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Plasmodium suppresses T cell responses to heterologous infections by impairing T cell activation

Chelsi Elizabeth White
University of Tennessee - Knoxville, cwhite44@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Chelsi Elizabeth White entitled "Plasmodium suppresses T cell responses to heterologous infections by impairing T cell activation." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Nathan W. Schmidt, Major Professor

We have read this thesis and recommend its acceptance:

Timothy Sparer, Vitaly V. Ganusov

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Plasmodium suppresses T cell responses to heterologous infections by impairing T cell activation

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Chelsi Elizabeth White
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DEDICATION

To my future husband and our two furry kids
Adam Short
Gus
Stella

To my parents and sister
Scotty, Teresa, and Molly White

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ABSTRACT

Malaria is a devastating disease caused by parasites of the genus *Plasmodium*. *Plasmodium falciparum*, which is responsible for most malaria related fatalities, suppresses host immune responses during heterologous coinfections or following vaccination. However the mechanisms responsible for this defect are not well defined. The mechanism and to what extent this immunosuppression is occurring was investigated. This study demonstrates that both dendritic cell and T cell activation are impaired following a *Plasmodium* infection, ultimately altering the adaptive T cell response to secondary infections. T cell suppression is evident early on following a secondary infection and continues throughout the peak of parasitemia. To address the mechanism of *Plasmodium* T cell suppression of heterologous infections, both T cell activation and expression of costimulatory molecules on dendritic cells were analyzed. It was found that costimulatory molecules on dendritic cells were downregulated, as well as the activation markers CD25 and CD69 on CD8 T cells. These data demonstrate that *Plasmodium* impairs T cell activation during heterologous infections. These data provide insight into how *Plasmodium* may be altering immune responses to coinfections in malaria endemic regions and how this could potentially change the administration of vaccines that elicit CD8 T cell responses.

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Introduction

Malaria has been one of the deadliest infectious diseases throughout history and continues to be a threat across the globe today. This disease can be extremely debilitating, with symptoms including fever, chills, anemia, and even coma [1-4]. Throughout most tropical and subtropical regions the parasite responsible for this disease, of the genus *Plasmodium*, continues to infect up to 500 million people every year resulting in approximately one million deaths [2, 4]. The most susceptible populations in these areas are young children under the age of five and pregnant women [4]. Protection against this disease only arises following repeated exposure to *Plasmodium*, and sterilizing immunity is never achieved [5, 6].

There are five species of *Plasmodium* that are capable of infecting humans. *Plasmodium ovale* tends to be asymptomatic and results in a clinically silent infection. Similarly, *Plasmodium malariae* also results in very few symptoms or complications. *Plasmodium vivax* is the most common cause of acute febrile illness and in some cases results in anemia, however this infection is rarely lethal [4]. *Plasmodium knowlesi* is a zoonotic species that is specific to

macaques, but has been reported to infect humans in southeast Asia. The deadliest by far of all *Plasmodium* species is *Plasmodium falciparum* [4]. The blood stage of this disease oftentimes can result in death and has been shown to display an ever increasing resistance to drug treatment [4]. Consequently, *P. falciparum* is the target of extensive vaccine development and novel anti-malaria drugs.

The life cycle of *Plasmodium* species is quite complex and is one of the many tactics that this parasite employs to evade the immune system. Avoiding the immune system and preventing clearance from the host enables the parasite to establish infection and continue the spread of disease. The complex life cycle of *Plasmodium* requires two hosts: a mosquito vector and a vertebrate host (Figure 1). The transmission of *Plasmodium* species begins with development of the parasite in the midgut of a female mosquito of the genus *Anopheles.* When the mosquito takes a blood meal, it injects sporozoites with its saliva into the dermis of the vertebrate host [7]. The sporozoites enter circulation and traverse directly to the liver via an actinomysin motor, initiating the liver stage of disease [1, 8]. During this stage, sporozoites rapidly invade hepatocytes and begin differentiation and asexual replication [1]. The extent of the liver stage can last anywhere from 10-12 days in humans and is clinically silent [1, 4, 9].

As the liver stage progresses, sporozoites develop into merozoites and are released into the blood stream as merosomes, which are blebs of hepatocyte membrane filled with merozoites [1]. Merosomes function as a “Trojan horse” to

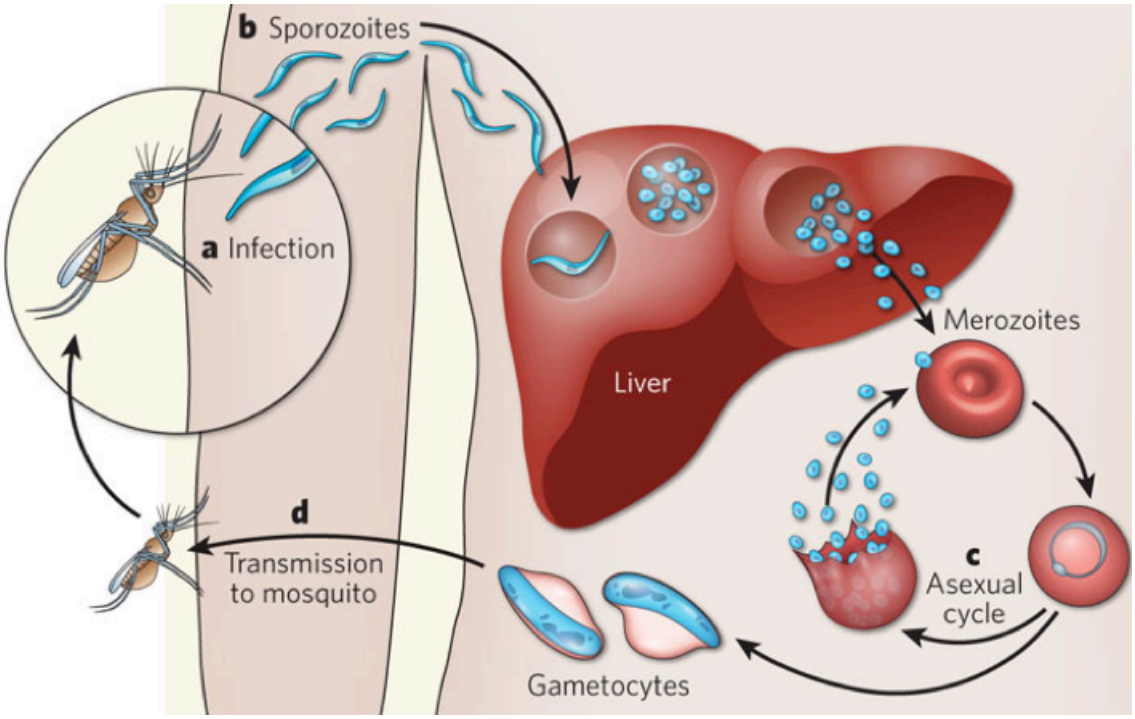


Figure 1. Life cycle of *Plasmodium* parasite. Malaria: Evolution in vector control. Yannis Michalakis & François Renaud. *Nature* **462**, 298-300 (19 November 2009).

carry the merozoites out of the liver where they eventually reach the lung and rupture to release the merozoites [1]. Once in the blood stream, merozoites infect red blood cells (RBCs) which results in the clinical disease, malaria [1, 4].

To infect a RBC, merozoites attach to a RBC and orient themselves into a position to subsequently invade it [10]. Inside the parasitized red blood cell (pRBC), the merozoite undergoes asexual reproduction, eventually rupturing the RBC and releasing dozens more merozoites into the blood stream to infect more RBCs [10]. This cycle of infectivity perpetuates the blood stage and can ultimately lead to anemia and other hallmark signs of malaria [10].

During the maturation of the merozoites, the parasite will begin expressing adherent ligands, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which allows it to bind to multiple ligands on the vasculature, thus preventing passage through the spleen [1, 10]. The spleen is a secondary lymphoid organ that acts as a blood filter and removes pRBCs from the bloodstream [11]. Consequently, expression of these adherent proteins provides the parasite with another means of evading the host's immune system and continuing infection. Sequestration of pRBCs can also contribute to cerebral malaria and complications during pregnancy, which occurs when pRBCs obstruct blood vessels in the brain or placenta, respectively [1]. During the blood stage of disease, merozoites may also differentiate into gametocytes, the sexually reproductive form of the parasite. Gametocytes can be taken up by another mosquito during a blood meal and undergo sexual reproduction. These forms of

the parasite will also differentiate again into sporozoites in the mosquito, allowing their transmission into another vertebrate host (Figure 1) [1].

Although malaria vaccine development has made significant strides toward a successful vaccination regimen, there still exists no licensed vaccine. *Plasmodium falciparum* poses many problems when it comes to vaccine development, including its polymorphic nature and antigenic variation [3, 12-16]. Vaccines have been targeted toward multiple stages throughout the parasite's life cycle including the pre-erythrocytic stage, the erythrocytic stage, and the sexually reproductive stage [17-19]. In the past ten years, over 40 different vaccine candidates targeting these various stages of disease have undergone clinical trials [20]. However there has been limited success with the most advanced candidate being the RTS,S/AS01 (RTS,S) vaccine [21]. This vaccine targets the pre-erythrocytic stage, or the liver stage, of the parasite and is currently in a large Phase III clinical trial in Africa. The RTS,S is comprised of pieces of the circumsporozoite protein fused to the hepatitis B surface antigen (HBsAg) in the adjuvant AS01 [17, 22]. The circumsporozoite protein is the major surface protein found on the sporozoite that binds to liver heparan sulfate proteoglycans [1]. Unfortunately, the RTS,S provided only modest protection against malaria in young infants 6 - 12 weeks of age, which is the intention-to-treat population [21]. The vaccine efficacy was only 30%, and the efficacy waned over time [21]. These discouraging results only further emphasize the need for an efficacious malaria vaccine.

Other methods of malaria elimination and drug treatment are somewhat effective although drug resistance has become increasingly recurrent. There are several drugs available for malaria treatment, with one of the more common being chloroquine. Because chloroquine-resistant strains of *Plasmodium falciparum* have become a widespread problem, the most effective treatment currently is artemisinin-based combination therapy (ACT). This involves a combination of the drug artemisinin, or derivatives of artemisinin, and other antimalarial drugs [23, 24]. By using combinations of antimalarial drugs, the chance of the parasite developing drug resistance is much lower than using a single drug. Vector control has also been implemented in many areas to control transmission and to limit exposure to mosquitoes. Examples include using insecticide-treated bed nets as well as residual indoor insecticide spraying [23]. Unfortunately, insecticide resistance is on the rise [23].

Although malaria is not a pandemic problem, it still occurs in practically all tropical and subtropical regions. The Global Malaria Eradication Programme (GMEP) was implemented in 1955 to prevent malaria transmission and ultimately eradicate the disease [23, 25]. Seventy-nine countries eliminated malaria between 1945 and 2010 [25]. However there has recently been a resurgence of malaria transmission events, with 61 countries identifying new resurgent events. These countries participated in the GMEP, but failed to reach complete elimination [25]. Multiple programs such as this one have been put into action, yet malaria transmission is still rampant across the globe and presents a major

threat to large populations, especially in sub-Saharan Africa. These data only further support the need for malaria elimination and control.

In malaria endemic regions, there are multitudes of coinfections that can occur with *Plasmodium* [26]. Malaria itself can be an extremely morbid and devastating disease, however when infection occurs along with a secondary infection, complications ensue within the host. Multiple studies have indicated that when a *Plasmodium* infection occurs with a secondary infection, suppression of the immune response to the secondary infection occurs [27-34]. It is during the blood stage of infection when parasitemia is high that this immunosuppressive activity occurs [26, 27, 29, 31-33].

This conundrum has been implicated with multiple types of infections, including both bacterial and viral infections [34]. *Plasmodium* has been shown to significantly increase susceptibility and mortality to multiple bacterial infections including non-typhoid *Salmonella*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Acinetobacter* species, *E. coli*, and *Pseudomonas aeruginosa* [26, 31, 32]. The mechanisms behind this increase in susceptibility has not been fully evaluated, although it was demonstrated that *Plasmodium* impairs resistance to non-typhoid *Salmonella* by disrupting neutrophil function [35].

