Investigation of Acute Neutrophil Response in Malaria Infection and Characterization of the CS Tag

by
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Abstract

Much progress has been made in malaria research and malaria-related mortality has been declining throughout the years, yet malaria remains to be a global threat given the lack of an effective vaccine and spread of reoccurring drug-resistant parasites. Hence, better understanding of how malaria infection progresses and how human immunity response combats infection is paramount in achieving malaria elimination. This thesis has been divided into two parts. One is to explore how neutrophil infiltration impacts the earliest phase of *Plasmodium* infection. Although the role of neutrophils combating different pathogens has been investigated extensively, no study has explored their roles in *Plasmodium* sporozoites in the skin phase, which is critical in understanding the innate immune response against malaria infection in the pre-erythrocytic stage. We used topical application of arachidonic acid to illicit a more rapid neutrophil infiltration in mouse ears prior to sporozoite inoculation by mosquito bite. We found that arachidonic acid treated mice showed decreased susceptibility to sporozoite infection as measured by parasite liver load. Furthermore, intravital imaging with LysM-EGFP transgenic mice expressing enhanced green fluorescent protein (EGFP) in neutrophils and monocytes was also used to observe the influx of EGFP+ cells. The other part of the thesis investigates whether a tag composed of a portion of the circumsporozoite protein can be used as a more sensitive and specific protein tag. Many commonly used protein tags, such as His (HHHHHHHH), HA (YPYDVPDYA), FLAG (DYKDDDDK), and Myc (EQKLISLEEDL) tags, provide satisfactory results but unable to reach the required sensitivity and specificity for better work flow. Hence, a novel CS tag with potentially better sensitivity and specificity can benefit greatly in the biotechnology field.

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INTRODUCTION

Malaria is an ancient infectious disease that dates back to between 3200 and 1304 B.C. in which malaria antigens were found in ancient Egyptian mummies by using the dual antibody immunoassay, ParaSight-F test (Miller et al.1994). Possibly originating from Africa or Southeast Asia, malaria has spread across the globe in the nineteenth century, reaching throughout North America, Europe, Africa, and Asia (Carter and Kamini 2002; Schlagenhauf 2004). With extensive efforts in malaria research and advances in public health measures, the mortality related to malaria has declined. (Oaks et al.1991). According to the World Malaria Report 2015, both numbers of malaria cases and malaria deaths in 2015 have decreased substantially, recording a decline of 18% and 48% respectively since 2000. Most cases and deaths are currently concentrated in sub-Saharan Africa and Southeast Asia. Despite efforts of developing novel prophylactic regimes and implementing vector control measures, there are still 214 million estimated malaria cases and 438,000 estimated deaths recorded in 2015 (WHO 2015). Furthermore, problems of drug-resistant parasites, insecticide-resistant mosquitos, and lack of effective malaria vaccines remain to be formidable hurdles toward malaria elimination. (Arrow, Panosian, and Gelband 2004)

The *Plasmodium* Life Cycle

The *Plasmodium* life cycle is complicated as it involves the vector, where sexual cycle occurs, and the human host, where asexual cycle takes place. Among all *Plasmodium* species, five species – *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* – are known to

infect humans. It is known that *Plasmodium falciparum* constitutes the most deaths from malaria in the world and is the most prominent malaria species in Africa (Ibid.).

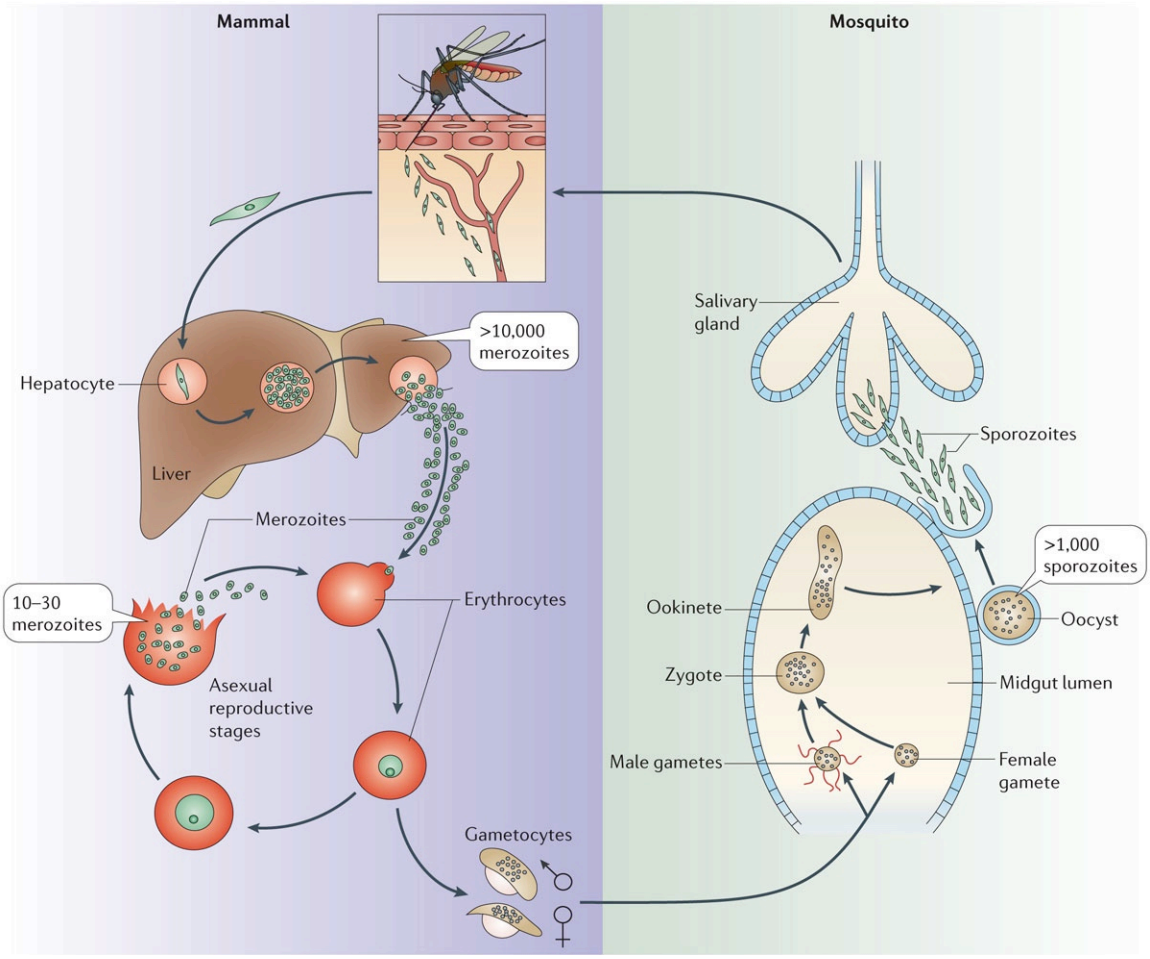


Figure 1. The *Plasmodium* life cycle
The life cycle of *Plasmodium* parasite infecting a mammalian host. The cycle begins with the skin phase, followed by sporozoites reaching the liver and invading the hepatocytes. There, they generate thousands hepatic merozoites, which can later enter the bloodstream and invade the erythrocytes. Multiplication in the erythrocytes results in symptoms of malaria. Later, intra-erythrocytic parasites form male or female gametocytes, which can taken up by a mosquito during blood meal and later fuse together and form zygotes. Zygotes then develop into oocysts, which contain thousands of sporozoites. Sporozoites are then released into the body cavity of the mosquito and enter the salivary ducts.
Source: Ménard et al. 2013

The life cycle begins with a female *Anopheles* mosquito taking a blood meal, ingesting gametocytes from the blood circulation of the infected human host as shown in Figure 1. Male and female gametocytes then fuse together and form zygotes, which later

develop into invasive ookinetes, transversing the midgut and transforming into oocysts (Kappe, Buscaglia, and Nussenzweig 2004; Cowman and Crabb 2006). A mature oocyst contains thousands of sporozoites, which leave the oocysts and enter the hemolymph, invading the salivary glands and remaining there until they are injected into the mammalian host skin during a blood meal (Sinnis and Zavala 2008). As the mosquito probes, it introduces its saliva and sporozoites from the salivary glands into the skin (Ibid.). Instead of being injected directly to the blood vessels, most sporozoites initially reside in the skin for a period of time (Matsuoka et al. 2002; Medica and Sinnis 2005; Yamauchi et al. 2007). It is known that a single mosquito can introduce few hundreds of sporozoites into the skin during a blood meal (Jin, Kebaier, and Vandergerg 2007), yet the number of sporozoites inoculated can be largely variable among mosquito bites (Medica and Sinnis 2005). It has been shown that a single mosquito infected with *Plasmodium yoelii* inoculates a median of 18 sporozoites and a mean of 123 sporozoites, ranging from 0 to 1,297 sporozoites (Ibid.). During this skin phase, the sporozoites use gliding motility and randomly navigate in the skin until they have reached a blood vessel (Sinnis and Coppi 2007; Hopp and Sinnis 2015). After the sporozoites reach and penetrate the blood vessels, the blood circulation carries them to the liver. The spozoroites are arrested once they reach the liver, likely via the binding of the sporozoite's major surface protein, the circumsporozoite protein (CSP), and the heparan sulfate proteoglycans (HSPGs) of the liver (Cerami et al. 1992; Frevert et al. 1993; Pinzon-Ortiz et al. 2001; Pradel, Garapaty, and Frevert 2002; Sinnis and Coppi 2007).

In the liver, sporozoites develop into exo-erythroctic forms, which grow and differentiate into thousands of hepatic merozoites (Kappe, Buscaglia, and Nussenzweig

2004). When the infected hepatocytes rupture, these merozoites are then released and invade the erythrocytes, beginning the symptomatic blood stage infection where they progress to ring, trophozoite, and schizont stages (Cowman and Crabb 2006; Prudêncio, Rodriguez, and Mota 2006). The free merozoites can invade other erythrocytes to carry on with the asexual blood stage life cycle, which is responsible for all clinical symptoms (Cowman and Crabb 2006). In the blood, intra-erythrocytic merozoites develop into male and female gametocytes that can be taken up by the mosquitoes during blood meal and fuse to form zygotes (Kappe, Buscaglia, and Nussenzweig 2004; Ibid.), returning back to the beginning of the life cycle.

Immune System and Neutrophil Infiltration Response to *Plasmodium* Sporozoites in the Skin

Understanding the underlying mechanism of protective immunity and the role of innate immune response against *Plasmodium* sporozoites in the skin would be helpful in malaria vaccine design. The skin is the largest human organ that serves as the first defense line against foreign pathogens and toxic subtances (Nestle et al. 2009; Grice and Segre 2011). The human skin is composed of epidermis, followed by dermis and subcutaneous tissue (Heath and Carbone 2013). The epidermis largely consists of keratinocytes, which comprise more than 90% of the epidermal cell population (Nestle et al. 2009). Underneath this, the dermis layer is relatively more loosely packed (Heath and Carbone 2013), consisting of elastin fibers and collagen, the dermis is interspersed with a network of capillary beds and lymphatic vessels, establishing a network that allows immune cells to transverse in and out of the dermis layer (Salmon 1994; Ibid.; Hopp and Sinnis 2015). The dermis contains most of the lymphocytes, about 90% of cutaneous T lymphocytes, and other leukocytes such as macrophages, mast cells, innate lymphoid

cells, different dendritic cells subsets, and T cells, which include memory αβ T cells, γδ T cells, natural killer T cells, and CD4+ type 1, type 2, type 17 helper T cells (Macleod and Havran 2011; Heath and Carbone 2013; Hopp and Sinnis 2015). Studies have suggested that innate immunity response has the potential to provide protection from malaria (Stevenson and Riley 2004). Given that the skin is a crucial barrier for sporozoites in which even at optimal conditions, over 50% of sporozoites remain at the inoculation site (Sinnis and Zavala 2012), this window of opportunity could allow innate immune cells to provide an effective first line defense against malaria.

The process of mosquito probing produces acute inflammation in the skin, resulting recruitment of immune cells to the area of the bite site (Kolaczkowska and Kubes 2013). Neutrophils are believed to be one of the first immune cells recruited to inflammatory sites, and are known to kill pathogens through phagocytosis, neutrophil extracellular traps (NETs), and release of proteases as well as other toxic compounds (Ibid.; Sinnis Lab unpublished data). Although the importance of neutrophils for combating pathogens has been previously researched, their potential role in fighting off *Plasmodium* sporozoites is yet to be explored.

