Preclinical optimization of a prime-and-trap malaria vaccine

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Abstract

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Malaria is a deadly parasitic disease that disproportionally impacts infants and children under five years old. A more effective vaccine is urgently needed to reduce the global burden of malaria. The liver stage (LS) is a critical bottleneck in the parasite lifecycle that if blocked, would prevent blood stage infection, clinical disease, and transmission. Radiation-attenuated sporozoite (RAS) vaccines are critical for sterile LS protection in mice, non-human primates (NHP), and humans by inducing antibodies and $CD8^+$ T cells, including liver resident memory $CD8^+$ T cells (Trm) that are critical for long-term protection. Such T cells can also be induced by a novel two-step vaccine strategy called prime-and-trap that was designed to simplify and improve upon RAS-only vaccination. Prime-and-trap combines DNA priming against the immunodominant circumsporozoite protein (CSP) with a subsequent intravenous (IV) dose of liver-homing RAS to “trap” the activated and expanding T cells in the liver. This strategy induces durable protective CSP-specific CD8$^+$ liver Trm cells in mice, and efforts are underway to translate this vaccine strategy to NHPs and humans. Reducing the RAS dose and/or determining if RAS must be strictly administered by the IV route would further enhance the translational potential of the prime-and-trap malaria vaccine. This dissertation provides preclinical research aimed at enhancing the efficacy of the RAS trapping component of the prime-and-trap vaccine.

This dissertation aimed to optimize the translational potential of RAS trapping by co-administration with the potent glycolipid adjuvant 7DW8-5 to 1) reduce the dose, and 2) improve the administration route to be more suitable for clinical use. Additionally, since these studies revealed drastic differences between protection in male and female mice, this dissertation also explored if the addition of the 7DW8-5 adjuvant could enhance protection in male mice. First, this study found that in female mice, IV-administered freshly-dissected RAS could be replaced with the more desired but less immunogenic cryopreserved RAS and that the dose of IV-RAS could be reduced four-fold by co-administration with 7DW8-5. Next, the study found intradermal (ID) co-administration of RAS and 7DW8-5 in ultra-low volumes (2.5 µL) was completely protective and dose sparing compared to standard ID volumes (10-50 µL) and induced protective levels of CSP-specific CD8$^+$ T cells in the liver. The finding that adjuvants and ultra-low volumes are required for ID-RAS efficacy may explain why prior reports about higher volumes of unadjuvanted ID-RAS proved less effective. Finally, the study found that male mice were not protected from any prime-and-trap vaccine regimen (IV-RAS or ID-RAS) with or without 7DW8-5. Further, the final studies found significant sex differences in cytokine

expression induced by 7DW8-5 in mice. Additional studies are required to understand the mechanism of protection induced by IV- or ID-RAS and 7DW8-5 and to understand the dramatic protection differences observed between male and female mice. The ID route may offer significant translational advantages over the IV route and may improve RAS vaccine development. Taken together, this research found that the translational potential of the prime-and-trap malaria vaccine could be improved by the co-administration of 7DW8-5. These studies pave the way for further evaluation of prime-and-trap in NHPs and humans and future studies should continue to include sex as a biological variable.

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ABBREVIATIONS

α-GalCer = alpha-galactosylceramide
7DW8-5 = [(2S,3S,4R)-1-O-(α-D-galactopyranosyl)-N-(11-(4-fluorophenyl)undecanoyl)-2-amino-1,3,4-octadecanetriol)]
aa = amino acid
Alum = aluminum potassium sulfate
AMA = apical membrane antigens
APC = antigen-presenting cell
AP2-G = apicomplexa-specific transcription factor
AS01 = adjuvant system 01
BH = Benjamini-Hochberg correction
bp = base pair
BS = blood stage
CD = cluster of differentiation
CHMI = controlled human malaria infection
CI = confidence interval
CSP = circumsporozoite protein
CSP (FL) = circumsporozoite protein full-length
CSP (FL NR) = circumsporozoite protein full-length no repeats
CPS = chloroquine prophylaxis and spz
Cryo = cryopreserved
Cryo-RAS = cryopreserved radiation attenuated sporozoites
CQ = chloroquine
CXCR = CXC chemokine receptor
DC = dendritic cell
DEG = differentially expressed genes
dLN = draining lymph node
DMSO = dimethyl sulfoxide
DNA = deoxyribonucleic acid
EARD = early arresting replication deficient
EDTA = ethylenediaminetetraacetic acid
EMP = erythrocyte membrane proteins
ELISA = enzyme-linked immunosorbent assay
ELISPOT = enzyme-linked immunosorbent spot
EP = electroporation
FBS = fetal bovine serum
FDA = federal drug administration
FDR = false discovery rate
Fresh-RAS = freshly-dissected radiation attenuated sporozoites
GAP = genetically attenuated parasite
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GDP = gross domestic product
gg = gene gun
ggCSP = gene gun cluster prime (Day 0 and Day 2)

GM-CSF = granulocyte-macrophage colony-stimulating factor
GMP = good manufacturing practice
Gy = gray
HK-spz = heat-killed sporozoite
hr = hour
hpi = hours post injection
IACUC = Institutional Animal Care and Use Committee
ID = intradermal
IFN = interferon
IL = interleukin
*i*NKT = invariant natural killer T cell
IM = intramuscular
IP = intraperitoneal
ITN = insecticide-treated net
IV = intravenous
IVIS = in vivo imaging system
KLRG = Killer cell lectin-like receptor subfamily G member
LARC = late arresting replication competent
LS = liver stage
LT = heat-labile toxin
Luc = luciferase
ND = not detected
NHP = non-human primate
NK = natural killer cell
NKT = natural killer T cell
ns = non-significant
mAb = monoclonal antibody
MB = mosquito bite
MHC = major histocompatibility complex
MPEC = memory precursor effector cells
MSP = merozoite surface proteins
Pb = *Plasmodium berghei* (*P. berghei*)
Pf = *Plasmodium falciparum* (*P. falciparum*)
Pk = *Plasmodium knowlesi* (*P. knowlesi*)
Pm = *Plasmodium malariae* (*P. malariae*)
Po = *Plasmodium ovale* (*P. ovale*)
Py = *Plasmodium yoelii* (*P. yoelii*)
Pv = *Plasmodium vivax* (*P. vivax*)
PfSPZ = Sanaria's cryopreserved radiation attenuated sporozoite vaccine
PfSPZ-cVac = Sanaria's cryopreserved chemoprophylaxis-attenuated sporozoite vaccine
PBS = phosphate buffered saline
PE = pre-erythrocytic
PO = popliteal
P&T = prime-and-trap
PV = parasitophorous vacuole

RAS = radiation attenuated sporozoite
RBC = red blood cell
RNA = ribonucleic acid
RDT = rapid diagnostic test
RT-PCR = reverse transcription polymerase chain reaction
RTS,S = World Health Organization recommended malaria subunit vaccine
R21 = malaria subunit vaccine undergoing phase 3 clinical trials
ROI = region of interest
SC = subcutaneous
SCRI = Seattle Children’s Research Institute
SD = standard deviation
SLEC = short-lived effector cells
STV = standard volume
SPU = spot forming units
Spz = sporozoite
Tcm = T central memory
Tem = T effector memory
Trm = T resident memory
Th = T helper
TNF = tumor necrosis factor
TRAP = thrombospondin-related adhesion protein
ULV = ultra-low volume
UW = University of Washington
VLP = virus-like particle
WSV = whole sporozoite vaccine
WT = wildtype

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DEDICATION

I would like to dedicate this dissertation to my family who have always supported me.

Chapter 1. INTRODUCTION

1.1 OVERVIEW OF MALARIA

1.1.1 *Global Burden*

Plasmodium parasites are unicellular obligate eukaryotes that infect vertebrates and insects and cause malaria disease. Over 200 species of *Plasmodium* have been described, but only five commonly cause malaria disease in humans: *P. falciparum* (Pf), *P. vivax* (Pv), *P. malariae* (Pm), *P. ovale* (Po; *P. ovale curtisi* and *P. ovale wallikeri*) *and P. knowlesi* (Pk) [1-3]. Of these five species, Pf and Pv are responsible for most of the global burden of malaria. Pf infections are the deadliest and are generally concentrated in Sub-Saharan Africa, while Pv infections occur more ubiquitously throughout tropical and subtropical regions [2,4,5]. The World Health Organization (WHO) 2022 Annual Malaria Report estimated that in 2021 there were 247 million malaria cases and 619,000 deaths, and that over half of the world's population lived in regions at risk of malaria [2]. Approximately 95% of malaria deaths occurred in Sub-Saharan African regions and 80% of these deaths occurred in children under five years of age [2]. Over the past 20 years, global malaria cases have plateaued or declined [2,4,5]. However, disruptions in prevention measures due to the COVID-19 pandemic have abrogated this progress and in 2020 the number of malaria deaths increased by 12% [5]. In addition to the social and global health toll of malaria, there is also a significant economic burden with an estimated reduction of 1.3% Gross Domestic Product (GDP) per person per year in countries with high malaria transmission (reviewed in [6]). Thus, the prevention, treatment, and the ultimate elimination of malaria remains a top priority for the WHO to reduce the social, global health, and economic burdens of malaria worldwide.

1.1.2 *Prevention*

Malaria prevention efforts are critically important to reduce the global burden of malaria [2]. Preventative measure categories broadly include vector control (*i.e.*, indoor residual spraying of insecticides and/or the elimination of mosquito larvae and breeding habitats), prophylactic and transmission blocking antimalarial chemotherapies (*i.e.*, mass drug administration and/or seasonal and vulnerable population chemoprophylaxis), and insecticide-treated bed nets (ITN) [7,8]. The use of ITN alone has led to a 27% increase in survival of children under 4 years of age [9] and this survival benefit was maintained into adulthood [10]. In 2020, COVID-19 pandemic disruptions led to only 58% of planned ITN campaign being completed [5], which may have contributed to the increase in mortality observed in the same year. A combination of these prevention and intervention measures is advantageous, and the benefits of these efforts should not be understated.

Recently, novel prevention strategies have been introduced for malaria: monoclonal therapies and vaccines. Monoclonal antibodies (mAb) are designed to passively block parasite infection, ideally preventing both malaria disease and further transmission [11]. Several mAb are currently undergoing clinical development and will be initially prioritized for use in infants and children [2]. A small USA based phase 1 clinical trial of a promising mAb called L9LS protected 88% of participants from *Plasmodium* challenge [12]. Additionally, a phase 2 study found that another mAb called CIS43LS protected 75-88% of participants during a 6-month malaria season in Mali [13]. These are novel therapies still undergoing clinical trials, but mAb have the potential to play a large role in malaria reduction efforts as they are administered in single doses, are cost effective, and could be highly protective in both healthy adults and high-risk groups such as infants and pregnant people [11,12]. This data is encouraging and suggests that mAb can provide another effective tool to prevent malaria, however unlike vaccines, mAb are essentially drugs that provide passive immunity and do not induce memory immune responses.

Vaccines can have immense global health impacts as they can generate long-lasting memory immune responses, but historically malaria vaccine development efforts have been challenging. On October 6, 2021 the WHO recommended the use of an antibody-inducing malaria subunit vaccine called RTS,S for the prevention of Pf in children living in regions with moderate to high malaria transmission [5]. This was a groundbreaking announcement that is expected to reduce severe malaria disease by 30% in vaccinated children [14-16]. Although RTS,S is a great addition to the malaria prevention toolkit, it does not prevent *Plasmodium* infection or prevent further transmission. Thus, better vaccines will be required for malaria elimination efforts. Malaria vaccines will be discussed in further detail in **Section 1.3**.

1.1.3 *Diagnostics and treatment*

Early, rapid, and accurate diagnosis of malaria in the clinic and field is critical. Clinical diagnosis involves healthcare personnel recognizing signs and symptoms of malaria (flu-like symptoms including chills, fever, and muscle aches), but this is complicated by the numerous asymptomatic malaria cases [17]. Other diagnostic tools include detection of blood stage parasites by microscopy, parasite antigen detection by a rapid diagnostic test (RDT), or molecular assays such as reverse-transcription polymerase chain reaction (RT-PCR) (reviewed in [18]). Molecular assays are more sensitive than RDTs and can detect lower parasite densities including asymptomatic infections [17,19]. The most widespread RT-PCR targets are the abundant and conserved *Plasmodium* 18S rRNA, which can be detected from single parasites in a 0.05–1 mL blood sample [19].

After accurate diagnosis, malaria can be treated with antimalarial drug regimens, which are complex and often involve combination therapies [20,21]. Which combination treatment an individual receives can depend on many factors including the infecting parasite species, the patient history and health status, and the geographic region where the infection occurred [20,21]. Most of the approved drugs target asexual parasites in the blood, which are rapidly acquiring drug resistance (reviewed in [22,23]). The increase of drug resistant parasite strains is a major concern in the field as it jeopardizes malaria elimination and eradication efforts. Thus, the emergence of drug resistance is closely monitored, and in 2022 the WHO launched the "strategy to respond to antimalarial drug resistance in Africa" initiative to directly address the growing concern [2]. In addition, efforts to identify novel antimalarial drugs are underway, with research expanding to also include host-targeted therapies as a promising new strategy (reviewed in [24,25]).

1.1.4 *Lifecycle and biology*

Plasmodium parasites have a complex lifecycle that requires an insect and vertebrate host and are spread to humans through the bites of female *Anopheles* mosquitoes. *P. falciparum*-infected female mosquitoes bite human skin and deposit tens to hundreds of sporozoite (spz) stage parasites into the dermis (reviewed in [26]). Inoculated spz in skin have several fates: 1) differentiation or phagocytosis in the skin [27,28], 2) migration through lymphatics to draining lymph nodes (dLN), or 3) invasion of vascular system for migration to the liver [29-31]. Spz motility in the dermis is required for vascular or lymphatic invasion and occurs via a contact-dependent method called gliding motility [32,33]. Only spz that reach the liver can continue their lifecycle and further differentiate, but the other skin stages are of increasing interest in the field and will be discussed in more depth in **Chapter 3**.

Spz that successfully enter the vascular system home to the liver and traverse several cells before sensing and selecting a single hepatocyte to invade for further differentiation [34] (**Figure 1.1**). During hepatocyte invasion, the spz forms a parasitophorous vacuole (PV) composed of hepatocyte plasma membrane that shields and protects the parasite from the host [35]. The Pf parasite rapidly transforms and replicates inside the PV for ~7-10 days in humans, during which time tens of thousands of exoerythrocytic merozoites form [36]. Once the merozoites are mature and infectious, the PV ruptures and releases thousands of merozoites packaged into merosomes to initiate the blood stage infection [37].

The blood stage (BS) is the part of the lifecycle that is responsible for clinical malaria disease symptoms and transmission of the parasite. In the bloodstream, merozoites invade red blood cells (RBC) for further development and then follow one of two paths: 1) asexual replication for further RBC invasion of the host, or 2) sexual stage differentiation into male and female gametocytes for transmission and completion of the lifecycle (reviewed in [38]). The asexual replication cycle completes in 1-3 days (the length is dependent on the *Plasmodium* species), producing billions of parasites through repeated rounds of RBC invasion and replication cycles [38,39]. The molecular mechanisms surrounding commitment to asexual or sexual differentiation pathways are still poorly understood, but the Apicomplexa-specific transcription factor (AP2-G) is required to initiate gamete development [40]. Full gametocyte formation varies greatly by *Plasmodium* species and occurs in 1-12 days [38,41]. Mature gametes then circulate in the blood and are picked up by a mosquito during her feeding to continue the *Plasmodium* lifecycle inside the insect host [42]. Inside the definitive mosquito host, parasite development into infectious sporozoites occurs in 14-21 days: male and female gametes recombine to form zygotes, develop into oocysts in the mosquito midgut, spz form inside the oocysts, and then finally migrate to the salivary gland as infectious spz (reviewed in [43,44]). Infectious spz are then transmitted back to humans in the next mosquito blood meal. The various lifecycle stages of the *Plasmodium* parasites complicate malaria prevention, vaccination, and elimination/eradication efforts. A complete understanding of host-parasite interactions (for both mosquitoes and humans) is required to design better and more targeted interventions to disrupt the malaria lifecycle.

