased scores on behavioral and neurological tests. Malaria also induced stunted growth in the infected pups and a 60% mortality. There was no significance difference in cytokine production in memory cells between pups that were re-infected and adult mice. Upon transfer of immune splenocytes into immunocompromised RAGKO mice, mice that received pup cells looked healthier and active throughout the experiment, compared to their adult cell recipient counterparts. Mice that received adult cells also lost more weight than the pup cell recipients. There was no difference in both central and effector memory CD4+ T cell subsets at day 60 post-infection in the RAGKO mice, but the adult cell recipients had significantly more IFN-γ and TNFα. These data suggest that pup cells protect immunocompromised mice from death, but do not develop well into functional memory in chronic infection. Finally, we demonstrate that pup splenocytes proliferate faster than adult cells, when stimulated *in vitro* by plate bound anti-CD3/CD28, as measure by CFSE dilution. But purified CD4+ T cells from pups spleens proliferate slower compared to CD4+ T cells from the adult mice. This suggests that there is yet to be described immune population in the pup splenocytes that is protective and proliferate better than the CD4+ T cells. Therefore, future studies will determine other immune cells other than CD4+ T cells that could be protective in young mice. These studies will open avenues for vaccine design for this deadly malaria disease and other chronic infections.

Keywords

Plasmodium chabudi; Malaria; Immunology; Pups; CD4$^+$T cells; IFN-gamma; TNF-a; Baby Mice.

Terms Defined

CD11b+: Surface marker for macrophage cells that phagocytose parasites

CD4+ T cells: Helper T cells that regulate immune response

iRBCs: infect red blood cells

RAGKO: Recombinant activation gene knockout mice

Sporozoite: a motile stage of the parasite *Plasmodium falciparum*

IFN-γ (interferon gamma): Inflammatory cytokine important activator of macrophages

CD8+ T cells: Cytotoxic T cell subset that kills cells are that infected

Interleukin 12 (IL-12): Important cytokine for T helper 1 differentiation

Tumor necrosis factor (TNF): Inflammatory cytokine produced by T helper 1 cells

Interleukin 10 (IL-10): Anti-inflammatory cytokine protective in malaria

Interleukin 6 (IL-6): Acts as both a pro-inflammatory cytokine

Gamma Delta T Cells (γδ T cells): A type of T cell with a distinctive T-cell receptor found mainly in younger mammals compared to older ones.

Introduction

According to the World Health Organization, malaria accounted for 445,000 deaths in 2016, of which 70% occurred in children under the age of 5 [1]. Although there are four strains of malaria that affect humans, *Plasmodium falciparum* is the deadliest strain, accounting for 90% of the world's malaria cases and 91% of malaria deaths [1, 2]. Children who have contracted malaria show symptoms of high fever accompanied by chills and headaches [3]. Many children in Africa also suffer stunted growth due to malnutrition and malaria [4, 5, 6]. Children suffer from severe malaria the first time they contract the disease compared to adults that are asymptotic due to multiple infections throughout their life [2]. Other symptoms presented are diarrhea, weakness, abdominal pain, myalgia, and pallor, which can frequently be misdiagnosed as a viral syndrome or acute gastroenteritis [3]. Severe disease in children also leads to behavioral and neurological changes during malaria infection [7]. Some of the neurological effects are coma, altered state of consciousness and severe metabolic derangement [7]. For children who receive adequate treatment, the fatality rate is lower than those children who live in rural areas where access to hospitals are limited. Although there have been improvements in the survival rate for children between 2010 to 2016, malaria continues to claim the life of one child every 2 minutes [1].

Currently the focus for eradicating malaria is to decrease the amount of cases malaria first before eradication efforts begin. The major control efforts currently in place are long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and treatment with artemisinin-based combination therapy (ACT) [8]. WHO recommends that for prevention of malaria, children of 5 years and lower should use LLINs [9]. If a child has contracted malaria the only pediatric recommended medicine by WHO is ACT [9]. Long lasting insecticidal nets were first used in rural Kenya starting in the 1980s, but since the mid-2000s there has been a resurgence of malaria cases among communities with high ownership of LLINs [10]. This could be due to the emergence of resistance to the insecticide and improper use of ITNs. In South-East Asia there

are now accounts for malaria being resistant to ACT [11]. Although ACT shows effectiveness with *Plasmodium falciparum*, there is concern that there may be development of resistance to this drug as reported in South-East Asia.

Geography has played a crucial role in helping eradicate malaria by mapping mosquito breeding sites near human habitats. By knowing where a large mosquito breeding site is located, governments can determine where to distribute drugs and insecticide-treated bed nets [12]. Model-based geostatistical methods and Geographic Information System (GIS) are major techniques used to map malaria. Geographers are trying to predict how malaria will spread with climate change by looking at variables such as temperature and rainfall. The current findings predict that with warmer temperatures and more rainfall, a larger proportion of the world's population will be at risk of malaria [12]. With the rising temperatures throughout the world, geographers have started to focus on mapping wetlands, as they can facilitate malaria transmission by safeguarding mosquitos during dry seasons [13]. With the rising temperatures throughout the world, geographers are focusing on mapping wetlands, as they can assist malaria transmission by allowing the mosquito to lay larvae in water during dry seasons [14]. By mapping locations of wetlands and their proximity to human habitats this can help identify places that require a larger amount of eradication efforts due to the wetlands containing a large amount of *Anopheles*.

The life cycle of malaria begins when the female *Anopheles* mosquito injects *Plasmodium* sporozoites into the skin of a human. The sporozoites then migrate to the liver, where they grow in the hepatocytes, rupture the hepatocytes and enter the bloodstream, causing anemia and eventual death if the patient is not treated. Using rodent models of malaria, scientists have shown that within the first 15 minutes, some *Plasmodium* sporozoites begin the migration from skin to the liver. This process can last up to a few hours [15]. If sporozoites do not transverse through the dermis to the liver quickly, they are trapped in the dermis and removed by CD11b+ phagocytic macrophage cells [16].

Upon reaching the liver, the parasite can be eliminated by CD8+ cytotoxic T cells which kill the infected hepatocytes [15, 17, 18]. If the sporozoites survive and make it to the blood, then CD4+ T cells play a major role in producing cytokines such as IFN-γ and IL-12, that facilitate differentiation of CD4+ T helper cells to help in eliminating the parasite by producing more proinflammatory cytokine including interleukin (IL)-12, tumor necrosis factor alpha (TNFα) and interferon gamma (IFN-γ) [17, 19-24]. This helps to limit the progression of malaria from becoming severe. However, if the immune system produces too much TNFα, this could result in increased pathology. One study reported lower amounts of IL-12 and IFN-γ in African children suffering from severe malaria, compared to those with mild malaria [25]. A child suffering from severe malaria or anemia had a higher amount of TNFα and IL-10 detected in their blood [25]. In another study, a child that succumbed to the disease was found to have a higher amount of IL-6, IL-10, and TNFα in their blood compared to those who survived [26]. This suggests that malaria infection induces production of a variety of cytokines.

Most human hosts who are infected with malaria survive the acute infection. However, they are considered to be chronically infected afterwards due to the inability of the host body to produce a sizable immune population without repeated infections [27]. *Plasmodium falciparum* is able to elude the immune system by changing the expression of certain surface proteins [28]. The parasite has around 60 var genes, which allows it to maintain the chronic stage of the infection [29]. If the parasite only expressed one to five var genes, then the immune system would be able to eliminate the it [29]. However, as the immune system does this, it weakens its response against erythrocyte membrane protein (pfEMP1) proteins, which are found on the infected red blood cells [29]. This allows the parasite to live longer causing chronic stage malaria in the host. To evade the immune system is the infection erythrocytes can also cytoadhere in human endothelium, this is why pfEMP1 has been identified as key towards humoral immunity [30, 31].