The process of neutrophil recruitment from the blood to the infected tissue site in other infections is known to involve several steps: inflammatory signals lead to changes in the endothelial cells that in turn lead to adhesion and transmigration of circulation neutrophils (Figure 2 and Kolaczkowska and Kubes 2013). One critical event for neutrophil recruitment is the upregulation of adhesion molecules, such as P-selectin, which is pre-stored in Weibel-Palade bodies, and E-selectin, which is synthesized *de novo*, activated by the pattern-recognition receptor-mediated detection of pathogens in

the endothelial cells (Ley 2007, Petri 2008, Ibid.). Both of these selectins maximize neutrophil recruitment by providing glycosylated ligands on the endothelial surface, allowing tethering of the free-flowing neutrophils from the blood to the endothelial surface as well as neutrophil rolling along the blood vessel in the blood flow direction (Ley et al. 2007; Zarbock et al. 2011; Kolaczkowska and Kubes 2013). With the help of integrins, cell adhesion molecules, junctional adhesion proteins, and epithelial cell adhesion molecule, neutrophils then transmigrate across the endothelium and enter into the tissue (Ley et al. 2007; Phillipson and Kubes 2011; Kolaczkowska and Kubes2013).

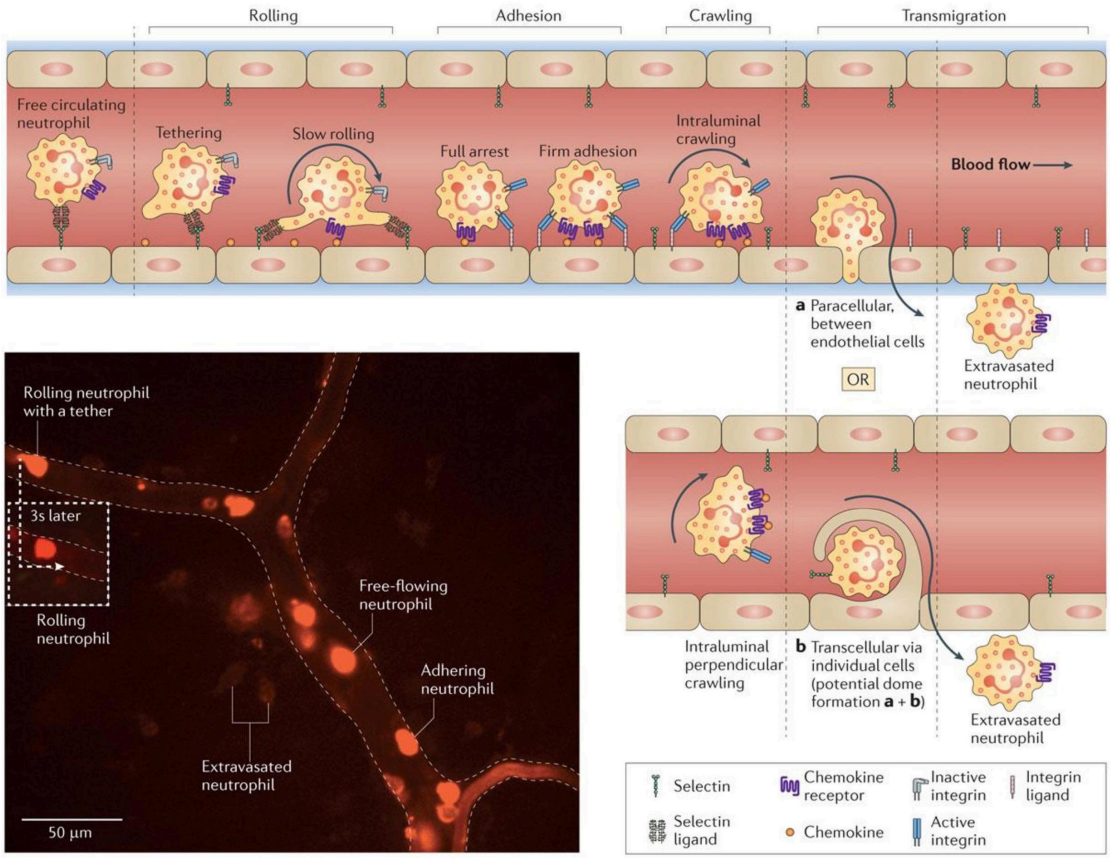


Figure 2. The process of neutrophil recruitment cascade
The process of neutrophil recruitment from the blood to the infected tissue site involves in several steps – tethering, rolling, adhesion, crawling, and transmigration. Upregulation of adhesion molecules, such as P-selectin and E-selectin, play an important role in neutrophil recruitment.
Source: Kolaczkowska and Kubes 2013

Although the impact of neutrophils on *Plasmodium* sporozoites in the skin and subsequent liver infection is not fully understood, their role in another a parasitic disease, *Leishmania*, another protozoan parasite, has been studied (Peters et al. 2008; de Almeida et al. 2003). *Leishmania* is transmitted by sand flies (de Almeida et al. 2003). During biting, the sand fly proboscis causes laceration of the blood vessels, creating hemorrhagic pools that they feed on (Ribeiro 1987). Parallel to malaria infection, such hemorrhage produces local inflammation response (Kamhawi et al. 2000; Belkaid et al. 2000). As shown in Figure 2A and 2B, significant neutrophil infiltration and macrophage recruitment were seen in the skin after sand fly bite (Peters et al. 2008). By intravital imaging, it was also found that neutrophils began to accumulate into the skin and localize around the bite site as early as 30 seconds after sand fly bite (Ibid.). Interestingly, as shown in Figure 3C-F, neutrophils rapidly accumulate around the bite site by both infected and uninfected sand flies after an hour, suggesting that the potential trigger of rapid neutrophil infiltration is the salivary contents and/or physical damage of the skin (Ibid.). Remarkably, neutrophil depletion reduced the ability of parasites to develop infection, which leads to the hypothesis that *Leishmania* parasites evolved to exploit the host innate response to promote infection.

The research in *Leishmania* parasites provides a valuable framework on which to speculate the causes and potential role of neutrophil infiltration for malaria infection. Certainly the physiology of *Leishmania* parasites and *Plasmodium* sporozoites are not the same since *Leishmania* parasites remain in the skin whereas *Plasmodium* sporozoites exit the dermal inoculation site. Furthermore, there are significant differences in the nature of wounding at the insect bite site between *Leishmania* and *Plasmodium*. Nonetheless, these

Leishmania studies can still provide valuable clues for understanding the neutrophil infiltration at the mosquito bite site and its subsequent impact on malaria infection.

Metabolic Pathways of Arachidonic Acid in Inflammation and Neutrophil Recruitment

A common method to study neutrophil infiltration in the skin involves topical application of the polyunsaturated fatty acid arachidonic acid (AA), which is known to induce an acute immune response (Needleman et al. 1986). AA is metabolized via two major pathways: the cyclo-oxygenase (COX) and the lipoxygenase pathway (Figure 3; Malmsten 1986). The COX pathway involves a two-step reaction catalyzed by prostaglandin H (PGH) synthases 1 and 2, converting AA to prostaglandins, such as PGE_2 and prostacyclin (PGI_2) (Samuelsson 1991; Rocha, Plumb, and Coffman 2003). The lipoxygenase pathway involves converting AA into leukotriene by 5-lipoxygenase, followed by the conversion into leukotriene A_4 (LTA_4) (Samuelsson 1991). LTA_4 hydrolase and LTC_4 synthetase can then catalyze further reactions, in which LTA_4 can be hydrolyzed to form LTB_4 or conjugate with glutathione and form LTC_4, an intermediate for LTD_4 (Rocha, Plumb, and Coffman 2003). Importantly, it has been discovered that PGE_2, LTC_4, and LTD_4 are all mediators of inflammation (Bach 1982; Opas, Bonney, and Humes 1985). Furthermore, LTC_4, and LTD_4 were found to increase vascular permeability (Samuelsson 1991). PGE_2, LTC_4, and LTD_4 have all been implicated in edema through their synergistic effects on the blood vessels (Opas, Bonney, and Humes 1985).

One of the major players in neutrophil infiltration is LTB_4, which was found to cause an acute amplification of local cell death signals, which augments neutrophil

recruitment (Lämmermann et al. 2013). In addition to the role of inducing chemotaxis, LTB_4 is also found to activate integrin receptors and regulate the neutrophil adhesiveness (Ford-Hutchison et al. 1980; Palmblad et al. 1981). Furthermore, LTB_4 can serve as a critical signal for intercellular communication between neutrophils, inducing a rapid neutrophil recruitment response through the tissue (Lämmermann et al. 2013). Hence the mechanism of AA induction of neutrophil infiltration has been largely elucidated, making topical application of AA a reliable and valuable tool to study neutrophil in filtration in the laboratory.

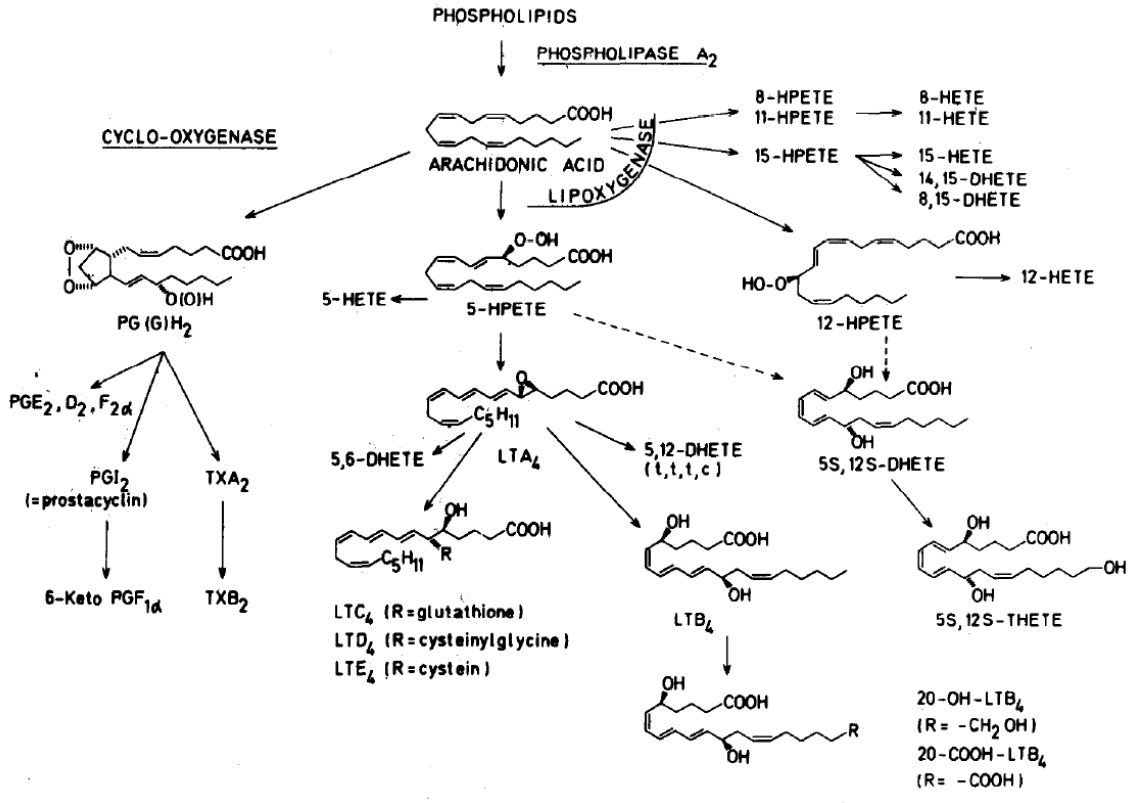


Figure 3. The metabolic pathways of arachidonic acid
Major pathways of arachidonic acid metabolism are depicted here. LTB_4 is known to play a major role in neutrophil infiltration.
Source: Malmsten 1986

MATERIALS AND METHODS

Mosquito Rearing

Anopheles stephensi mosquitos infected with WT *Plasmodium berghei* parasites were reared and maintained at the Johns Hopkins Insectary Core Facility at 27°C and 80% humidity. Adult mosquitoes were allowed to feed on Swiss Webster mice infected with *Plasmodium berghei*. During the progression of parasite development, the mosquitoes were maintained on 10% sucrose. All experiments were performed using parasites from mosquitoes between 19 and 22 days post infection.