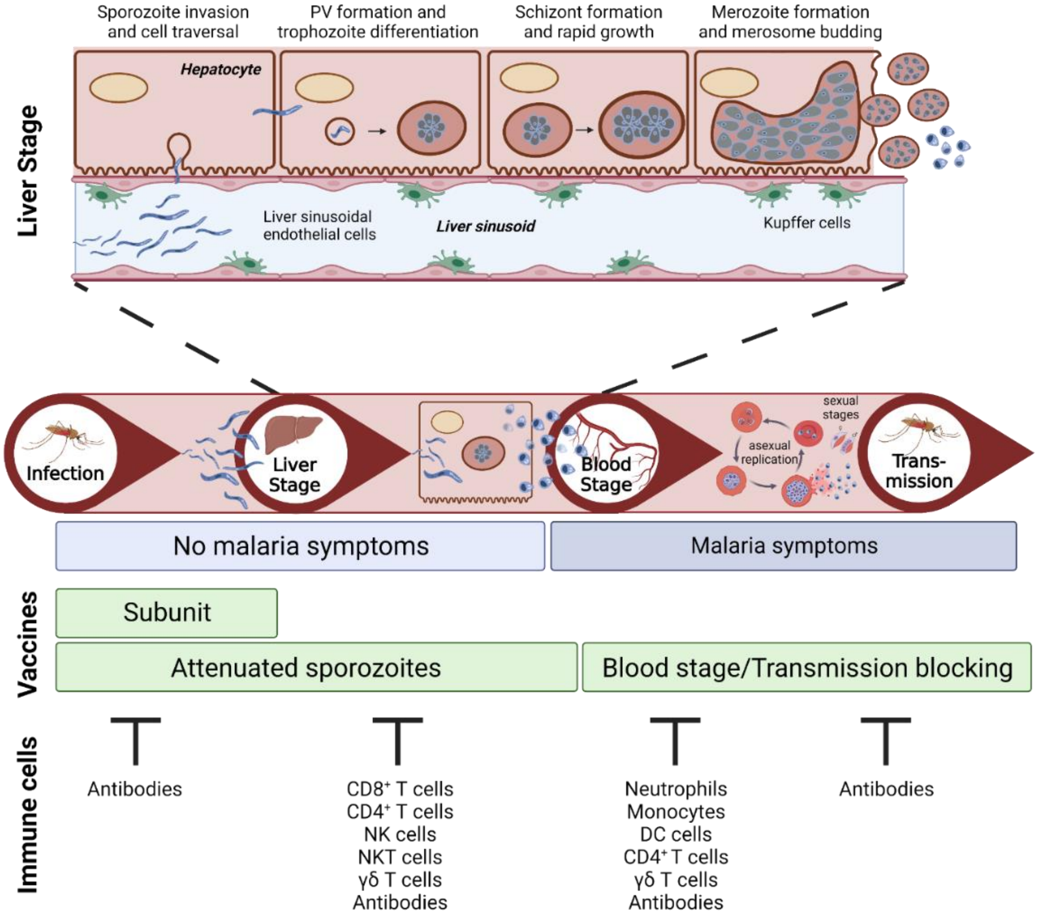


Figure 1.1. An overview of the *P. falciparum* lifecycle, immunology, and vaccines. Female *Anopheles* mosquitoes deposit spz into the skin where they migrate into blood vessels and home to the liver to initiate the liver stage infection. The spz traverses several cells before selecting a final hepatocyte to invade, forming a protective PV composed of the host plasma membrane. Parasites differentiate and rapidly grow inside the PV for ~7-10 days before mature merozoites bud out of merosomes and initiate the BS infection. BS parasites replicate asexually inside red blood cells or commit to sexual differentiation into male and female gametocytes. Mature and circulating gametocytes can be transmitted back to mosquitoes during blood feeding to continue the *Plasmodium* lifecycle. The various stages of the parasite lifecycle induce different host immune responses and can be targeted by different types of vaccines. Image adapted from a BioRender figure created by Caroline J. Duncombe.

1.2 MALARIA IMMUNOLOGY

The complexity of the malaria lifecycle is compounded by the complexity of the parasite itself. *Plasmodium* contains over 5000 genes that are expressed in stage specific manners [44], which leads to stage specific host immune responses. These are described in detail below and summarized in **Figure 1.1**.

1.2.1 *Blood stage immunology*

Host immune responses induced by BS have been extensively studied in humans and are better characterized compared to the other stages due to controlled human malaria infection (CHMI) studies and the accessibility of sampling human blood. However, BS immune responses are influenced by prior malaria exposures and the severity of the clinical disease [45,46]. The BS infection can be acute or chronic and asymptomatic or symptomatic (ranging from mild to severe disease). Severe malaria is associated with a dysfunctional host immune response and can lead to complicated malaria (*e.g.,* cerebral malaria or severe anemia) that if left untreated, often results in death [47]. Repeated malaria exposure does not generally result in sterilizing immunity (inhibition of all BS infection), but rather reduces the severity of BS malaria disease [45,46].

Different *Plasmodium* spp. preferentially infect RBC of a specific age class (*e.g.*, Pv and *P. berghei* (Pb) preferentially infect young RBCs known as reticulocytes) [48,49]. This blood stage immunology section will focus on the human-infecting Pf parasites, which are promiscuous and infect RBCs of any age [48]. The RBC is a protected niche in the human host that allows BS parasites to develop and replicate without exposure to the innate immune system. However, when schizonts are ruptured, RBC-free merozoites and other antigenic parasite debris (*e.g.,* DNA, RNA, heme, and digestive vacuoles with hemozoin) are released into the blood stream and accessible to the host immune system (reviewed in [50]). Innate immune cells including neutrophils, dendritic cells, monocytes, and macrophages are quick to respond as they are stimulated by the foreign debris [51-53]. These cells phagocytose debris and produce reactive oxygen species [54,55], take up antigen for presentation to adaptive immune cells, and rapidly produce inflammatory cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, and GM-CSF [50-53]. This early cytokine production is critical for shaping the subsequent adaptive response and can differ between malaria naïve and pre-exposed individuals and vary with age [56,57].

Humoral immune responses to BS parasites are especially important for controlling infection [58,59]. Parasite proteins that are exported and expressed on the surface of infected RBCs or the merozoite surface are accessible to the immune system and thus are targets for humoral immune responses [58,60-62]. Putative exported and dominant antibody targets include erythrocyte membrane proteins (EMP1), merozoite surface proteins (MSP), and apical membrane antigens (AMA1) [63-65]. However, although the roles of EMP1, MSP, and AMA1 are well studied, other proteins like ribosomes can also be exported to the infected RBC surface and targeted by antibodies, but their mechanism of export and role in BS immunity are less clear [66,67]. Moreover, these exported but non-dominant ribosomal antigens may make more ideal therapeutic targets as they are highly conserved, but possibly not under the same immune pressures as the dominant antigens [66,67].

With a huge number of parasite proteins, the humoral immune system responds to multiple antigenic targets, some of which are only expressed for brief periods of time due to antigenic variation. Antigenic variation is a key immune escape mechanism utilized by BS parasites to evade antibody responses [68]. In Pf infection, ~60 *var* genes encode EMP1 family proteins and each have slightly differing cytoadherence properties, which gives the parasites high plasticity and the ability to escape immune detection [63,68]. However, despite the highly polymorphic nature of EMP1, many antibody-mediated vaccine development efforts have targeted this protein family and others like it [69]. Thus, due to the many potential BS vaccine targets and antigenic diversity, multicomponent vaccines that target several BS antigens will likely be required for durable BS vaccination [60].

Mature RBCs do not express MHC-I molecules and cannot express Pf $CD8^+$ T cell epitope targets. Thus, other adaptive immune responses including antibodies and non-conventional T cells (*i.e.*, γδT cells) play a more important role in Pf BS immunity than $CD8^+$ T cells. γδT cells are innate-like lymphocytes that secrete high levels of IFN-γ and IL-17 upon activation, which are important for recruiting innate cell responders [45]. In addition, these cells are highly cytotoxic and are known to inhibit merozoite invasion capacity [70,71]. Taken together, a combination of cellular and humoral immune responses contributes to BS immunity. Novel therapeutic interventions to control BS infection are critically important as findings have demonstrated that BS infections can detrimentally impact the host immune responses to subsequent liver stage infection [72].

1.2.2 *Pre-erythrocytic innate immunology*

Pre-erythrocytic (PE) stages include skin, spz, and liver stages (LS). Overall, PE immune responses are less well-characterized in humans compared to the BS due to the asymptomatic nature of the PE stages and the inaccessibility of the sampling the human liver. Furthermore, systematic immune signatures in the blood do not always correlate with immune responses in the PE organs of interest: skin, dLN, or liver [73,74]. Thus, most PE immunology data comes from studies in mice and non-human primates (NHP) utilizing *Plasmodium* species that are suitable for animal models, such as *P. yoelii* (Py) or Pb in mice or Pk in NHPs.

Spz are deposited into the dermis by mosquitoes, but only a fraction of those spz result in successful liver invasions: 60% remain in the skin, 15% go to the dLN, and 25% go to the liver (reviewed in [26,75]). The majority of spz are cleared by immune responses in the skin via innate cells or antibodies [27,28,75,76]. Spz in the skin are rapidly phagocytosed by neutrophils and then by infiltrating inflammatory monocytes, dermal dendritic cells (DC), and other antigen presenting cells (APC) [75,77]. These cells then migrate to the dLN and hand off antigens to dLN resident DCs or directly cross present antigens to $CD8^+$ T cells [78,79]. Depletion studies in mice have demonstrated that $CD11c^+$ DC cross presentation to $CD8^+$ T cells in the dLN is critical for LS immunity [79]. The minority of spz that exit the skin via lymphatics also end up the in the dLN where they can be phagocytosed and still contribute to $CD8^+$ T cell priming in the dLN [29-31].

In the liver, innate responses are characterized by inflammatory pathways primarily induced by spz infected hepatocytes, neutrophils, NK, and NKT cells [73,80]. Infected hepatocytes sense replicating parasites and initiate type I IFN and IFN-γ responses to recruit other inflammatory cells to the liver to kill the infected cells [73,80]. The contribution of IFN responses and the involvement of specific cell types is unclear and complicated (reviewed in [81]). Some reports suggest IFN signaling or NKT cells are necessary to limit and control infection [74], while others suggest they are dispensable or even damaging [82-84]. Interestingly, potent inflammatory cytokines induced by α-galactosylceramide (α-GalCer), or recombinant IL-12 or IFN-γ injection are protective in mice and NHPs from spz challenge at an acute but not memory timepoint [85-87]. Thus, the timing of inflammation in the liver seems to be a key component for protective PE immunity. Overall, the consensus in the field is that liver innate immune responses reduce some of the parasite liver burden but are likely not enough to control infection without adaptive memory immune response assistance.

1.2.3 *Pre-erythrocytic humoral immunology*

Antibodies interact with spz in many ways but are generally thought to directly neutralize spz in the skin and inhibit liver invasion [76,88]. However, antibodies exhibit other functions including complement and Fc-mediated functions [89,90], and can also act in the liver [91]. Additionally, antibody maturation and avidity are critical to produce protective antibodies in mice and humans [89,92,93]. Anti-spz antibody responses significantly contribute to PE immunity and are used as correlates or surrogates of protection in clinical settings. High titers of antibodies alone can protect from malaria, but antibodies wane over time and the threshold required for sterile protection (inhibition of all BS infection) is high [94,95]. In general, higher anti-spz antibody titers correlate with increased protection from malaria, however this is not always the case [89,96]. Thus, antibody functional readouts may be more reliable indicators of protective status then antibody titers [97].

Many PE stage proteins stimulate antibody responses, but abundant antigens expressed on the spz surface are immunodominant, including circumsporozoite protein (CSP). Anti-CSP antibodies are induced by the only WHO-approved malaria vaccine, RTS,S [98] and are detectable in individuals previously exposed to *Plasmodium* [99,100]. Immune responses to Pf CSP elicit diverse responses to many epitopes, but the most potent spz neutralizing antibodies bind the central region composed of a four amino acid repeat (NANP) [89,101]. Pf CSP-NANP neutralizing antibodies are well characterized and targeted by the most advanced and promising interventions including the RTS,S vaccine (discussed in detail in **Section 1.3.1**) and mAb therapy [98,102]. The adoptive transfer of high titers of CSP antibodies or antibodies endogenously induced by CSP-targeted vaccination conferred complete protection from spz challenge in mice [96,103,104]. Inducing potent anti-CSP antibodies is an effective malaria prevention method; however, as described above, these protective antibodies wane quickly. Thus, an ongoing challenge in the field is the development of vaccination strategies to induce robust *and* durable anti-CSP and other anti-spz antibodies. Other types of anti-spz antibodies are also important for protection, including non-NANP, non-neutralizing, and non-CSP antibodies, but the significance of these antibodies is not well understood [96,101,105]. A recent study found that a non-neutralizing CSP antibody could inhibit the binding of a CSP neutralizing antibody and reduce the efficacy of antibody-induced LS malaria protection [96]. Thus, more studies are warranted to understand the role of both neutralizing and non-neutralizing PE antibodies to understand how they interact. Overall, anti-spz antibodies are critical components of the protective PE immune response to reduce the number of infected hepatocytes in the liver.

1.2.4 *Pre-erythrocytic cellular immunology*

The role of $CD4^+$ T cells during LS malaria is complex, but these cells are known to be essential to help establish protective cytotoxic $CD8^+$ T cells [106]. $CD4^+$ T cells are critical for licensing APCs in the liver and dLN and are also responsible for direct cytotoxic effector mechanisms (reviewed in [107-109]). For example, Th1-like $CD4^+$ T cells secret IFN-γ and induce various immune responses including innate cell activation, antibody class switching, and APC licensing [108]. Proper APC licensing is vital to establish cytotoxic $CD8^+$ T cells. Licensing occurs “classically” via $CD4^+$ T cell:APC interactions or “alternatively” via NKT:APC interactions (**Figure 1.2**) [110]. In malaria LS, both classical and alternative APC licensing is likely occurring [106,109]. Classical $CD4^+$ T cell APC licensing is mediated though TCR:MHC II interactions and alternative NKT licensing is mediated through TCR:CD1d interactions. Non-professional APCs, including hepatocytes, do not express MHC II molecules; thus, only NKT cells can license infected hepatocytes that may present malaria antigen [109]. Furthermore, NKT cells are abundantly found in the liver [111], and therefore, along with CD4$^+$ T cells, they are important contributors to establishing cytotoxic CD8$^+$ T cell responses.