Plasmodium species can impair immunity to viral infections as well [30, 32, 36-38]. Human immunodeficiency virus (HIV) is prominent throughout practically all of sub-Saharan Africa. Individuals coinfected with *Plasmodium* and

HIV demonstrate higher viremia [26, 32, 39]. It has also been documented that Epstein-Barr virus (EBV), when combined with a *Plasmodium* infection, can result in endemic Burkitt's lymphoma (eBL) [37]. Children coinfected with malaria and EBV demonstrate higher viral loads and increased susceptibility to eBL [30, 36, 37]. Endemic Burkitt's lymphoma is hypothesized to develop because of the loss of T cell control of these EBV-infected B cells. Without the control of EBV-infected B cells, they are more likely to undergo abnormal proliferation and subsequent transformation [30, 36].

Furthermore, immunity generated by vaccines is impaired by malaria. Multiple vaccines including *Salmonella typhi*, tetanus toxoid, and meningococcal polysaccharide exhibit reduced antibody responses when vaccinated individuals are infected with *Plasmodium* [29, 33]. There is also diminished protection against *Plasmodium* infection following vaccination with the malaria vaccine candidate, RTS,S, in adults in malaria endemic regions compared to malaria naïve adults [21]. Thus malaria is somehow impairing the immunity to vaccines elicited to *Plasmodium* infected individuals, which further emphasizes the effects this parasite may have on heterologous immune responses.

In addition to *Plasmodium*, other pathogens are also capable of suppressing immune responses to coinfections. Of these, the most prominent is HIV. HIV depletes the host of its CD4 T cells and eventually eliminates T cell help [38]. There are several other pathogens that are immunosuppressive including measles virus [40-42], helminth infections [43-47], viral hepatitis [48-

50], and herpes simplex virus (HSV) [51, 52]. Measles virus induces a lymphopenic environment, leading to a reduction in lymphocyte proliferation and ultimately suppressing the immune response to subsequent pathogens [41, 42]. Although HIV and measles virus immunosuppression is better understood mechanistically, the immunosuppressive characteristics of these other pathogens are still unknown.

These data provide insight into a significant problem that has yet to be fully addressed. There are rarely cases where an individual is infected with a sole pathogen, especially in malaria endemic regions where coinfections are quite common. Yet, there has been limited analysis in the ramifications that one infection may impose on another. Malaria immunosuppression could potentially have major implications on vaccines administered in malaria endemic areas. Determining to what extent and by what mechanism this immunosuppression is occurring will provide crucial information that could have a major affect on vaccine delivery.

To examine *Plasmodium*-induced immunosuppression, two rodent-specific species of *Plasmodium* (*Plasmodium chabaudi* and *Plasmodium yoelii*) were employed to demonstrate immunosuppression with various heterologous (i.e., bacterial – *Listeria monocytogenes* and viral – lymphocytic choriomeningitis virus (LCMV)) infections. Immunosuppression was observed following both bacterial and viral coinfections. The mechanism by which this suppression occurs is intrinsic to the activation of dendritic cells (DCs) and the priming and activation of

T cells. Collectively, these data provide detailed information into the immunosuppressive capabilities of *Plasmodium* species and other infectious pathogens, specifically LCMV. By examining immunosuppression in *Plasmodium* specifically, it was determined that *Plasmodium* imparts a deleterious effect on T cell activation resulting in suppression of the immune response to subsequent infectious pathogens.

Section II

Materials and Methods

Mice and Infections

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Mice were housed at the University of Tennessee animal care facility under the appropriate biosafety level. For *Plasmodium* infections, mice were infected with either 10^5 *Plasmodium yoelii* 17XNL pRBCs or 10^6 *Plasmodium chabaudi chabaudi* AS pRBCs. Mice that were infected with *Plasmodium* species were infected at the indicated times with either 5 x 10^6 *actA*-deficient *Listeria monocytogenes*-OVA (Lm-OVA) CFUs or 2 x 10^5 LCMV-armstrong PFUs. Mice that were infected with wild type *Listeria monocytogenes* were infected with 1 x 10^4 Lm 10403s CFUs and infected at the indicated time with 2 x 10^5 LCMV-armstrong PFUs. Mice that were infected with LCMV followed by *L. monocytogenes* were infected with 2 x 10^5 LCMV-armstrong PFUs or 2 x 10^6 LCMV-clone 13 PFUs followed by 5 x 10^6 *actA*-deficient *Listeria monocytogenes*-OVA CFUs at the indicated times. LCMV-armstrong infections were performed intraperitoneally. All other infections were done intravenously. The Institute Animal Care and Use Committee approved all animal experiments.

Quantification of Ag-specific T cells

Spleens and inguinal lymph nodes were disrupted to generate single-cell suspensions in Hyclone RPMI media (Thermo Fisher Scientific Inc, Waltham, MA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA) and 3% supplementary complement. Supplementary complement included HEPES (Thermo Fisher Scientific Inc, Waltham, MA), L-Glutamine (Research Products International Corp., Mt. Prospect, IL), Pen/Strep (Invitrogen, Grand Island, NY), Gentamicin Sulfate (Invitrogen, Grand Island, NY), and 2-Mercaptoethanol (Thermo Fisher Scientific Inc, Waltham, MA). Livers were perfused with cold PBS through the hepatic portal vein and made into single cell suspensions using 35% Percoll/HBSS. Lungs were perfused through the left ventricle with cold PBS and treated with DNase/collagenase for one hour prior to generation of single-cell suspension. Single suspension cells were treated with ammonium chloride potassium (ACK) to lyse red blood cells. Blood was collected in heparinized collection natelson tubes and treated with ACK to obtain peripheral blood mononuclear cells (PBMCs). Tissues were harvested as indicated at the number of days post infection.

OVA-specific, LLO-190-specific, and LCMV-specific CD8 and CD4 T cells were detected using intracellular cytokine staining (ICS) for IFN-γ. Cells were incubated five hours with brefaldin A (Biolegend, San Diego, California) with or without respective antigenic peptides (CD8 = 200 nM, CD4 = 4 μM) in Hyclone

RPMI media supplemented as described above. After incubation, cells were stained with Fc block (anti-CD16/32; clone 2.4G2) and indicated antibodies (Abs) resuspended in FACS buffer (1x PBS, 2% fetal calf serum, 0.02% sodium azide), fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Diego, California), stained for intracellular proteins, and washed with BD Perm/Wash (BD Biosciences), San Diego, California). OVA-specific, NP396-specific, and GP33-specific CD8 T cells were also detected by tetramer staining. Cells were incubated with tetramers and Fc block in FACS buffer for 45 minutes. Antibodies to cell surface markers were then added. Tetramers were generated from biotinylated monomers (generously provided by John Harty, University of Iowa) conjugated with streptavidin-APC (Invitrogen, Eugene, OR).

Antibodies

The following Abs were used from Biolegend (San Diego, California): CD8-PerCP/Cy5.5, CD4-PerCP/Cy5.5, CD25-PE, CD69-FITC, CD40-PE, CD80-PE, CD86 PE, Thy1.1-APC, and IFN-γ-FITC.

Parasitemia

Percent parasitemia was calculated by performing thin blood smears. Blood was obtained by performing tail snips. Slides were fixed with methanol for ten minutes and then stained using Giemsa stain (Thermo Fisher Scientific Inc, Waltham, MA) diluted in PBS for 30 minutes.

BrdU assay

Mice were injected with 200 µL 10 mg/mL bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO) solution intravenously three hours prior to spleen removal. BrdU was put into solution with 1X sterile PBS. The BrdU Flow Kit (BD Pharmingen, San Diego, California) was used for labeling BrdU+ cells. Single-cell suspensions were generated and cells were stained with specific fluorescent Abs. Cells were then fixed with BD Cytofix/Cytoperm, washed with BD Perm/Wash, and incubated with BD Cytoperm Permeabilization Buffer Plus. Cells were then treated with DNase and incubated for one hour. The cells were then stained for BrdU with fluorescent α-BrdU antibodies and incubated for 20 minutes at room temperature. BrdU+ cells were analyzed using flow cytometry.

Caspase 3/7 assay

Spleens were disrupted into single-cell suspension. The Caspase-3 and -7 Assay Kit (Invitrogen, Grand Island, NY) was used to detect apoptosis. A 30X FLICA working solution was added to cells and incubated for 1 hour at 37°C and 5% CO_2. Cells were washed with 1X wash buffer and stained with a tetramer followed by cell surface fluorescent antibodies. Detection of caspase-3 and -7 was detected using flow cytometry.

Adoptive transfer of OT-I TCR Transgenic CD8 T cells

Thy 1.1+ transgenic mice were used as sources of OT-I's. Spleens and lymph nodes were removed and disrupted into a single-cell suspension. OT-I's were isolated using a CD8α+ T cell isolation kit II (Miltenyi Biotec, Cologne, Germany). OT-I transgenic T cells were quantified and injected intravenously at the indicated amount.

Statistical analysis

All statistical analyses were performed using the Prism 6.0c software. Significant data were represented by the following: * p=0.05-0.01, ** p=0.01-0.001, *** p=0.001-0.0001, ****p<0.0001

Section III

Results

Plasmodium yoelii *17XNL suppresses T cell responses to a bacterial coinfection*

Previous studies have indicated that *Plasmodium falciparum* impairs resistance and increases susceptibility to multiple diseases, including both bacterial and viral infections [26, 27, 31, 32, 37, 38]. In one particular study, it has been demonstrated that *P. falciparum* causes neutrophil dysfunction during non-typhoid *Salmonella* coinfections [35]. This bacterial infection is most commonly associated with malaria in several regions throughout sub-Saharan Africa [31, 32]. Therefore the objective was to determine to what extent adaptive immunity to heterologous coinfections was affected following a *Plasmodium* infection. The non-lethal strain of *Plasmodium yoelii* (*P. yoelii* 17XNL) was employed to serve as the *Plasmodium* model due to it causing an acute infection with high parasitemia. C57BL/6 mice were infected with 10^5 *P. yoelii* parasitized red blood cells (pRBCs) at day 0. Seven days following the *P. yoelii* infection, both naive and *P. yoelii* infected mice were infected with 5 x 10^6 *actA*-deficient *Listeria monocytogenes* CFUs specifically expressing the protein, ovalbumin (Lm-OVA). The Lm-OVA strain was an attenuated strain deficient in the *actA* protein,

preventing the bacteria from spreading from cell to cell [53]. Using this strain provided a means to monitor OVA-specific CD8 T cells that responded to the Lm-OVA infection. Parasitemia of this *P. yoelii* strain was also monitored in order to determine the magnitude of infection (Figure 2). Parasitemia was found to peak at approximately day 18 post-*Plasmodium* infection.

A reduction in OVA-specific CD8 T cells was evident the first two weeks following Lm-OVA infection (Figure 3A,B). CD4 T cells specific for the LLO-190 peptide were also suppressed following the Lm-OVA infection, although only significantly through day 14 (Figure 3C,D). These data provided insight into how extensive immunosuppression was occurring, with CD8 T cells being significantly depleted through day 21 of the experiment (Figure 3B). Although CD4 T cells were not suppressed to the degree that CD8 T cells were (7-fold and 11-fold, respectively), there was still a significant ($p = 0.0001$) reduction in their numbers at day 14 (Figure 3D). Both CD8 and CD4 T cell numbers recovered by day 35, indicating that these immunosuppressive events occur early following the *P. yoelii* infection. The timing of T cell recovery corresponded with parasite clearance, indicating that the presence of the parasite was playing a role in T cell suppression.