Source of Mice

C57BL/6 LysM-EGFP mice, expressing enhanced green fluorescent protein (EGFP) in neutrophils and monocytes under the control of endogenous murine lysozyme M promoter (LysM-EGFP), were used for intravital imaging (Faust et al. 2000). Female C57BL/6 mice were purchased from Taconic Laboratory. All mice were housed in the animal facility at the Johns Hopkins Bloomberg School of Public Health and 5 to 11 week old mice were used for experiments. For each experiment, the same age, batch, and breed of mice were used. All mouse work was done in accordance with the recommendations listed in the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals. Animal protocol was approved by the Johns Hopkins University Animal Care and Use Committee (Protocol #M014H363), which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Topical Application of Arachidonic Acid

Arachidonic acid (AA), ≥98% purity in liquid, was purchased from MP Biomedicals (Product number: 02150384). AA was protected from light and stored at -20°C in container filled with desiccants to keep dry. AA was freshly prepared by making a final concentration of 400 mg/ml in acetone as vehicle (4mg AA in 10ul volume) or a final concentration of 200 mg/ml in acetone as vehicle (2mg AA in 10ul volume). Based on previous studies, 4mg of AA was determined to be the optimal dose level to induce adequate neutrophil infiltration without introducing excessive edema (Carlson et al. 1985; Doherty et al. 1988). Hence only 4mg of AA/ear was tested in all experiments. For all experiments, 4mg of AA per ear was applied to mice that had been anesthetized with ketamine (70-175 mg/kg body weight) and xylazine (6-15 mg/kg body weight). Depending on the experiment, topical application of AA was done either by applying 4mg of AA in 10 microliters of acetone to the ventral side of each ear or 2mg of AA in 10 microliters of acetone to each side of the ear. Maximal care was taken to distribute the AA agent by pipetting evenly onto the ear pinnae and to minimize any spreading of the applied solution into the inner ear or onto the head of the mice.

Edema Measurements of Mouse Ears

At specific time points after AA topical application of C57BL/6 mouse ears, while mice were under anesthesia, the thickness of each ear was measured at the middle of the pinna using a digital fractional caliper with a rated accuracy of 0.02mm. Following this, mice were sacrificed by cervical dislocation to minimize any suffering inflicted

when ear biopsy was performed. A 6 mm-diameter disc of ear was removed with a metal biopsy punch and weighed.

Spinning-Disk Confocal Microscopy of Arachidonic Acid Treated Mouse Ears

C57BL/6 LysM-EGFP knock-in mice were anesthetized with ketamine and xylazine and placed in a lateral recumbent position on an imaging platform, allowing the ventral side of the ear pinna to be placed on a coverslip. A strip of surgical tap was placed gently over the side of ear to attach it to the coverslip and another strip of tape was used to secure the mouse to the microscope platform. Maximal care was taken to minimize pressure on the mouse ear. All images were captured towards the top half the ear pinna where hair follicles are relatively sparse.

Images were acquired using the enhanced 3i Marianis/ Yokogawa Spinning Disk Confocal (Zeiss AxioObserver.Z1 inverted microscope) enclosed in a temperature/ humidity/ CO2 regulated chamber that was maintained at 35-37°C. In some experiments, mice were maintained at 35-37°C on a slide warmer prior to imaging to prevent significant drop of body temperature due to anesthesia. All images were acquired using the 10x air objective with channel set as 1. The lazar with wavelength at 488 nm was used for EGFP excitation. The exposure time was set at 35 ms. The gain was set to 3 and the intensification was set to 219. The neutral density filter, which controls the lazar light intensity, was set to 100. Three-dimensional stacks of 20 to 30 microns were captured with step size of 2-3μm with 10 planes for each Z stack image. Images were taken in dimensions of 408X512X10 in one experiment set or 416X512X10 in the other experiment set, representing the number of pixels across X axis, Y axis, and the number of planes. SlideBook software was used to create “Maximum Z projection” for all

images. Then the images were exported to 16-bit TIFF files, which were used specifically for fluorescence quantification purposes. Lastly, Fiji software was used to measure the mean fluorescent intensities covering the entire area of each image.

Mosquito Feeding on Arachidonic Acid Treated Mice

For routine monitoring of mosquito infections, either detection of midgut oocysts or salivary gland squashes for detecting sporozoite was performed prior to mosquito bite challenge experiments, except the first experiment. More specifically, for detection of oocysts in midguts, eleven days after receiving an infected blood meal, midguts were dissected from 20 female mosquitoes and all midguts were checked for the presence of oocysts by phase-contrast microscopy. For salivary gland squashes, salivary glands were dissected on 20 female mosquitos and squashed eighteen days after receiving an infected blood meal. Number of salivary glands that contained free sporozoites was recorded by phase-contrast microscopy. The proportion of mosquitos infected within a cage was defined by having at least one oocyst in the midgut or high salivary gland sporozoite load. These oocysts and sporozoite numbers served as rough estimation of the infection prevalence and intensity of each cage. Experiments were performed using mosquito cages that at least had 70% infection prevalence. With respect to the intensity of infection, sporozoite counts per mosquito were recorded by dissecting salivary glands from twenty female mosquitos on the day of mosquito challenge.

One day prior to mosquito bite challenge, female *Anopheles stephensi* mosquitos were anesthetized on ice and sorted into individual plastic feeder tubes. Different number of mosquitos were placed into each feeder tube, ranging from one, two, or even five

mosquitos per tube to achieve the desired number of mosquito bites for each mouse. All tubes were then covered with fine mesh netting over the tube opening. The mosquitoes were then returned to the incubator and were deprived of sugar overnight to encourage biting on the day of the experiment.

On the day of the experiment, the water soaked cotton pads were removed 5-6.5 hours prior to mosquito bite challenge. Mice were anesthetized and the starved mosquito(s) were placed onto the ventral side of the ear that had previously been treated with AA. The mice were placed in a lateral recumbent position on the slide warmer that was maintained at 35-37°C. Special care was taken to position the feeder tube in a way that minimizes any space between the mesh netting and mouse ear to allow easy access for mosquitos to probe on the skin. The probing events were carefully observed and number of mosquitos that took a blood meal on the mouse ears was recorded. The number of mosquitos that probed on the ear varied by each experiment and is listed in detail in Table 1. The success of the blood meal was determined by observing the mosquitos moving their proboscis in and out of the ear surface as well as the engorgement and/or redness of the mosquitoes' abdomen. The timing of the probing event was not strictly regulated but ranged from approximately 15-20 minutes per ear. If the mosquitoes did not probe for couple of minutes, new batch of mosquitos was used to ensure successful feeding took place.

Quantification of Mouse Liver Stage Parasite Burden

Following infection by mosquito bite challenge as described above, mice were anesthetized and livers were harvested at 38.5-39 hours post-infection. Total RNA was

isolated from livers using Tri-reagent (Molecular Research Center). The liver parasite burden was then quantified by using reverse transcription (RT), which was performed by using 1μg of total RNA and random hexamers, followed by real-time polymerase chain reaction (qPCR) that was described in Bruña-Romero et al. (Bruña-Romero et al., 2001). Primers that recognize *Plasmodium berghei* specific sequences within the 18S rRNA (forward primer: 5'-GGAGATTGGTTTTGACGTTTATGTG-3" and reverse primer: 5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3') were used together with Applied Biosystems SYBR Green PCR Mastermix (ThermoFisher Scientific). The cycling profile was 95°C for 15 minutes followed by 40 cycles of 95°C, 30 seconds; 58°C, 30 seconds; 72°C, 30 seconds. The profile for the melt curve was 95°C, 15 seconds; 60°C, 60 seconds; 95°C, 15 seconds. Ten-fold dilutions of a plasmid construct containing *Plasmodium berghei* 18S rRNA were used to create the standard curve.

Statistical Analysis

To determine whether significant differences in liver stage burden appear between AA treated and untreated mice, unpaired Mann-Whitney test was used, ranking all data values from low to high, with a statistical significance cutoff set at two-tailed $p < 0.05$. All statistical tests were computed with GraphPad Prism.

RESULTS

Rapid Neutrophil Infiltration is Induced by Arachidonic Acid in a Time Dependent Manner

Neutrophils are recognized as one of the first responders to pathogens at inflammatory sites (Kolaczkowska and Kubes 2013) and their role in *Leishmania* infection has been well studied. However in contrast to *Leishmania* parasites, *Plasmodium* sporozoites are more motile and actively search for blood and lymphatic vessels. Although we acknowledge that neutrophils are "quick" responders to pathogens, our unpublished data that neutrophils typically have a negligible effect on *Plasmodium* sporozoite infection. We previously showed that in both *Plasmodium berghei* and *Plasmodium yoelii* sporozoite liver burdens of untreated and neutrophil-depleted mice showed insignificant differences (Sinnis Lab unpublished data). Thus, we hypothesized that motile sporozoites have already left the skin prior to the peak of neutrophil infiltration. To test this, we attracted neutrophils to the bite site prior to inoculation of sporozoites and determine whether in this case they had an impact on malaria infection. We used topical arachidonic acid treatment to stimulate neutrophil infiltration at the bite site prior to *Plasmodium* sporozoite infection by mosquito bite.

We first used intravital imaging with LysM-EGFP transgenic mice, which express enhanced green fluorescent protein (EGFP) in neutrophils and monocytes (Peters et al. 2008) to observe the influx of EGFP+ cells at different time points following topical treatment of AA to the ear. As shown in Figure 4, aggregation of EGFP+ cells was observed by two hours after AA application and the signal progressively increased over time. In contrast, the vehicle-alone treated control mice showed significantly lower numbers of EGFP+ cells. Quantification of these results are shown in Figure 5.

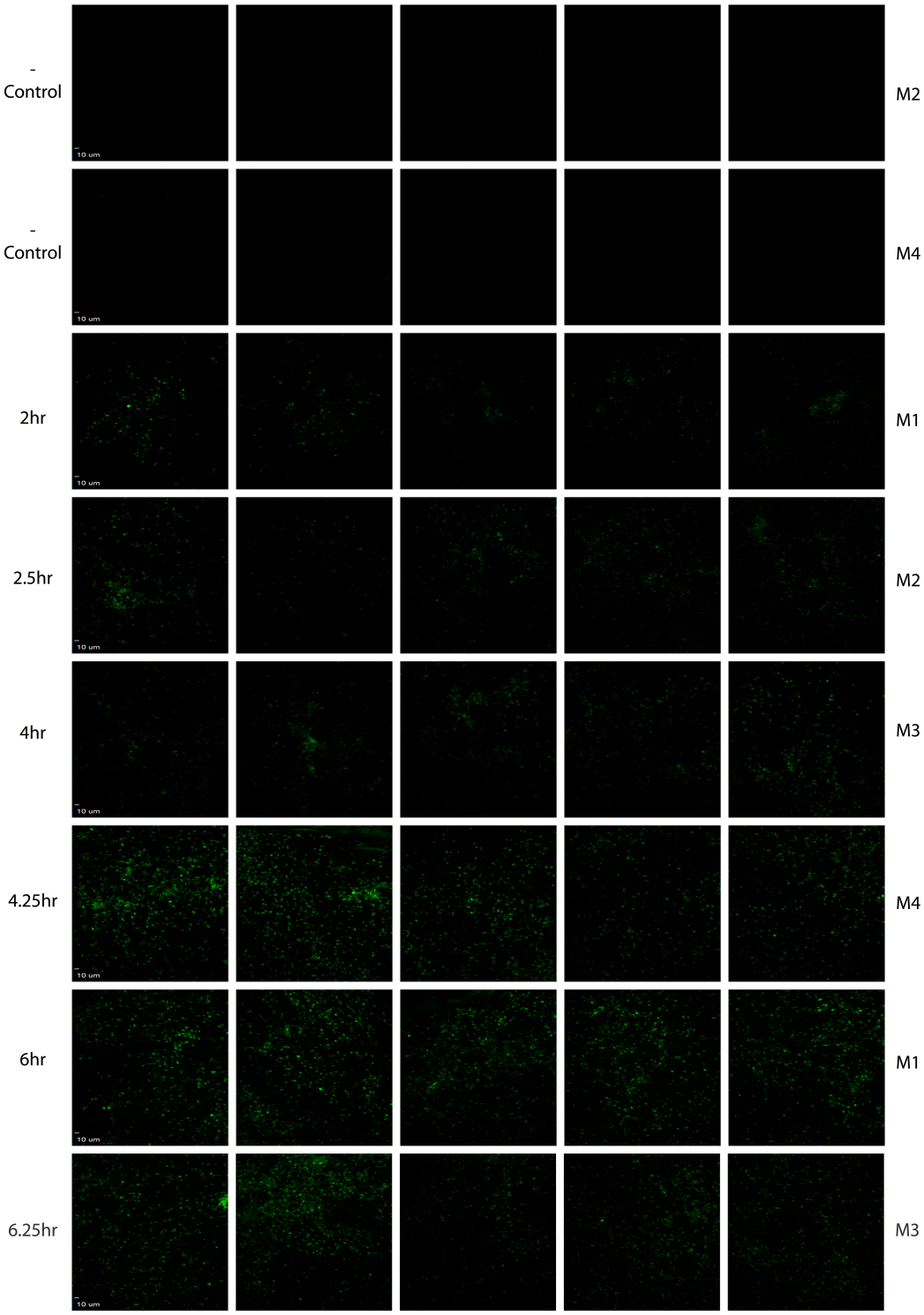


Figure 4. Increasing neutrophil recruitment after arachidonic acid treatment at successive time points.