The proposed vaccine for malaria is the RTS,S/AS01E, which is in the fourth stage of clinical trial. This vaccine candidate targets the pre-erythrocytic cycle of *Plasmodium falciparum* in humans because this stage allows the parasite to avoid the immune system [32]. RTS,S/AS01E halts the life-cycle of the parasite in the pre-erythrocytic phase, and therefore blood stage malaria does not occur. A recent trial of RTS,S/AS01E in infants showed the vaccine to be 65% effective [33]. However, young children between the ages of 3 to 5 years old, who engage in more outside play time, are at greatest risk of contracting malaria, but the vaccine has yet to be tested on children at this age. One paper contradicted most studies and argued that the effect of the vaccine was not as apparent or significant when adjusting for previous episodes of malaria, such as the child acquiring natural immunity from contracting the disease [30].

Most of what is known about malaria is based on an adult mouse model, there has yet to be a mouse model that mimics malaria in children [2]. Despite these elegant studies, malaria in children is understudied, and there is no established model for childhood malaria in mice. Lack of such a model limits progression of studies to understand the pathogenesis of malaria in the most susceptible population Thus, it is important to identify a pup model that reflects malaria in children. The studies in this thesis aimed to provide novel information and establish a timeline for childhood malaria using a C56Bl/6 mouse model.

MATERIALS AND METHODS

Parasite

Our experiment used the rodent strain of *Plasmodium chabaudi chabaudi* (AS). This parasite induces chronic malaria infections and imitates symptoms of human malaria induced by *Plasmodium falciparum*, such as anemia, weight loss and chronic infection. The parasite was provided to us by Dr. Robin Stephens (University of Texas Medical Branch), with permission from her postdoctoral mentor Dr. Jean Langhorne (Francis Crick Institute, UK).

Animals

Types of animals used and conditions

A breeding colony of wildtype (C57BI/6) mice were purchased from Charles River Laboratories (Wilmington, MA), and maintained in our animal facility at Appalachian State University, to produce young mice (pups) for the experiments. RAGKO ($Rag1^{tm1Mom}$) mice were purchased from Jackson Laboratories (City, state), and a breeding colony was also maintained in the animal facility with filtered tops to avoid exposure to the environment as they are immunocompromised. RAGKO mice were used to determine if pup or adult splenocytes could protect RAGKO from death. Wildtype pups were used for experiment between days 14 - 17 of age, while wildtype adults and RAGKOs were at 8 to 12 weeks when infected with malaria for all experiments. The housing conditions for animals were maintained at a constant room temperature with 12 hour light and dark cycles. The animals were allowed free access to water and food at all times. The experiments performed on the mice are described below and mice were euthanized with isoflurane or pentobarbital. All experiments were carried out in accordance with the protocols approved by The Institutional Animal Care and Use Committee and The Appalachian State University Institutional Biosafety Committees.

Techniques Used

Flow cytometry surface staining

Spleens were collected from mice and placed in labeled tubes filled with Iscove's Modified Dulbecco's Medium (IMDM). The spleens were mashed through a 70-micron mesh screen and then gathered back into the respective tubes. The tubes were spun at 1200 rpm for 8 minutes, decanted and vortexed. Cells were then incubated for 2 minutes with red blood cell (RBC) lysis buffer to remove the RBCs. Lysis of RBC was stopped by addition of phosphate-buffered saline (PBS) and centrifugation to pellet the remaining white blood cells. Cells were

resuspended in Iscove's media and an aliquot was taken for counting at 1 to 100 dilutions in PBS and trypan blue to determine viable cells. After counting, 3 x10^6 cells were aliquoted into 5 mL polystyrene tubes. One mL of florescent activated cell sorting (FACS) buffer was added to each tube and spun at 1200 rpm for 5 minutes. Cells were stained with 50 μL of antibody cocktail containing CD4- fluorescein isothiocyanate (FITC), CD44- phycoerythrin (PE) and CD62L phycoerythrin-Cy7 (PE-Cy7) (Tonbo Biosciences, San Diego, CA), surface T helper markers to determine CD4 T cell activation status. The tubes were incubated on ice for 40 minutes, then washed with FACS buffer. The cells were then re-suspended in 300 μL of FACS, and filtered using cell strainers, and collected on a FC500 flow cytometer (Beckman Coulter Inc, Indianapolis, IN). Here we are measuring the amount of effector T cells, and memory-T cells.

Flow cytometry intracellular cytokine staining

An aliquot of 3 x 10^6 cells from each initial spleen tube was placed in a 24-well plate and incubated for 5 hours. Two additional wells were used as controls with one being an isotype control with stimulation and the other being a negative control without stimulation. After the 5 hours incubation, the cells were harvested into 5 mL polystyrene tubes and washed with 1 mL of FACS buffer. The cells were stained with a cocktail of 50 μL containing CD4- FITC, and incubated on ice for 40 minutes. After incubation, cells were washed in FACS buffer, and fixed by incubating in 2% paraformaldehyde (PFA) for 30 minutes. Cells were spun down and permeablized by addition of 1 mL of Perm-Wash buffer (Tonbo Biosciences) and 30 minutes incubation on ice. Cells were spun down and incubated for 20 minutes in Fc-block, before addition of a cocktail containing IFN-γ-PE and TNFα-PE-Cy7 (BioLegend, San Diego, CA,) antibodies, followed by a further 40 minutes incubation. Cells were then washed three times with Perm-Wash and resuspended in 300μl then collected on a FC500 Beckman Coulter flow

cytometer. The samples were analyzed by FlowJo to determine proportions of IFN-γ and TNFα in the splenocytes.

SHIRPA

SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment (SHIRPA) was conducted to characterize the behavioral and neurological changes in pups during three stages with a series of individual tests that provide quantitative data about the pup's individual performance. SHIRPA has previously been used to predict the severity of malaria disease in mice and the behavioral and neurological that are seen children [34]. Scores were determined based on descriptions in protocol for each task that was provided by the creator of SHIRPA. Two students were trained by the professor before the experiment began and were allowed to conduct the experiment by themselves once they received approval from the professor. Looking at a wide range of tests such as motor strength and neural control define the presence of specific deficits. When a pup showed a poor performance in locomotor test this reflected a muscular weakness due to a dysfunction in the central nervous system. The tests were run in a simple manner that allowed the mouse to rest for 10 minutes before the next task began. Transfer arousal is when the mouse is transferred quickly into a new environment with little to no human contact in order to observe the animal's immediate reaction. A score of 5 showed that the mouse did not freeze when transferred to the new environment while a lower score indicates a longer pause before moving. As the mouse explores its' new environment, the tail elevation is observed during the animal's forward motion with a 2 showing the tail is elevated, a 1 indicating it is horizontally extended, and a 0 represents the tail is being dragged. The mouse was placed into a beaker for 5 minutes before body position was recorded. A score of 4 indicated the mouse was rearing on the hind legs, a 3 represent the mouse sitting, a 2 was lying prone and when the mouse was lying on its' side it received a score of 1. While the mouse is being observed in a viewing jar the spontaneous activity of the mouse was observed with a