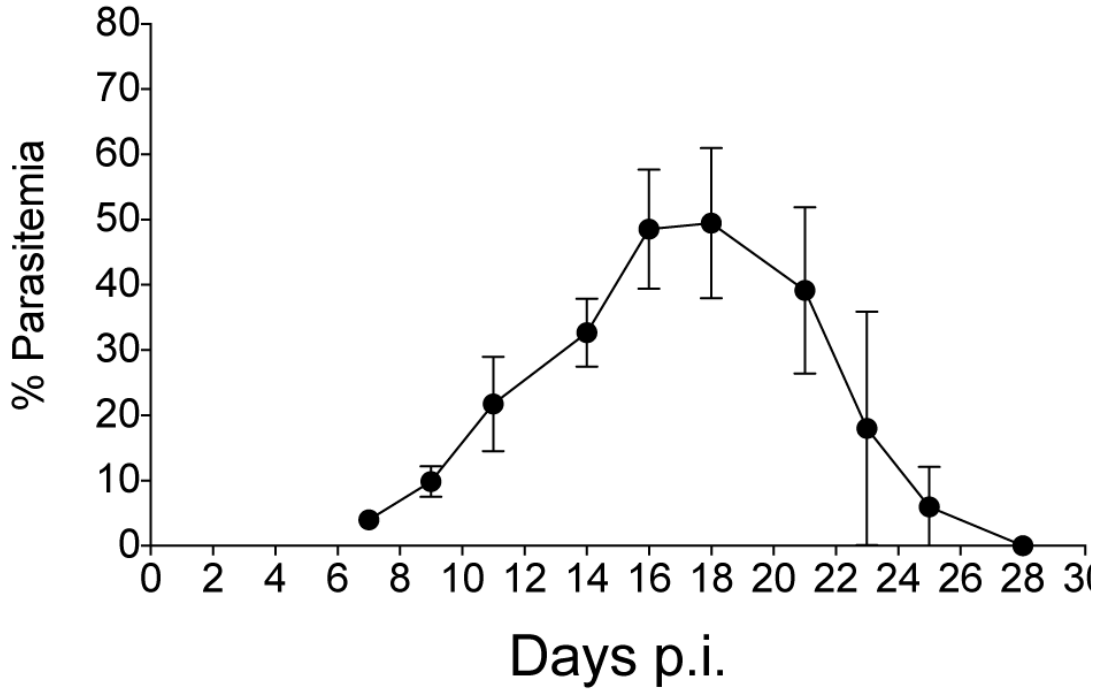


Figure 2. Percent parasitemia following *P. yoelii* infection. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL (Py) pRBC. Blood smears were taken starting at day 7 and continued throughout infection. Percent parasitemia was calculated by Giemsa staining blood smears and performing parasitized red blood cell counts.

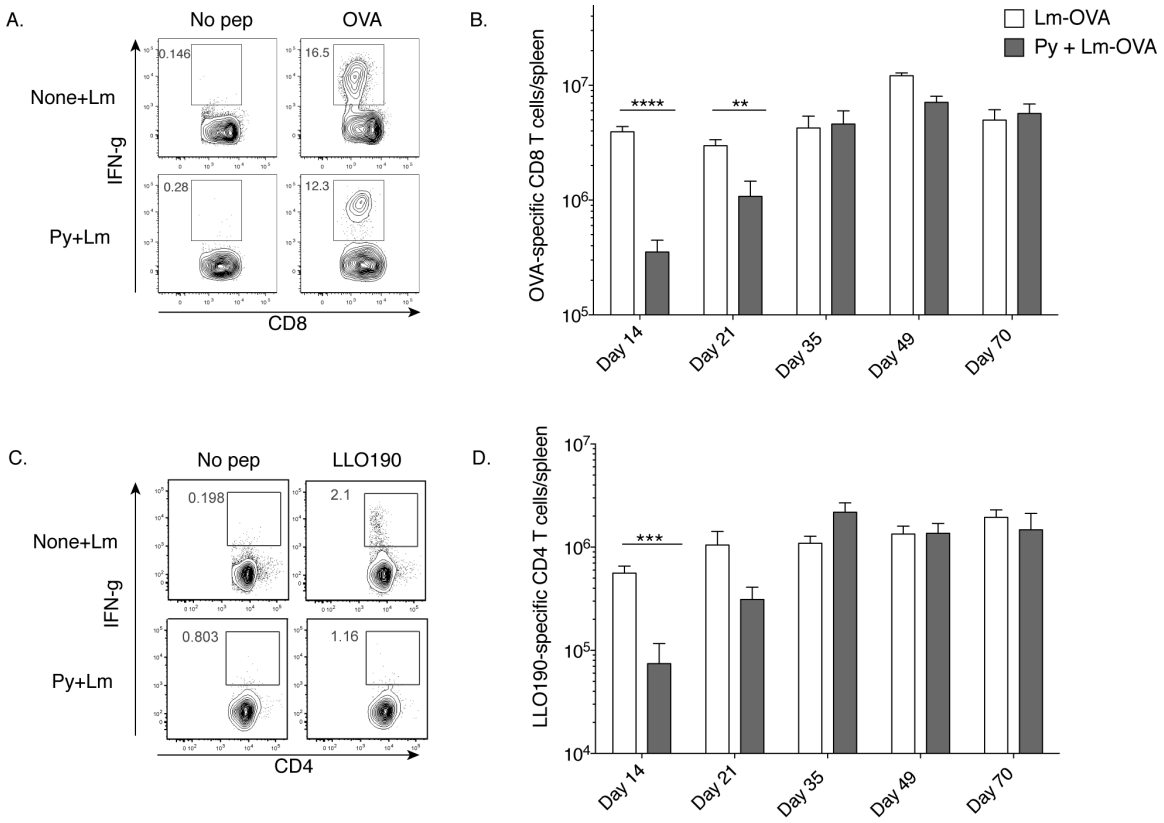


Figure 3. *P. yoelii* 17XNL induces short-term suppression of T cell responses to secondary *L. monocytogenes* infection. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL (Py) pRBC. At various times post Py infection both naïve and Py infected mice were infected with $5x10^6$ *actA*-deficient *L. monocytogenes* expressing ovalbumin (Lm). Lm-specific T cells were analyzed one-week post Lm infection. Representative contour plots showing percent of CD8 T cells (A) or CD4 T cells (C) that are IFN-γ positive following stimulation with indicated peptide and intracellular cytokine stain (ICS). Number of OVA-specific CD8 T cells (B) and LLO190-specific CD4 T cells (D) per spleen were quantified at the indicated time points. Data (mean+S.E.M.) are cumulative results from at least three independent experiments. LLO190-specific CD4 T cell numbers at day 21, 35, and 70 are from one experiment. Data were analyzed by Student's unpaired two-tailed *t* test.

P. chabaudi *induces suppression of T cells responding to secondary Lm-OVA infection*

There are multiple species and strains of *Plasmodium* that cause malaria. Therefore, it is important to understand to what degree other *Plasmodium* species suppress T cell responses to coinfections. *Plasmodium chabaudi* AS is a widely used murine *Plasmodium* model because it induces a chronic infection, which is similar to infection with *P. falciparum* infection in humans [54]. However peak parasitemia is lower than that observed in *P. yoelii*. This provided another means to examine variable levels of parasitemia and how parasite loads could potentially impact the T cell response to coinfections.

To determine whether *P. chabaudi* also impaired T cell responses to a bacterial coinfection, C57BL/6 mice were infected with 10^6 *P. chabaudi* AS pRBCs on day 0. On day 7, naïve and *P. chabaudi* infected mice were subsequently infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. Similar to *P. yoelii*, *P. chabaudi* infected mice exhibited reduced OVA-specific CD8 T cells and LLO-specific CD4 T cells on day 14, however this was the only time point that showed significant differences (Figure 4). Although there was a significant decrease in T cell numbers (p=0.0040), the decrease in OVA-specific CD8 T cells was not as severe as the *P. yoelii* infection (Figure 4A). This could potentially be due to *P. chabaudi* causing a lower level of parasitemia (data not shown) compared to that of *P. yoelii*. By day 21, T cell numbers were still lower

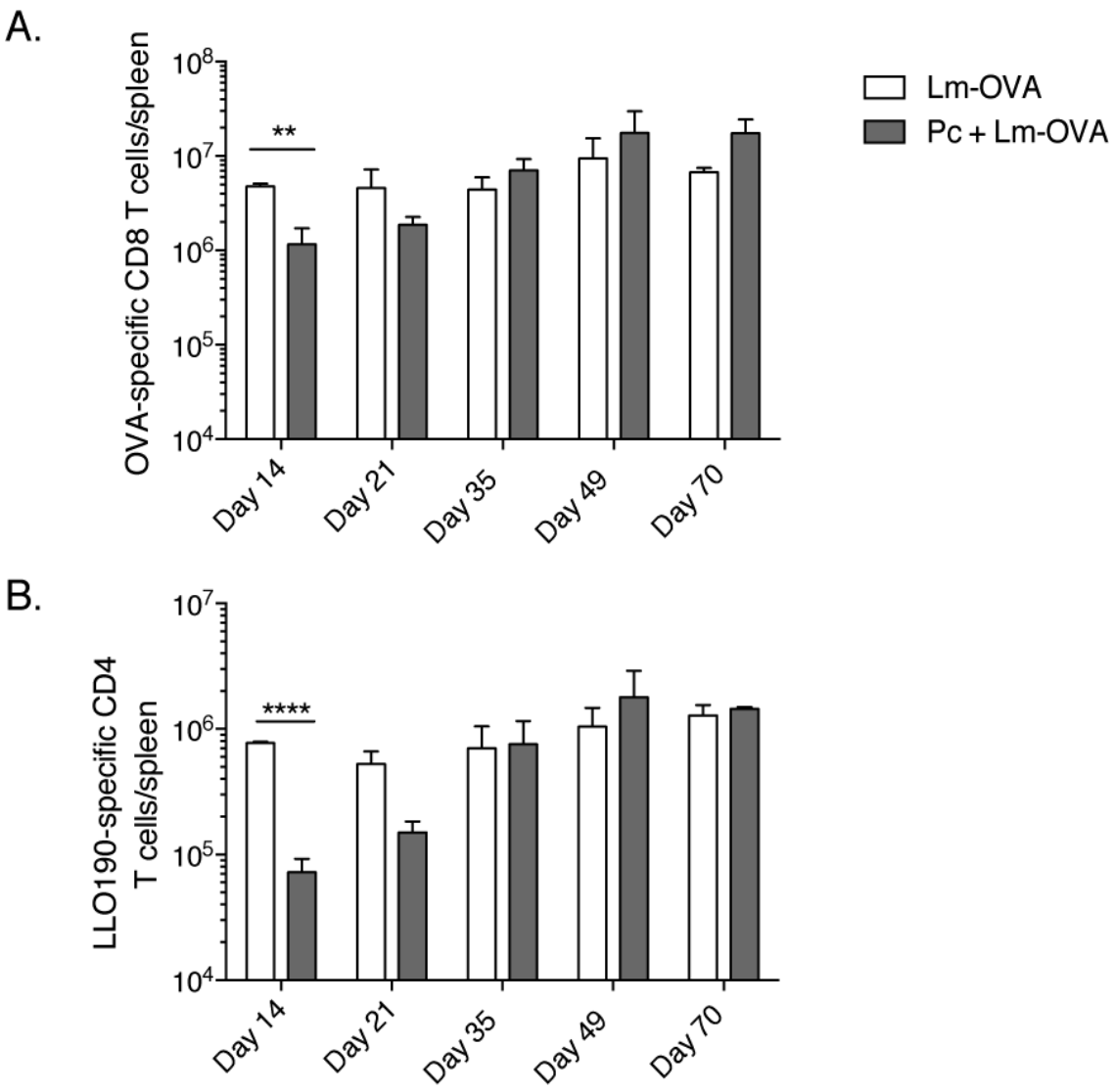


Figure 4. Suppression of T cells responding to secondary *L. monocytogenes* infection is observed in *P. chabaudi* AS infected mice. C57BL/6 mice were infected with 10^6 *P. chabaudi* AS (Pc) pRBC. At various times post Pc infection both naïve and Pc infected mice were infected with $5x10^6$ *actA*-deficient Lm. Lm-specific T cells were analyzed one-week post Lm infection. OVA-specific CD8 T cells (A) or LLO190-specific CD4 T cells (B) were determined by ICS for IFN-γ. Data (mean+S.D.) are from three mice per group. Data were analyzed by Student's unpaired two-tailed *t* test.

in *P. chabaudi* infected mice, although neither OVA-specific CD8 nor LLO-specific CD4 T cell numbers were significantly reduced (Figure 4). As demonstrated previously with *P. yoelii*, T cell numbers returned to normal by day 35 following the *P. chabaudi* infection. By examining both of these murine *Plasmodium* species, suppression of T cell responses to secondary bacterial infections was demonstrated to occur with various species and strains. Thus, *Plasmodium* species were capable of imparting immunosuppressive effects on T cell responses to secondary infections in the first two weeks following a *Plasmodium* infection.