Maximum intensity projection images across Z dimensions derived from 3i spinning disk confocal microscopy of the ears of LysM-EGFP mice (green) at successive time points after AA treatment starting from 2 hours (third row) to 6.25 hours (bottom row) post treatment. Each row represents 5 images for each specific timepoint that were taken on the same ear from one experiment. M1, M2, M3, M4 (shown in the right) are abbreviations for mouse 1, 2, 3, and 4. For the - control, images of ears of Lysm-EGFP mice treated with acetone taken at >6.5 hours post-treatment are shown in the first two rows. Scale bars; 10 μm.

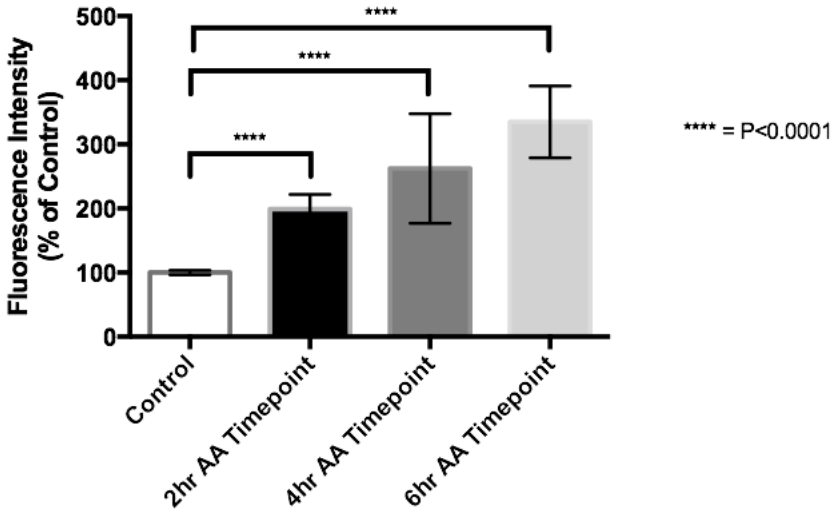


Figure 5. Increasing infiltration of EGFP+ cells after arachidonic acid treatment over time.

Average fluorescence intensities of the entire area of each image were measured. Total of 10 images (5 images for each mouse, 2 mice for each timepoint) are included for each timepoint. Data are expressed as percent mean fluorescence intensity relative to control. Values are mean ± SD. Two-tailed unpaired t test was used to assess the statistical significance between groups. Significant differences are denoted by symbol: p≤ 0.0001.

Arachidonic Acid Treatment Induces Edema in the Mouse Ear

Topical application of arachidonic acid on mouse ears was previously studied by Opas et al., who suggested that AA induces synthesis of leukotrienes C_4, D_4, and prostaglandin E_2, which are mediators of inflammation cause both edema and increased vascular permeability (Opas, Bonney, and Humes 1985). To investigate whether topical application of AA may create excessive edema to the point where it inhibited mosquito probing or parsite motility, we made observations and quantitive measurements of edema in the mouse ear. Qualitative observation during the mosquito challenges described later suggested that AA did not deter mosquitos from probing the ear since mosquitos were observed to insert their proboscis into the AA treated mouse ears normally. We suspected that the treatment of AA did not seem to affect the mosquitos from probing but mosquitos were observed to be cleaning their proboscises after blood meal, which was not the case in acetone treated ears. Furthermore, it was observed that mosquitos in AA group took a little longer to probe than those in control group. For experiment 1-4, time duration for mosquitoes to probe usually lied between 7-10 minutes/ mouse ear in all control groups and 10 – 15 minutes/ mouse ear in AA groups (few took longer 20 minutes/ mouse ear in AA groups). In experiment 5, mosquitoes were observed to be somehow lethargic in the AA group, even before the mosquito bites. Hence, more mosquito tubes were used to acquire the wanted number of mosquitoes probed, making the probing time longer than usual. The time duration for mosquitoes to probe in all five independent experiments are shown in Figure 6.

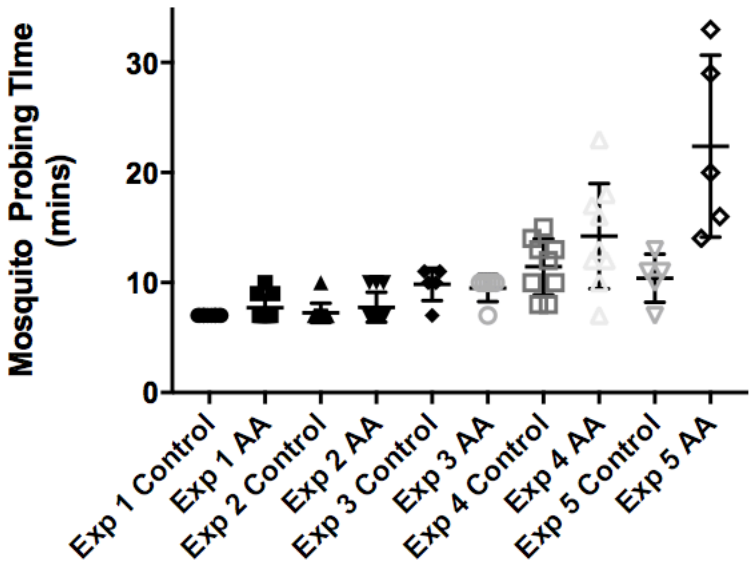
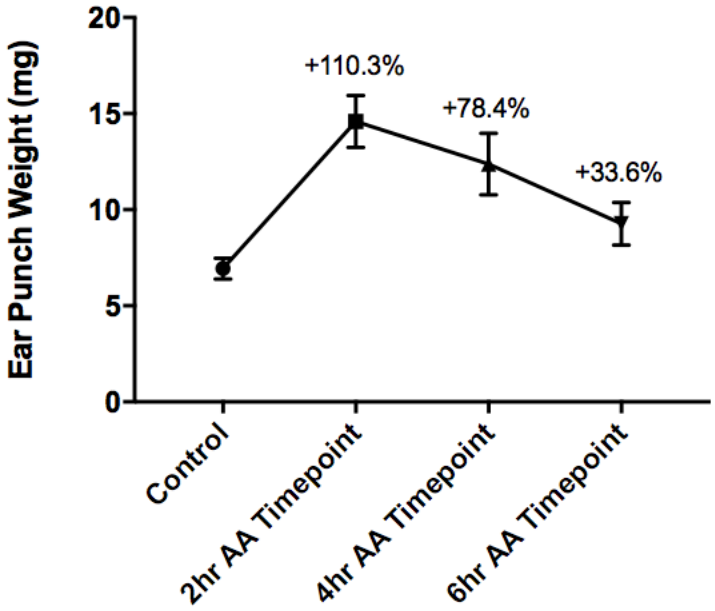


Figure 6. Mosquito Probing Time
This graph shows the mosquito probing time for all five independent experiments. All controls were mice treated with acetone alone. Values are mean ± SD.

To more quantitatively study the potential changes by AA treatment, we measured edema by examining changes in ear punch weight and thickness. 2mg AA was topically applied to each side of the ear of C57BL/6 mice and at designated time points, mice were sacrificed by cervical dislocation and the thickness of each ear was measured using a caliper and the weight of 6 mm-diameter disc of ear tissue was recorded. Data shown in Figure 7 shows that topical application of AA increased edema in mouse ears. The edema was maximum at 2 hour after treatment and later decreased in successive timepoints. Nonetheless, Figure 8 demonstrates that AA treatment did not increase ear thickness markedly, as no clear linear relationship was observed between ear thickness and time. At no time point were mouse ears observed to have signficant observable changes in appearance. Thus although the possbility of edema influencing sporozoite motility could

not be excluded, with these results, we decided to test liver burden in mice treated 3 hours after AA treatment in later experiments.

Experiment 1; 2+2mg AA:



Experiment 2; 4mg AA:

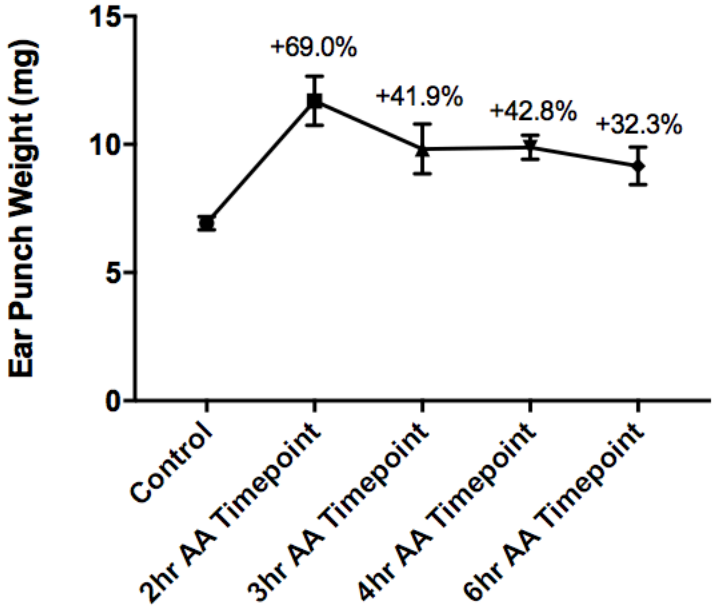
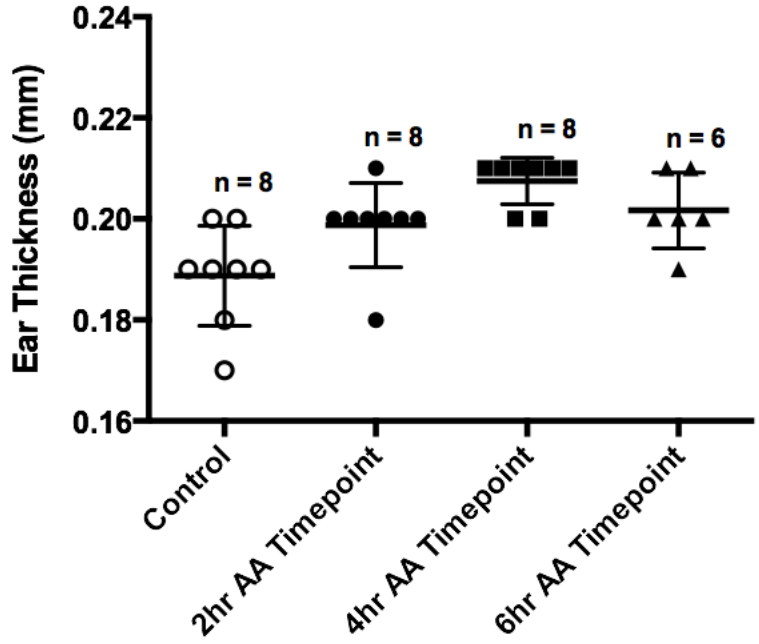


Figure 7. Increased edema in response to AA at different time points.
The top graph shows each point representing the mean of 8 ear punch biopsy measurements and the point at 6 hour post-treatment represents the mean of 6 ear punch biopsy measurements. Application of 4mg AA in 10ul acetone to just the ventral side of each ear is denoted as 4mg AA. The bottom graph shows each point representing the mean of 8 ear punch biopsy measurements. Percentage values listed above the data points represent the percent increase in ear biopsy punch weights between AA treated groups and vehicle alone controls. Application of 2mg of AA in 10ul acetone to each side of the ear is denoted as 2+2mg AA. All controls in both independent experiments were mice treated with acetone alone. Values are mean ± SD.

Experiment 1; 2+2mg AA:



Experiment 2; 4mg AA:

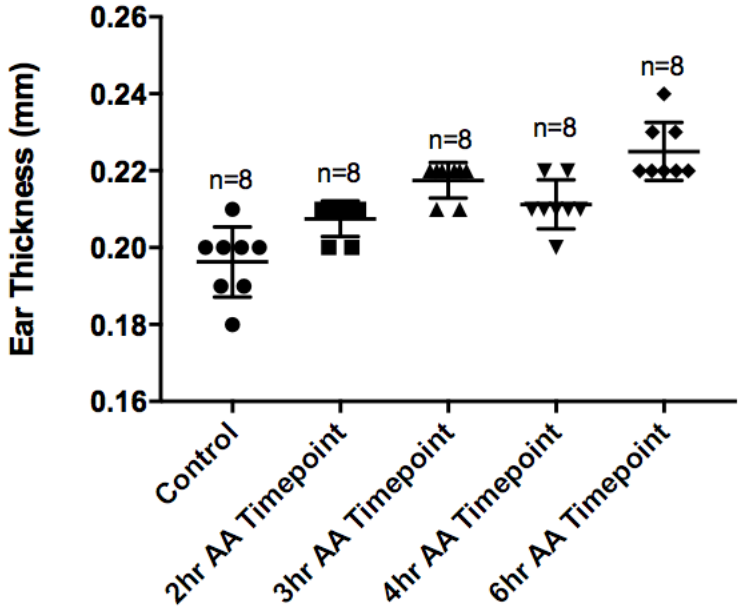


Figure 8. AA treatment did not increase ear thickness.
The top graph shows data representing the ear thickeness of mice with 8 ears per group except for the 6 hour post-treatment group, which includes 6 ears. The bottom graph shows data representingthe ear thickeness of mice with 8 ears per group. All ear

thickneness was measured using a caliper. All controls in both independent experiments were mice treated with acetone alone. Values are mean ± SD.