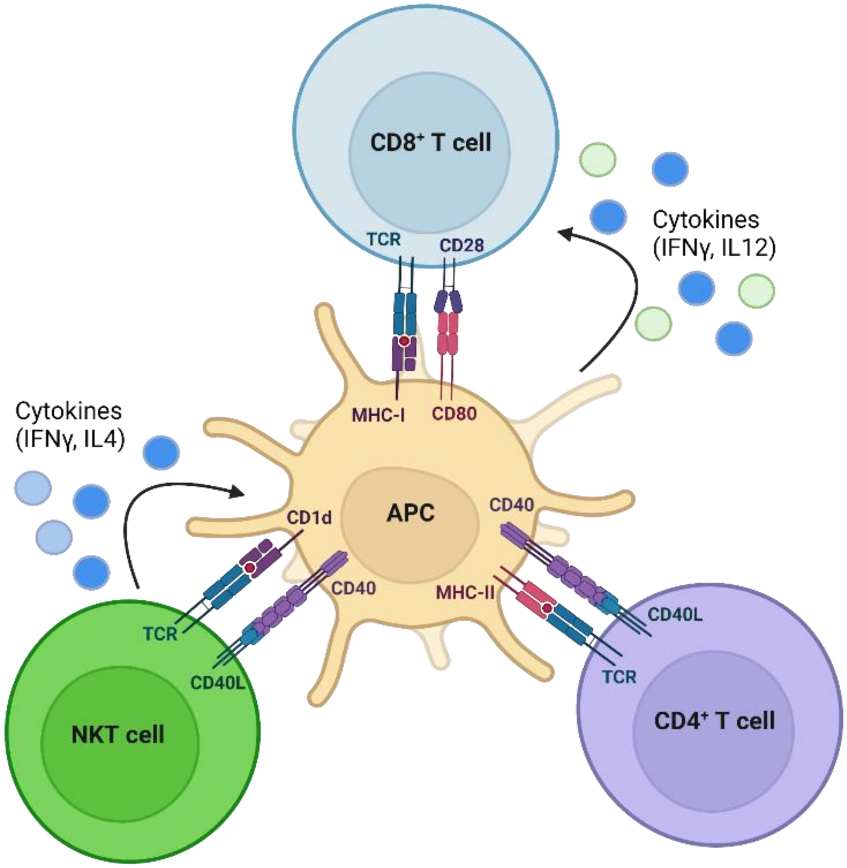


Figure 1.2. Classic and alternative APC licensing and CD8$^+$ T cell activation. Multiple signals are required for malaria cytotoxic CD8$^+$ T cell activation. APCs must be properly stimulated by malaria antigen and "licensed" by helper lymphocytes, CD4$^+$ T cells or NKT cells. Activated APCs secrete cytokines to recruit and activate CD8$^+$ T cells. Classic licensing involves CD4$^+$ T cells and alternative licensing involves NKT cells. CD4$^+$ T and NKT cells can license separate APCs or synergize and bind the same cell as depicted here. Figure inspired by [110] and created in BioRender.

CD8$^+$ T cells play an essential role in LS immunity. CD8$^+$ T cells alone can confer sterile protection against malaria spz challenge, which has been demonstrated by depletion and adoptive transfer experiments in mice [112-114]. Cytotoxic CD8$^+$ T cells rapidly target and kill infected hepatocytes in the liver, completely preventing BS infection (sterile protection). CD8$^+$ T cells are primed by CD11c$^+$ APCs in the dLN or liver and then differentiate into short-lived effector cells (SLEC) or memory precursor effector cells (MPEC) depending on transcription factors and the cytokine signals received during priming [115-117]. These primed and activated CD8$^+$ T effector cells rapidly clonally expand in the dLN after spz exposure in a dose-dependent manner [115], then migrate back to the liver to kill off infected hepatocytes. In the liver, malaria specific CD8$^+$ T cells cluster around infected hepatocytes resulting in death of the infected cell [118]. After clearing spz infection, SLEC rapidly die, while MPEC live on and differentiate into memory CD8$^+$ T cells [119]. Malaria-specific memory CD8$^+$ T cells provide long-term protection and are rapidly re-activated upon encounter with malaria antigen. In general, memory CD8$^+$ T cells can be subdivided into 3 classes based on the expression of surface markers: central memory CD8$^+$ T cells (Tcm), effector memory CD8$^+$ T cells (Tem), and tissue resident memory CD8$^+$ T cells (Trm). Tcm cells (CD62L$^+$ CCR7$^+$) are found circulating and in primary lymphoid tissues, Tem cells (CD62^{lo} CCR7^{lo}) are found in the spleen and circulating through the tissues, and Trm cells (KLRG1^{lo} and often CD103$^+$) are non-circulating and reside in tissues (*i.e.*, gut, skin, liver) [117]. Although malaria antigen-specific Tcm and Tem cells contribute to malaria PE immunity [120], Trm cells may be the most important memory subset for sterile protection.

Studies in mice have demonstrated the critical importance of non-circulating liver CD8$^+$ Trm cells for the killing of infected hepatocytes in the liver [82,121-123]. These cells patrol liver sinusoids and have defined phenotypic surface markers (KLRG1^{lo} CD69$^+$ CXCR6$^+$ CXCR3$^+$) that are distinct from Tem or Tcm cells [82]. The exact mechanism of cytotoxicity of these cells remains unclear, but many studies suggest that direct IFN-γ secretion and indirect induced IFN-γ-effector functions are important for protection (reviewed in [108,117,124]). Malaria-specific CD8$^+$ Trm cell frequencies correlate with protection from spz challenge, and mouse studies found that the cells are very durable with a half-life of over 400 days [82,123]. Further, antigen-specific CD8$^+$ Trm cells were the only cell population found to be critical for protection following cell depletion experiments [82]. However, although CD8$^+$ Trm cells alone can confer sterile protection, very high numbers of cells are required in the liver [125]. In naturally exposed individuals, malaria-specific CD8$^+$ T cells can be detected in the periphery, but these cells occur at such a low frequency that it is unclear if they are sufficient for protection or reflect liver tissue responses [126]. Thus, efforts to specifically boost cytotoxic CD8$^+$ Trm cells in the liver through vaccination are of great interest.

1.3 MALARIA VACCINES

Natural immunity to malaria generally wanes quickly, but the mechanism behind this short-lived memory is unclear. Vaccines can supplement natural immunity and improve the longevity of protection against malaria (reviewed in [127-129]). However, the complexity of *Plasmodium* and the mosquito vector stages of the parasite lifecycle have complicated vaccine development efforts. In general, there are three types of vaccines: 1) infection blocking, 2) disease blocking, and 3) transmission blocking. Malaria vaccine development efforts have included all three of these types and have targeted different stages of the parasite. Although vaccines can be made against any stage of the parasite lifecycle, the most advanced vaccines target the PE stages [127-129]. PE vaccines are especially attractive as they aim to block parasite infection, which subsequently prevents malaria disease and transmission (reviewed in [130,131]). PE vaccines also target a bottleneck in the parasite lifecycle - there are only tens to hundreds of LS parasites to kill off compared to the billions of parasites in the BS [130,131]. However, if even one infected hepatocyte is missed by the LS immune response, BS infection is induced, and clinical disease may occur. Thus, significant efforts have also gone into the development of BS or transmission blocking vaccines (TBV) (reviewed in [132]). Additionally, next-generation vaccine development efforts are focused on multi-stage vaccines designed to protect against more than one *Plasmodium* lifecycle stage; but to date, no such vaccines have been approved for widespread use. Currently, the most advanced and promising malaria vaccines are PE vaccines, which can be grouped into two types: subunit or whole sporozoite vaccine (WSV). This dissertation will focus on both types of PE malaria vaccines and next-generation strategies to combine them.

1.3.1 *Pre-erythrocytic subunit vaccines*

Subunit vaccines do not contain live pathogen and specifically target single or a limited number (*i.e.*, 1-5) of immunogenic pathogen antigens. Thus, subunit vaccines rely on validated antigen targets, which is complicated for malaria as the parasite contains ~5268 genes [133]. The malaria LS is especially challenging for vaccine development as few hepatocytes are infected and the human liver is inaccessible. Transcriptomic analysis of mouse LS parasites showed that ~2000 genes are active during the LS [133]. However, despite the large number of LS proteins, few antigens have demonstrated protective efficacy as subunit PE vaccine targets in the clinic [131,134]. The most effective subunit vaccines target spz surface proteins, like CSP or thrombospondin-related adhesion protein (TRAP) and can induce both humoral and cellular immune responses that aim to prevent spz infection of the liver [131,135]. However, these surface proteins are highly genetically diverse due to selection pressure from the host immune system; and despite some clinical success, Pf CSP and Pf TRAP have not yet achieved sterile protection in all populations [136-142]. Additionally, clinical trials involving CSP demonstrated reduced vaccine performance against heterologous challenge strains compared to homologous strains, which suggests that more protective antigen targets are needed [143,144].

Malaria subunit vaccine development efforts are vast and have included protein, peptide, virus-like particles (VLP), viral vectors, or nucleic acid vaccine strategies (reviewed in [127,129,145]).

In general, subunit vaccines are simple to manufacture and administer with standard methods. The overall trend suggests that many preclinical subunit vaccine strategies are immunogenic and protective in small animal models, but high levels of protection do not always translate into NHPs or humans. Further, few subunit vaccines have demonstrated protective efficacy in phase 2-3 trials. **Table 1.1** summarizes the advantages and disadvantages of subunit vaccines [127,129,145].

Table 1.1. Pros and cons of malaria vaccine strategies.

	Pros	**Cons**
Subunit*	Standard administration	Limited antigen targets (CSP or TRAP)
	Standard manufacturing	Low immunogenicity
	Effective in infants	Humoral immunity bias
	WHO recommended	Immunity wanes quickly
	Widely accepted by the public + safe	Repeated booster doses required
Whole sporozoite Vaccine	Many antigen targets	IV administration
	Highly immunogenic + safe	Unique manufacturing
	Liver specific infection	Liquid nitrogen storage
	Humoral and cellular immunity	Ineffective (but feasible) in infants
	Durable immunity	Repeated booster doses required
Heterologous Prime-and-Trap	Many antigen targets	IV administration
	Highly immunogenic + safe	Two-component manufacturing
	Humoral and cellular immunity	Liquid nitrogen storage
	Durable immunity	May be difficult to scale-up
	Two-dose vaccine	No human clinical trials completed

*The term “subunit” here refers to any vaccine that includes only subunits or antigens from the pathogen, including VLP recombinant protein vaccines.

The two most clinically advanced malaria subunit vaccines are RTS,S/AS01 (reviewed in [14,146]) and R21/Matrix-M [147]. These vaccines are very similar: they both utilize the hepatitis B VLP platform, and they are both designed to induce Pf CSP antibodies to block spz liver invasion. Both vaccines contain Pf CSP peptide fused to the hepatitis B surface antigen to form a VLP with both CSP and hepatitis B surface antigen on the surface. The two vaccines differ primarily in the adjuvant and the CSP valency on the surface of the VLP. RTS,S was the first malaria vaccine to undergo phase 3 clinical trials and to date is the only malaria vaccine to receive

WHO recommendation for pediatric use in endemic regions [5,15,16]. RTS,S was originally developed in the 1980s as a VLP vaccine that contains the Pf CSP carboxy-terminus and NANP repeat co-administered with an adjuvant (various RTS,S/adjuvant combinations have been tested) [148]. Over the years, the RTS,S vaccine has improved and been optimized for administration with the AS01 adjuvant [149], resulting in a 30% reduction of severe malaria cases in phase 3 clinical trials [5]. RTS,S induces potent anti-CSP antibodies, but these antibodies wane quickly and no titer threshold has been correlated with protection [98]. Anti-CSP antibodies primarily block spz liver invasion and infection, which typically results in a reduction of parasite numbers and less severe disease. $CD4^+$ T cells are also induced by RTS,S, but the roles of these cells are less clear [150]. Overall, RTS,S is a very safe vaccine that prevents malaria morbidity and mortality in children, but efforts to enhance protection are still needed [146].

R21 was developed in the 2010s and designed to induce higher titer anti-CSP antibodies than RTS,S by increasing CSP VLP surface valency and reducing antibodies directed against the VLP hepatitis B vehicle [147]. In phase 2a clinical trials, R21 was safe and highly efficacious (~77%) in African children at one year, and vaccine efficacy was associated with anti-CSP antibody titers [94,151]. Phase 3 clinical trials of R21 are underway and will be completed in 2024 (ClinicalTrials.gov ID: NCT04704830). Similar to RTS,S antibodies, R21 induced humoral responses waned over time, but were increased following booster vaccination [151]. Both RTS,S and R21 induce protective anti-CSP antibodies that aim to inhibit spz liver infection, however, once spz initiate a LS infection, the anti-CSP antibodies are ineffective. Furthermore, to prevent all spz liver invasion, antibody titers must be extremely high *and* durable, which has not been possible with RTS,S or R21. Thus, subunit vaccines that primarily induce anti-CSP antibodies are a great tool to reduce severe malaria, but they will likely be insufficient for malaria elimination and eradication. Subunit vaccine strategies that can induce cytotoxic cellular responses to kill off spz-infected liver cells or provide cross-stage immunity (by including BS antigens) are desirable.

1.3.2 *Pre-erythrocytic whole sporozoite vaccines*

Live attenuated WSV are highly effective PE vaccines as they contain thousands of immunogenic antigens, specifically home to the liver, and can induce sterile protection in mice, NHPs, and humans (reviewed in [75,152,153]). Additionally, WSV induce both protective antibodies and cytotoxic CD8$^+$ T cells to block liver infection and kill off infected cells, respectively [92,154-156]. A key advantage for WSV vaccines is that by using the whole spz organism, all parasite antigens are included in the vaccine, increasing the breath of PE immune responses compared to subunit vaccines. However, WSV are limited by non-standard manufacturing and IV administration requirements. To date, clinical WSV are produced in mosquitoes, hand dissected and purified, and cryopreserved in liquid nitrogen vapor [157]. **Table 1.1** summarizes the advantages and disadvantages of WSV [75,152,153].

There are three main types of WSV: 1) radiation-attenuated spz (RAS), 2) genetically-attenuated parasites (GAP), or 3) chloroquine prophylaxis and spz (CPS) (**Figure 1.3**) [152,158,159]. RAS are attenuated by irreversible DNA damage, GAPs are created by the genetic manipulation of genes essential for LS development, and CPS consists of injecting wild-type infectious spz in combination with chloroquine (or another anti-malarial) drug to selectively kill BS parasites. Within these three WSV types, two additional classifications are early arresting replication deficient (EARD) and late arresting replication competent (LARC) [160,161]. EARD parasites, including RAS and some GAPs, actively home to the liver and invade hepatocytes but then arrest development and do not replicate [162-164]. LARC parasites, including CPS and some newer

GAPs, actively home to the liver and invade, replicate, and differentiate inside hepatocytes but do not initiate BS infection or disease [160,161,165,166]. Many different GAPs have been developed over the years with varying single or combination gene deletions to yield EARD or LARC parasites [160,161]. LARC are more immunogenic than EARD as the replication ability generates more antigens for the immune system to target (**Figure 1.3**). However, LARC, but not EARD, induce type I IFN responses in the liver that can impair CD8$^+$ memory T cells [83]. Thus, there appears to be trade-offs between LARC and EARD parasites and variations in vaccine dose and schedule may overcome these hurdles, but more studies are needed to deconvolute the correlates of protection for WSV.