Immunosuppression of T cell response to viral infection occurs following a* P. yoelii *infection

While there are many bacterial infections that could accompany a *Plasmodium* infection in malaria endemic areas, there are also reports of individuals displaying a higher susceptibility to viral infections, including HIV and EBV, when infected with malaria [30, 36-38]. Thus, it was also necessary to determine whether suppression of the immune response to a viral infection could occur. For this experiment, lymphocytic choriomeningitis virus (LCMV) was utilized to serve as a viral infection. LCMV-armstrong strain induces an acute infection and is typically cleared from the murine host within 8 days. On day 0, C57BL/6 mice were infected with *P. yoelii* and one week later, naive and *P. yoelii* infected mice were infected with 2×10^5 LCMV-armstrong PFUs. Eight days after the LCMV-

armstrong infection (day 15), LCMV-specific T cells were analyzed. The major antigenic peptides are NP396, GP33, and GP276 for CD8 T cells and GP61 for CD4 T cells. For all three CD8 T cell responses analyzed, there was a significant reduction in numbers (Figure 5A,B). Similarly, GP61-specific CD4 T cells were lower in *P. yoelii* infected mice (Figure 5C,D). These data demonstrate that *P. yoelii* also suppresses T cell responses to viral coinfections.

Immunosuppression is not attributed solely to* Plasmodium *species

After demonstrating that *Plasmodium* could suppress the adaptive immune response to coinfections, it was hypothesized that other types of infection could exhibit immunosuppression of coinfections as well. Chronic viral infections can display immunosuppressive activity and induce functional impairment and suppression of virus-specific CD8 T cells, specifically in LCMV-clone 13 [55]. During persistent viral infections, immunosuppressive effects can be observed with altered immunodominance profiles and tissue distribution, ultimately altering the viral T cell response to infections [55]. Therefore, the objective was to determine whether immunosuppression of coinfections was attributed solely to *Plasmodium*, or whether this could occur with other infections as well. To test this, LCMV was used to serve as the viral infection. LCMV-armstrong strain and LCMV-clone 13 strain cause an acute and chronic infection, respectively. These two strains provided a means to observe differences in immunosuppression with

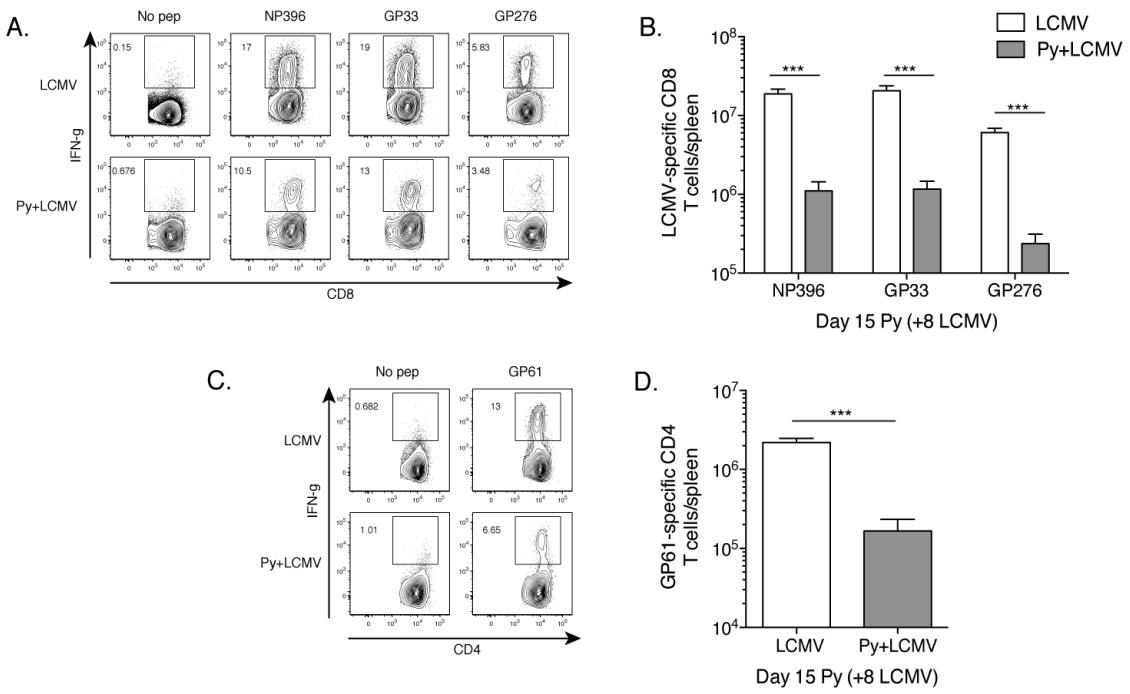


Figure 5. ***P. yoelii*** **17XNL suppresses T cell responses to a viral coinfection.** C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL (Py) pRBC. One-week after Py infection naïve and Py infected mice were infected with $2x10^5$ lymphocytic choriomeningitis virus (LCMV) Armstrong. LCMV-specific T cells were analyzed 8 days post LCMV infection. Representative contour plots showing percent of CD8 T cells (A) or CD4 T cells (C) that are IFN-γ positive following stimulation with indicated peptide and ICS. Number of NP396-, GP33- and GP276-specific CD8 T cells (B) and GP61-specific CD4 T cells (D) per spleen. Data (mean+S.E.M.) are cumulative results from two independent experiments. Data were analyzed by Student's unpaired two-tailed *t* test.

both an acute and chronic strain, as was seen with the *Plasmodium* strains (Figures 3, 4, and 5).

C57BL/6 mice were infected with either 2 x 10^5 LCMV-armstrong PFUs or 2 x 10^6 LCMV-clone 13 PFUs on day 0. One week later, naïve and LCMV infected mice were infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. It was determined that both strains were capable of inducing immunosuppression (Figure 6). Both LCMV-armstrong and LCMV-clone 13 demonstrated a substantial reduction in the Lm-OVA CD8 and CD4 T cell responses at day 14. Several mice in both LCMV-armstrong and LCMV-clone 13 infected groups had T cell responses below the limit of detection at day 14, therefore statistical analyses were not performed on these data for this time point (Figure 6B,D). LCMV-clone 13 demonstrated a much more severe suppressive capacity throughout the entire experiment for both CD8 and CD4 T cells. OVA-specific T cell responses in these mice were significantly suppressed through day 70, indicating a long-term suppressive effect elicited by this virus. LCMV-armstrong suppressed the *Listeria* T cell response through day 21, but the response returned to normal by day 35 (Figure 6B,D). These differences in immunosuppressive capabilities could be due to multiple factors that have yet to be examined. Because LCMV-clone 13 causes a much more systemic and chronic infection, it was expected that this strain would cause more T cell suppression. These data demonstrate that *Plasmodium* was not the only pathogen capable of suppressing immune responses to secondary infections, but a viral infection could as well.

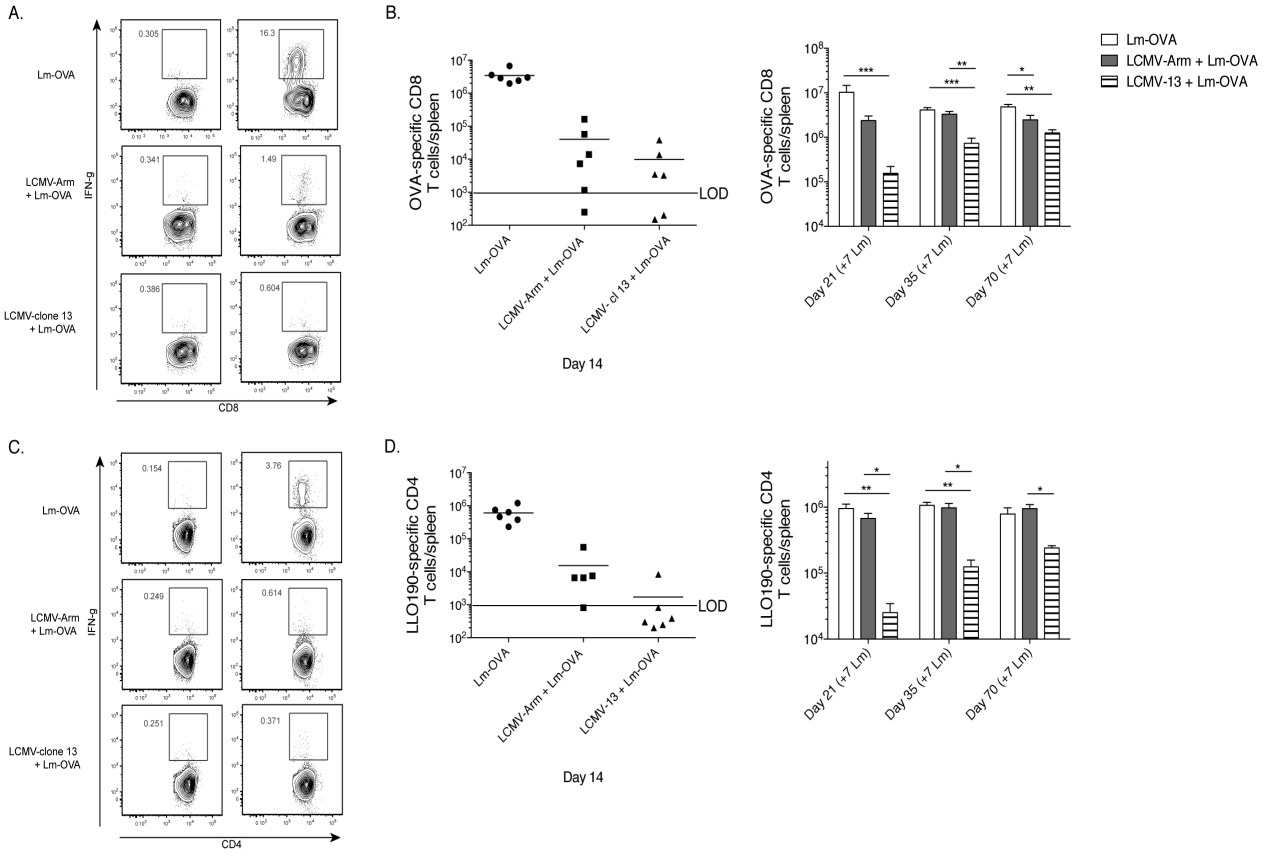


Figure 6. Acute and chronic viral infections suppress T cell responses to secondary *L. monocytogenes* infection. C57BL/6 mice were infected with either $2x10^5$ LCMV Armstrong (Arm) or $2x10^6$ LCMV clone 13 (cl 13). At various times post LCMV infection naïve and LCMV infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA CFUs. Lm-specific T cells were analyzed one-week post Lm infection. Number of OVA-specific CD8 T cells (A) and LLO190-specific CD4 T cells (B) per spleen at the indicated time points were determined by ICS for IFN-γ. LOD = limit of detection. Data (mean+S.E.M.) are cumulative from 2 separate experiments. Data for CD8 T cells on days 35 and 70 were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data for CD8 T cells on day 21 and data for CD4 T cells on days 21, 35, and 70 were analyzed by a Kruskal-Wallis test followed by Dunn's multiple comparison test.

Wild type Listeria *infection has little to no effect on the immune response to viral coinfection*

After demonstrating that a viral infection was capable of suppressing a bacterial immune response, immunosuppression by other types of pathogens was further investigated by examining whether a bacterial infection was able to alter the T cell response to a viral infection. To test this, a wild type strain of *Listeria monocytogenes* (Lm 10403s) served as a bacterial infection. This strain is more pathogenic than the strain used in previous experiments, as it is not deficient in the *actA* protein. C57BL/6 mice were infected with 10^4 Lm 10403s CFUs on day 0 and subsequently infected with LCMV-armstrong on days 7 and 14. On days 15 and 22, NP396-, GP33-, and GP276-specific CD8 and GP61-specific CD4 T cell responses were analyzed. At days 15 and 22, CD8 T cell responses were not altered in mice (Figure 7C,E). It was shown that, on day 15, CD4 T cell responses were slightly less than that of LCMV only infected, but there was no difference by day 22 (Figure 7D,F). Thus, a virulent *Listeria* infection had little impact on T cell responses to a viral coinfection. Therefore, suppression of T cell responses to coinfections is not a universal observation for all pathogens.