Sporozoite Infection in Mosquito Bite Challenged Mice Treated with Arachidonic Acid

As the goal of this project is to better understand the potential role of neutrophil infiltration against *Plasmodium* sporozoites at the mosquito bite site, we next compared infection in AA treated mice to untreated (acetone only) control mice. Mouse ears were treated with arachidonic acid at 2 or 3 hours prior to infection by mosquito bite. For the infection, *Anopheles stephensi* mosquitoes infected with *Plasmodium berghei* were allowed to probe on the ears of AA treated or control in C57BL/6 mice or C57BL/6 LysM-EGFP knock-in mice. Special care was taken to insure that similar numbers of mosquitoes probed on control and AA treated mice. The number of mosquitoes that probed the ear for each experiment is shown in Table 1. After 38.5-39 hours, the livers of all the mice were harvested and total RNA was isolated. The liver parasite burden was then determined by qPCR. Full details about each experiment are listed in Table 2. It should be noted that when LysM-EGFP knock-in mice with lower intensity of mosquito infection (≤14,000spz/mosquito), while other experiments tested normal C57BL/6 mice with relatively higher mosquito infection intensity.

In Figure 9, the liver stage parasite burden of AA treated and control mice is shown. All data are pooled and also shown as individual experiments. While one experiment showed no difference, all others had some decrease in infection in the AA treated mice. These experimental differences suggest there is an impact of neutrophils on sporozoite infection but that several factors are likely involved. First, liver *Plasmodium berghei* 18S rRNA copies were relatively lower in both vehicle-alone and AA groups that were challenged with lower sporozoite numbers (see experiment 1 and 5). This suggests that mosquitoes with lower sporozoite numbers induced lower infection than those with

higher sporozoite numbers. When lower sporozoite numbers were inoculated, neutrophils seemed to better decrease sporozoite infection than those with higher sporozoite number inoculum, suggesting neutrophils may better inhibit sporozoite infection in mice with lower levels of sporozoite inoculum. Here, we showed that the degree of sporozoite infection is relevant to the level of sporozoite number inoculated and that AA groups showed a larger decrease in sporozoite infection specifically in mice with lower sporozote numbers. Future research in the mechanism of how neutrophils inhibit infection under different levels of sporozoite inoculum could allow us to better understand this observation. Second, some heterogeneity in neutrophil infiltration was observed in images of AA treated LysM-EGFP mice. Neutrophils were observed to aggregate in lumps in some areas of the mouse ears while some were more loosely packed in other areas of the ear. We suspect that such phenomenon is related to where mosquitoes like to bite. Since the location mosquitos choose to probe on is based on the behavioral preference of each mosquito, it is a factor that is hard to take control of in our mosquito bite challenges. Third, as shown in Figure 9, few extreme outliers were seen in the AA group. We suspect that these outliers are due to the heterogeneity in neutrophil infiltration due to AA treatment. Perhaps we could postulate that these outliers are the results from changed mosquito probing behavior caused by AA. We acknowledged that these outliers could skew the data and generate statistically insignificant conclusion on the potential impact of neutrophils on sporozoite infection. Unfortunately we still do not fully understand the real cause of these outliers. In summary, we provide the first empirical data that suggests that AA treatment can help partially protect against *Plasmodium* berghei infection in mice in certain contexts.

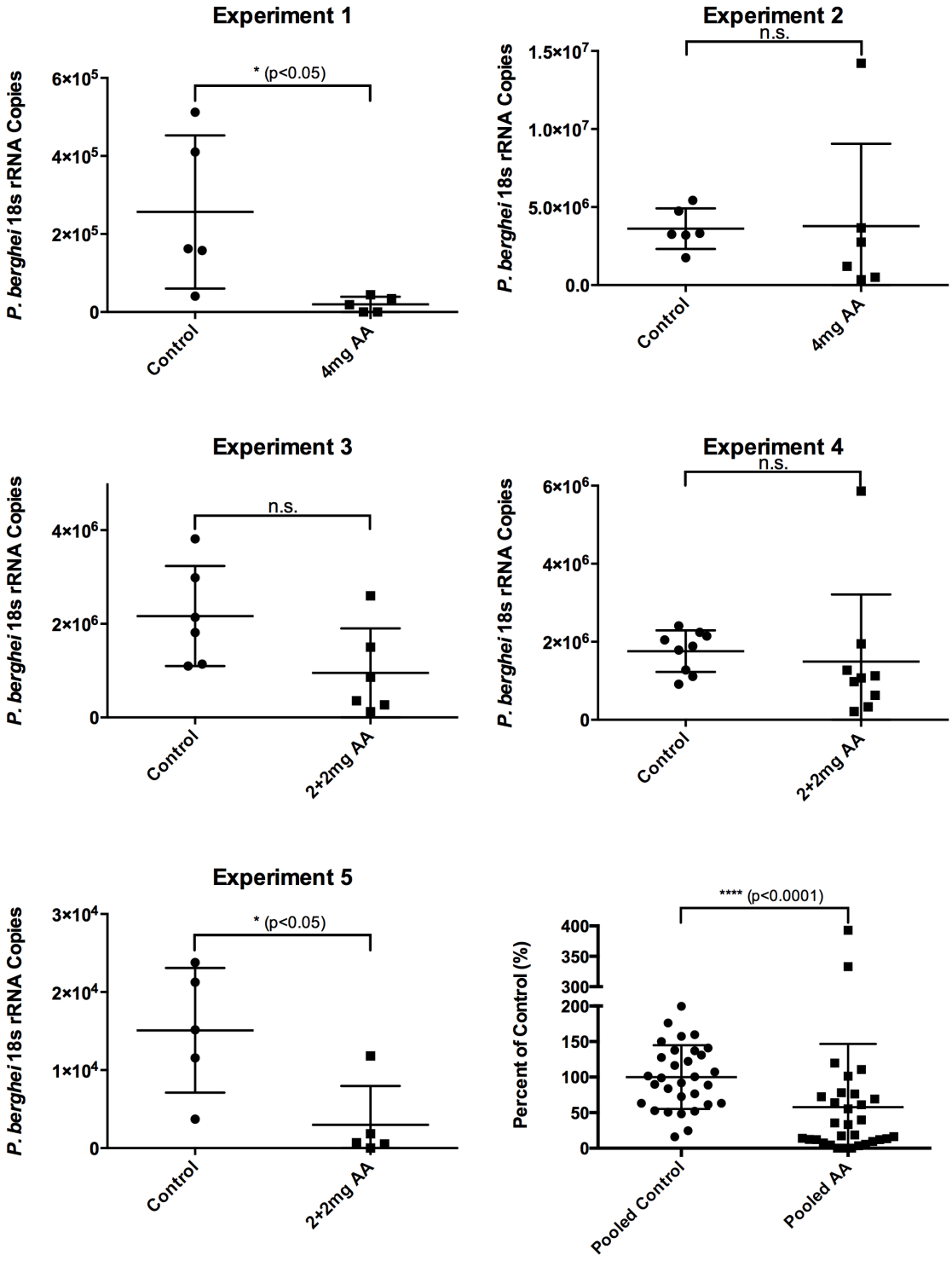


Figure 9. Liver Burden in Mosquito Bite Challenged Mice Treated with Arachidonic Acid

The amount of liver *Plasmodium berghei* 18S rRNA copies is represented as the percent of control. The percentages listed represent the mean of the percent of control in the AA treated group. Values are mean ± SD. Unpaired Mann-Whitney test was used, ranking all

data values from low to high with a statistical significance cutoff set at two-tailed $p < 0.05$, to assess the statistical significance between groups. The lower error bars of AA treated group are negative values and are not shown in the graphs. Significant differences are denoted by symbol: *$p \leq 0.05$; ****$p \leq 0.0001$.

Experiment 1

Mouse number	Number of mosquitos probed on right ear	Number of mosquitos probed on left ear
Control Group (Acetone alone)		
1	8	8
2	7	8
3	9	8
4	8	8
5	8	7
Arachidonic Acid Treated Group		
1	7	8
2	7	8
3	7	8
4	7	7
5	8	9

Experiment 2

Mouse number	Number of mosquitos probed on right ear	Number of mosquitos probed on left ear
Control Group (Acetone alone)		
1	7	8
2	7	7
3	7	11
4	8	10
5	7	7
6	7	8
Arachidonic Acid Treated Group		
1	9	9
2	8	7
3	8	7
4	7	8
5	8	10
6	8	7

Experiment 3

Mouse number	Number of mosquitos probed on right ear
Control Group (Acetone alone)	
1	8
2	7
3	9
4	9
5	9
6	8
Arachidonic Acid Treated Group	
1	8
2	7
3	7
4	8
5	8
6	8

Experiment 4

Mouse number	Number of mosquitos probed on right ear
Control Group (Acetone alone)	
1	9
2	9
3	9
4	9
5	9
6	9
7	9
8	10
9	9
Arachidonic Acid Treated Group	
1	9
2	9
3	9
4	9
5	9
6	9
7	9
8	9
9	9

Experiment 5

Mouse number	Number of mosquitos probed on right ear
Control Group (Acetone alone)	
1	8
2	7
3	7
4	7
5	7
Arachidonic Acid Treated Group	
1	8
2	7
3	6
4	7
5	7

Table 1. Number of mosquitoes probed in control and arachidonic acid treated mouse groups

	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5
Prevalence of mosquito infection (Oocysts in midguts or salivary gland squashes)	-	95% (Midguts)	90% (Salivary glands)	80% (Salivary glands)	70% (Salivary glands)
Intensity of mosquito infection (spz/mosquito)	~14,000	~17,000	~21,000	~31,000	~6,100
Average mosquitos that took blood meal on each mouse in the control group	15.8 (both ears)	15.6 (both ears)	8.3 (right ear)	9.1 (right ear)	7.2 (right ear)
Average mosquitoes that took blood meal on each mouse in the AA group	15.2 (both ears)	16 (both ears)	7.7 (right ear)	9 (right ear)	7 (right ear)
Mouse strain	LysM-EGFP	C57BL/6	C57BL/6	C57BL/6	LysM-EGFP
Hours post AA	2	2	3	3	3

Table 2. Experimental regime for testing the liver burden in mosquito bite challenged mice treated with arachidonic acid

DISCUSSION AND CONCLUSION

We investigated the potential impact of neutrophils on *Plasmodium* sporozoite infection. Although neutrophils have been extensively research in the context of many other pathogens, it has been reported that when *Plasmodium berghei* sporozoites are injected into the skin, neutrophils are suggested to participate in restraining skin sporozoites using NETs and to only engulf immobile sporozoites in the skin (Mac-Daniel et al. 2014). However, our lab reported that insignificant differences between both *Plasmodium berghei* and *Plasmodium yoelii* sporozoite liver burdens of untreated and neutrophil-depleted mice (Sinnis Lab unpublished data), which led to our suspicion that significant number of sporozoites have already left the skin prior to the peak of neutrophil infiltration. This led to our hypothesis that by using topical AA, a more rapid neutrophil infiltration prior to infection with *Plasmodium berghei* sporozoites into the bite site may impact *Plasmodium* sporozoites. First we determined the kinetics of neutrophil infiltration after AA treatment by intravital imaging. We found that neutrophil infiltration increased over the 6-hour time frame of the experiment. Quantitative measurements suggest that AA induces edema in mouse ears, similar to what was observed in previous study (Opas, Bonney, and Humes 1985) but this did not lead to significant changes in ear thickness possibly due to the relative low dose of AA. Although qualitative observation suggested that AA treatment did not prevent mosquitos from probing, a slight change in mosquito behavior during probing was observed as mosquitos tended to brush their proboscis after blood meal. Figure 6 shows that mosquitoes in AA group tended to take similar or slightly longer time to probe, except for experiment 5, which the mosquitoes were observed to be lethargic before the mosquito bite.