score of 3 showing that the mouse had a rapid and darting movement, a score of 2 showed vigorous grooming and moderate movement, 1 represented casual grooming or slow movement. When a mouse was resting or showed no movement it was given a score of 0. As the mouse was exploring the new environment the pelvic elevation was observed. If the pelvic region was more than 3mm in elevation the score of 3 was assigned, 2 represented a normal elevation of 3mm, 1 showed that the pelvic elevation was barely touching the floor and 0 was the pelvic flattened on the ground. For locomotor activity, a mouse was placed in an arena with equal lengths squares and a top was placed so the mouse would not run away. The number of times all four paws entered a square in 30 minutes was counted. Tremor was determined while the mouse was in the viewing jar. A score of 2 shows no tremor, a 1 represents that there was a mild tremor and a 0 show that an important tremor was observed. For negative geotaxis, the mouse was placed on a horizontal cage top and when the mouse moved in one direction the top was raised vertically so that the animal was facing the floor. A stopwatch was set for 30 seconds and the mouse was observed. A score of 4 showed that the mouse turned around and climbed up the grid, a 3 represents that although the mouse turned around it froze, when a mouse moved but did not turn around it was given a score of 2, 1 was given for the mouse that did not move for 30 seconds and 0 represents when the mouse fell off the grid. After a mouse had explored its' new environment the mouse was stroked by a finger. When stroked if the mouse vigorously escapes the finger stroke it was given a score of 3, if the mouse had a rapid response to a light stroke it was given a score of 2, a score of 1 indicated that a firm stroke was required to get an escape response. For visual placing, the mouse was held by its' tail and lowered to the cage top, and the extension of the forelimbs by the animal from a height of 15cm was observed. The score of 4 shows that the mouse had early vigorous extension around 25mm above the cage top, 3 represents an extension of forelimbs before vibrasse contact around 18mm, 2 shows the extension of forelimbs occurred upon vibrasse contact, 1 represents it occurred upon nose contact and 0 shows that there was no response. For SHIRPA tests, The

error bars represent the standard errors of the mean (SEM). The ¼ denotes that one of the 4 mice that died during the experiment died that day. ¾ denotes that on that day 3 of the 4 mice that died during the experiment died that day. A cross denotes that all the mice that died during the experiment had died.

In vitro culture

Splenocytes and CD4 T cells were cultured in complete culture media comprising of IMDM, 10% FCS, 2mM L-glutamine, 5mM Sodium Pyruvate, MEM NEAA, 10mM HEPES, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM of 2 microMercaptoethanol. A 24 well plate was coated with a cocktail of 100 mL of 1 μg/ml of anti-CD3 and anti-CD28 solution and incubated overnight at 4^{o}C in the fridge. On day 2, single cells suspensions were collected from the spleens of naïve day 14 pups or 8 weeks old adult mice using the normal procedure. The CD4+ T cells were purified using the EasySep CD4 purification kit according to the manufacturer's instruction (StemCell Technologies, Vancouver, Canada). Cells were incubated in Rat Serum for 10 minutes followed by addition of CD4 Isolation Cocktails. The tubes were then incubated for 10 minutes and vortexed for 30 seconds afterwards. RapidSpheres were added to the samples and then incubated for 2.5 minutes. The volume in the tubes was adjusted then placed into the magnet for a 5 minute incubation. Both enriched cell suspension and spenocytes were stained with Carboxyfluorescein succinimidyl ester (CFSE) and counted. An aliquot of 1 x 10^6 cells were placed into designated wells for culture. The cell culture plate was placed into a 37^{o}C incubator supplemented with 5% CO_2. Flow cytometry was performed on the cells at days 0, 2 and 4 to examine proliferation status of both CD4 T cells and splenocytes from the two groups.

Statistical analysis

Prism was used to statistically analyze the charts and graphs. A two-tailed t-test was performed for comparisons and signified with a ***** if there was significance. When no significance

was found NS was used to represent this. A ¼ or ¾ signified when deaths occurred of the 4 that occurred during SHIRPA. A cross symbolizes that all mice from the group had past.

Experimental Design

Monitoring progressive growth of healthy pups.

This project first looked at the growth pattern of pups without malaria infection. We weighed the pups starting at day 10 and continued to record the weights until they were 70 days old. This experiment included multiple litters of various sizes to calculate an average growth rate of both male and female pups.

How does malaria infection affect the health and growth kinetics of young mice?

This analysis used pups that were 15 days old and were infected with $1x10^6$ iRBCs, through the peritoneum. This experiment was carried out for 60 days post infection with blood being collected every day from day 8 to day 30 and at day 60. Survival and growth rate of infected pups and control adults were determined by calculating new weight minus the old weight divided by the old weight times 100 to get a percentage. SHIRPA was conducted on these mice to determine neurological and behavioral deficits between the infected pups, adults and the uninfected controls. The SHIRPA technique is described above. Once euthanized, flow cytometry was used to collect data, which was analyzed using the FlowJo software.

Does malaria infection affect functionality of memory cells after pups grow to adulthood?

After the successful infection of pups and replication of stunted growth and mortality seen in humans, some experiments where the pups survived the first infection were re-infected at day 60 post infection to look at memory cells. We re-infect mice to observe the difference in activation of memory cells from pups that were previously infected to mice that have never been infected. The now adults and older adults were infected with $1x10^5$ iRBCs and euthanized 7

days later to look at cytokine production. Data was collected by flow cytometry and analyzed using the FlowJo software.

How does malaria infection affect activation and functionality of effector cells in pups?

To address this question, an experiment was conducted to determine the phenotype and cytokine secretion by CD4+ T cells during the peak of the infection. Day 15 pups and 8-week-old adults were infected and euthanized at day 8 post infection. Cells were collected from the spleens, processed using FACS protocol, and analyzed using flow cytometry and the FlowJo software.

Can splenocytes from pups protect immunocompromised mice?

Day 15 pups and 8-week-old adults were infected with $1x10^5$ iRBCs. At 8-day post infection, the pups and adults were euthanized, and spleens were collected. Single cell suspensions were obtained by mashing the spleens through a mesh screen and plunger, and red blood cells were lysed using the red blood cell lysis buffer (Tonbo Biosciences, CA). The spleen cells, (splenocytes) were transferred into RAGKO mice the same day. The next day, the RAGKO mice were infected with a lethal dose of malaria. The recipient mice were then monitored and weighed for 60 days. Blood smears were collected until day 30 post infection for parasite counting. Percent weight change was calculated to determine the effect of malaria infection on host mice upon receiving pup or adult cells. On day 60 post-infection, the recipient mice were sacrificed, and phenotype and functionality of the donor cells were determined using flow cytometry and FlowJo.

Are pup cells defective in their ability to proliferate?

This *in vitro* experiment was designed to test the difference in proliferation capability of pup and adult CD4+ T cells and all splenocytes after observing that the RAGKO recipients of

pup cells did not lose as much weight as adults' recipients, but they survived through day 60 post-infection. The cells were cultured *in vitro* to determine how well they proliferate at day 2 and day 4. The technique of *in vitro* is described above.