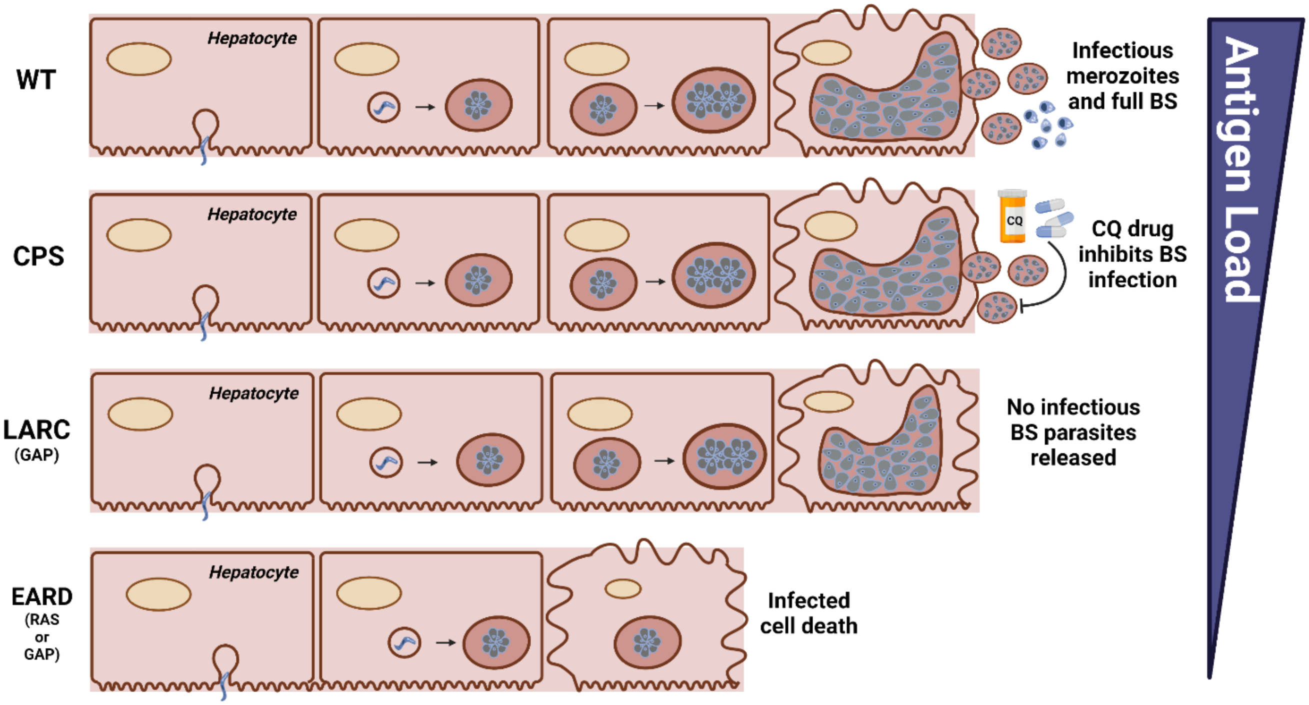


Figure 1.3. Liver infection of whole sporozoite vaccines for malaria.
Different whole sporozoite vaccine types induce differential liver infections. Wildtype (WT) spz infections and CPS vaccination both under undergo full liver stage and initiate a BS infection. In CPS vaccination, CQ drug selectively kills BS parasites with no effect on spz or LS parasites. LARC vaccination results in a longer liver stage than EARD vaccination, but both arrest in the liver and do not initiate a BS infection. Longer liver stage infections yield higher antigen loads, which may be advantageous for the host immune response. Figure inspired by [167] and created in BioRender. CQ=chloroquine.

This dissertation will focus on RAS, an EARD parasite that has been extensively researched and characterized. RAS vaccines were developed in the 1960s and are immunogenic and induce protective efficacy in mice [162,164,168,169]. From the 1960-2010s, RAS have been tested in mice, NHPs, and humans and are now the gold-standard WSV in the clinic. Decades of research on RAS vaccination have demonstrated that RAS induce both protective antibodies and cytotoxic $CD8^+$ T cells (reviewed in [124,153]), but that $CD8^+$ T cells are critical for RAS vaccine efficacy [92,112,170,171]. Specifically, RAS induces non-patrolling liver $CD8^+$ Trm cells that are critical for targeting and killing infected hepatocytes in the liver [82,121].

A key attribute of successful RAS vaccination is the ability of the spz to actively migrate and invade the liver. Non-motile inactivated or heat killed spz are not effective WSV [172,173]. The route of RAS administration is also extremely important for effective vaccination [174]. First generation RAS were administered through the bites of X-ray irradiated mosquitoes [175]. Mosquito bite vaccination is not practical and vaccine doses are too inconsistent and low for widespread clinical use. In 2010, Sanaria, Inc. patented an approach to aseptically purify infectious spz from mosquitoes, irradiate them, and then cryopreserve the spz for later administration via syringe [157]. Sanaria's cryopreserved RAS vaccine is called PfSPZ. Preclinical and clinical trials with PfSPZ demonstrated that intravenous (IV) administration achieved higher vaccine efficacy compared to intradermal (ID) or mosquito bite (MB) vaccination [92,155]. Thus, the current gold-standard RAS vaccination strategy consists of 3-4 doses of IV-administered PfSPZ.

To date, PfSPZ vaccines have exhibited variable levels of sterile protection in adult humans, ranging from ~50-100% sterile protection depending on the population demographics (reviewed in [152]). Despite the variable clinical success, the numerous clinical trials have demonstrated that RAS vaccination is feasible, safe, and can be highly effective in humans [92,154,155,176].

However, unlike subunit vaccines, RAS have many unique challenges that limit their widespread use, including strict liquid nitrogen vapor storage requirements, repeated high dose booster immunizations, IV-administration, and mosquito rearing (**Table 1.1**). Additionally, RAS vaccine efficacy is higher in malaria-naïve individuals [177] and data suggests that spz vaccination in the presence of an existing malaria infection reduces the WSV efficacy [178]. Thus, novel strategies to improve and simplify RAS and the other WSV for malaria endemic regions are urgently needed.

1.3.3 *Adjuvants*

One of the major challenges for PE vaccine development (for both subunit and WSV) is improving the rapidly waning vaccine induced immune responses. Combining a suboptimal vaccine with a potent adjuvant is one strategy to improve efficacy. Adjuvants are designed to enhance overall vaccine induced immune responses and can be dose sparing and/or improve the longevity of vaccine responses (reviewed in [179]). Adjuvants are classified by their mechanism of action and many different types of adjuvants have been identified [179]. Aluminum potassium sulfate (Alum) is the most widely utilized adjuvant in the clinic [180]. Decades of research with Alum have demonstrated its safety and efficacy and studies suggest that Alum preferentially induces a Th2-like biased immune response with low Th1-like or cytotoxic responses [179,180]. Different adjuvant classes induce diverse immune responses by triggering various innate or adaptive response pathways that bias the response toward a specific type of immunity (*e.g.*, Th1- or Th2-like). Thus, each vaccine requires individual optimization of the adjuvant component, and finding the right combination and ratio of vaccine and adjuvant is critical. A major challenge in the adjuvant field is optimizing immunogenicity while minimizing off-target effects.

Malaria subunit vaccines do not induce durable immune responses and thus adjuvants are required to improve immunogenicity and protective efficacy (reviewed in [181,182]). Multiple adjuvants were tested with RTS,S to determine which type yielded the optimal immune response [146,181]. Unlike subunit vaccines, WSV are live-attenuated organisms that contain many "endogenous adjuvants" that are typically not exogenously adjuvanted in the clinic [179]. However, the PfSPZ vaccine is highly purified, and thus the vaccine immunogenicity could be improved by the addition of an adjuvant. Many adjuvants have been tested with malaria vaccines [181,182], but direct comparisons of vaccine responses against multiple adjuvants is lacking. One head-to-head comparison showed that the adjuvant classification drastically impacted malaria $CD8^+$ Trm responses in the liver [183]. This data suggests that not all adjuvants induce favorable immune responses and understanding the impact of an adjuvant with an individual vaccine is important.

As described above, sterile protection from spz challenge requires cytotoxic $CD8^+$ cell responses [112]. Thus, adjuvants that specifically induce cytotoxic $CD8^+$ T cell responses are attractive for malaria vaccination. Glycolipid adjuvants like α-GalCer bind to CD1d on APCs, stimulate *i*NKT cells, and induce a cascade of immune cell activation including $CD8^+$ T cells (**Figure 1.4**) [184-186]. In 1944, α-GalCer was isolated from a marine sponge and shown to induce the activation of *i*NKT cells [187]. *i*NKT cells are abundant in mouse livers and are thus suggested to play an important role in malaria LS immunity, especially for liver-targeted vaccination strategies. In mice, α-GalCer has shown promising results as an adjuvant by increasing $CD8^+$ T cell responses [85,188-190], but clinical trials have been suboptimal [191]. The difference in efficacy between mouse and human studies have been attributed to the differences in the frequencies of *i*NKT cells in mice and humans [192,193]. To enhance α-GalCer adjuvant effects, many synthetic analogs were developed that can bind to CD1d with varying affinity and thus have different toxicological profiles and/or immunostimulatory effects [194]. Many of these novel synthetic analogs have even demonstrated enhanced effects at a lower dose [195]. One group found that synthetic glycolipid-peptide conjugate vaccines induce malaria specific CD8$^+$ Trm cells in the liver and superior levels of protection against spz challenge compared to unadjuvanted vaccines [196]. Another group reported that 7DW8-5 (**Figure 1.4**) is a promising synthetic analog of α-GalCer that has significant translational potential and increases the efficacy of both malaria subunit and WSV [189,190,195,197,198]. Based on these findings, this dissertation will further investigate 7DW8-5 as a malaria vaccine adjuvant to increase protective CD8$^+$ Trm cells in the liver and enhance vaccine protection.

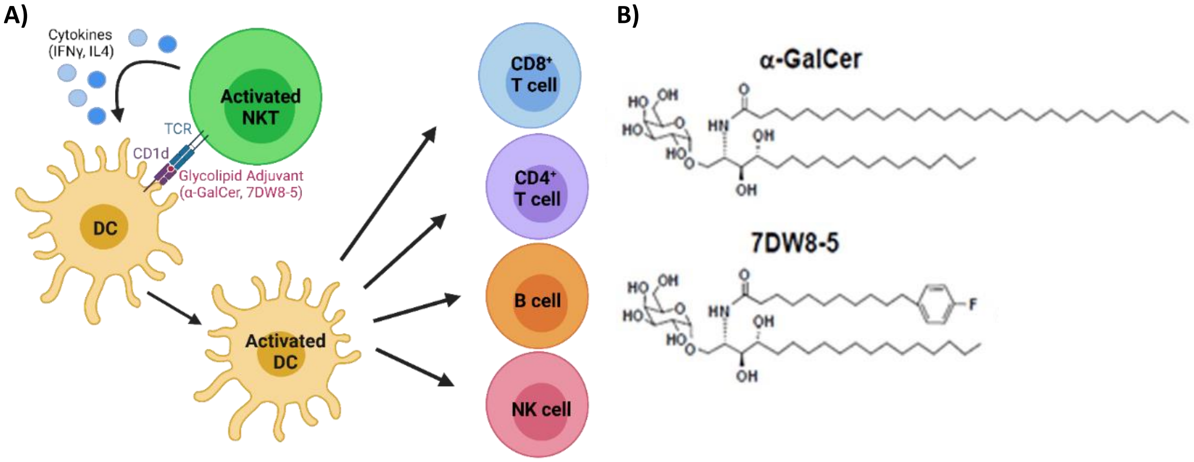


Figure 1.4. Glycolipid adjuvants induce the activation of innate & adaptive immune cells. A) Schematic of glycolipid adjuvant mechanism. Glycolipid adjuvants bind to CD1d expressed on non-professional and professional APCs, like DCs. Adjuvant bound CD1d binds the invariant TCR to activate NKT cells. Activated NKT cells secrete cytokines, including IFN-γ and IL-4, that aid in licensing and activating DCs. Activated DCs induce a cascade of other immune cell activation including CD8$^+$ and CD4$^+$ T cells, B cells, and NK cells. Figure inspired by [199] and created in BioRender. **B)** Structure of α-GalCer and 7DW8-5 [199].

1.3.4 *Heterologous prime-boost*

Traditionally, the same vaccine is administered as a series of homologous prime and boost doses. However, the prime and boost vaccines can be different or heterologous (reviewed in [200,201]). Previous studies have demonstrated that for complex pathogens, heterologous prime-boost strategies were more immunogenic and induced higher numbers of protective humoral and cellular responses compared to homologous strategies [202-205]. Further, Masopust et al 2006 tested various heterologous prime-boost strategies (*e.g.*, combinations of DNA, viral, and bacterial vectors) and found that they primarily led to an increase in CD8$^+$ Tem cells [205]. As the specific induction of robust and durable CD8$^+$ Tem cells are known to be important for malaria LS immunity, many PE vaccine development efforts have focused on heterologous prime-boost strategies (reviewed in [206-208]).

Studies in mice, NHPs, and humans have demonstrated superior immune responses when two different malaria subunit vaccines were combined into one vaccine strategy [142,209-211]. Specifically, the use of DNA-encoded malaria vaccines for prime-boost strategies has been extensively investigated [212-215], with data suggesting that plasmid DNA priming followed by a heterologous subunit booster vaccine induced higher CD8$^+$ T cell responses compared to homologous vaccination strategies [207,212,216-221]. However, despite the induction of higher cytotoxic immune responses in the periphery, heterologous prime-boost vaccine efficacy in clinical trials remained suboptimal [142,209,211,222-224]. Methods to enhance heterologous prime-boost approaches for malaria LS vaccine development are desired.

Preclinical and clinical studies have reported that a high number of malaria specific CD8$^+$ Trm cells must be positioned in the liver to achieve sterile protection from spz challenge [82,123,176]. Thus, designing prime-boost vaccines to specifically induce cellular responses in the liver may be advantageous. Several heterologous prime-boost strategies (herein called ‘prime-and-trap’) have been designed to target malaria specific CD8$^+$ T cells to the liver and can achieve 100% sterile protection in mice [82,118,121,123,225] and some NHPs (Shears et al 2023, unpublished). Prime-and-trap vaccines combine subunit vaccine priming in the periphery and boosting with a liver trafficking vaccine expressing the cognate antigen to pull and trap expanding immune cells in the liver. A promising preclinical prime-and-trap vaccine involves priming with DNA encoding CSP followed by a single IV dose of liver homing RAS (**Figure 1.5**) [121]. This strategy combines the two main types of malaria vaccines, subunit and WSV, in a heterologous prime-boost strategy [121]. This strategy reliably induces 100% sterile protection in female BALB/cJ mice and induces higher levels of CSP-specific CD8$^+$ Trm cells in the liver than DNA vaccine or RAS alone (**Figure 1.5** and [121]). However, although prime-and-trap confers sterile protection in mice, the vaccine is complex and requires unique components that complicate its translation from small to large animal models or humans. Pros and cons for prime-and-trap malaria vaccines are summarized in **Table 1.1**. This dissertation will focus on methods to simplify and optimize the translational potential of a promising prime-and-trap vaccine.