Our investigation into sporozoite infectivity in AA treated mice provide the first empirical data suggesting neutrophils decrease liver infection in mice. Indeed, two of the five experiments showed significant decrease in liver infection in AA treated mice, agreeing our hypothesis that rapid neutrophil infiltration induced by AA treatment was able to reach a certain threshold that decreases the infectivity of *Plasmodium* sporozoites in the liver. Interestingly, the other three experiments only showed a trend of decreased liver infection in AA treated mice that did not reach statistical significance. When all of the data were combined, the result was statistically significant. One possible explanation is that the experiments demonstrating a large decrease in infection after AA treatment were experiments in which LysM-EGFP mice were used. Though these are C57BL/6 mice, they are transgenic mice that have been cloned and further inbred. It is possible that their neutrophils, by expressing EGFP, are somehow more potent. However, the more likely explanation is that these mice are significantly less susceptible to sporozoite infection. As shown in Figure 9, LysM-EGFP mice have significantly lower liver stage parasite burdens compared to non-transgenic C57BL/6 mice. This suggests that possibly neutrophils have a certain capacity to inhibit sporozoites but that it is more difficult to observe at high sporozoite inocula. It would be interesting to conduct more infection studies in C57BL/6 mice with decreased numbers of infecting mosquito bites. Since in the field, malaria infection is initiated by a single mosquito bite, our findings are likely relevant to what occurs in natural infection.

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Part II: Characterization of A Novel Tag Derived from Circumsporozoite Protein

INTRODUCTION

Antibody Recognition to the Plasmodium Circumsporozoite Protein and Its Potential Tool for Biotechnology

Protein tags are one of the most powerful tools for protein purification, expression, and localization. Protein tags are peptide sequences that are added onto proteins by introducing a short genetic sequence encoding the tag in frame with the gene of interest. Commonly used protein tags include His (HHHHHHHH), HA (YPYDVPDYA), FLAG (DYKDDDDK), and Myc (EQKLISLEEDL) tags. Since the repeat region of the *Plasmodium* circumsporozoite protein (CSP) has an antibody that binds to it with very high affinity, we hypothesized that this repeat sequence, NANPNANPNANP, could be used as a protein tag.

CSP is a distinctive protein found uniquely in *Plasmodium*. CSP covers the entire surface of sporozoites and plays many major roles in immunogenicity, parasite invasion, and organization of parasite surface (Kappe, Buscaglia, and Nussenzweig 2004). It is known that among different *Plasmodium* species, CSP has a common structure that includes a signal peptide, amino acid repeat rich central domain, and a hydrophobic C terminus (McCutchan et al. 1996; Ibid.). The conserved motifs of CSP consist of region I, II-plus, and III (Kappe, Buscaglia, and Nussenzweig 2004). In relation to my project, repeated NANP short sequences are found in central domain of CSP. Due to the nature of this epitope and the high affinity of a commonly used monoclonal antibody in our lab that binds to it (Yoshida et al. 1980), we hypothesize that the CS tag can be used as a protein tag with enhanced specificity and sensitivity compared to the commercially made His tags. Conventionally, protein purification can be performed by running a mixture of

proteins including the targeted protein through a column that is composed of agarose coupled with the antibody that binds specifically to the tag of your protein of interest. Protein that is bound to the column non-specifically can then be washed off, leaving the protein of interest specifically bound to the column. The desired protein can then be washed with a low-pH buffer to detach the proteins from the antibody on the column. In my project, the insertion of His tag alongside the CS tag allows us to compare both tags side by side in all the experiments. Using MBP as the fusion protein, we then investigated whether the CS tag could be used as a biotechnology tool and tested if it was a more sensitive and specific tag compared to some of the other commonly used tags.

MATERIALS AND METHODS

Construction of Recombinant Plasmid that Encodes for Maltose Binding Protein with CS Tag and His Tag Attached

Two oligonucleotides corresponding to the repeat region of the *Plasmodium falciparum* CS protein, $(NANP)_3$, were ordered from Integrated DNA Technologies (forward oligo: 5'-AATTCAACGCCAATCCGAATGCCAACCCGAACGCAAACCCGTAAA -3' and reverse oligo: 5'-AGCTTTTACGGGTTTGCGTTCGGGTTGGCATTCGGATTGGCG TTG-3'). Both forward and reverse oligonucleotides (100μM) were mixed and heated to 94°C for 2 minutes, followed by gradual cooling to room temperature in order to allow both oligonucleotides to anneal together. This double stranded oligo insert was cloned into the Eco RI and Hind III restriction sites of pMALcHT vector, which was designed to produce cytosolic expressed fusion protein with the maltose-binding protein (MBP) at the amino-terminus (Muench et al. 2006). The pMALcHT vector has a linker region that contains a nucleotide sequence encoding the tobacco etch virus (TEV) protease cleavage site followed by 6xhistidine tag sequence, which was in front of the Eco RI restriction site (Muench et al. 2006). The construct was later verified by sequencing in both directions to ensure its fidelity and was then transformed into *E.coli* DH5α cells by heat-shock 60 seconds in 42°C prior to protein expression. This newly constructed recombinant plasmid will henceforth be referred to as "pMAL-MBP-(His)6-$(NANP)_3$". The recombinant plasmid is shown in Figure 10 and the cloned DNA sequence can be found in the supplementary data.

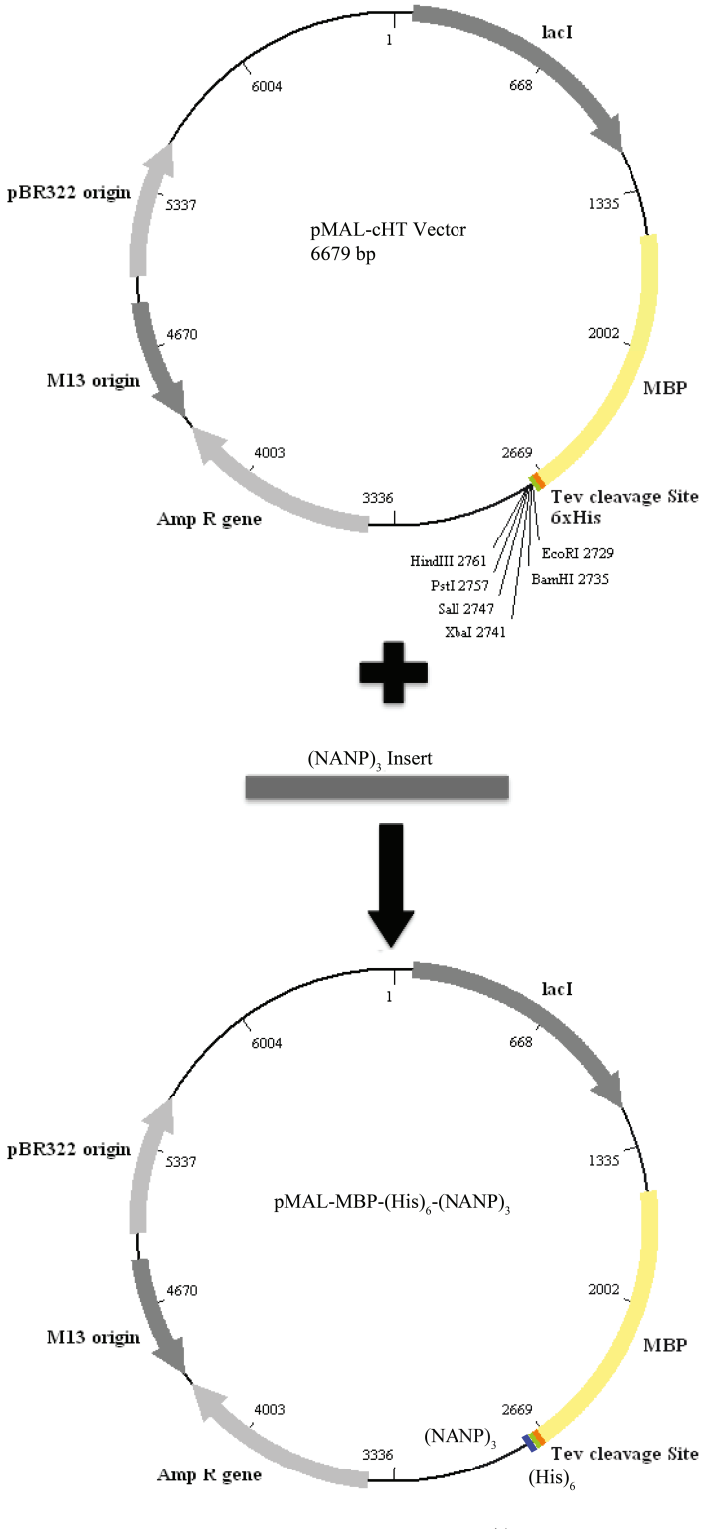


Figure 10. Cloning of the Recombinant Plasmid pMAL-cHT-MBP-$(His)_6$-$(NANP)_3$
The diagram shows the $(NANP)_3$ insert cloned into the pMAL-cHT vector, making a recombinant plasmid with both His tag and CS tag.

Protein Expression of pMALcHT-MBP-$(His)_6$-$(NANP)_3$

The recombinant plasmid pMALcHT-MBP-$(His)_6$-$(NANP)_3$ was transformed into TRIL cells by adding 1µl of the plasmid into 50 µl of TRIL cells for 30 minutes on ice, followed by 40 seconds at 42°C and later 2 minutes on ice. The TRIL cells are *Escherichia coli* cells, expressing TEV protease and contain the RIL plasmid (Muench et al. 2006). A single colony of E. coli expressing pMAL-MBP-(His)6-$(NANP)_3$ in TRIL cells was inoculated into 5ml of LB broth supplemented with 5µl of each 1000x chloramphenicol, ampicillin, and kanamycin prior to growing overnight with shaking. The starting culture was then inoculated into 50ml of lysogeny broth (LB) medium supplemented with 50µl of each 1000x chloramphenicol, ampicillin, and kanamycin and grown at 37°C with shaking for 4.5 hours, reaching OD600=1.5, which was too high comparing to the optimal OD600=0.5-0.6. Hence, 5ml of the overgrown culture medium was inoculated into a new 50ml of LB medium supplemented with 50µl of each 1000x chloramphenicol, ampicillin, and kanamycin and grown at 37°C with shaking for 1 hour to reach OD600=0.5-0.6. 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and was incubated with 250 rpm shaking overnight at 25°C. The cells were harvested by centrifugation at 4200 rpm for 30 minutes. The cell pellet was then re-suspended for cell lysis in PBS/2 Complete Mini, EDTA-free Protease inhibitor cocktail tablets (Roche Diagnostics, Lot #: 11100100)/ 1mg/ml lysozyme and incubated for 30 minutes at room temperature, followed by sonication at 50% power four times consecutively with each repetition composed of 2 minutes of 1 second pulses. (The sonicator was purchased from Misonix, Model CV26). The sonicated cell lysate was then

centrifuged at 16,000rpm for 30 minutes. Supernatant was collected and aliquots were frozen at -80°C.

SDS-PAGE and Western Blot

The proteins in the cell lysate were separated by SDS-PAGE on 10% polyacrylamide gel and transferred onto methanol activated polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with blocking buffer without milk (5%BSA in Tris Buffered Saline and Tween 20) for 1 hour with constant shaking. The membranes were probed separately with 1μg/ml mAb 6B10 and 1μg/ml mAb anti-CSP 2A10, which both recognized the CS epitope, (Yoshida et al. 1980) as positive controls as well as monoclonal anti-His from different manufacturers. The anti-His antibodies used were as follow: 1:2000 ThermoFisher Scientific (Catalog #: MA1-21315); 0.15μg/ml Genscript (Catalog #: A00186-100); 1:750 Bio-Rad (Catalog #: MCA1396GA); 1:2000 Sigma (Catalog #: H1029). The membranes were then washed with tris-buffered saline and Tween-20 (TBST) three times and incubated for 1 hour with anti-mouse IgG horseradish peroxidase linked to secondary antibody diluted 1:10,000 (GE Healthcare). Membranes were then washed with TBST three times and incubated in ECL Western Blotting Detection Reagent (GE Healthcare; Catalog #: 9589151) for several minutes at room temperature, then exposed to Amersham Hyperfilm^{TM} ECL High Performance Chemiluminescence Film for detection.