RESULTS

Plasmodium chabuadi infection leads to stunted growth in infected young mice

To understand the effect of malaria on growth of young mice, we first determined the kinetics of growth in uninfected young mice by looking at a litter of pups starting on day 10 post birth. Both male and female pups progressively gained weight up to day 60, when the weights began to level of (Figure 1A). Based on recent studies comparing mice and human ages, it was determined that 2.60 days old pups would represent human ages 1 year. Therefore, we reasoned that 5-7 year old humans would be represented by young mice age range of 14 days to 17 days post birth [35]. We then infected 15 day old pups and 8 weeks old control adult mice to look at their response to malaria. Of the young mice that were infected, there was a dramatic decrease in weight gain compared to the uninfected control littermates. The dramatic decrease in weight gain occurred between days 9 to 14 post-infection (Figure 1B). The drastic difference in weight gain in the young mice reflects stunted growth shown in children that live in malaria-endemic areas [36]. The pups also had a higher parasitemia count than infected adults (Figure 1D). Sixty percent of the infected pups also succumbed to the infection between days 24-26 post-infection (Figure 1C). The young mice that survived the infection had decrease weight gain up to 6 weeks post-infection, but later caught up in growth with their uninfected control littermates. The 8-week old adults lost weight at the peak of infection but regained quickly thereafter after. This data suggest that malaria does delay the growth of infected pups.

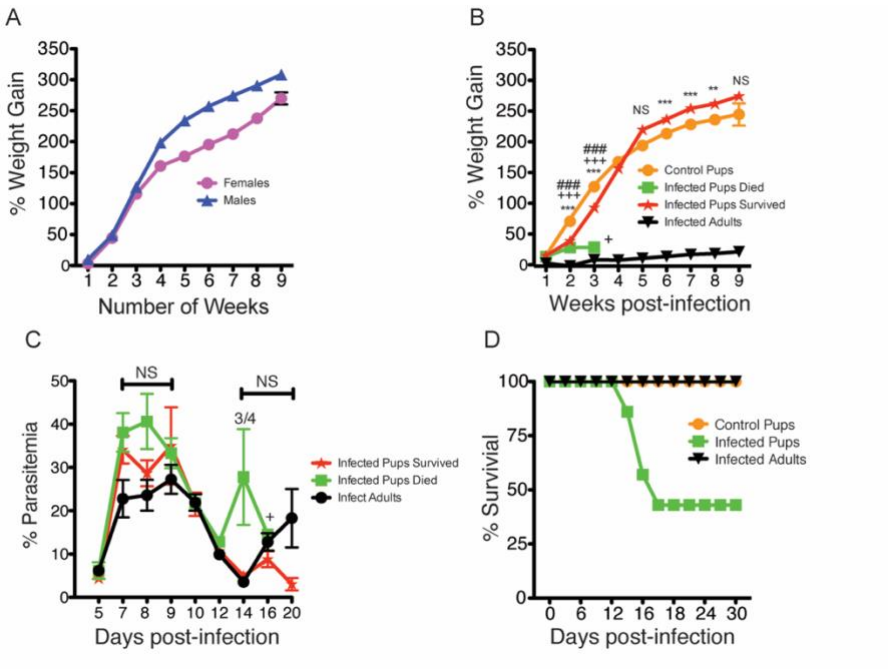

Fig. 1. Malaria infected pups have a slower growth and some die. Day 14 pups or week 8 adult mice were infected with 10^5 *P. chabaudi* and weights were taken over time. **(A)** Weight of control pups from day 10 to day 70 post birth (**B**) Weights over 9 weeks after infection. (**C**) Show parasitemia counts for pups and adults from day 5 to day 20. **(D)** Survival curve of pups and adults after infection with malaria.

Malaria infected pups experience severe disease compared to infected adult mice

To understand how malaria affects the behavior of pups, we conducted SHIRPA on infected young mice and adults. Day 16 old pups and 8-week-old adult mice were infected with $1x10^5$ iRBCs, then a cognitive and behavioral test was conducted each day starting at the peak of infection. We observed a difference in the pups who died from malaria compared to those that survived the infection. Infected pups that died (IFD) had lower scores in tests such as body position, spontaneous activity and tremor compared to infected pups that survived (IFS) and the control pups that were not infected (CP) (Figure 2A, 2B, 2C). Although the IFD began to recover, after day 11 post-infection, they once again began to decline at day 13, while IFS and CP remained constant (Figure 2A, 2B, 2C). Similar observations were seen in other tests such as transfer arousal, tail elevation, touch escape and pelvic elevation (Figure 3A, 3B, 3C, 3E).

Using SHIRPA, we also examined neurological functions in pups after malaria infection. Neurological deficits are seen in human children, as malaria renders them docile with a febrile fever [37]. On days 9 and 10, IFD and IFS were averaging the same on locomotor activity test, as they only moved around in a small number of squares while the CP were active and moved in many squares. At day 11 we saw the difference in IFS and IFD squares counted. After day 10 we observed that the IFS were now moving in the same number of squares as the CP, but the IFD never reached the same number of squares as the CP (Fig 3D). This is an important indicator because it shows that both IFS and IFD suffered severe disease, but IFS were able to recover to the level of the uninfected control healthy pups. This shows that the IFD suffered neurological deficits that hindered their ability to recover from the disease. These results are similar to what was found in the grip strength and visual placing test which determined the ability of the mouse to visualize the cage grid wire at 25mm, 18mm and 10mm away from the cage top (Figure 4A and 4B).

We next determined the ability of mice to recognize their body positioning through the negative geotaxis and righting reflex tests. IFD is the only category that showed deficit in these tests. On both tests the IFD showed a decrease in performance compared to others on day 12. The IFD were able to recover to their IFS and CP at day 13 but afterwards decreased in their ability to perform this function again (Figure 5A and 5B). The IFD entered into a coma like stage the day that they died and were unable to perform either of the tasks by either falling off the grid or failed to right itself when on its back. These data suggest that pups that were infected with malaria suffered severe neurologic damage from the disease.

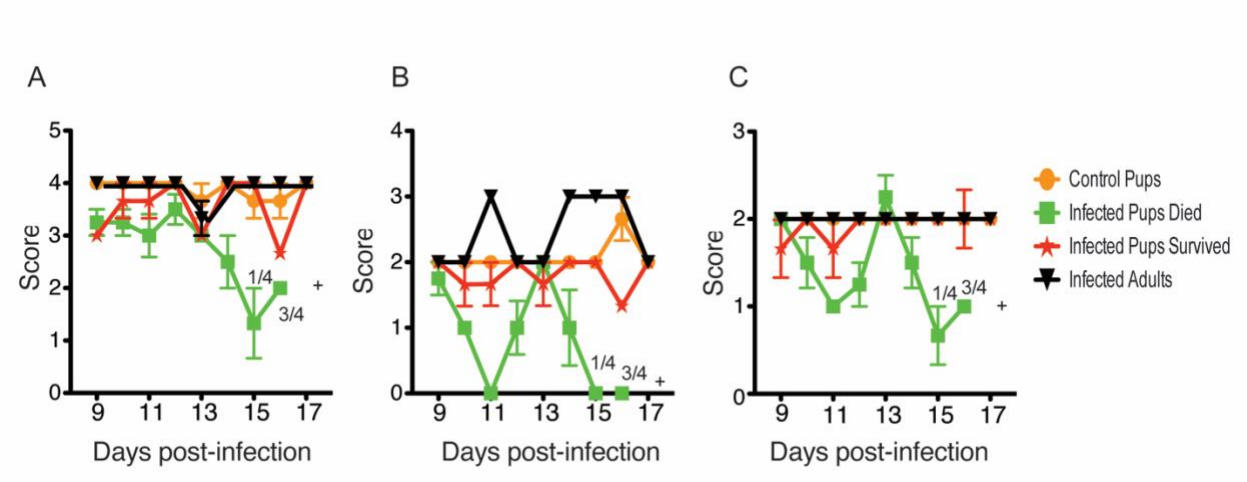

Fig. 2. Malaria infected pups suffer severe disease. Infected mice were placed in on an open platform and observed for (**A**) Body Position, (**B**) Spontaneous activity, and (**C**) Tremor during the peak of infection through recrudescence, as described in the methods and materials section.