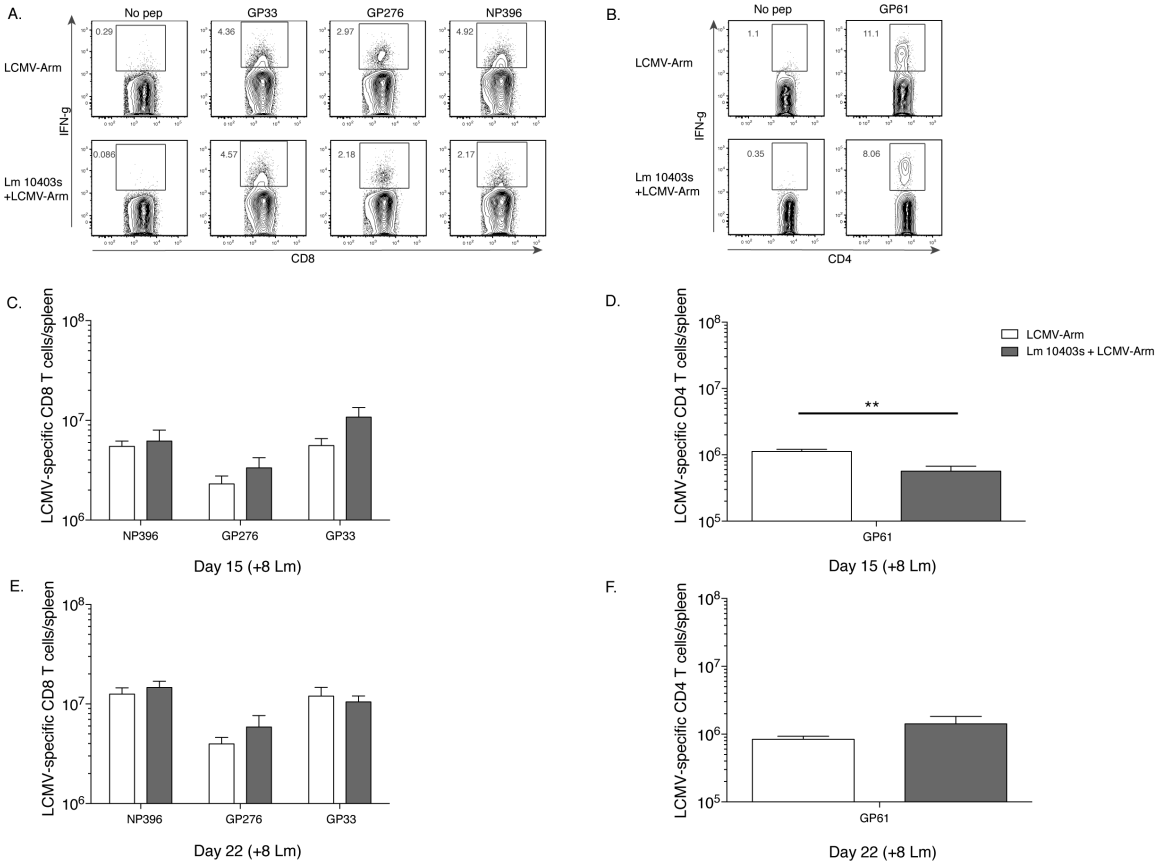


Figure 7. Virulent *L. monocytogenes* infection exhibits little to no impact on T cell responses to secondary LCMV infection. C57BL/6 mice were infected with 0.5-1x10^4 virulent *L. monocytogenes* (strain 10403s) (Lm). At various times post Lm infection naïve and Lm infected mice were infected with 2x10^5 LCMV Armstrong. LCMV-specific T cells were analyzed 8 days post LCMV infection. Number of NP396-, GP33- and GP276-specific CD8 T cells (A) and GP61-specific CD4 T cells (B) per spleen were determined by ICS for IFN-γ at the indicated time point. Data (mean+S.E.M.) are cumulative results from two independent experiments. Data were analyzed by Student's unpaired two-tailed *t* test.

P. yoelii ***infection results in a loss of*** **Listeria-*specific CD8 T cells in multiple tissues***

In order to examine the mechanism of immunosuppression, it was important to determine if there was a loss of coinfection-specific T cells in other tissues besides the spleen. It was critical to ensure that T cells being presented antigen in the spleen were not migrating to other tissues upon activation, thus contributing to the loss of T cell numbers in the spleen that was seen in previous experiments. To address this possibility, C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs on day 0. On day 7, naive and *P. yoelii* infected mice were infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. One week later, the spleen, inguinal lymph nodes (iLN), liver, lungs, and blood were collected and OVA-specific CD8 T cells were quantified using tetramer staining. There was a significant loss of T cell numbers in multiple tissues throughout the body, which demonstrated that T cell migration to tissues outside the spleen was not contributing to the impaired response (Figure 8). These data demonstrated that depletion of the OVA-specific CD8 T cell response was accurately detected in the spleen and that migration of the T cells was not contributing to a loss in numbers in the spleen (Figure 8). This suggests that a loss of OVA-specific CD8 T cells in the spleen was not being compensated by an increase in other tissues and that a reduction in the T cell response was occurring throughout the body. Therefore, *P. yoelii* induces systemic suppression of *Listeria*-specific T cells during a coinfection.

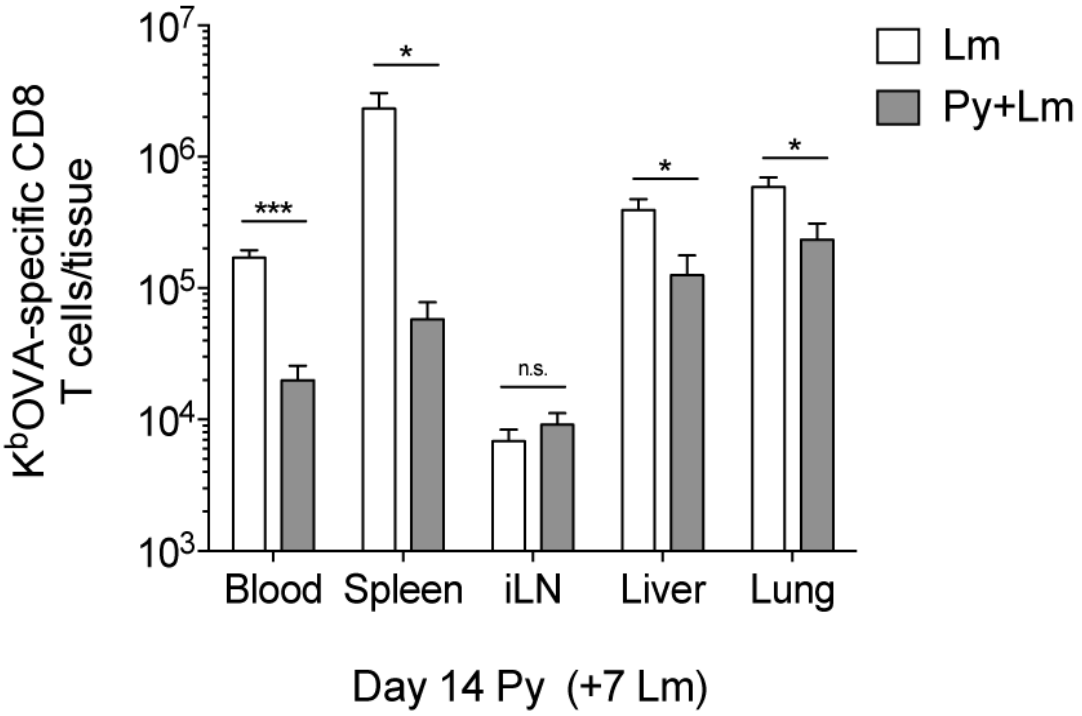


Figure 8. Loss of *Listeria*-specific CD8 T cells during *Plasmodium* infection occurs in multiple tissues. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL (Py) pRBCs at day 0. One week later, naïve and Py infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA. Spleens, livers, inguinal lymph nodes (iLN), lungs, and blood were taken from mice and analyzed 14 days post Lm infection. OVA-specific CD8 T cells were quantified using tetramer staining. Data (mean+S.E.M.) are cumulative from two independent experiments. Data were analyzed using Student's unpaired two-tailed *t* test.

Defect in T cell response occurs early after Lm-OVA infection

One explanation for the observed decrease in *Listeria*-specific T cells in *Plasmodium* infected mice is altered kinetics of the T cell response. To address this possibility *Listeria*-specific CD8 T cell responses were quantified at multiple time points. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs on day 0 and infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs one week later. OVA-specific CD8 T cells were quantified daily day 4 through day 10 post Lm-OVA infection. Suppression of OVA-specific CD8 T cells occurred as early as day 4 following the Lm-OVA infection and lasted through day 7 (Figure 9). The early decrease in OVA-specific CD8 T cells in *P. yoelii* infected mice suggests there is a defect in the early stages of T cell priming that causes the decrease of *Listeria*-specific T cells in *P. yoelii* infected mice.

Loss of CD8 T cells is not due to decrease in proliferation

The decrease in *Listeria*-specific T cells in *P. yoelii* infected mice is likely attributed to either a decrease in proliferation or increased apoptosis. To examine the proliferative potential of OVA-specific CD8 T cells in *P. yoelii* + Lm-OVA infected mice, C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs on day 0 and one week later infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. Beginning at day 4 post-Lm-OVA infection and continuing through day 9, mice were injected with 10 mg/mL bromodeoxyuridine (BrdU). BrdU is a thymidine analog that is incorporated into the DNA of dividing cells and can be

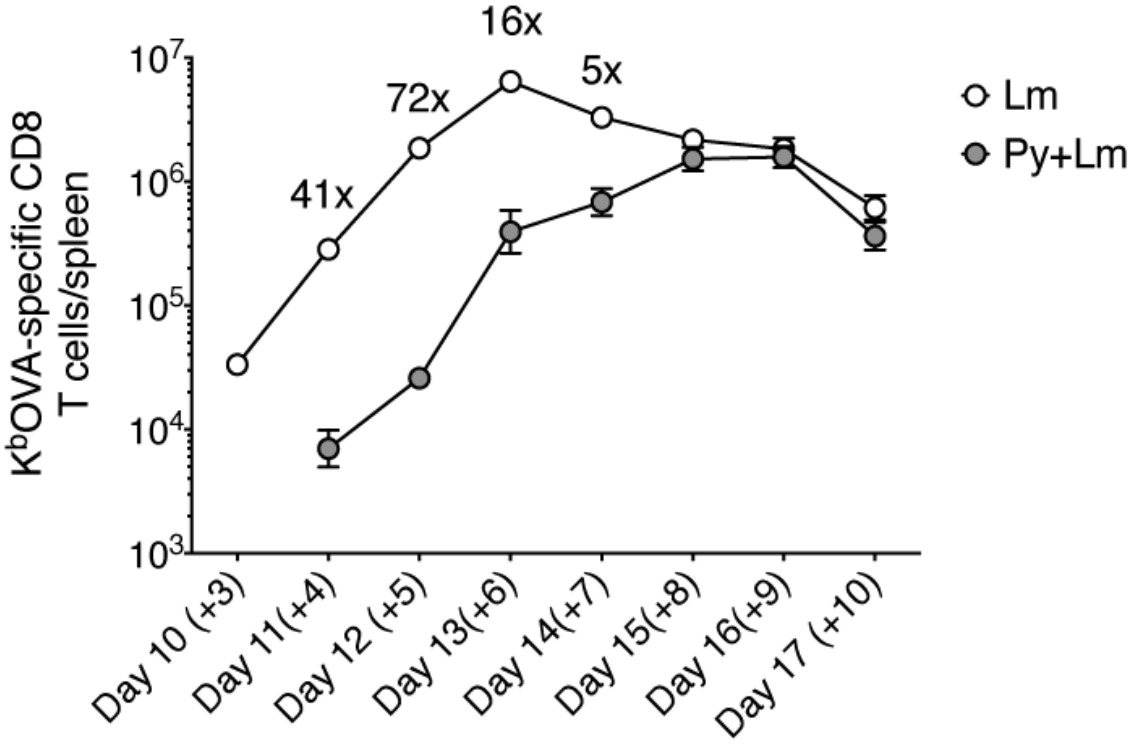


Figure 9. Suppression of T cells occurs early following *Listeria* infection. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs at day 0. On day 7, naïve mice and Py infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA. (A) OVA-specific CD8 T cells were detected starting at day 4 post Lm infection. OVA-specific CD8 T cells were quantified using tetramer staining. Data (Mean+S.E.M.) are cumulative results from four separate experiments. Data were analyzed by Student's unpaired two-tailed *t* test.

tracked with BrdU-specific antibodies. Three hours after injection of BrdU, spleens were removed for analysis of BrdU incorporation in OVA-specific CD8 T cells. The data indicated there was no significant difference in proliferation in OVA-specific CD8 T cells early in the response (days 4 and 5) (Figure 10). Surprisingly, OVA-specific CD8 T cells in *P. yoelii* + Lm-OVA infected mice exhibited higher rates of BrdU incorporation on days 6 through 8 (Figure 10). These results demonstrate that *P. yoelii* does not impair *Listeria*-specific CD8 T cells through decreased proliferation. This observation could help explain why there is a delay in the peak OVA-specific CD8 T cell response in *P. yoelii* infected mice as demonstrated in Figure 9.