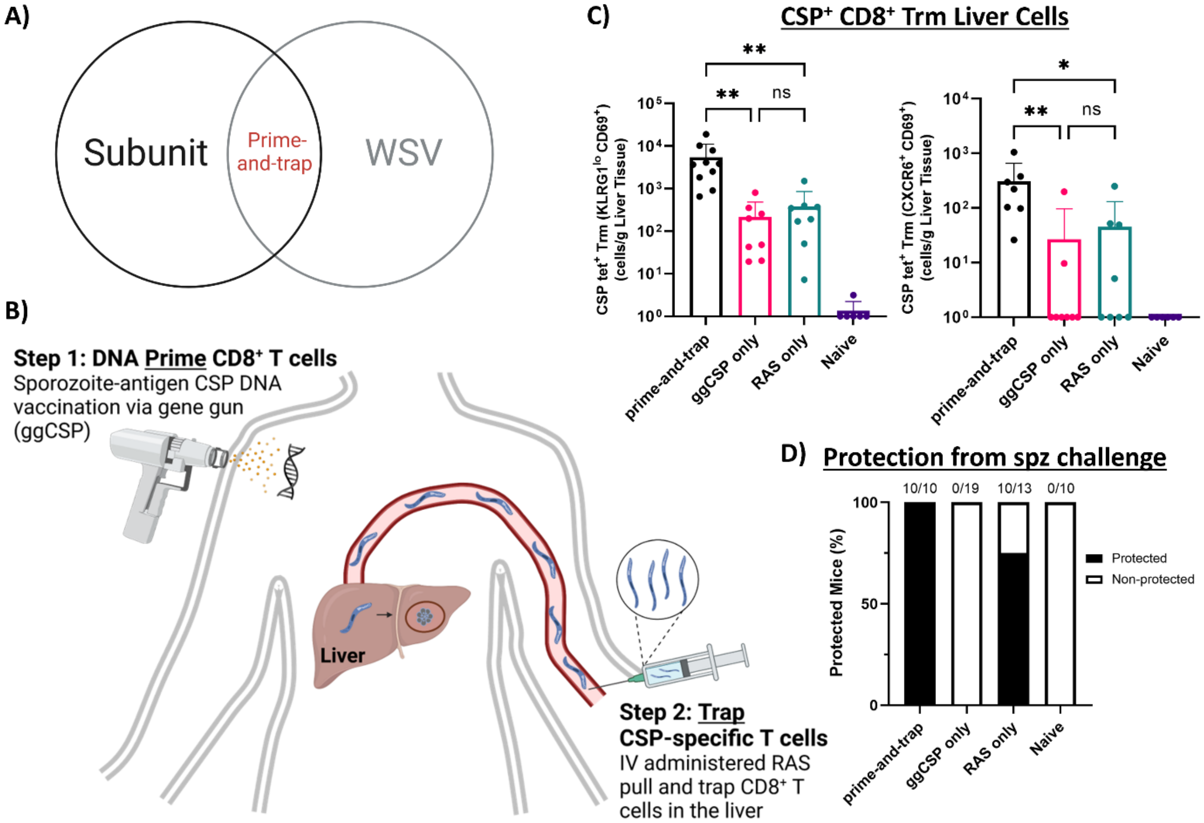


Figure 1.5. Heterologous prime-and-trap preliminary data.
A) Prime-and-trap combines subunit and WSV components into one heterologous prime-boost vaccine strategy. **B)** Schematic of the prime-and-trap strategy: CSP-encoded DNA vaccine

priming via gene gun followed four weeks later by IV administered RAS trapping. **C)** Flow cytometry of CD69^{hi}/KLRG1^{lo}/CSP-tetramer^{+} **(left)** or CD69^{hi}/CXCR6^{hi}/CSP-tetramer^{+} **(right)** CD8^{+} liver Trm cells from vaccinated mouse livers four weeks post vaccination. **D)** Results of parallel protection studies after challenge with 1x10^{3} WT purified Py spz four weeks post vaccination. Data in **(C)** and **(D)** adapted from [121] and analyzed with Kruskal-Wallis test, **p<0.01, *p<0.05, ns=p>0.05. Figures in **(A)** and **(B)** were created in BioRender.

1.4 DISSERTATION AIMS

1.4.1 *Dissertation overview*

The development of next-generation malaria vaccine strategies is critical to induce robust and durable protective immune responses. RAS vaccines can be highly effective and multiple IV-RAS doses can confer sterile protection in mice, NHPs, and malaria-naïve humans [92,154,155,226]. However, while RAS are highly effective in animal models and malaria-naïve humans, vaccine efficacy decreases in endemic regions and implementation is hindered by the requirement for repeated high dose IV injections. A heterologous prime-and-trap vaccine strategy was specifically designed to overcome these limitations of RAS-only vaccines. Prime-and-trap combines epidermal priming with DNA encoding the well-characterized CSP antigen followed by a single IV dose of RAS to direct and "trap" the activated T cells in the liver [121]. This vaccine strategy induces robust CSP-specific liver CD8^{+} Trm cell responses and confers durable protection against spz challenge in a rodent malaria model (**Figure 1.5**). However, the prime-and-trap vaccine is potentially too complicated for large scale vaccine implementation and decreasing the trapping dose and/or removing the need for IV administration would further enhance the translation of prime-and-trap to larger animal models or humans. This dissertation seeks to investigate methods to improve the translational potential of a heterologous prime-and-trap malaria vaccine.

1.4.2 *Dissertation specific aims*

This dissertation aims to investigate methods to decrease the RAS trapping dose by co-administration with the glycolipid adjuvant 7DW8-5 and seeks to understand why ID-RAS administration has historically been less protective than IV-RAS. The overall hypothesis is that the IV-RAS trapping dose can be reduced or substituted for ID-RAS by the co-administration of RAS with 7DW8-5. Additionally, ultra-low volume inoculation of ID-RAS is expected to overcome spz motility-related barriers to effective ID-RAS vaccination. Together, these rational vaccine modifications are expected to improve the translational potential of prime-and-trap. This will be accomplished through the following specific aims summarized in **Table 1.2**.

Table 1.2. Dissertation chapters and specific aims.

	Specific Aims
Chapter 2	**Improve the translational potential of IV-RAS trap in Prime-and-Trap.** **a)** Replace freshly dissected RAS with a more translational alternative (cryopreserved or heat-killed spz) and **b)** reduce the IV-RAS trapping dose.
Chapter 3	**Understand why ID-RAS has previously been less protective than IV-RAS administration and investigate methods to improve ID-RAS.** **a)** Determine how the ID-RAS inoculation volume impacts spz liver infectivity and protective efficacy and **b)** determine how the glycolipid adjuvant 7DW8-5 modulates local and systemic immune responses and impact protective efficacy following ID-RAS.
Chapter 4	**Understand the significant differential sex-specific responses to 7DW8-5.** Determine how **a)** local or systemic adjuvant administration impacts the expression of cytokines induced by 7DW8-5 in inbred mice, **b)** in outbred mice, and **c)** determine if 7DW8-5 improves the prime-and-trap vaccine efficacy in male BALB/cJ mice.

Chapter 1 has summarized the challenges associated with malaria vaccine development and discussed next-generation approaches including heterologous prime-boost and adjuvants to improve existing malaria vaccines. The remaining chapters in this dissertation will investigate

methods to improve a promising heterologous prime-and-trap malaria vaccine to improve the accessibility and translation of the vaccine to NHPs and humans. **Chapter 2** will investigate methods to enhance RAS vaccine efficacy by adjuvanting and investigate methods to enhance vaccine implementation by replacing freshly dissected RAS with cryopreserved RAS. **Chapter 3** will seek to eliminate IV-RAS administration requirements and understand the inferior immune responses associated with non-IV administration. **Chapter 4** will investigate the differential vaccine efficacy observed in male and female mice and explores sex specific responses to the glycolipid adjuvant 7DW8-5. Finally, **Chapter 5** will summarize the content of the dissertation and discuss limitations and future directions for the project.

Chapter 2. CRYOPRESERVED SPOROZOITES WITH AND WITHOUT THE GLYCOLIPID ADJUVANT 7DW8-5 PROTECT IN PRIME-AND-TRAP MALARIA VACCINATION

This chapter was previously published in the "American Journal of Tropical Medicine and Hygiene" in 2022 and has been reproduced and adapted here with permission [173].

2.1 CHAPTER SUMMARY

Repeated intravenous (IV) administration of radiation attenuated sporozoite (RAS) vaccines induces *Plasmodium*-specific CD8$^+$ liver resident memory T (Trm) cells in mice and achieves sterile protection against challenge. The heterologous 'prime-and-trap' vaccine strategy was previously shown to simplify and improve upon RAS vaccination. Prime-and-trap vaccination combines epidermal priming by DNA-encoded circumsporozoite protein (CSP) antigen followed by a single IV dose of freshly-dissected RAS (fresh-RAS) to direct and trap activated and expanding CD8$^+$ T cells in the liver. Prime-and-trap vaccination protects mice against wild-type sporozoite (spz) challenge. Assessment of prime-and-trap vaccines in non-human primate (NHP) models and/or humans would be greatly enabled if fresh-RAS could be replaced by cryopreserved RAS (cryo-RAS). This study investigated if fresh-RAS could be replaced with cryo-RAS for prime-and-trap vaccination in BALB/cJ mice. Despite a reduction in spz vaccine liver burden following cryo-RAS administration compared to fresh-RAS, cryo-RAS induced a similar level of *P. yoelii* (Py) CSP-specific CD8$^+$ liver Trm cells and completely protected mice against Py spz challenge 112 days after vaccination. Additionally, when the glycolipid adjuvant 7DW8-5 was co-administered with cryo-RAS, 7DW8-5 permitted the dose of cryo-RAS to be reduced four-fold while still achieving high rates of sterile protection. In summary, cryo-RAS with and without 7DW8-5 were compatible with prime-and-trap malaria vaccination in a mouse model, which may accelerate the pathway for this vaccine strategy to move to NHPs and humans.

2.2 INTRODUCTION

Malaria is caused by *Plasmodium* parasites and was responsible for an estimated 247 million cases, and 619,000 deaths in 2021 [2]. Human *Plasmodium* infection begins when an individual is bitten by an infectious female *Anopheles* mosquito and spz are transmitted into the skin. Spz home to the liver and infect hepatocytes, replicating and differentiating for approximately 7-10 days before merozoites egress into the bloodstream to invade and begin replication within red blood cells. Blood stage infection is responsible for symptomatic malaria and further transmission [227,228]. Candidate malaria vaccines target different stages of the parasite lifecycle with the most successful vaccines thus far targeting the pre-erythrocytic (PE) spz and liver stages (reviewed in [229]). *Plasmodium* PE vaccines are particularly attractive as they would not only prevent blood stage infection and all clinical manifestations, but also stop further transmission of the parasite.

Sanaria, Inc. PfSPZ Vaccine and PfSPZ-CVac are live-attenuated whole spz vaccines (RAS and CPS, respectively) that can routinely induce greater than 90% sterile protection in humans [154,155,158,176,230-232]. Repeated IV administration of RAS vaccines can achieve sterile protection in mice, NHPs, and humans [92,155,165,166,170,233]. These RAS vaccines induce both humoral and cellular responses, which block spz invasion of hepatocytes [155,165,234] and kill infected cells, respectively [235]. The importance of cellular immune responses in the liver has been increasingly recognized as necessary for achieving sterile protection after RAS vaccination in mice [112,117,171,236,237]. The induction of $CD8^+$ T cells, specifically liver-resident memory $CD8^+$ T (Trm) cells, appears to be critical for long-term protection [82,92,155,226,238]. While the production of aseptic, purified, cryopreserved PfSPZ vaccines has seen significant advances in manufacturing, reducing the dosage or number of PfSPZ booster immunizations would nonetheless significantly reduce the cost of goods.

It was previously reported that a prime-and-trap vaccine strategy simplified and improved upon repeated RAS immunization of mice while maintaining the immunogenicity and protection of whole spz vaccines [121]. Prime-and-trap here comprises a two-step heterologous vaccine strategy that combines epidermal priming of DNA encoding the well-characterized CSP spz antigen with a single IV dose of RAS to direct and trap the activated and expanding CD8$^+$ T cells in the liver. This strategy induces robust malaria-specific CD8$^+$ Trm cell responses in the liver and confers sterile protection in the *P. yoelii* rodent malaria model [121]. However, it is not possible to use freshly-dissected RAS to immunize humans as any human vaccine must go extensive quality control release assay testing before administration.

Sanaria has pioneered the manufacture and use of aseptic, purified, cryopreserved PfSPZ products for use in humans including the radiation-attenuated PfSPZ [92,154,155,176,177,231,239], the chemo-attenuated Pf-CVac [165,231], the genetically-attenuated PfSPZ-GA1 vaccine [240], and infectious PfSPZ challenge parasites used in controlled human malaria infections [230,241]. Studies in mice have shown that it requires approximately seven times as many cryopreserved Py spz (cryo-RAS) as freshly dissected Py spz (fresh-RAS) to comparably infect mice [241] and three times as many cryo-RAS as freshly-RAS to comparably protect mice after immunization [92]. The cryo-RAS PfSPZ vaccine achieved sterile protection in many malaria-naïve individuals [155,226] and some malaria-exposed individuals [177,230]. These findings suggested that cryo-RAS may be used as an alternative to fresh-RAS in prime-and-trap vaccination.

This study investigated if fresh-RAS can be replaced with Sanaria-produced irradiated, purified, cryopreserved PySPZ (termed ‘cryo-RAS’) in the heterologous prime-and-trap vaccine. Mice primed with DNA encoding the PyCSP antigen administered via gene gun followed by trapping with fresh-RAS or cryo-RAS developed similar numbers of durable PyCSP-specific liver CD8$^+$

Trm cells and were equivalently protected against Py spz challenge. Moreover, the prime-and-cryo-RAS-trap vaccine can be further improved with the addition of a dose-sparing glycolipid adjuvant. These findings demonstrate that prime-and-trap is a versatile malaria vaccine that can induce protective liver-resident memory CD8^{+} T-cells using a single cryo-RAS dose, thereby making the strategy more feasible for translation into NHPs and humans.

2.3 METHODS AND MATERIALS

2.3.1 *Mice*

Female BALB/cJ mice (4-6 week old) were obtained from Jackson Laboratories (Barr Harbor, ME) and housed at University of Washington in an Institutional Animal Care and Use Committee (IACUC)-approved facility. All mice were used under IACUC protocol 4317-01 to SCM.

2.3.2 *Freshly-dissected spz vaccination and challenge*

Female *Anopheles stephensi* mosquitoes infected with wild-type (WT) Py (strain 17XNL) were reared at Seattle Children's Research Institute (Seattle, WA). Fresh spz were obtained by salivary gland dissection 14-18 days post-infection followed by Accudenz gradient purification as described [242]. Fresh-RAS were generated by dissecting spz from mosquitoes, purifying them as above, and then irradiating by x-ray exposure (10,000 rads; Rad Source). As a control, heat-killed spz (HK-spz) were generated by incubating WT Py spz in a 55°C water bath for 30 minutes. All spz (WT, RAS, or HK) were administered IV in a volume of 100 µL of Schneider's insect media (Gibco, Thermo Fisher Scientific). 1x10^{3} freshly dissected WT Py spz were used for mouse challenge experiments, unless otherwise specified. Blood stage protection after spz challenge was assessed by Giemsa-stained thin blood smear microscopy on Days 3-14 post-challenge. Mice were deemed protected if blood smears remained negative for parasites up to Day 14.

2.3.3 *DNA vaccination by gene gun*

The PyCSP minigene DNA vaccine encoding the SYVPSAEQI epitope was constructed in the pUb.3 vector and tagged with an N-terminal ubiquitin tag as described [121,243,244]. For all vaccinations, *Escherichia coli* heat-labile toxin (LT)-encoding plasmid was used as an adjuvant in a 1:10 ratio with the PyCSP-minigene vector [245]. DNA was purified using an endotoxin-free purification kit (Qiagen, #12362), loaded onto 0.8-1.5 μm diameter gold powder (Technic Inc., #12-507), and coated on tubing as cartridges as described previously [244]. Abdominal fur was trimmed, and mice were vaccinated on the abdomen using a PowderJect-style gene gun [246] by priming using two cartridges per day on Days 0 and 2 (0.5 μg DNA per cartridge). This method of priming with PyCSP-minigene/LT-encoding plasmid via gene gun is referred to as ggCSP.