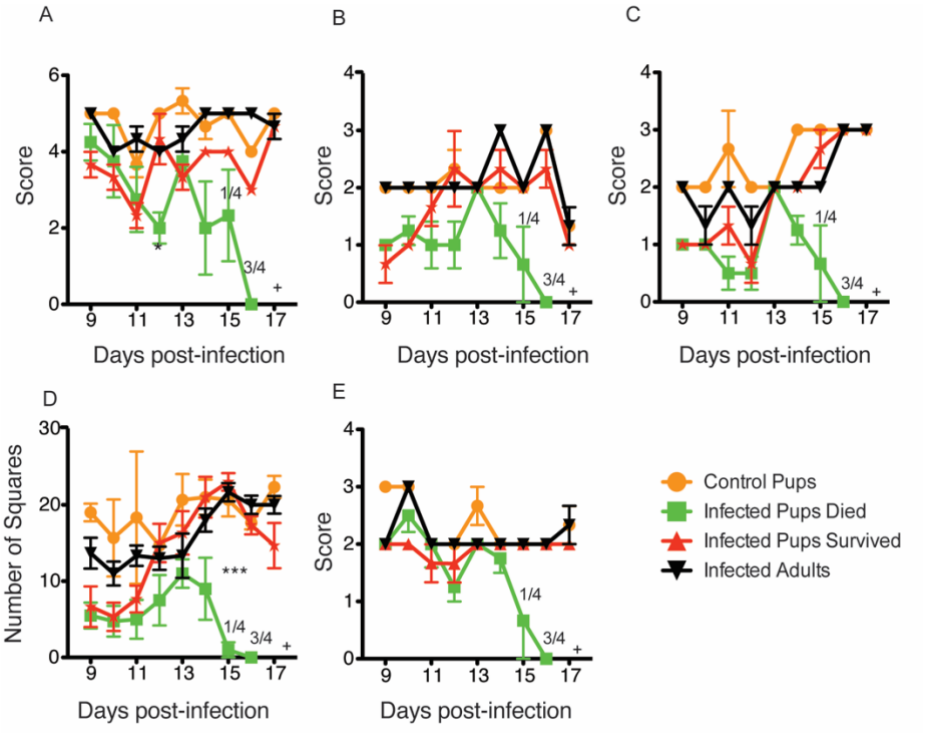

Fig. 3: Malaria infected pups have decreased behavior. Infected mice were placed on an open platform and observed for (**A**) Transfer Arousal (**B**) Tail Elevation **(C)** Touch Escape **(D)** Locomotor activity **(E)** Pelvic elevation during the peak of infection through recrudescence, as described in the methods and materials section.

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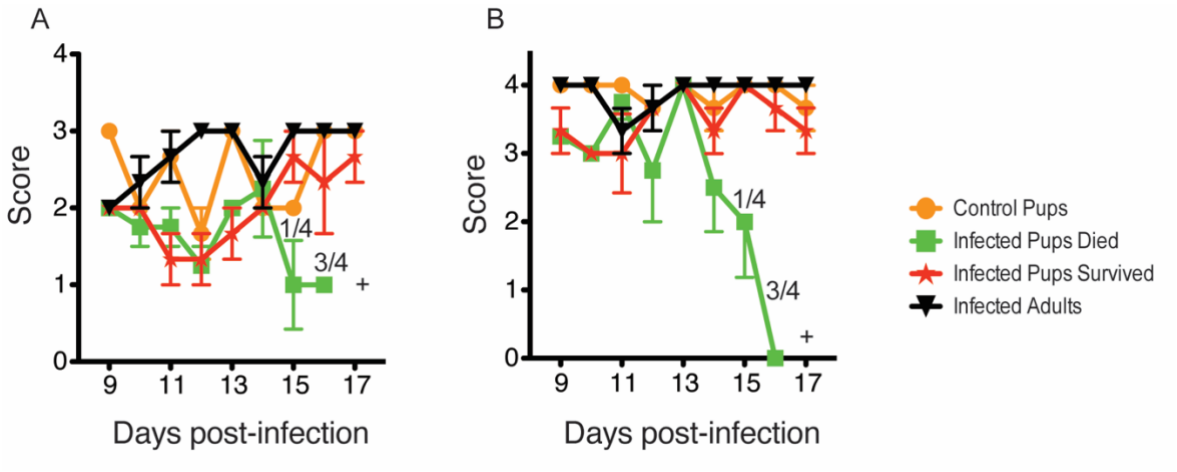

Fig. 4. Infected young mice have impaired grip strength and vision. Mice were tested for their **(A)** grip strength and **(B)** ability to visualize the cage grid wire at 25mm, 18 mm and 10mm between peak infection and recrudescence, as described in the methods and materials section.

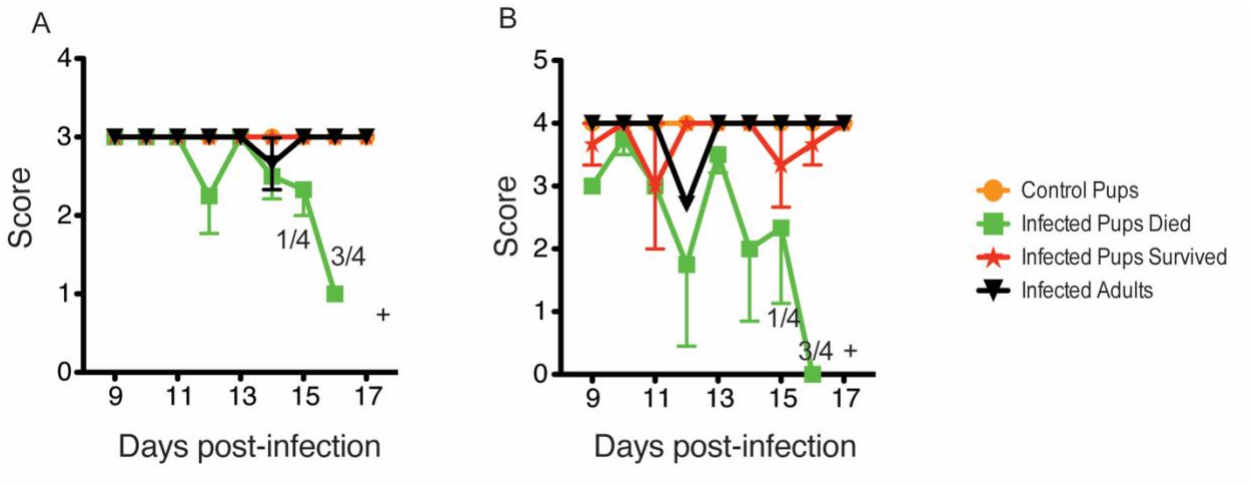

Fig. 5. Young mice that die have defective brain function. Mice were placed on a cage lid and (**A**) Negative Geotaxis was tested. Or mice were placed in a restricted container and turned upside down then (**B**) Righting reflex was determine between the peak of infection until recrudescence as described in the methods and materials section.