An increase in apoptosis occurs in OVA-specific CD8 T cells during a P. yoelii *plus* Lm-*OVA infection*

Since there was no reduction in proliferation, this suggested differences in apoptosis might contribute to decreased T cell responses in *P. yoelii* infected mice. To determine if OVA-specific T cells exhibited different rates of apoptosis, a caspase-3/7 assay was used to monitor apoptotic cells. This assay measures the activity of caspases-3 and -7, two proteins that are proteolytically cleaved and activated during apoptotic signaling events [56]. By measuring the activation levels of these two proteins, apoptotic cells can be enumerated via flow cytometry. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs at day 0

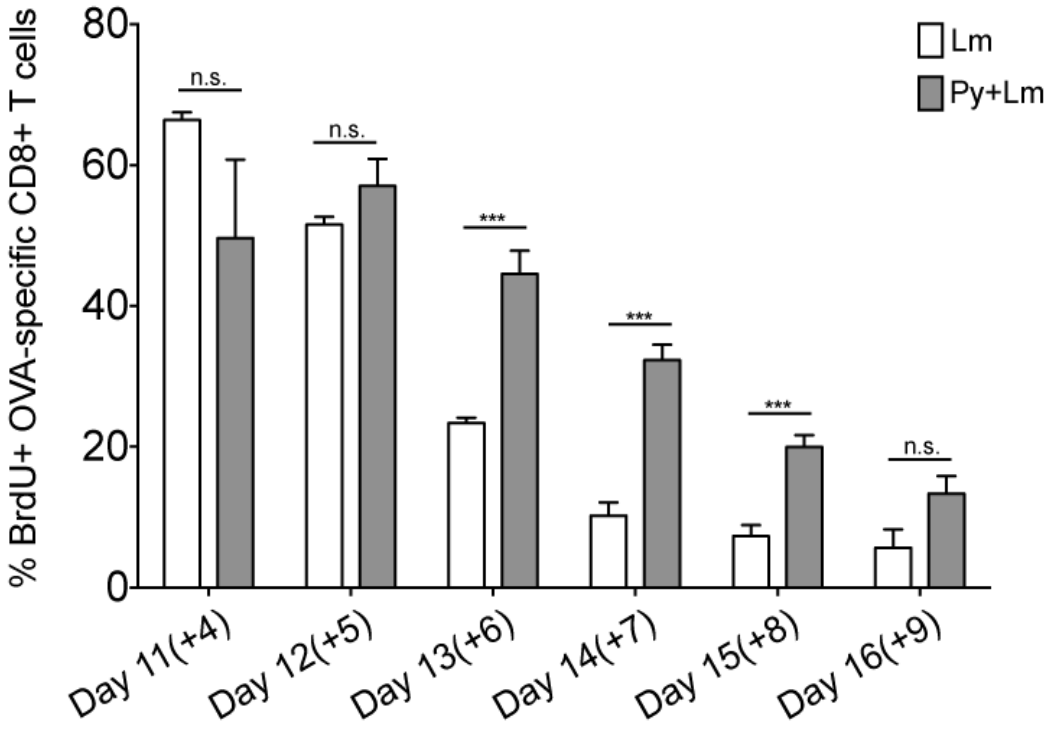


Figure 10. Suppression of CD8 T cell response is not due to a decrease in proliferation. C57Bl/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs at day 0. On day 7, naïve mice and Py infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA. At days 4 – 10 post Lm infection, mice were injected with BrdU 3 hours prior to spleen removal to determine proliferation of OVA-specific CD8 T cells. Data (mean+S.E.M.) are cumulative results from four separate experiments. Data were analyzed by Student's unpaired two-tailed *t* test.

and one week later infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. Starting at day 11, apoptosis was monitored through day 15 for detection of active caspase-3/7+ cells.

There were significant differences in apoptosis six and seven days post-*Listeria* infection in *P. yoelii* plus Lm-OVA infected mice (Figure 11). On day 11, OVA-specific CD8 T cells in *P. yoelii* infected mice were undetectable. By day 12 there were enough cells to be able to detect caspase 3/7+ cells, although apoptosis only proved to be significantly higher starting at day 13 (Figure 11). Both days 13 and 14 were significant (p=0.0041, p=0.0002 respectively) and demonstrated that *P. yoelii* infected mice had higher levels of OVA-specific apoptotic cells. By day 15, there were no differences in apoptosis. From these data, it can be concluded that apoptosis may be contributing to a reduction in the OVA-specific T cell response following a *P. yoelii* infection.

OT-I TCR Tg T cells behave similarly to endogenous CD8 T cell response

Collectively, the data suggest alterations in antigen presentation and T cell priming likely contribute to decreased T cell responses and increased susceptibility to apoptosis. To address these mechanisms requires the analysis of T cells early after T cell priming, which can only be accomplished through adoptive transfer of large numbers of T cell receptor transgenic (TCR-Tg) T cells specific for ovalbumin (OT-I TCR Tg T cells). Prior to utilizing OT-I TCR Tg T cells, it was important to demonstrate that they behaved similar to the

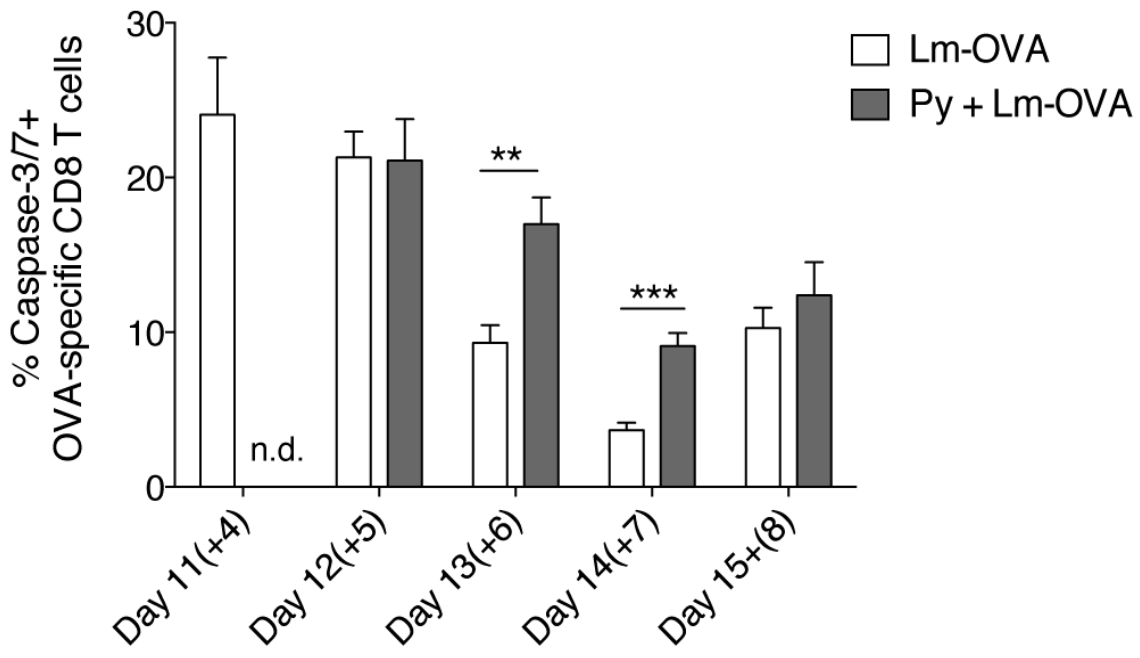


Figure 11. Apoptosis contributes to loss of CD8 T cell response. C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs at day 0. On day 7, naïve mice and Py infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA. At days 4 – 8 post Lm infection, OVA-specific CD8 T cells were stained for Caspase 3/7 to determine apoptotic cell numbers. Data (mean+S.E.M.) are cumulative results from two separate experiments. Data were analyzed by Student's unpaired two-tailed *t* test. n.d. = not detectable.

endogenous response in *P. yoelii* + Lm-OVA infected mice. C57BL/6 mice were injected with 250 OT-I TCR Tg T cells that were adoptively transferred from a Thy1.1+ transgenic mouse into Thy1.2+ C57BL/6 mice. A low number adoptive transfer was performed in order to mimic the endogenous naïve T cell pool. Twenty-four hours later, mice were infected with 10^5 *P. yoelii* 17XNL pRBCs, and one week later, naïve and *P. yoelii* mice were infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. Starting at day 12, OT-I TCR Tg T cells were quantified over a five day span.

Importantly, the kinetics of the OT-I TCR Tg T cell response (Figure 12) were comparable to the endogenous response (Figure 9). Suppression of the Tg T cell response was detected as early as day 12, although T cell numbers were not analyzed prior to this time point. However, *P. yoelii* plus Lm-OVA infected mice displayed similar kinetics of the T cell response to that of the endogenous response (Figure 9). The peak of the *P. yoelii* response was delayed by 24 hours. These results demonstrate that OT-I TCR Tg T cells followed similar kinetics as seen in the endogenous response of *P. yoelii* + Lm-OVA infected mice. Consequently, OT-I TCR Tg T cells provide a sufficient model for monitoring T cell priming and activation following a Lm-OVA infection.