2.3.4 *Cryopreserved spz vaccination and challenge*

Radiation-attenuated (100 Gy by C0-60), purified, vialed, cryopreserved 17XNL PySPZ (cryo-RAS) were produced by Sanaria Inc. (Rockville, MD) as described [92,247]. The vials were shipped to Seattle and stored in vapor phase liquid nitrogen per manufacturer recommendations. Cryo-RAS were thawed in a 37°C water bath for 30 seconds, diluted in 100 μL of Schneider's insect media, and administered IV within 30 minutes. Spz counts were confirmed on a hemocytometer within one hour of injection.

2.3.5 *Glycolipid adjuvant preparation*

Good manufacturing practice (GMP) grade 7DW8-5 powder was dissolved in DMSO, aliquoted, and stored at -20°C. Single use aliquots were thawed at 56°C for 10 min and then sonicated in an ultrasonic water bath for five min to break micelle formation. The 7DW8-5 was mixed with diluted RAS immediately before administration to mice. All mice received 2 μg of 7DW8-5 adjuvant.

2.3.6 *Mouse plasma ELISA*

IFN-γ or IL-4 cytokine levels were determined by commercial ELISA kit according to manufacturer's instructions (BioLegend, #430801, #431104). Blood was collected via submental bleed into tubes containing EDTA, and plasma was isolated and frozen. Plasma was diluted in kit diluent, and absorbance was read on the CLARIOstar Plus plate reader (BMG Labtech) according to kit instructions. Standard curves and concentrations were calculated in Microsoft Excel.

2.3.7 *CD1d depletion*

Mice were injected intraperitoneally (IP) with 10 μg of anti-mouse CD1d (CD1.1) (BioXcell, #BE0000) or matched isotype control (BioXcell, #BE0088) 24 hours before 7DW8-5 injection. Mouse blood was collected via submental bleed and plasma was isolated 12- and 24-hours post-adjuvant administration. Plasma IFN-γ levels were analyzed by ELISA as described above.

2.3.8 *Liver burden reverse transcription polymerase chain reaction (RT-PCR)*

Four hours post-RAS immunization, mice were sacrificed, and half of the liver was excised and pulverized by bead beating (1.0mm zirconia beads, BioSpec) in 5 mL NucliSENS lysis buffer (bioMérieux, Durham, NC). Total RNA was extracted by processing 50 μL of the NucliSENS buffer-treated sample diluted 1:20 in NucliSENS lysis buffer on the EasyMag system (bioMérieux) as described for whole blood [248]. RNA was subjected to RT-PCR using the SensiFAST™ Probe Lo-ROX Kit (Bioline, London, UK) using a predesigned HEX-labelled mouse GAPDH RT-PCR assay (IDT Inc, Coralville, IA) multiplexed with a Pan-*Plasmodium* 18S rRNA assay as described [249]. Conditions of 45 °C for 10 min, 95 °C for 2 min and 45 cycles of 95 °C for 5 s, 50 °C for 35 s were run on a QuantStudio 5 real-time PCR machine (ThermoFisher). Data were normalized to mouse GAPDH and copy numbers per reaction were determined using custom lot of quantified Armored RNA encoding full-length *Plasmodium* 18S rRNA (Asuragen, Austin, TX).

2.3.9 *Liver lymphocyte isolation and flow cytometry*

Liver lymphocytes were isolated by mechanical dissociation and Percoll density gradient as previously described [121,250]. Briefly, livers were perfused, mashed into a single cell suspension, and intrahepatic lymphocytes were isolated. Final liver lymphocyte pellets were resuspended in 150 µL 1X MACS buffer (PBS, 1 mM EDTA, 0.5% Fetal bovine serum (FBS)) and transferred to a U-bottom 96-well plate for blocking and staining for flow cytometry. All antibodies and flow cytometry analyses were as previously described [121] with the minor modification of the addition of a live/dead dye (Zombie NIR Fixable Viability Kit, BioLegend) to enable exclusion of dead cells from downstream analysis. All reagents used for flow cytometry are listed in **Appendix A**. Briefly, liver lymphocytes were treated with an Fc block and live/dead dye for 30 min (anti-CD16/32, clone 2.4G2; BD Biosciences), stained with antibody cocktail for 45 min, and fixed for 20 min (Cytofix/Cytoperm reagent; BD Biosciences) [121]. Flow cytometry was conducted on the LSRII instrument (BD Biosciences), and data were analyzed with FlowJo version 10.7.1 (BD Biosciences). A representative flow cytometry gating strategy is shown in **Appendix A**.

2.3.10 *Ex vivo IFN-γ ELISPOT*

PyCSP peptide (SYVPSAEQI) was synthesized by Genemed Synthesis and reconstituted in DMSO. Mouse IFN-γ ELISPOT (eBioscience) was conducted by stimulating 5×10^5 splenocytes with CSP peptide (or DMSO vehicle control) at 1 µg/ml for 18 hr at 37°C and developed following manufacturer guidelines as reported previously [121,251]. The number of spot-forming units

(SFU) in each well was calculated using an ImmunoSpot 5.1 Analyzer (Cellular Technology Limited, OH). SFU were normalized to DMSO control and SFU/million splenocytes was reported.

2.3.11 *DNA vaccination by electroporation*

Isoflurane anesthetized mice were intradermally (ID) injected with 2.5 µg DNA plasmid vaccines encoding PyCSP-Luciferase or phosphate buffered saline (PBS) in 30 µL on the lower back (near the base of the tail) with a 31G syringe (BD Veo Insulin Syringe with Ultra-Fine needle 6 mm × 31 G 3/10 mL/cc; #324909). Immediately after ID injection, a 4-needle array electrode (BTX™ #470040) was inserted ID over the injection site and *in vivo* electroporation (EP) was performed as described (2 pulses, 1,125 V/cm, 50 µs + 8 pulses, 275 V/cm, 10 ms) [252] with the BTX™ AgilePulse Waveform electroporator. Two ID DNA injections/EP were performed per mouse.

2.3.12 *Live in vivo imaging of protein expression*

Live *in vivo* imaging of Luc protein expression following EP vaccination was similarly performed as previously described [121,253]. Briefly, mice were EP immunized with 2.5 µg PyCSP plasmids expressing a luciferase reporter (PyCSP-Luc) and LT plasmid (1:10 ratio). Twenty-four hours later, bioluminescence was evaluated by IP administration of 30 mg/mL D-luciferin (Gold Biotechnology, St. Louis, MO) followed by isoflurane anesthesia and IVIS imaging. IVIS images were quantitatively evaluated using Living Image 3.0 software (Perkin-Elmer) with regions of interest (ROI) placed around the shaved ID injection and EP sites.

2.3.13 *Statistics*

Comparisons of liver burden RT-PCR and ELISA groups were done using non-parametric Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparisons test. Comparisons of flow cytometry cell counts, ELISPOT, and IVIS data were done using the non-parametric two-tailed Mann-Whitney *U* test. Protection data was evaluated using Fisher's exact test. All groups were compared against the ggCSP prime and 2×10^4 fresh-RAS trap positive control (unless otherwise specified). Error bars in figures are reported as standard deviation (SD) of the mean with individual mouse samples shown if applicable (unless otherwise specified). All p-values are listed in corresponding figure legends. Statistical significance was defined as $p < 0.05$. Prism GraphPad Prism 9.1.2 Software (San Diego, CA) was used for all calculations.

2.4 RESULTS

2.4.1 *DNA prime and fresh- or cryo-RAS trap leads to substantial parasite liver burden*

Based on earlier work, prime-and-trap vaccination using RAS for trapping is predicated on achieving a liver burden upon RAS trapping that is immunogenic and favorable for $CD8^+$ Trm cell formation [121,244]. The working hypothesis is that if the vaccination phase RAS liver burden was significantly reduced, $CD8^+$ Trm cell formation may also be hindered, and protection could be lost. This was a possibility since the liver-homing ability of cryo-RAS was previously shown to be somewhat reduced compared to fresh-RAS [254]. To investigate the liver trapping potential of cryo-RAS, BALB/cJ mice were DNA primed with ggCSP and then administered 2×10^4 Py fresh- or cryo-RAS trap four weeks later (**Figure 2.1**). Non-viable Py HK-spz were used as a control. Four hours post-trapping, livers were harvested for RT-PCR to compare parasite burdens. This timepoint was selected to capture the number of parasites that initially invaded the liver, allowing time for the majority of circulating spz to home to the liver but not enough time to be targeted and/or killed there [255]. The parasite burden in cryo-RAS immunized mice was significantly reduced compared to fresh-RAS but was still very high relative to HK-spz (**Figure**

2.1). Cryo-RAS 18S rRNA copy numbers per mouse liver were approximately nine-fold lower than after fresh-RAS. This was in striking contrast to the HK-spz liver burden, where 18S rRNA was not detectable in any mouse. Thus, it was hypothesized that the known reduction in viability and motility of Py cryo-RAS compared to fresh-RAS led to approximately 1 log reduction in parasite liver burden. The next aim was to determine if this difference would alter the suitability of cryo-RAS as a trapping candidate.

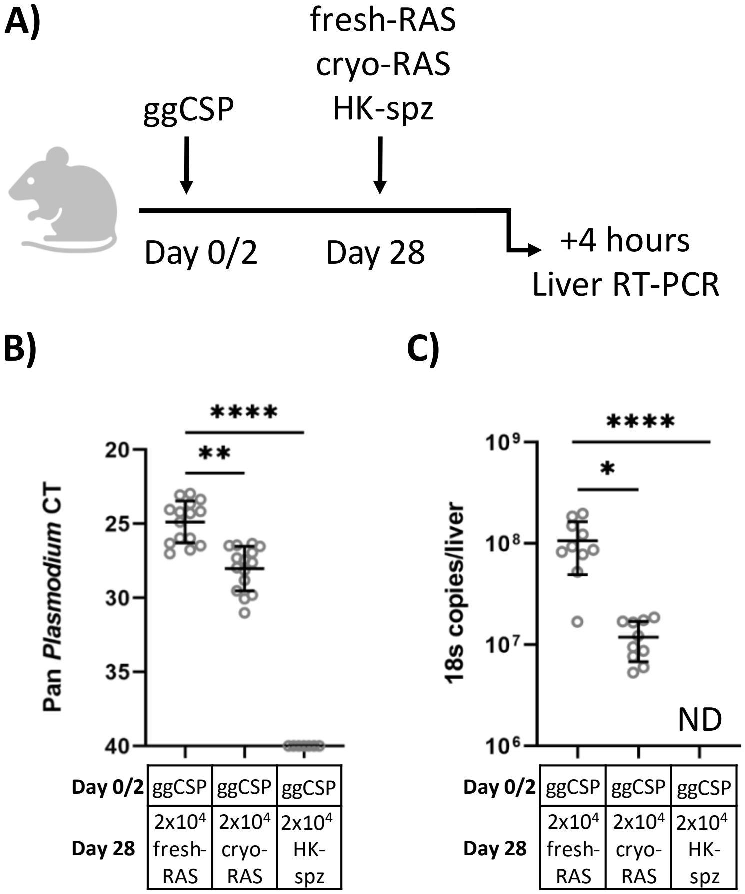


Figure 2.1. Prime and fresh- or cryo-RAS trap leads to substantial parasite liver burden. A) Experimental design of prime-and-trap liver burden studies. **B)** Four hours after trapping with fresh-RAS, cryo-RAS, or HK-spz, mouse livers were excised and processed for real-time RT-PCR to measure liver stage parasite burden with 18S pan *Plasmodium* primers. Data are shown as cycle thresholds **(B)** for all mice and for absolute 18S rRNA quantification for a subset of the mice where absolute calibrators were used **(C)**. Error bars represent SD of mean of N=15 mice across two experiments (N=7 mice across two experiments for HK-spz). Data points correspond to individual mice. Kruskal-Wallis with Dunn's multiple comparisons test: fresh-RAS vs all other groups. ND=not detected. $^{*}p<0.05$ $^{**}p<0.005$ $^{****}p<0.0001$.

2.4.2 *Py fresh- or cryo-RAS trap induce comparable levels of PyCSP-specific liver CD8⁺ Trm cells*

Despite the reduction of parasite burden following cryo-RAS trap, the cryo-RAS present in the liver was hypothesized to be sufficient to induce protective PyCSP-specific liver $CD8^+$ Trm cells. To investigate, mice were primed with ggCSP and administered $2x10^4$ fresh- or cryo-RAS as above, then mouse livers were harvested four weeks post-trap for flow cytometry to compare PyCSP-specific $CD8^+$ T cell frequencies. Previous data showed that prime-and-trap induced high frequency PyCSP-specific liver $CD8^+$ Trm cells at this timepoint [121]. To define liver PyCSP tetramer-stained $CD8^+$ Trm cells, cells were gated on either $CD69^+$/$KLRG1^{lo}$ or $CD69^+$/$CXCR6^{hi}$ expression as previously described [82,121,256]. The levels of PyCSP-specific $CD8^+$ Trm cells in livers from cryo- versus fresh-RAS trapped mice were significantly higher despite the differing parasite liver burdens observed by RT-PCR (**Figure 2.2**). The frequency of PyCSP-specific $CD8^+$ Trm cells in the livers of cryo-RAS trapped mice were also higher than in either of the control groups, which received ggCSP prime only or were completely naïve. Furthermore, increasing the cryo-RAS trap dose 10-fold to $2x10^5$ cryo-RAS only modestly increased the PyCSP-specific liver $CD8^+$ T cells (**Figure 2.2**). Taken together, this data suggests that despite the reduced parasite liver burden observed following cryo-RAS trap, this immunization strategy induces high frequency PyCSP-specific liver $CD8^+$ Trm cells.