Pup immune cells are stimulated in malaria infection

After the establishment of a successful infection in young mice, this model was used to study the immunology aspects of pup cells. Studies have suggested that children have a lower immune function than adults [38]. This may make them more susceptible to malaria than adults. To test the immune response to the infection between the pups and adults, we infected 8-week-old adult and 15-day old young mice with $1x10^5$ iRBCs to look at the activation status and

functionality of effector T cells at day 8 post-infection. When analyzing the splenocytes from pups and adults, there was a higher proportion of IFN-γ production by CD4 T cells in adults compared to pups (Figure 6A, 6B). There was no difference in TNFα secretion. This data suggests that due to the lower amounts of IFN-γ producing CD4 T cells, the pups may not induce a robust immune response to the infection hence succumb to the disease.

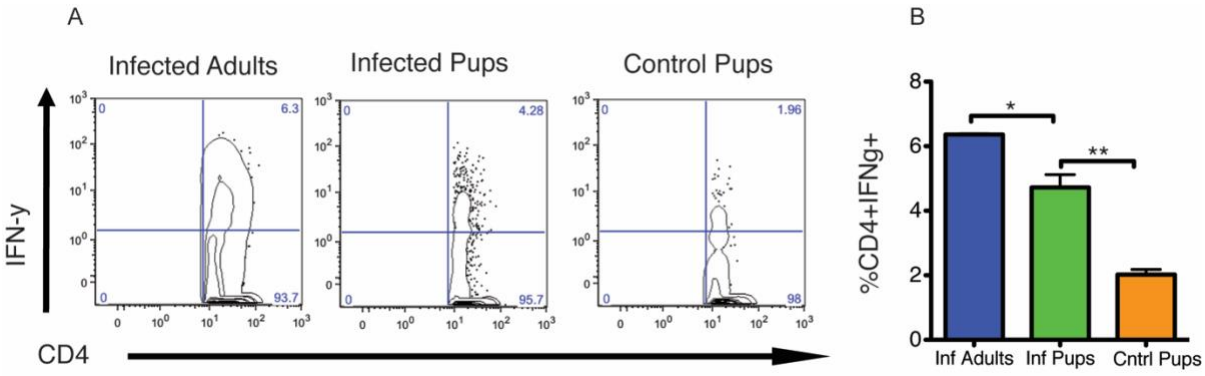

Fig. 6. CD4 T cells from adult mice produce more IFN-γ. Mice were infected and euthanized at day 8 post infection then cytokines were determined by ICS**. (A)** Plots of IFN-γ cytokine producing CD4 T cells in infected adults, Infected pups and uninfected pups. **(B)** Quantification of cytokine production in all the mice.

Mice that were previously infected as pups show no defect in cytokine production

To determine if there was a functional defect in memory CD4+ T cells generated in pups, we infected day 14 pups and 8-week-old adult mice for 60 days for memory cells to develop. We then re-challenged the pups that survived the original infection and adult controls at day 60 with $1x10^5$ iRBCs. We sacrificed the mice 7 days after the second challenge to determine cytokine production by the CD4 memory cells from these mice. We observed no significant difference in the total number of CD4 T cells at this time-point (Figure 7A). Looking at cytokine production, there was no significance difference between the two groups in the production of both IFN-γ and TNFα (Figure 7B and 7C). This data suggests that there is no defect in the ability of pup cells to develop into functional cytokine producing memory T cells.

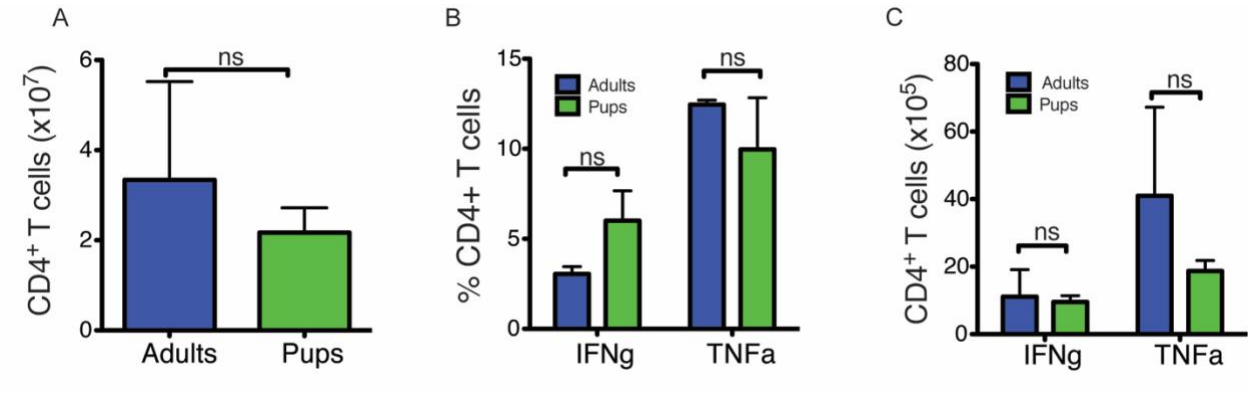

Fig. 7. There is no defect in cytokine production by memory cells in mice infected as pups. Adult and pup mice were infected at week 8 or day 15 of age. Mice were then infected on day 60 and cytokine production was determined 7 days later. (A) Total CD4 T cell numbers. (B) Percent of CD4 T cells producing IFN-g and TNF-a and (C) Number of cytokine producing CD4 T cells after a secondary infection.

Pup cells protect immunocompromised mice from death and weight loss

Since we observed lower cytokine production by the pup cells during the acute phase of infection, but similar cytokine production at the memory phase, we wondered if the effector CD4 T cells from pups would protect immunocompromised RAG knockout (RAGKO) mice, that do not have any adaptive response immune cells. We therefore infected day 15 old pups and 8-week-old adult mice with $1x10^5$ iRBCs. At day 8 post-infection, we harvested the spleens, lysed red blood cells and transferred all the splenocytes into RAGKO mice. The RAGKO recipient mice were then infected the following day with a lethal dose of malaria. Two RAGKO control mice that did not receive any cells were also infected with *Plasmodium chabaudi* (Figure 8A). These control mice died 24 days post-infection. The mice that were given pup splenocytes looked healthier and active throughout the experiment compared to the mice given adult splenocytes. The mice given adult splenocytes lost more weight than the pup cell recipients throughout the experiment, which lasted 60 days post infection (Figure 8B). The adult cell recipients also had a higher amount of parasitemia than pup cell recipients (Figure 8C). At day 60 post-infection, we euthanized the mice to determine the proportions and number of effector and central memory CD4+ T cells. Surprisingly, there were no differences in the effector and

central memory CD4 T cells in pup cells recipients compared to the adult cell recipients (Figure 9A, 9B, 9C).

We therefore reasoned that the adult cells must be producing more inflammatory cytokines, which would explain the drastic weight loss suffered by the adult host mice throughout the infection. We therefore determined cytokine production at d60 post-infection in the two groups. As expected, there was a higher production of both IFN-γ and TNF-α by the adult splenocyte recipients than pup splenocyte recipients (Figure 10A, 10B). By having a higher amount of IFN-γ and TNFα in adult cell recipients this shows that adult cells were more functional than pup cells in this experiment.