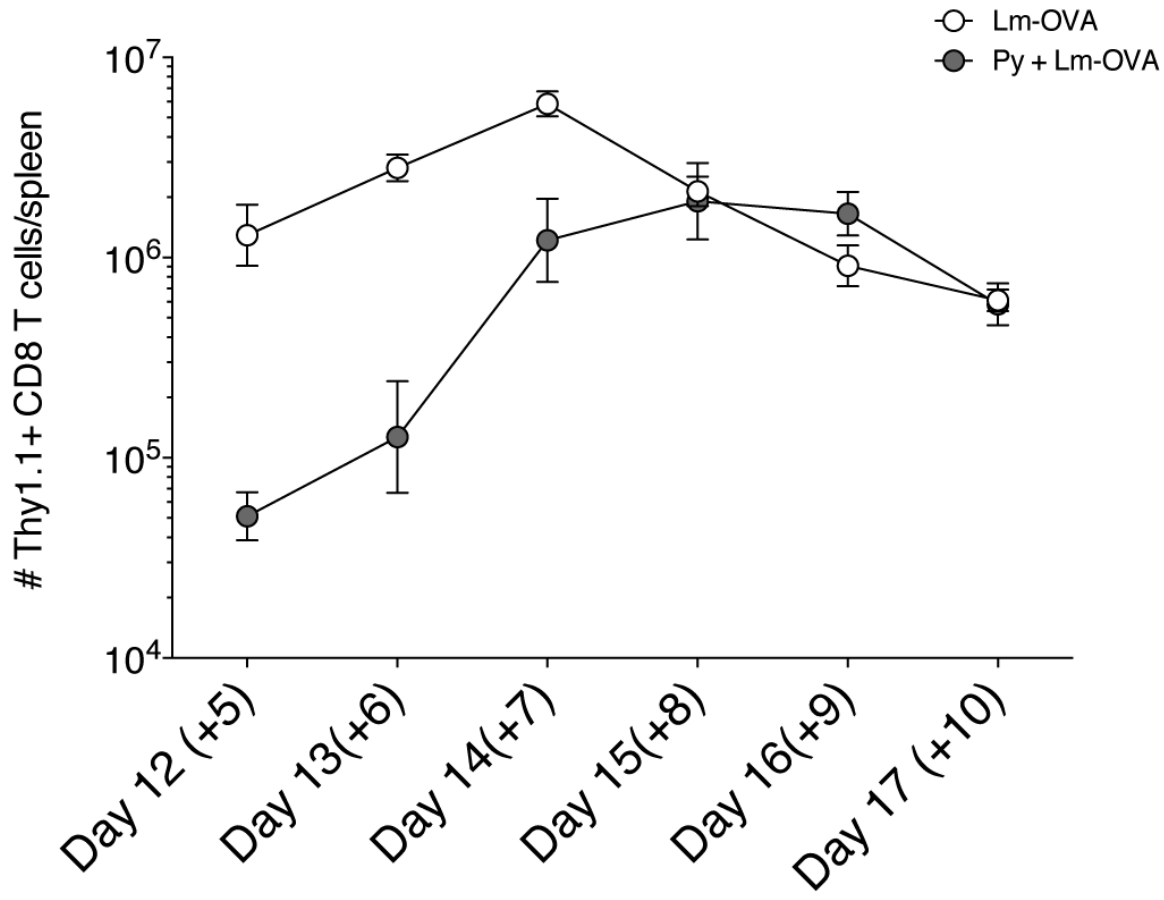


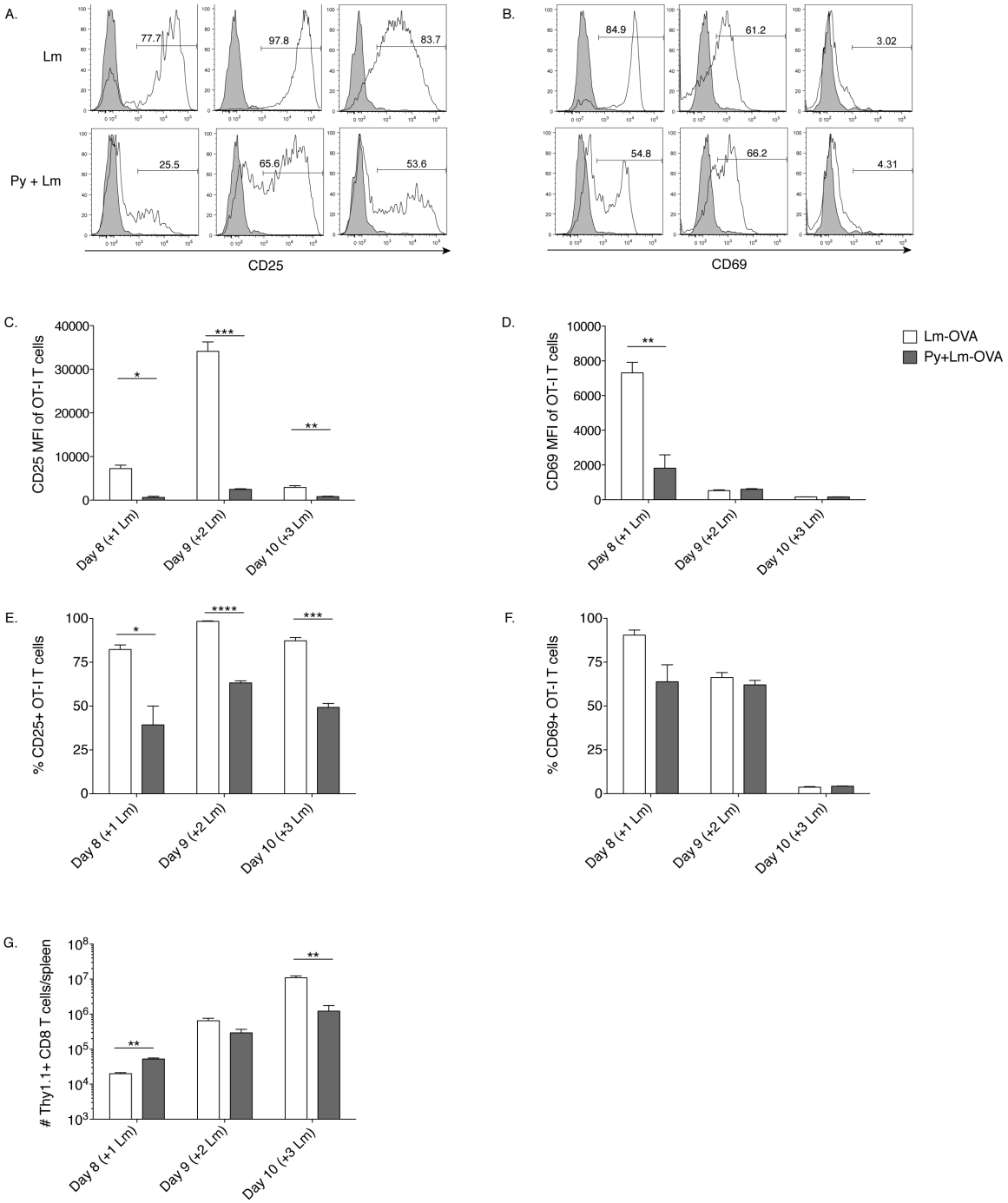
Figure 12. Adoptively transferred OT-I's behave similarly to the endogenous CD8 T cell response. Two hundred fifty OT-I's were adoptively transferred into naïve C57BL/6 mice. Half of these mice were infected with 10^5 *P. yoelii* 17XNL pRBCs one day later (day 0). At day 7, naïve and Py infected mice were infected with $5x10^6$ *actA*-deficient Lm-OVA. At days 12 – 17, OT-I cell numbers were quantified by cell surface staining. Data (mean+S.E.M.) are cumulative results from two separate experiments. Data was analyzed by Student's unpaired two-tailed *t* test.

Plasmodium *causes impaired activation of coinfection-specific T cells*

Due to immunosuppression being observed early following a *Listeria* infection, it was imperative to investigate whether initial priming of T cells was affected in some way by a *Plasmodium* infection. Following both TCR stimulation and costimulation, the cell surface markers CD69 and CD25 are upregulated and serve as an indicator for T cell activation [57-59]. Therefore, induction of CD69 and CD25 were examined to determine whether *P. yoelii* impaired T cell activation events following Lm-OVA infection. To examine these two activation markers, C57BL/6 mice (Thy1.2+) received ~2 x 10^6 adoptively transferred OT-I TCR Tg T cells from a Thy1.1+ TCR Tg mouse at day 0. Mice were then infected with 10^5 *P. yoelii* 17XNL pRBCs later on the same day. One week later naive and *P. yoelii* infected mice were infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. Expression of CD25 and CD69 was analyzed on days 1, 2, and 3 post Lm-OVA infection. Mean fluorescence intensity was quantified to determine the level of expression for both CD25 (Figure 13C) and CD69 (Figure 13D). CD25 expression was reduced in the *P. yoelii* infections for three days following the Lm-OVA infection (Figure 13C). Similarly, the percentage of CD25+ OT-I T cells was also significantly lower in *P. yoelii* infected mice three days following Lm-OVA infection (Figure 13E).

CD69 mean fluorescence intensity was significantly (p=0.0095) impaired one day following the Lm-OVA infection but expression in both groups of mice was reduced the following two days (Figure 13D). The percentage of CD69+ OT-I

Figure 13. Activation markers are downregulated on T cells. At day 0, 1.5 – 1.8 x 10^6 OT-I's were adoptively transferred into naïve C57BL/6 mice. Mice were infected with 10^5 *P. yoelii* 17XNL pRBC and one week later infected with $5x10^6$ *actA*-deficient Lm-OVA CFUs. On days 8, 9, and 10, OT-I cells were stained for CD25 and CD69. Histograms for CD25 (A) and CD69 (B) were quantified to determine CD25+ and CD60+ OT-I T cells. Mean fluorescence intensity of CD25 (C) and CD69 (D) in OT-I T cells was quantified using flow cytometry. Percentage of CD25+ (E) and CD69+ (F) OT-I T cells was also determined by fluorescent staining and flow cytometry. Total cell numbers of Tg OT-I T cells were quantified by cell surface staining for Thy 1.1 (G). Data is representative of two separate experiments. Data were analyzed by Student's unpaired two-tailed *t* test.


A.
Lm
Py + Lm
77.7
97.8
83.7
25.5
65.6
53.6
CD25
B.
84.9
61.2
3.02
54.8
66.2
4.31
CD69
C.
CD25 MFI of OT-I T cells
40000
30000
20000
10000
0
*
***
**
Day 8 (+1 Lm)
Day 9 (+2 Lm)
Day 10 (+3 Lm)
D.
CD69 MFI of OT-I T cells
10000
8000
6000
4000
2000
0
**
Lm-OVA
Py+Lm-OVA
E.
% CD25+ OT-I T cells
100
75
50
25
0
*
****
***
F.
% CD69+ OT-I T cells
G.
Thy1.1+ CD8 T cells/spleen
10^8
10^7
10^6
10^5
10^4
10^3
**

T cells on day 8 was lower in *P. yoelii* infected mice, although this time point did not prove to be significant (Figure 13F). At day 9, there were similar numbers of CD69+ OT-I T cells which was synonymous with reduced MFI in both groups of mice at the same time point (Figure 13D,F). By day 10, the MFI and percentage of CD69+ OT-I T cells were both extremely low and there were no differences between the groups (Figure 13D,F). From these data it can be concluded that T cell activation is impaired following TCR engagement as shown by the reduction in activation markers.

Costimulatory molecules on dendritic cells in P. yoelii *infected mice are not upregulated following* Listeria *infection*

There have been contradictory studies thus far on how *Plasmodium* species affect dendritic cell function and whether altered antigen presentation could be playing a role in suppression of T cell responses [60]. While reports have demonstrated that DCs are affected by malaria infection in the field [61, 62], there are inconsistent results from mouse models of malaria [63-69]. To address how dendritic cells were being affected by a *P. yoelii* infection, activation of dendritic cells was observed by investigating the level of expression of three costimulatory molecules: CD40, CD80, and CD86. Full activation of T cells is dependent on these costimulatory molecules [70], thus this provided a means to look at DC function.

C57BL/6 mice were infected with 10^5 *P. yoelii* 17XNL pRBCs on day 0. One week later, both naïve and half of the *P. yoelii* infected mice were infected with 5 x 10^6 *actA*-deficient Lm-OVA CFUs. At 24 hours post-Lm infection, DCs were monitored for expression of costimulatory markers.

All three molecules were downregulated following Lm-OVA infection, with CD86 being the most significantly impaired. CD86 exhibited the greatest decrease in expression in *P. yoelii* only infected mice and *P. yoelii* plus Lm-OVA infected mice (Figure 14A). CD40 and CD80 expression were significantly impaired as well, though not to the extent of CD86 (Figure 14B). These data suggest that *Plasmodium* is capable of compromising the activation of dendritic cells by reducing the expression of costimulatory molecules on the DC's surface, thus inhibiting T cell activation as well.