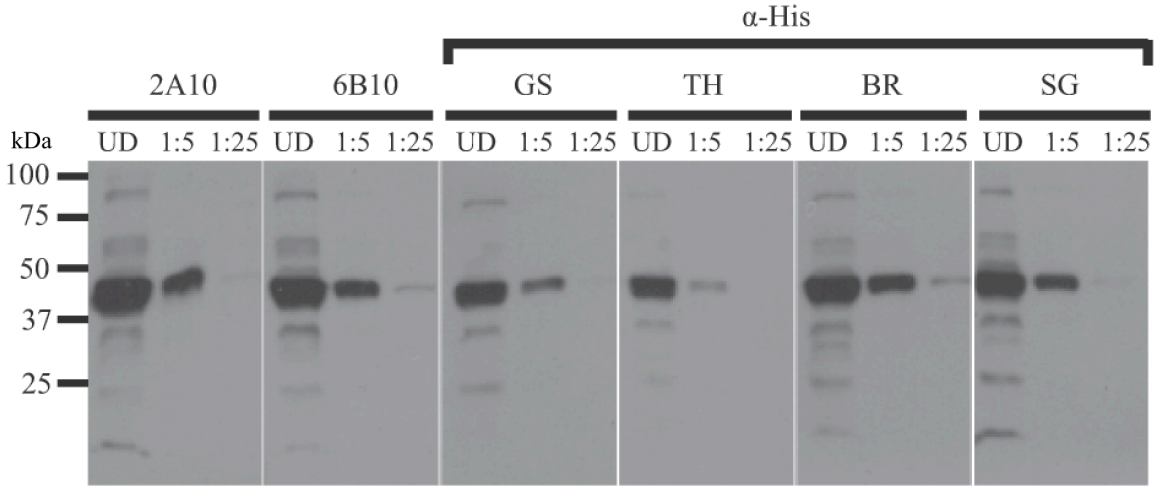
Dot Blot Analysis

Before performing comparisons of CS tag and His tag with immunoprecipitation, the required concentrations of antibodies to incubate and fully saturate the Dynabeads® M-280 Sheep Anti-Mouse IgG magnetic beads (Product number: 11201D) were needed to be determined. First, the "naked" anti-mouse magnetic beads were washed in 1ml of 0.1% bovine serum albumin (BSA)/ 2mM EDTA/ PBS washing buffer. Next, in total volumes of 30µl each, (10µg/ml, 50µg/ml, and 100µg/ml) mAb 2A10, mAb 6B10(10µg/ml, 50µg/ml, and 100µg/ml), anti-His ThermoFisher Scientific (5µg/ml, 25µg/ml, and 50µg/ml), anti-His Genscript (50µg/ml, and 100µg/ml), anti-His Bio-Rad (50µg/ml, and 100µg/ml), and anti-His Sigma (50µg/ml, and 100µg/ml) were incubated with Dynabeads® M-280 Sheep Anti-Mouse IgG for 2 hours and 20 minutes at 4°C with tilting and rotation. Then, tubes were placed in magnet for 2 minutes and supernatants that contained the unbound antibodies were collected. Serial dilutions of each supernatant were performed and added directly to the PVDF membrane in order to find the optimal concentrations for each antibody to fully saturate the magnetic beads. After air drying all the membranes, the membranes were blocked with 5% milk/ 1%BSA/ TBS overnight with shaking at 4°C in the cold room. Membranes were then washed with TBST three times and incubated for 1 hour with anti-mouse IgG horseradish peroxidase linked to secondary antibody diluted 1:10,000 (GE Healthcare) in 2% BSA/TBST. After five washes with TBST, 5 minutes per wash, membranes were incubated in ECL Western Blotting Detection Reagent (GE Healthcare; Catalog #: 9589151) for several minutes at room temperature, and then exposed to Amersham Hyperfilm™ ECL High Performance Chemiluminescence Film for detection.

RESULTS

Comparison of the CS and various His Tags for Protein Detection Through Western Blot

The first aim of this project is to assess whether our monoclonal antibodies against our novel CS tag can allow better detection comparing to various anti-His antibodies against the His tag. In order to perform the best side-by-side comparison between these two systems, MBP fusion protein was first reengineered to include a His tag and CS tag and then compared the levels of protein detection by western blot as described previously in the methods section. The inclusion of a TEV protease cleavage site is critical for our potential success of making a successive tag. The reason is that it has been observed that protein without the TEV protease cleavage site could not be eluted off with a low-pH buffer due to its strong affinity between our monoclonal antibody and the CS tag (data not shown). To solve this problem, we included the TEV protease cleavage site so that the proteins with our CS tag could be easily eluted off with the help of a protease. In addition, both mAb 6B10 and mAb 2A10 were tested as they recognize the repeat region of CSP from *Plasmodium falciparum* (Yoshida et al. 1980; Nardin et al. 1982; Anker, Zavala, and Pollok 1990). As shown in Figure 11, western blot analysis shows that the anti-CSP mAb 6B10 and anti-His from Bio-Rad had the best signals. This indicates that the CS tag and anti-His (Bio-Rad) gave the highest detection signal. Hence we can conclude that CS tag has comparable detection sensitivity compared to anti-His (Bio-Rad) and better detection than anti-His (Genscript, ThermoFisher Scientific, Sigma) in performing western blot.



GS, Genscript; TH, ThermoFisher Scientific; BR, Bio-Rad; SG, Sigma
UD, Undiluted protein lysate

Figure 11. Comparison of the CS tag and His tags for protein detection by western blot analysis

MBP-(His)6-$(NANP)_3$ protein was run on an SDS-PAGE gel. The figure shows a western blot probed with either antibodies to the CS tag or His tag. A dilution series of the lysate is performed to better measure intensity of binding.

Comparison of the CS and various His Tags Through Dot Blot Analysis

Prior to performing comparisons of CS tag and His tag with immunoprecipitation, we must first optimize the required concentrations for different antibodies to cross-link and fully saturate the Dynabeads® M-280 Sheep Anti-Mouse IgG. Here we have tested anti-CS tag antibodies mAb 2A10, mAb 6B10 and various anti-His antibodies from different manufacturers (Genscript, ThermoFisher Scientific, Bio-Rad, Sigma).

In Figure 12, the top panels for each antibody are the positive controls where antibody was directly placed on the membrane and probed, to ensure that anti-mouse secondary antibodies recognized all anti-CS and anti-His antibodies. The lowest concentrations of these positive controls that can be recognized by anti-mouse secondary antibodies are listed in Table 3.

Antibodies (+ controls)	Lowest detectable concentrations
2A10	0.2 to 1μg/ml
6B10	0.2 - 1μg/ml
anti-His (Genscript)	100μg/ml
anti-His (ThermoFisher Scientific)	5μg/ml
anti-His (Bio-Rad)	20 - 100μg/ml
anti-His (Sigma)	1 to 2μg/ml

Table 3. Lowest Concentrations of Antibodies detected by anti-mouse secondary antibodies

We then wanted to test how well each antibody bound to anti-mouse magnetic beads. We incubated each antibody at the indicated concentration with 50ul slurry volume anti-mouse conjugated to magnetic beads and after this, dilutions of the supernatant, containing unbound antibodies was placed on the membrane and probed as

above. The lowest concentrations of antibodies required to saturate the magnetic beads are listed in Table 4. Knowing these concentrations for each antibody not only gives us an estimation of how much antibody is needed to saturate the beads but also help us select the suitable anti-His antibody candidate for comparing the anti-CS antibodies in the later immunoprecipitation experiments. This results shows that anti-His antibodies from manufacturers Genscript, ThermoFisher Scientific, and Bio-Rad require large amount of antibodies to fully saturate the beads comparing to the anti-His (Sigma). From an economical standpoint, anti-His (Sigma) proves to be the best candidate and will be used to cross-link anti-mouse magnetic beads for later immunoprecipitation.

Antibodies	**Required concentrations**
2A10	5μg/ml
6B10	1 to 5μg/ml
anti-His (Genscript)	50μg/ml
anti-His (ThermoFisher Scientific)	25μg/ml
anti-His (Bio-Rad)	100μg/ml
anti-His (Sigma)	5μg/ml

Table 4. Optimal Concentrations of Antibodies Required for Saturating Magnetic Beads Conjugated to Anti-mouse IgG

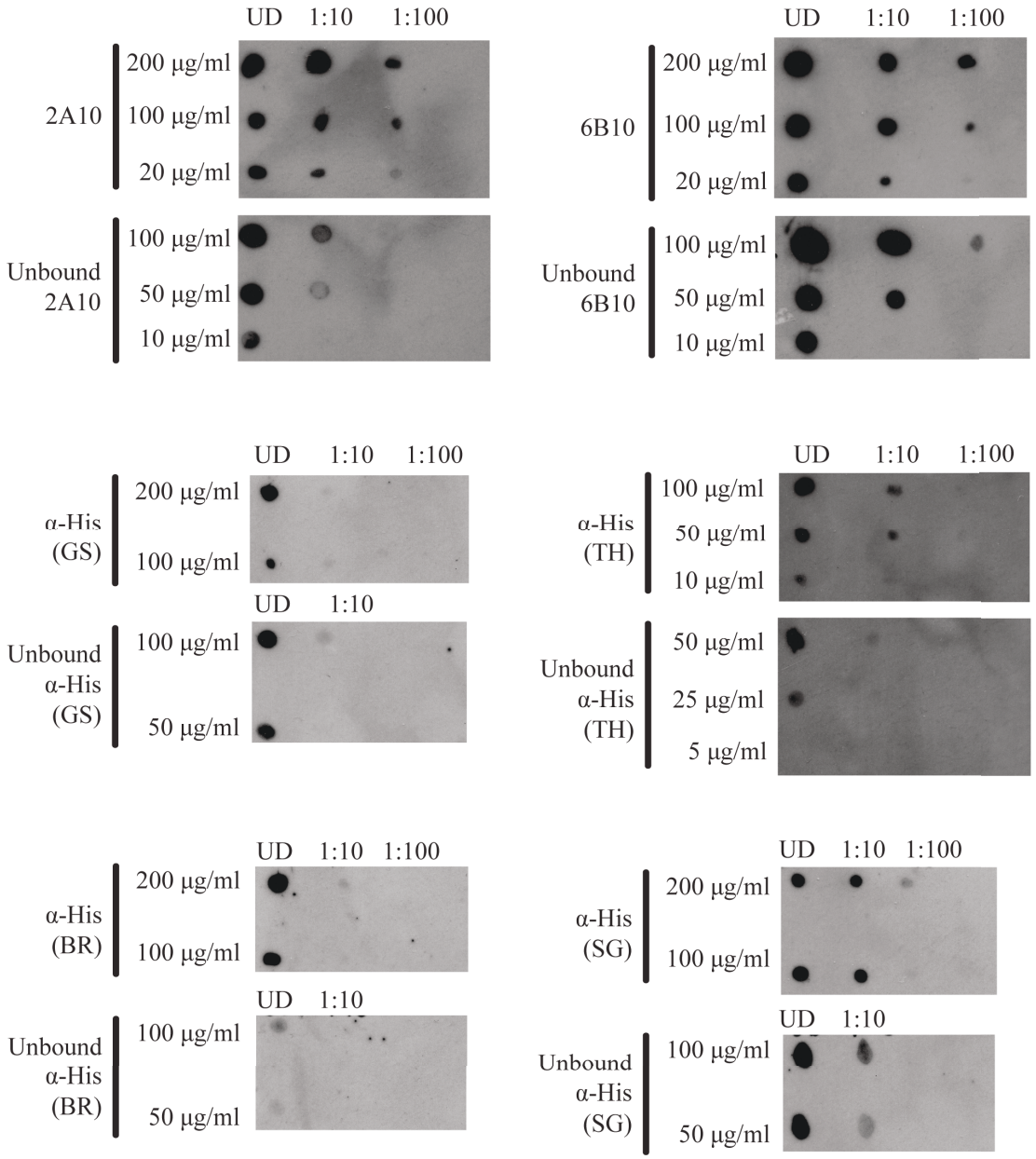


Figure 12. The Making of α-mouse Beads Conjugated to Different Antibodies
The top panels for each antibody are positive controls. Antibodies in their indicated concentrations were directly added to the PVDF membranes. The lower panels for each antibody are the excess unbound antibodies present in the supernatant after 2 hours and 20 minutes of incubation at 4°C with tilting and rotation.

DISCUSSION AND CONCLUSION

The second project investigated the potential of CS tag being used as a biotechnology tool. We decided to compare our novel CS tag to the commonly used His tag. To do this, we reengineered a MBP fusion protein to include both His tag and CS tag. This allows us to compare the performance between our anti-CSP monoclonal antibodies and anti-His antibodies against their respective epitopes. First we compared levels of protein detection by western blot analysis. Our result showed that anti-CSP mAb 6B10 and anti-His (Bio-Rad) have comparable sensitivity, which both gave the highest yield of target protein with high detection signal. More importantly, the western blot result demonstrated that mAb 6B10 has better protein detection than several anti-His antibodies (Genscript, ThermoFisher Scientific, Sigma). Next, we determined the optimal concentrations for all anti-CS and anti-His antibodies to cross-link to the Dynabeads® M-280 Sheep Anti-Mouse IgG. As expected, there was a high variation in binding between different antibodies and anti-mouse conjugated to magnetic beads. This result lays the cornerstone for the next step, which will be an immunoprecipitation experiment to compare the efficiency with which our CS-tag versus a His-tag functions in protein purification protocols. Here, we were able to provide evidence that the CS-tag system had better performance than most anti-His systems that are available in the market in detecting target protein through western blot. A direct comparison of immunoprecipitation of MBP-(His)6-$(NANP)_3$ protein using the CS-tag and His-tag is still in working progress and is needed to fully demonstrate that the CS tag system is potentially one of the best protein tags in today's research field.