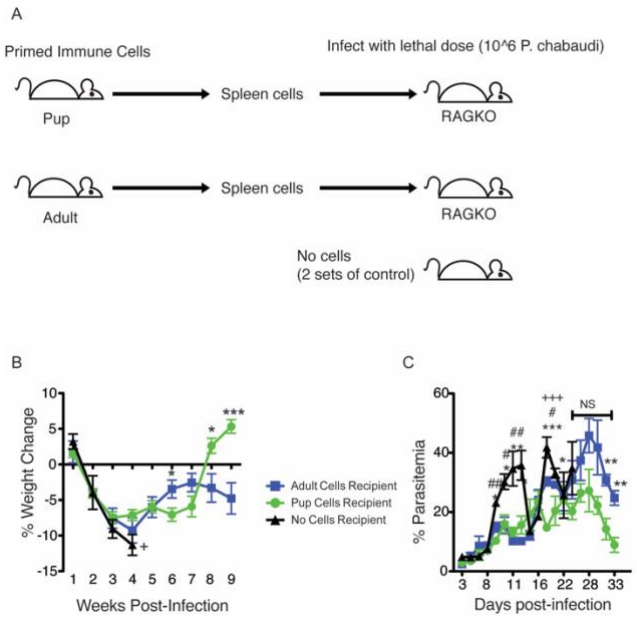

Fig. 8: **RAGKO mice that receive adult cells loss significant weight compared to mice that receive pup cells.** Splenocytes were harvested from adults or pups on day 8 post-infection and transferred into RAGO. The recipient mice were infected, and weights were determined. (A) The experimental scheme (B) Weights over 9 weeks (C) Parasitemia count till day 33.

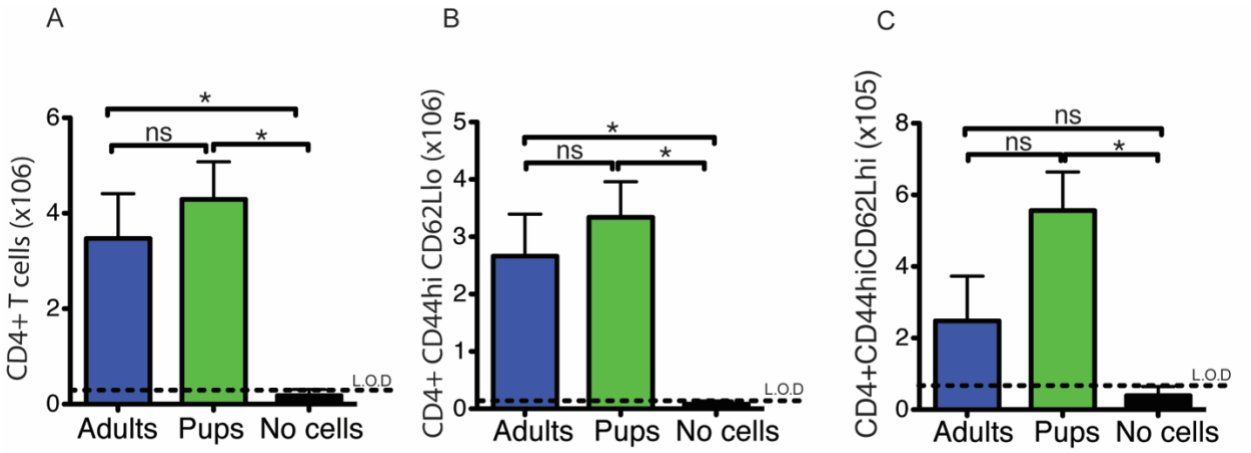

Fig. 9. No difference in the effector and central memory CD T cells in pup cells recovered in RAGKO mice. Activation status of CD4+ T cells recovered on d60 post-infection was determined. (**A**) Total CD4 memory T cells in adult, pup and no cell recipients. (**B**) Numbers of CD4 effector memory T cells and (**C)** central memory T cells in recipient hosts.

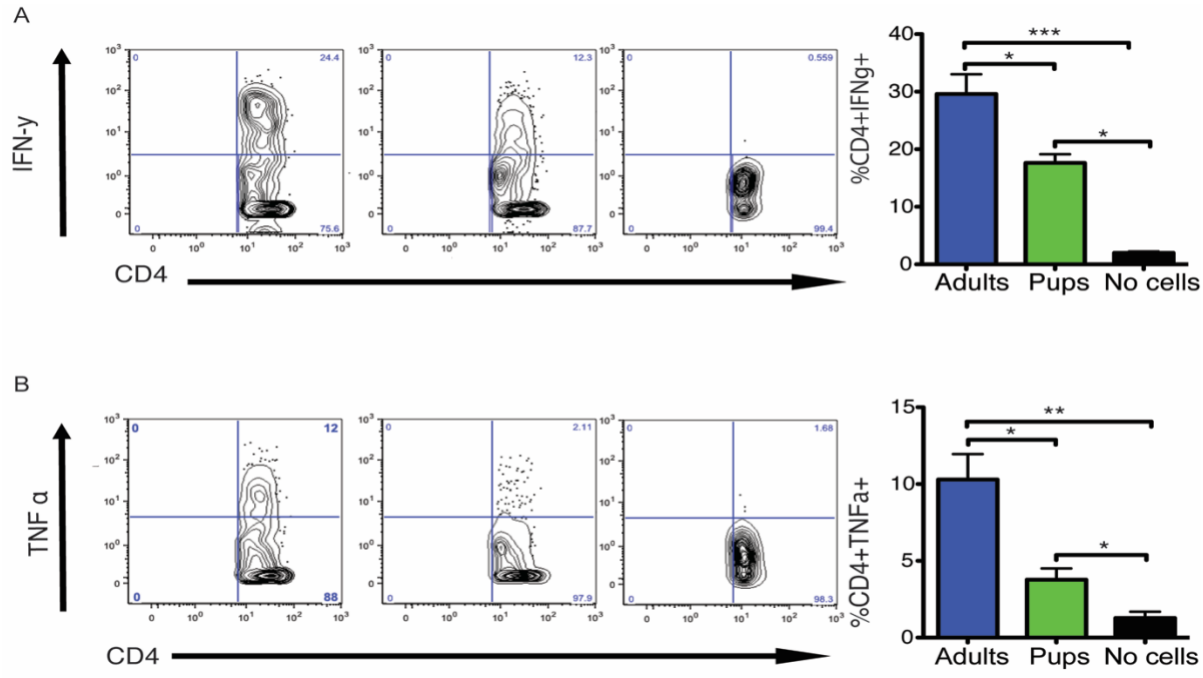

Fig. 10. Adult cells produce more inflammatory cytokines in immunocompromised animals. Spleen samples were harvested on day 60 post infection. **(A).** Plots quantification of IFN-γ. (**B**) Plots and quantification of TNFα producing CD4+ T cells from recipient hosts.

Proliferation

To better understand the functional ability of pup cells, we looked at the proliferation of both adult and pup cells. This experiment looked at the proliferation of immune cells upon stimulation *in vitro*. Cells were harvested from 14 days old pups and 8-week-old adult mice and stimulated in wells bound with anti-CD3 and CD28. CFSE levels were determined to observe cell proliferation. On day 4 post culture, pup splenocytes had more $CFSE^{lo}$ cells, meaning that the cells had proliferated more than adults (Figure 11A). However, the pup CD4+ T cells were slower to proliferate than adult CD4+ T cells at day 4 (Figure 11B). We also noticed that we collected less pup CD4 T cells on day 2 and 4 than we did for adult cells. Taken together, these data suggest that while the immune CD4 T cell population in the pups proliferate slower, there is yet undefined cell population in the pup splenocytes that proliferate faster and could be responsible for malaria protection.