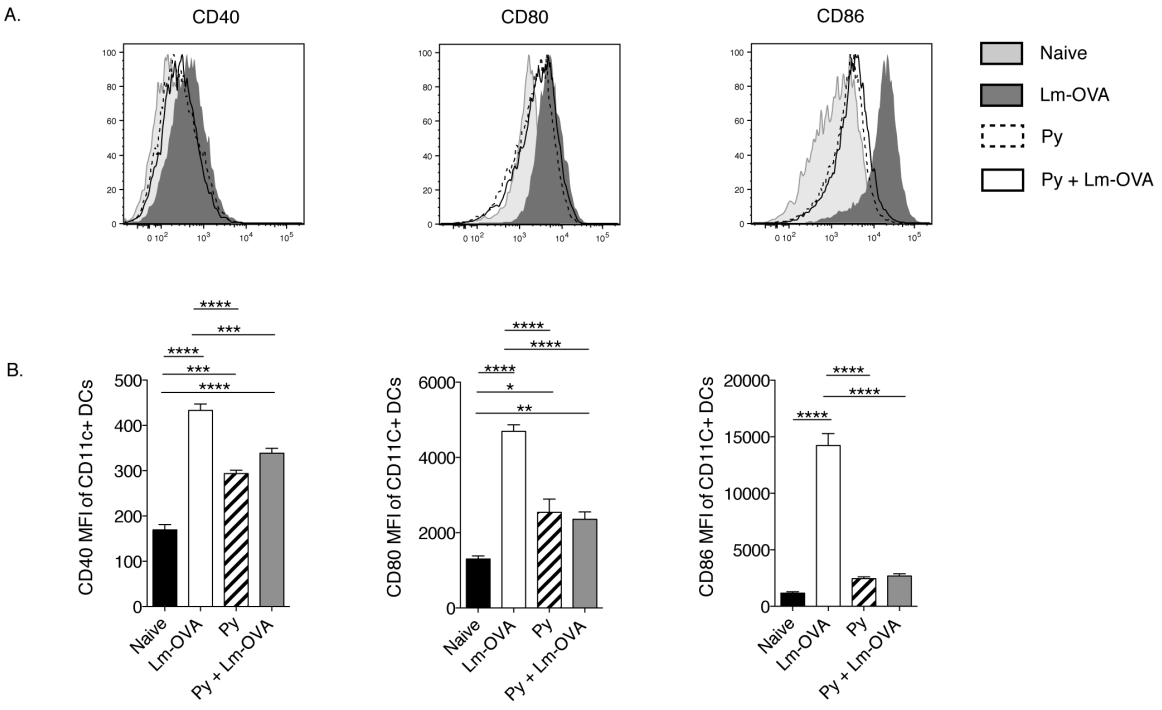


Figure 14. Costimulatory molecules are downregulated on dendritic cells. Mice were infected with 10^5 *P. yoelii* 17XNL pRBC on day 0 and one week later infected with $5x10^6$ *actA*-deficient Lm-OVA CFUs. On day 8, dendritic cells were stained with CD40, CD80, and CD86 (A) and mean fluorescence intensity (MFI) was quantified for each costimulatory molecule (B). Data is representative of two cumulative experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Section IV

Conclusion

The host's immunity to disease has mainly been studied in the context of tracking the immune response to a single infectious pathogen. However individuals are often infected by more than one pathogen. While there have been reports documenting suppression of immune responses following coinfections [27, 32-34, 38, 39, 41, 50, 51, 71-73], the mechanisms and extent to which immunosuppression occurs remains unknown for most pathogens. This lack of information presents a critical knowledge gap that needs to be addressed, especially in malaria endemic regions where coinfections are extremely prominent. Evaluating immunosuppression and its effects will provide much needed information for disease treatment and vaccine delivery in these malaria endemic regions.

To address this problem, an experimental system was devised to examine immunosuppression in heterologous coinfections. By utilizing multiple pathogens (e.g., *Plasmodium*, LCMV, and *Listeria*) the extent of immunosuppression was evaluated in multiple coinfections. The results generated from these experiments demonstrate that immunosuppression is most severe in *P. yoelii* and LCMV-clone 13 infections. This could potentially be due to the magnitude and/or duration of infection with these two pathogens. The data demonstrated that their suppressive effects were greater than that of *P. chabaudi* and LCMV-armstrong,

which were short-lived infections. For example, although *P. chabaudi* exhibited immunosuppression early after infection, it did not occur to the degree of *P. yoelii*. *P. chabaudi* generates a chronic infection but with much lower levels of parasitemia. Parasitemia levels in the *P. chabaudi* only reached 7% (data not shown), while *P. yoelii* reached up to 50% pRBCs. Likewise LCMV-armstrong did not display the same magnitude of immunosuppression as did LCMV-clone 13. LCMV-armstrong results in an acute infection that is cleared from the host within eight days [55]. On the contrary LCMV-clone 13 causes a chronic infection that can result in viremia that persist in some tissues indefinitely, which could perpetuate immunosuppression as shown in these data [55].

Whereas *P. yoelii* and LCMV-clone 13 demonstrated greater levels of immunosuppression, it appeared that wild type *L. monocytogenes* was not immunosuppressive (Figure 7). Although this virulent strain was lethal to one-third of the mice infected (data not shown), immunosuppression did not occur. These data suggest that not all infections suppress T cells responding to heterologous infections, however it is not known what dictates the differential outcome. Importantly, the model system employed here will allow for mechanistic studies to determine why some infections impair T cell responses to subsequent infections while others do not.

These data may also provide information for the treatment of coinfections in *Plasmodium*-infected individuals. Disease treatment and vaccine administration in *Plasmodium*-infected individuals comes into question if

immunosuppression alters the immune response to an infection. Furthermore, if the immune system is incapable of mounting an appropriate response, then administering a vaccine could be dramatically impaired. Indeed, there has been much debate on whether individuals infected with malaria should be given a vaccine if they are already present in the clinic [32, 34, 74]. A major problem in developing countries is getting people into the clinic to actually receive vaccines, therefore some suggest if individuals are already in the clinic then they should be vaccinated regardless of whether they have malaria or not [75]. This is especially relevant in vaccination regimens that require multiple boosters over the course of several months or years. However, it is unknown if there is a correlation between parasite burden and impaired host immunity in humans. Thus determining the mechanism and extent of immunosuppression in humans could provide valuable information that would determine when a vaccine should be administered to someone with malaria.

Previous studies have indicated that *P. falciparum* alters the adaptive immune response, increases susceptibility to heterologous infections, and modulates dendritic cell function [26, 28-33, 37, 39, 44, 48, 63]. However, there are conflicting reports about the mechanism behind immunosuppression, which have primarily focused on dendritic cells using malaria mouse models. Some studies state that dendritic cell cytokine secretion and stimulation of T cells is only altered in lethal infections, while they function normally in nonlethal infections, such as *P. yoelii* 17XNL and *P. chabaudi* AS [65]. Leisewitz *et al*.

(2004) observed the response of the dendritic cell population to a *P. chabaudi* AS infection and demonstrated that costimulatory markers on dendritic cells are upregulated following infection [67]. Other research shows that *Plasmodium* induces systemic maturation of dendritic cells, thereby altering their antigen presentation capabilities and impairing the T cell response [66, 76]. Conversely, other reports have shown that *P. yoelii* 17XNL infections inhibit dendritic cell maturation and reduce the expression of costimulatory markers ([77, 78]). These reports are similar to the findings presented here that a nonlethal strain of *P. yoelii* does alters dendritic cell activation and proper T cell priming and costimulation. However, one important caveat is our study looks at how DCs in *Plasmodium* infected mice respond during subsequent infections, which has not been addressed in prior studies.

When examining the costimulatory molecules on DCs, further evidence was provided that stimulation of the T cells by the DCs is also impaired. These findings are similar to other studies that have shown that DC activation and T cell activation is compromised following a *Plasmodium* infection. Urban *et al*. (1999) show that *Plasmodium falciparum*-infected erythrocytes affect the maturation of dendritic cells and that activation markers on T cells are downregulated [63]. They also demonstrated that costimulatory molecules on DCs exposed to pRBCs are downregulated. This study was the first to demonstrate that DCs could be affected by pRBCs. Similarly, Millington *et al*. (2007) demonstrated that in a *Plasmodium* infection, DCs are capable of eliciting a normal “signal 1” to T cells,

or normal TCR engagement with the MHC I, but impair T cell clustering and priming [69]. However, this study only analyzed CD40 on DCs and did not examine any activation markers on the T cells themselves, therefore activation of the T cells was not assessed.

Examining costimulatory molecules on dendritic cell populations provided evidence that *Plasmodium* induced immunosuppression was not solely intrinsic to T cell activation, but in dendritic cell activation as well. Expression of CD40, CD80, and CD86, all costimulatory molecules, were downregulated 24 hours following a *Listeria* infection (Figure 14). Curiously, the expression of CD40 and CD80, but not CD86, was higher than that on DCs from naïve mice (Figure 14), which suggests that DCs in *Plasmodium* infected mice were unable to further upregulate expression of these costimulatory molecules following infection with *Listeria*.

Although it has been shown that immunosuppression was induced during DC activation, it is not clear what the causative agent is that is inducing immunosuppression and altering the T cell response. Multiple studies have attributed the suppressive effect of *Plasmodium* on DCs to hemozoin, the malaria pigment [64, 68, 79-84]. When a *Plasmodium* parasite invades a red blood cell, it digests hemoglobin, which in turn releases heme. Heme is toxic to the parasite, thus it must convert heme into another compound, hemozoin, in order to promote cell survival, thus promoting its own survival. Research shows that DCs that have phagocytosed pRBCs containing hemozoin demonstrate immunosuppressive

capabilities and diminished T cell activation along with a decrease in phagocytic activity [64, 68]. Although that aspect was not examined in this study, hemozoin is likely to have some effect on antigen presenting cells that have phagocytosed pRBCs and may contribute to the alteration of the immune response to secondary infections.

Upon T cell priming and activation, T cells will upregulate certain activation markers. CD69 is the earliest detectable activation marker following TCR engagement with the major histocompatibility complex (MHC). Following TCR activation, CD69 is the first activation marker to be upregulated and expressed, typically within 3 hours of stimulation [57, 58]. Effective T cell activation also requires costimulation that involves the costimulatory molecules, CD80 and CD86, expressed on the dendritic cell. These costimulatory molecules are capable of ligating receptors CD28 and CTLA-4 on the T cell, however only CD28 ligation induces T cell activation. In contrast, CTLA-4 ligation downregulates the immune response [57, 59]. CD25, another T cell activation marker, is expressed following induction of CD69. CD25 expression is dependent on costimulatory events and is only expressed following costimulation of T cells [59]. Alternatively, other studies have shown that this marker is dependent on “signal 3” of T cell priming, i.e., IL-12 stimulation [85]. Nevertheless, both of these signals occur downstream of TCR engagement, thus T cell activation must be compromised following TCR engagement as shown here by a reduction in CD25 and CD69 expression (Figure 13).

It is also possible that T cell suppression is not due to a sole contributing factor, but a network of components that impair T cell activation and dendritic cell activity. Several studies have attributed LCMV's suppressive capabilities to the secretion of IL-10, an anti-inflammatory cytokine, by immunoregulatory antigen presenting cells [86-89]. These studies demonstrate that dendritic cells specific to LCMV secrete IL-10, thus downregulating the immune response and perpetuating the viral infection [86-89]. Wilson *et al*. (2012) showed that during a chronic LCMV infection immunoregulatory APCs (iAPCs) are expanded and sustained, and they contribute to diminishing the antiviral T cell response [89]. These iAPCs were the major source of IL-10 during persistent viral infection and simultaneously expressed multiple immunosuppressive molecules (PDL1, PDL2, and IDO), while downregulating T cell stimulatory factors, such as IL-12. Therefore, these cell types more than likely play a role in diminishing the antiviral T cell response to prevent immunopathology during a persistent infection. On the contrary, an acute infection induces the expansion of immunoregulatory APCs but this population rapidly declines [89]. Although not examined in this study, these observations suggest that *Plasmodium* may impair coinfection-specific T cells through elevated production of IL-10 and immunoregulatory APCs. Further investigation is needed in order to determine whether immunosuppression during these two types of infections are similar or are occurring via distinct regulatory pathways.

It has been shown here that immunosuppression is not intrinsic to only dendritic cells, but in the activation of T cells as well. Consequently, these immunosuppressive events have been deemed responsible for the reduced expansion of CD8 and CD4 T cells responding to a secondary infection. This study has provided vital information that furthers the understanding of how *Plasmodium* impairs host immune responses during heterologous infections.

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Vita

Chelsi Elizabeth White was born on April 15, 1988 in Ripley, Tennessee. She graduated from the University of Tennessee with a Bachelor of Science in Microbiology and Ecology & Evolutionary Biology. She is currently completing her Master of Science degree in Microbiology at the University of Tennessee.