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Kappe, S. H., C. A. Buscaglia, and V. Nussenzweig. "Plasmodium Sporozoite Molecular Cell Biology." *Annual Review of Cell and Developmental Biology* 20 (2004): 29-59.

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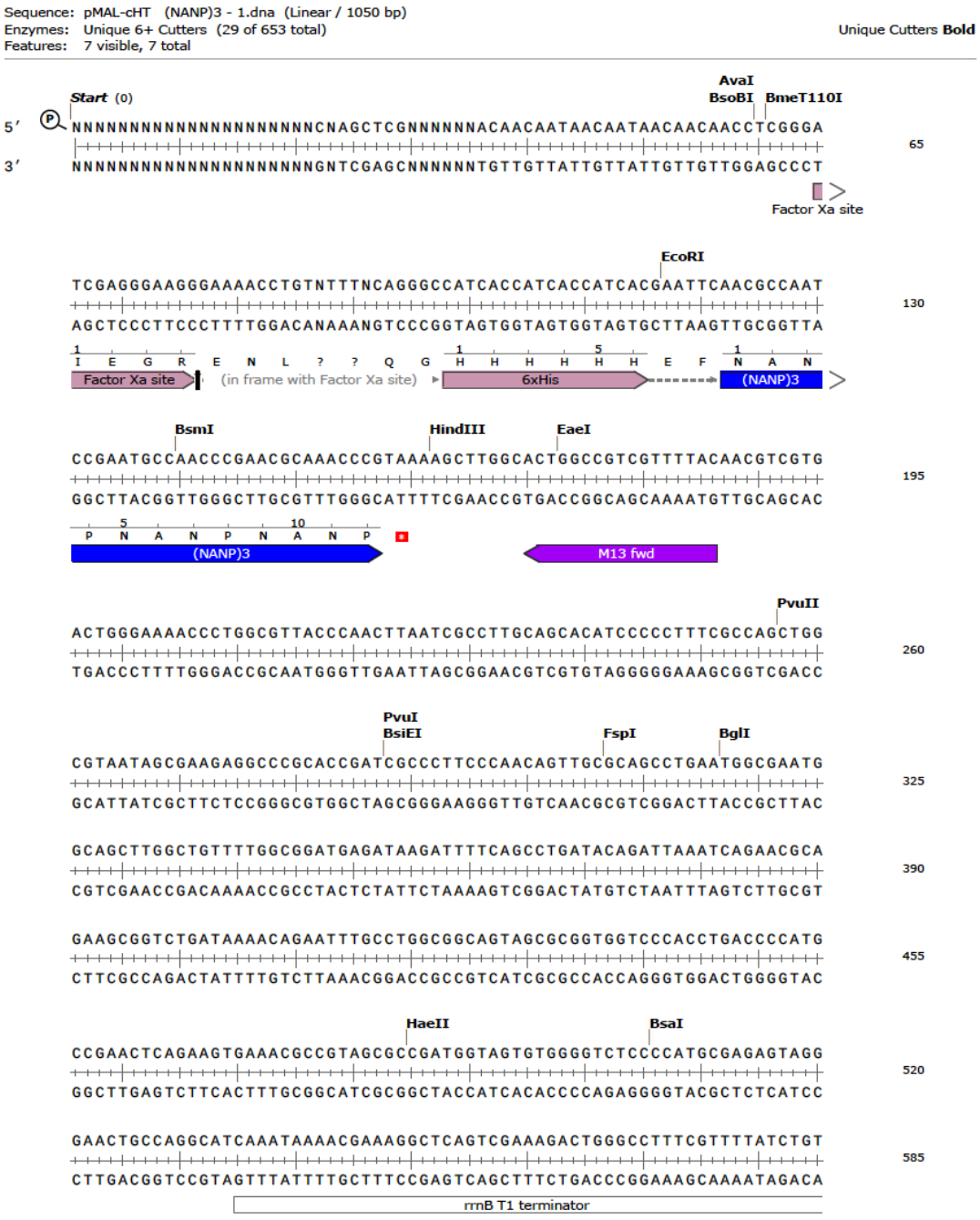
Muench, S. P., et al. "Expression, Purification and Preliminary Crystallographic Analysis of the Toxoplasma Gondii Enoyl Reductase." *Acta crystallographica.Section F, Structural biology and crystallization communications* 62.Pt 6 (2006): 604-6.

Nardin, E. H., et al. "Circumsporozoite Proteins of Human Malaria Parasites Plasmodium Falciparum and Plasmodium Vivax." *The Journal of experimental medicine* 156.1 (1982): 20-30.

Yoshida, N., et al. "Hybridoma Produces Protective Antibodies Directed Against the Sporozoite Stage of Malaria Parasite." *Science (New York, N.Y.)* 207.4426 (1980): 71-3.

SUPPLEMENTARY DATA



S1. Cloned DNA Sequence of pMALcHT-MBP-(His)6-$(NANP)_3$

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Education and Awards

JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH, JOHNS HOPKINS UNIVERSITY
BATIMORE, MD
Master of Science, Biochemistry and Molecular Biology **Present**

- **Certificate in Vaccine Science and Policy**
- **Certificate in Good Clinical Practice**
- Masters thesis for ScM requirement – Impact of Neutrophil Infiltration at the Mosquito Bite Site on *Plasmodium berghei* Infection & Investigation of a More Sensitive and Specific Tag – The CS Tag
- Masters thesis for MHS requirement – Prognostic and Therapeutic Biomarkers for Non-Small Cell Lung Cancer
- Selected and presented "Implication of Neutrophil Infiltration at the Mosquito Bite Site on *Plasmodium berghei* sporozoite infection" at the JHMRI 2016 Future of Malaria Symposium

UNIVERSITY OF ROCHESTER
ROCHESTER, NY
Bachelor of Science, Cell and Developmental Biology with Minor in Music **May 2013**

- Completed pre-medical course requirements, Public Health Cluster.
- Inducted into National Society of Collegiate Scholars in recognition of academic excellence and class standing.
- **2011 Undergraduate Writing Colloquium Contest Winner:** Voted Best Essay of the year in WRT 105/E, a compulsory writing seminar for all undergraduate students at the University of Rochester.

Research/Work Experience and Training

McCrone Research Institute
Chicago, IL
January 2016

- Received the certification for Microscopical Identification of Asbestos granted by the institute. Trained using polarized light microscopy (PLM) to identify and study the crystallographic and optical properties of asbestos minerals, including serpentine and amphibole asbestos types, as well as a variety of common substances that may be confused with asbestos. Acquired skills in sample preparation and fiber identification.

UNIVERSITY OF ROCHESTER
Research Assistant, Department of Pathology and Lab Medicine
Fall 2011 - Summer 2011, Spring 2014

- Assisted with elucidating the Ectropic Virus Integrating site 1 (EVI1) apoptotic pathway critical developing novel targeted therapy and further rational drug design against poor prognostic Evi1-positive Acute Myeloid Leukemia (AML).
- Created several mutant EVI1 expression constructs that are defect in leukemic function.
- Selected and presented "EVI1 is Critical in Myeloid Leukemia Cell Apoptosis" at the 2012 National Conference of Undergraduate Research in Utah and the 2012 University of Rochester Undergraduate Research Exposition.

Independent Research, Department of Clinical and Social Sciences

Fall 2010 - Spring 2013

- Assisted with project relying upon brain-computer interfaces to collect brain activity data as it relates to self-controlled health behaviors, such as exercise and diet.
- Used electroencephalography (EEG) techniques to record brain activities of test subjects and question subjects about their health and emotions.

Independent Research, Department of Microbiology and Immunology

Fall 2010 - Spring 2011

- Designed a Gemin5-Flag tagged construct for transient over-expression of the tagged protein in A549 and 293T cells.
- Performed various techniques such as PCR, ligation and transformation vector, and site directed mutagenesis.
- Submitted a final written report and attended regular group meetings.

Research Assistant, Physical Medicine and Rehabilitation Department

Fall 2009 - Fall 2010

- Filed and sourced patient records as well as medical records.
- Worked with scheduling patients with different therapists.

Healthcare, Shadowing and Volunteering Experience

ROCHESTER GENERAL HOSPITAL
ROCHESTER, NY

Shadowing Opportunity **Spring 2014**

- Shadowed doctor in the Department of Pulmonary and Critical Care. Observed morning rounds, in-patient, out-patient, and medical grand rounds.

NEW YORK UNIVERSITY AT BELLEVUE HOSPITAL CENTER
NEW YORK, NY

Project Healthcare Emergency Department Intern **Summer 2012**

- Assisted during rotations in the Emergency Department, including Adult, Pediatric and Psychiatric Emergency Services, Urgent Care, Social Work, operation and recovery rooms.
- Presented on lung cancer for the annual health fair and had team presentations of research study results about methadone study at the Department of Emergency Medicine conference.
- Participated in autopsy observation, ambulance ride-along and the Child-Life program that aids the physical, emotional, cognitive, and social needs of children and their families, as well as "Reach Out and Read" Program, a program consisting of pediatricians, volunteers, parents, and educators working together to develop literacy skills for children.
- Attended informational lectures and weekly meetings to discuss medical issues and practical problems that patients, medical providers and hospitals encounter.
- Observed various surgeries including abdominal hysterectomy, open-heart surgery, hip replacement surgery and cataract surgery.
- Obtained certification in CPR and First Aid.
- Monitored patient's length of stay and assisted during interpretations with Chinese Mandarin, Cantonese, and English languages.

Healthcare, Shadowing and Volunteering Experience Continued

BRIGHTON VOLUNTEER AMBULANCE
ROCHESTER, NY

Assistant Intern **Summer 2011 – Spring 2012**

- Completed approximately 80 hours working with Field Training Officer and applying in-class scenarios and actual emergency calls during ride-along.

UNIVERSITY OF ROCHESTER MEDICAL CENTER
ROCHESTER, NY

Shadowing Opportunities **Fall 2010-Spring 2011**

- Shadowed doctors in the Department of Radiation Oncology. Observed day-patient and learned about assessing patients by clinical evaluation and imaging.
- Observed open-heart surgery in the Department of Cardiac Surgery.

Leadership, Teaching and Co-curricular Activities

JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH, JOHNS HOPKINS UNIVERSITY
BATIMORE, MD

Johns Hopkins Taiwanese Student Association Representative of the JHU Medical Institutions
(School of Public Health)

Summer 2015 – May 2016

- As one the executive boards, worked closely with other executive boards from various divisions such as the East Baltimore campus, Homewood campus, as well as downtown Baltimore campus and ensured new students a smooth transition to Johns Hopkins.
- Organized orientation and various events
- Arranged club meetings.

UNIVERSITY OF ROCHESTER
ROCHESTER, NY

Teaching Assistant for US Life: Custom and Practices

Spring 2013

- Assisted instructor in presentation, led classes to promote discussion of course material, and graded essays.

Tutor for Center of Academic Support

Fall 2010 – Spring 2013

- On a one-on-one basis, assisted students with problem solving on assignments and preparation for exams.
- Major subject areas included Chemistry, Cellular Biology and Math.

Librarian Front Desk Attendant of Carlson Library

Summer 2010 – Spring 2013

- Sorted, charged, and discharged books.
- Provided general assistance to patrons.

Social Chairperson for the Charles Drew Pre-Health Society

Fall 2012 – Spring 2013

Planned various club events and coffee hours with individuals working in health-related fields, such as medical students and other graduate students.

- Publicized club meetings and events.

Executive Board, National Society of Collegiate Scholars

Fall 2010 - Spring 2013

- As Executive Vice President, oversaw the planning of the Induction convocation and organized meetings.
- Invited Richard Feldman, Ph.D., Dean of the College, to speak at the induction ceremony.
- Organized chapter general membership meetings and initiated collaboration with other organizations.

- As Vice President of Public Relations and Recruitment, organized and promoted philanthropic event programs benefitting Golisano Children's Hospital, made valentines for nursery home residents and volunteered with the Salvation Army.
- Planned the Induction convocation and recruited new members.
- Publicized chapter meetings, events, and member accomplishments.

Officer of Awareness and Activism for University of Rochester Genocide Intervention
Spring 2011 – Fall 2011

- Held educational workshops and presentations to raise awareness about genocides and human injustices in our global community and to empower individuals to make a difference in preventing genocide in the community of Rochester.

Chemistry 131 Workshop Leader, Learning Assistance Center
Fall 2010

- Held recitations to promote discussion of course material and address study needs of students.

Skills and Techniques

- Confocal Microscopy
- R Statistical Computing
- STATA
- Geographic Information System (GIS)
- Trained working in the Insect Core Facility (Johns Hopkins Bloomberg School of Public Health)
- Trained working with mice in the Animal Facility (Johns Hopkins Bloomberg School of Public Health)
- Language: Cantonese, Mandarin, Hakkanese, English