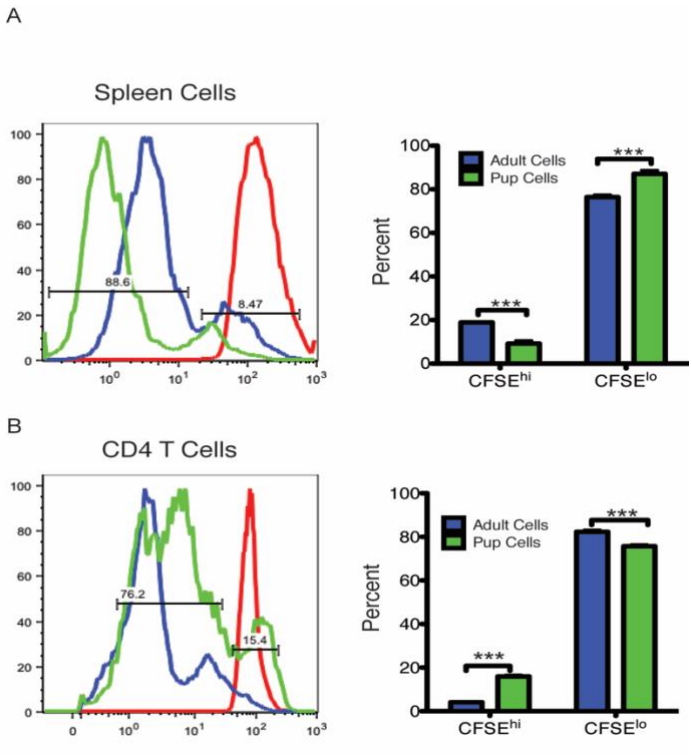

Fig. 11. Pup Splenocytes proliferate more, while CD4 T cells proliferate slower. All spleen cells or purified CD4+ T cells were stimulated *in vitro* using anti-CD3 and CD28. CFSE dilution was measured to determine cell proliferation. Plots and graph showing CFSE dilution **(A)** Spleen cells and **(B)** CD4 T cells.

DISCUSSION

While there has been great advancement in our understanding of immunity to malaria, most of this knowledge has focused on adult mice. With malaria accounting for over 70% deaths in children of five and younger [1], there is an urgent need for a young rodent model to help understand the mechanisms of immune development in this patient population. In this study, we developed a young mouse model to study the pathogenesis of the disease and immunity to malaria in young children. To our knowledge, this is the first rodent model design that has the ability to replicate childhood malaria in young mice. Just like children infected with malaria, we observe stunted growth of mice infected with malaria, and a mortality rate of close to 60%. Therefore, our proposed model is one of the first to match symptoms in childhood malaria disease.

We found that when RAGKO mice were given pup spleen cells, they looked healthier and did not lose as much weight as the RAGKO mice that received adult cells. Interestingly, the adult cell recipients also had higher parasitemia. It has been reported that gamma delta T cells ($\gamma\delta$ T cells), which are different from the conventional $\alpha\beta$ T cells, are protective in malaria infection and are found in high quantities in younger animals than adult mice [39]. To fight off an infection, the $\alpha\beta$ T cells must be primed to recognize antigens; $\gamma\delta$ T cells on the other hand have the ability to recognize patterns of dysregulation and can immediately respond and kill the infected cell [40]. Ohteki *et al.,* found that there was a higher amount of CD3+ $\gamma\delta$ T cells found in mice aged 2- 8 weeks old, but then declined afterward [39]. This could explain why immunocompromised mice that received pup cells performed better.

One characteristic of malaria infection is change in behavior and defects in motor neurons [41]. SHIRPA has been used previously as an indicator to predict the severity of malaria disease, and manifests behavioral changes seen in children with severe malaria

infection [34]. We therefore used this technique in this study to understand the neurological and behavioral aspects of malaria in our new mouse model. A difference in performance was noted between the pups that were infected and died compared to pups that survived. The pups that were infected and survived did decrease in their ability to complete the different tasks, but did recover compared to the pups that were infected and died. The pups that were infected and died reached a comatose status, which was a key sign that they were succumbing to malaria. With the compelling deficiencies in the SHIRPA test in infected baby mice, this suggests that malaria has the potential to affect neurological functions in young mice. Similar observations have been reported in humans, especially children that suffer from cerebral malaria [48]. Based on these observations, we propose that SHIRPA may be used as an indicator that malaria affects neurological functions in mice, and possibly predict those that will die.

One of our interesting observations was that pups are stunted when they are infected with malaria compared to pups that are never infected. Importantly, those that died never attained more than 30% growth rate compared to the uninfected controls or the pups that survived the infection. Pups require large amounts of transforming growth factors-beta (TGF-β) to grow; however, TGF-β is known to be immunosuppressive [42]. TGF-β suppresses the immune system downregulating IL-2 proliferative signals, thus inhibiting normal T cells proliferation [43]. With an unusual amount of TGF-β, this throws off the concentration of helper T cell subsets by favoring regulatory T cells (Tregs) [44]. Indeed, a recent study showed that Tregs are highly generated in young children infected with malaria [44]. This could be one explanation as to why the pups succumb to the disease more often than adults, as the inflammatory cytokines are inhibited by the effect of Tregs.

An intriguing result from our project was how pup splenocytes proliferated more than adult's splenocytes, but the opposite was found for pup CD4 T cells, in comparison to adult cells. In future studies, we plan to explore the different populations of cells in the spleens of

pups that could be expanding faster than adults. It is possible that they could be innate immune cells, or a yet to be discovered innate like lymphocyte cell population.
We will also investigate if this faster proliferation is associated with increased cytokine production in the spleens of the pups splenocytes.

While our young rodent model provides a good basis for studying malaria, we observed a high background of activated cells in our uninfected control mice. This is a caveat that affects the interpretation of our results on CD4+ T cell activation. One possible explanation for these results is that the air in the animal room was not filtered and the cages had no cover tops thus allowing mice to be exposed to open air. We also experience difficulty of consistency in infecting baby mice due to their small size. This resulted in some mice that were supposedly infected not showing signs of infection. Therefore, precision is critical when using this model for immunological studies.

We will also determine differences in the proportions and numbers of γδ T cells in the young mice. We will specifically investigate at what point these cells are most active, and their distribution in different tissues of malaria infected pups. Once we have determined the proportions of γδ T cells, we will next test their ability to protect immunocompromised RAGKO mice. As young mice require TGFβ for growth, we will evaluate if the TFG-β induces anti-inflammatory response thereby inhibiting the development of an effective immune system in pups infected with malaria. Such information will be important for vaccine designs that can overcome this hurdle in growing young mice. We were surprised by the ability of primed pup splenocytes to protect RAGKO. Therefore, we would like to determine the role that naïve splenocytes from pups play in RAGKO protection compared to adult naïve splenocytes.

CONCLUSION

We believe that this pup model is the closest model of childhood malaria for mice in the current scientific community. This model replicates the survival rate of children and also shows the potential loss of neurological and behavioral functions that are found in cerebral malaria. Importantly, there is stunted growth in infected pups, a very common feature for children in malaria endemic areas. With this model that replicates malaria in children, scientists will be able to explore the mechanisms underlying the susceptibility of children to malaria disease. While we use malaria as our infection model, we believe that this model could be applicable to other chronic infections such as Leishmania, LCMV and even tuberculosis.

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