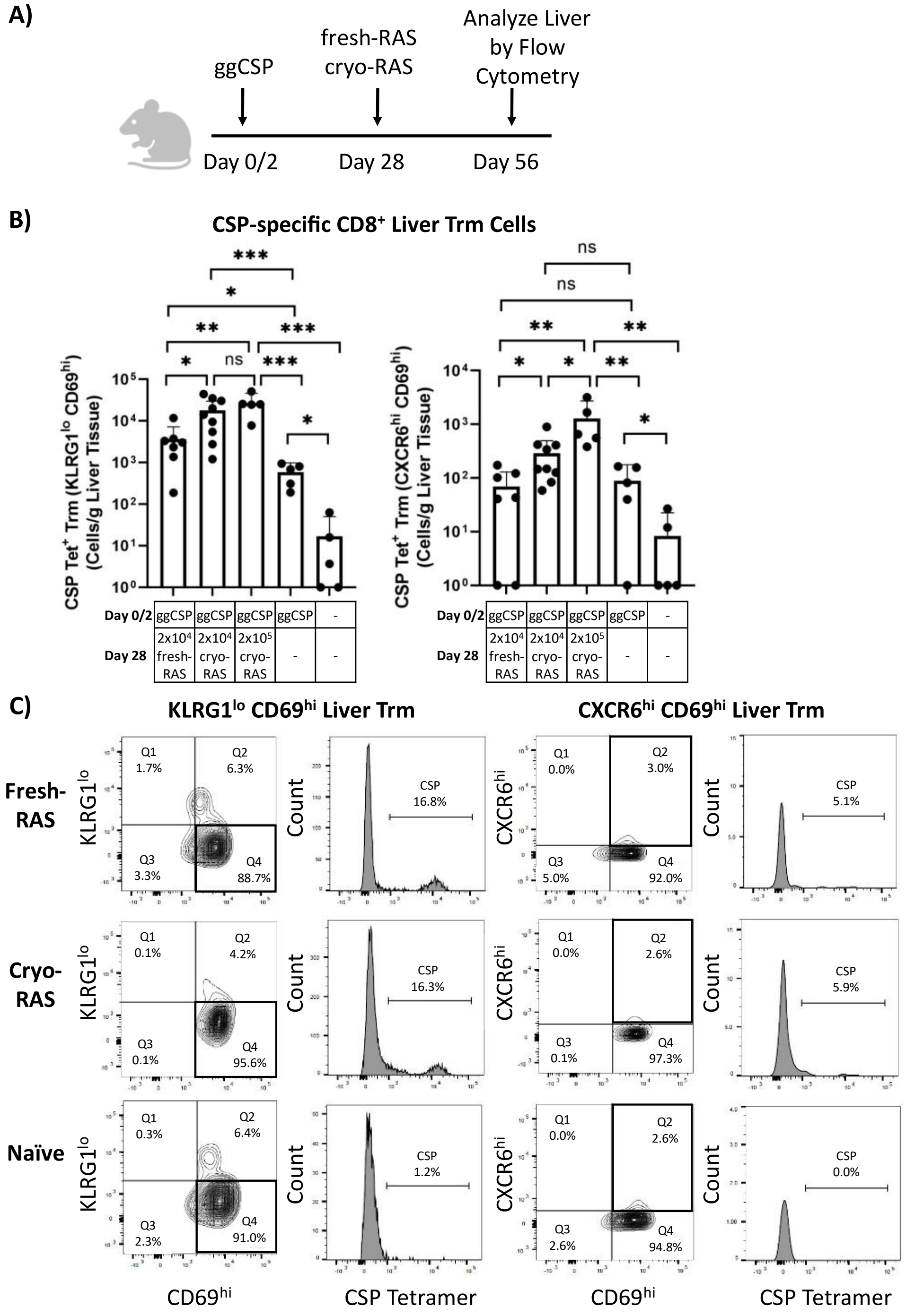


Figure 2.2. Prime and fresh- or cryo-RAS trap induce comparable levels of CSP-specific liver CD8$^+$ Trm cells.

A) Experimental design of prime-and-trap studies. **B)** Flow cytometry of CD69^{hi}/KLRG1^{lo} (**Left**) or CD69^{hi}/CXCR6^{hi} (**Right**) tetramer-stained, CSP-specific CD8$^+$ liver Trm cells from **(A)** livers. **C)** CSP-specific CD8$^+$ liver Trm cell gating strategies, one representative animal per group is shown. Error bars represent 95% CI of the mean of N=7-9 mice across two experiments (2x10^5 cryo-RAS, ggCSP only, and naïve groups N=5 mice from one experiment). Mann-Whitney two-tailed test. ns=p>0.05. *p<0.05. **p<0.008. ***p<0.0001.

2.4.3 *DNA priming followed by cryo-RAS trap protects mice against Py spz challenge 4-6 weeks post-vaccination*

Next, the study investigated if DNA priming followed by cryo-RAS trap could protect mice against spz challenge at a memory timepoint. Previous studies demonstrated that ggCSP prime and fresh-RAS trap completely protected mice when challenged at four weeks [121]. As CSP-specific liver Trm cell frequencies were similar in fresh- or cryo-RAS trapped mice, cryo-RAS trap was hypothesized to similarly protect mice against spz challenge at a memory timepoint. Mice were ggCSP primed and trapped with $2x10^4$ fresh- or cryo-RAS as before, then challenged with $1x10^3$ fresh wild-type infectious Py spz 4-6 weeks later. Both fresh- and cryo-RAS achieved comparable protection at the four-week challenge interval (**Figure 2.3**). Protection at six weeks was slightly reduced in the cryo-RAS trapped group compared to fresh-RAS, although this difference was non-significant. As expected, fresh- or cryo-RAS trap only or ggCSP prime only control group mice showed little to no protection. In addition, no protection was observed in mice that received ggCSP priming with HK-spz trap at either timepoint (**Figure 2.3**). Taken together, this data suggest that cryo-RAS can be substituted for fresh-RAS in prime-and-trap, can induce comparable levels of liver $CD8^+$ Trm cells, and can similarly protect mice against spz challenge at a memory timepoint.

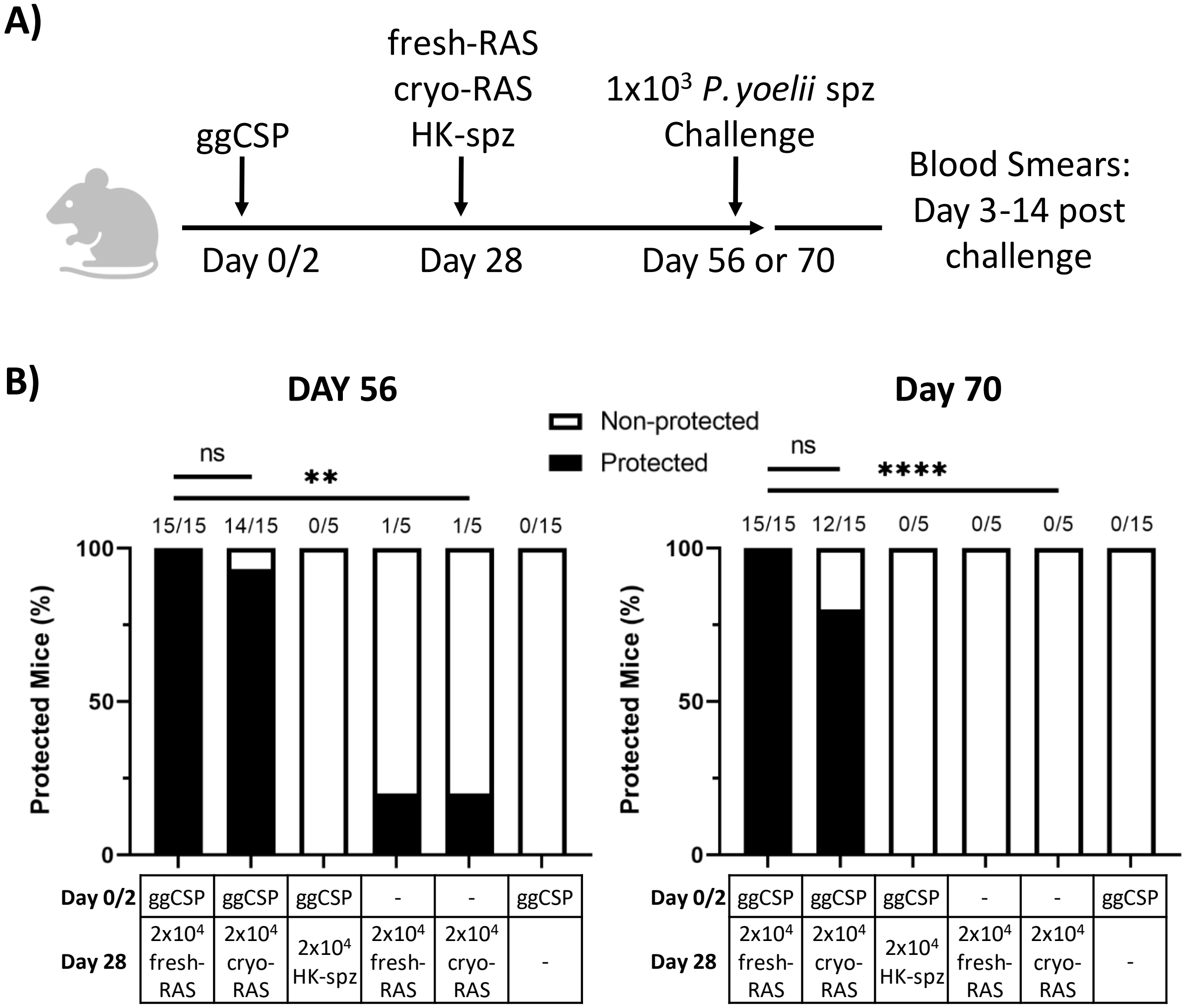


Figure 2.3. DNA priming followed by cryo-RAS trap protects mice against Py spz challenge 4-6 weeks post-vaccination.

A) Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with 1x10^3 WT purified *P. yoelii* spz administered four (**Left**) or six (**Right**) weeks after trapping. N=15 mice across two experiments (N=5 mice from one experiment for HK-spz and RAS trap only (no ggCSP prime) groups). Fractions above bars indicate number of animals protected out of total group size. Protection was assessed with thin blood smears on days 3-14 post challenge. Fisher Exact Test: 2x10^4 fresh-RAS vs all other groups. ns=$p>0.05$. **$p<0.005$. ****$p<0.0001$.

2.4.4 *Py cryo-RAS trapping can be dose de-escalated by co-administration of glycolipid adjuvant 7DW8-5*

In response to the trend for reduced vaccine efficacy following prime-and-trap with cryo-RAS versus fresh-RAS at the six-week challenge timepoint, the study investigated if the addition of an adjuvant could both increase vaccine efficacy and extend durability. Earlier studies identified the glycolipid adjuvant 7DW8-5 as a means to increase malaria vaccine efficacy in mice and NHPs [189,190,195]. 7DW8-5 is an α-galactosylceramide (α-GalCer) analog selected for its potent adjuvant activity and reduced toxicity compared to other earlier α-GalCer analogs [195]. Mechanistically, 7DW8-5 is presented on CD1d molecules to activate invariant natural killer T (*i*NKT) cells. When activated, *i*NKT cells produce Th1 and Th2 cytokines, including IFN-γ and IL-4 [257]. 7DW8-5 is an attractive adjuvant for malaria as it co-localizes in the draining lymph nodes with RAS and increases activation of $CD8^+$ T cells [189,190,195]. Here, the effect of 7DW8-5 on the required trapping dose and protection durability of prime and cryo-RAS trap was tested.

First, the cytokine responses induced by IV administration of 7DW8-5 were characterized. α-GalCer analogs containing phenyl groups were previously shown to induce Th1 skewed cytokine responses [194]. Here, IV administration of 7DW8-5 induced a potent transient spike of IFN-γ and to a lesser extent IL-4 in mouse blood (**Figure 2.4**). Consistent with the literature [189,190], IFN-γ expression peaked approximately 12 hours post-injection and returned to baseline levels by 48 hours. To demonstrate the specificity of the adjuvant response, mouse CD1d was transiently blocked prior to 7DW8-5 injection using an anti-mouse CD1d antibody. IFN-γ was significantly blocked by anti-CD1d, but not a matched isotype control (**Figure 2.4**). This data demonstrates that 7DW8-5 can be IV administered to mice and induces a specific and transient spike of IFN-γ in the blood that is cleared within 48 hours.

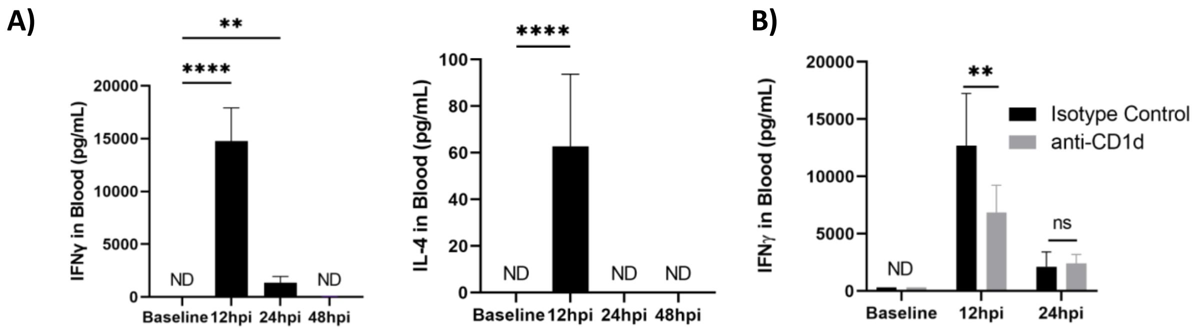


Figure 2.4. IV-administration of 7DW8-5 induces a systemic IFN-γ and IL-4 spike. **A)** Quantification of IFN-γ (**left**) and IL-4 (**Right**) in mouse blood plasma induced by 7DW8-5 injection over time measured by ELISA. Kruskal-Wallis with Dunn's multiple comparisons test, baseline vs all other groups. **B)** Results of anti-CD1d blocking studies after administration of 7DW8-5. Multiple unpaired t-tests. Error bars represent SD of mean of N=10 mice across two experiments. ND=not detected. ns=p>0.05. **p<0.005 ****p<0.0001.

Expression of IFN-γ is correlated with protection in NHP and human clinical trials [176]. Based on the preliminary mouse studies, the IFN-γ induced by 7DW8-5 administration was hypothesized to improve cryo-RAS efficacy and durability. Preliminary experiments investigated the durability of prime-and-trap with fresh-RAS trapping and found that sterile protection against spz challenge at four months could be achieved with a high dose of fresh-RAS ($5x10^4$) or with the $2x10^4$ fresh-RAS combined a ggCSP boost (**Supplementary Figure S2.8**). Thus, prime-and-trap with $2x10^4$ fresh- or cryo-RAS was hypothesized to protect a substantial portion of mice at four months and any reduced protection could be rescued by the addition of 7DW8-5 at the trapping step. To investigate this possibility, mice were ggCSP primed and then trapped with either $2x10^4$ fresh- or cryo-RAS with or without 7DW8-5. Four months post-trapping, mice were challenged with $1x10^3$ freshly dissected wild-type Py spz and protection was assessed. Somewhat surprisingly, given the data in **Figure 2.3** suggesting reduced protection with cryo-RAS trap at 6-weeks, prime-and-trap with fresh- or cryo-RAS achieved complete protection against spz challenge at four months with or without the addition of 7DW8-5 (**Figure 2.5**). The reasons for slight differences in protection in the 4-6 week challenge (86-100% protection) versus four-month challenge (100% protection) are unclear, but could include inter-operator variations in IV techniques and/or differences in batches of spz. Nonetheless, in summary, the data indicates that PyCSP DNA prime and cryo-RAS trap vaccination achieves long lasting sterile protection in mice.

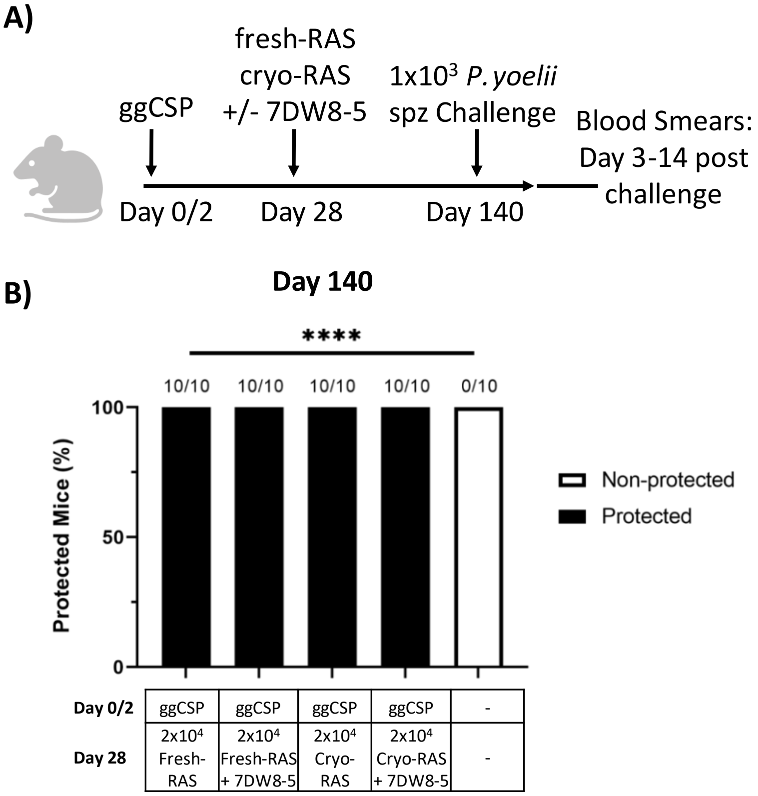


Figure 2.5. DNA priming followed by fresh- or cryo-RAS trap +/− 7DW8-5 induces durable protection in mice.

A) Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with $1x10^3$ WT purified *P. yoelii* spz administered four months (Day 140) after trapping. N=10 mice across two experiments. Fractions above bars indicate number of animals protected out of total group size. Protection was assessed with thin blood smears days 3-14 post challenge. Fisher Exact Test: fresh-RAS vs all other groups. ns=p>0.05. ****p<0.0001.

Next, the study determined if the cryo-RAS dose required for sterile protection could be decreased. Preliminary experiments investigating prime-and-trap with fresh-RAS dose de-escalation suggested that $2x10^4$ fresh-RAS were required for reliable sterile protection (**Supplementary Figure S2.9**). Thus, the study investigated if the cryo-RAS trapping dose could be reduced by the co-administration with the glycolipid adjuvant 7DW8-5. Mice were ggCSP primed followed by

$2x10^4$, $5x10^3$, or $5x10^2$ cryo-RAS with or without the addition of 7DW8-5. The full dose of $2x10^4$ cryo-RAS was required for complete protection in the absence of 7DW8-5, but the dose of cryo-RAS could be reduced four-fold by co-administration with 7DW8-5 while still maintaining ≥90% sterile protection (**Figure 2.6**). As expected, control mice receiving ggCSP prime followed by 7DW8-5 trap without any cryo-RAS, and those immunized with only cryo-RAS+7DW8-5 (no ggCSP prime) were not protected against spz challenge. In summary, prime-and-trap vaccination with cryo-RAS is feasible and durable, and the cryo-RAS trapping dose can be reduced by co-administration of 7DW8-5.

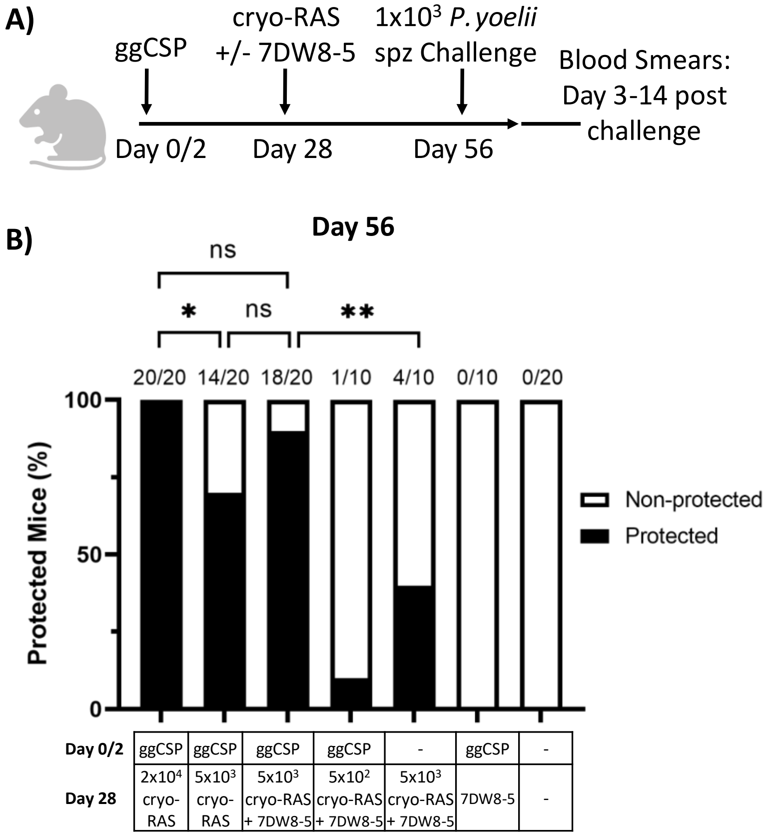
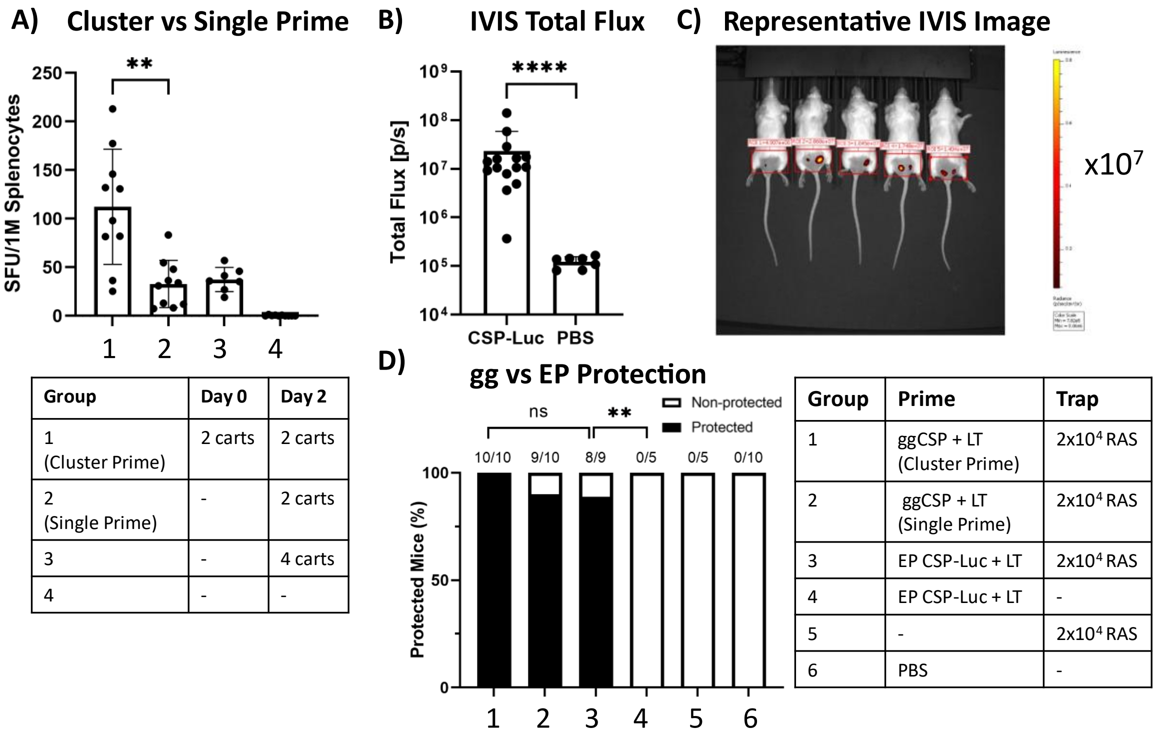


Figure 2.6. Cryo-RAS trap dose can be reduced by co-administration of 7DW8-5.
A) Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with $1x10^3$ WT purified *P. yoelii* spz administered four weeks (Day 56) after trapping. N=10-20 mice across 2-4 experiments. Fractions above bars indicate number of animals protected out of total group size. Protection was assessed with thin blood smears days 3-14 post challenge. Fisher Exact Test: $2x10^4$ cryo-RAS vs all other groups. ggCSP+($5x10^3$ cryo-RAS+7DW8-5) vs all other groups. ns=$p>0.05$. *$p<0.05$ **$p<0.01$.

2.4.5 *Gene gun or EP DNA priming are both protective in prime-and-trap vaccination*

The final set of experiments sought to better understand the priming requirements for prime-and-trap. All the prime-and-trap experiments in this section thus far have utilized ggCSP priming, which was previously reported to be immunogenic and induce reliable levels of protection against spz challenge in mice [121]. However, as ggCSP involves cluster priming on Days 0 and 2, it is not a realistic translatable strategy due to the schedule. Here, mice were immunized with gene gun (gg) cluster prime (two cartridges of 0.5 µg DNA/cartridge on Day 0 and 2) or single prime (two cartridges of 0.5 µg DNA/cartridge on Day 2) and the CSP-specific T cell frequencies were analyzed by ELISPOT. When directly compared, ggCSP cluster priming was found to be more immunogenic than ggCSP single priming (**Figure 2.7**). Additionally, doubling the dose for ggCSP single priming (four cartridges of 0.5 µg DNA/cartridge on Day 2) did not increase immunogenicity. However, despite differences in immunogenicity, both priming methods induced equivalently high levels of protection against spz challenge when utilized in the prime-and-trap strategy (**Figure 2.7**). Thus, a single gg prime is more translatable than cluster priming and is sufficient to induce high level of protection in mice.

Although a single gg prime was effective in prime-and-trap, there is currently no FDA approved gg for clinical use. Electroporation (EP) is another effective method for DNA vaccination and the path to the clinic may be simpler. Here, prime-and-trap with gg or ID EP priming was directly compared. A PyCSP-Luc reporter plasmid was used for EP priming to visualize and confirm the *in vivo* protein expression following priming (**Figure 2.7**). Prime-and-trap with gg or EP priming followed by $2x10^4$ fresh-RAS trapping induced protection against spz challenge (**Figure 2.7**). Taken together, this data suggests that the priming step in prime-and-trap is versatile and effective with various DNA priming regimens and administration methods.



Group	Day 0	Day 2
1 (Cluster Prime)	2 carts	2 carts
2 (Single Prime)	-	2 carts
3	-	4 carts
4	-	-

Group	Prime	Trap
1	ggCSP + LT (Cluster Prime)	2x10^4 RAS
2	ggCSP + LT (Single Prime)	2x10^4 RAS
3	EP CSP-Luc + LT	2x10^4 RAS
4	EP CSP-Luc + LT	-
5	-	2x10^4 RAS
6	PBS	-

Figure 2.7. Prime-and-trap with gene gun or EP priming is protective in mice. **A)** CSP-specific CD8$^+$ T cell IFN-γ ELISPOT from splenocytes collected day 28 post vaccination and stimulated with CSP peptide (SYVPSAEQI) or DMSO vehicle control. Data was normalized to vehicle control. Mice were vaccinated with PyCSP+LT plasmid DNA vaccines administered via gg with varying doses and schedules. ELISPOT data was analyzed with Mann Whitney test, ***p<0.001, **p<0.01 from N=7-10 mice/group from two independent experiments. **B-D)** Mice were vaccinated with DNA plasmids administered via gg or injected ID and followed by EP. **B)** IVIS imaging of mice 24 hours post vaccination with 2.5 µg PyCSP-Luc plasmid injected ID (dose split across two injections) followed by EP. IVIS flux data was analyzed with Mann Whitney test, ****p<0.0001, from N=7-15 mice/group from two independent experiments. **C)** Representative IVIS image of PyCSP-Luc mice from **(B)**. gg or EP primed mice were then trapped with 2x10^4 Py fresh-RAS. **D)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks after RAS trapping. Protection data was analyzed with Fisher Exact Test, **p<0.01, ns=p>0.05 from N=9-10 mice across two independent experiments (N=5 from one experiment for groups D4-D5). Cart=0.5 µg DNA cartridge.

2.5 DISCUSSION

In this study, a two-step malaria prime-and-trap vaccine strategy was optimized for translation to NHPs and humans. The original prime-and-trap vaccine combined epidermal priming with DNA encoding the PyCSP antigen followed by a single IV dose of freshly-dissected liver-homing RAS and a concurrent ggCSP DNA boost [121]. This study investigated the potential substitution of fresh-RAS trap with cryo-RAS, which can be moved to humans based on progress with PfSPZ Vaccine [155,176], PfSPZ—CVac [165], and PfSPZ Challenge [241]. The study demonstrated that prime-and-trap can be improved with the use of cryo-RAS in the place of fresh-RAS, and that a lower trapping dose can be used if cryo-RAS are co-administered with the glycolipid adjuvant 7DW8-5. This trapping approach is therefore quite versatile in that liver-homing cryo-RAS can be administered either with or without immunostimulatory 7DW8-5. The study further demonstrated that ggCSP cluster priming and ggCSP boosting during trapping, which were utilized in the original vaccine strategy [121], are dispensable for achieving protection by prime-and-trap. Additionally, EP priming is also compatible with prime-and-trap, further improving the versatility of the vaccine strategy.

Fresh-RAS trap was previously shown to position protective CD8$^+$ Trm cells in the liver [121]. This study demonstrated that despite the reduced liver burden observed following cryo-RAS trap compared to fresh-RAS, both RAS formulations (fresh or cryopreserved) are sufficient for inducing durable sterile protection in mice. The reduction in parasite liver burden is likely due to reduced cryo-RAS infectivity, as previous studies in mice have shown a seven-fold decrease in cryo-RAS liver infectivity compared to fresh-RAS [241,258]. However, despite reduced infectivity, cryo-RAS trapping induced higher levels of protective PyCSP-specific liver CD8$^+$ Trm cells compared to fresh-RAS at four weeks and similarly achieved sterile protection at four months in mice. Differences in spz purification methods (fresh-RAS purified by Accudenz gradient and cryo-RAS purified at Sanaria) could be responsible for the discrepancy observed between liver burden and liver CD8$^+$ Trm formation. Since it is known that mosquito debris can reduce spz immunogenicity [249], it is tempting to speculate that ultra-purified aseptic cryo-RAS are more immunogenic than fresh-RAS and can thus induce more $CD8^+$ Trm cells in the liver.

In contrast, HK-spz did not show any measurable liver burden in ggCSP DNA-primed mice, and their use as a trapping vaccine did not provide any protection against spz challenge. The lack of protection induced from non-viable HK-spz trapping offers further evidence that liver invasion by RAS is likely key for protective $CD8^+$ Trm formation. A prior study evaluated HK-spz immunization in C57Bl/6 mice previously adoptively transferred with parasite-specific PbT-1 cells. Unadjuvanted HK-spz conferred no sterile protection in such mice, and the addition of α-GalCer protected only 1/8 mice [122], suggesting that viable spz are critical for protection. RAS have limited intrahepatic development but do actively invade hepatocytes. This study found that RAS trapping induces liver $CD8^+$ cells and achieves sterile protection but HK-spz do not. Thus, the results suggest that hepatocyte invasion is important for liver immunity. Recent studies have also demonstrated that liver $CD8^+$ Trm cells are long-lasting and can have a half-life of ~425 days and still maintain protective efficacy 200 days post-vaccination [259]. Consistent with this data, this study observed no loss of protective efficacy with our prime-and-trap vaccine using fresh- or cryo-RAS trap four months (112 days) post trap. This data suggests that the durability of CSP-specific liver $CD8^+$ Trm cells induced from fresh- or cryo-RAS trap are similarly long-lasting.

Many studies have revealed the beneficial adjuvanting effects of glycolipids in mice [189,198,259]. The α-GalCer analog 7DW8-5 has emerged as a promising candidate adjuvant for malaria vaccines, demonstrating efficacy in both mice and NHPs [189,190,195]. Mechanistically, 7DW8-5 is presented on CD1d molecules to *i*NKT cells (reviewed in [260]), which are found normally patrolling liver sinusoids in mice [111]. Although *i*NKT cells are conserved between mice and humans [193], many differences exist in *i*NKT cell frequencies, functions, and behaviors

[192]. However, despite over 25 years of research, many cancer and infectious disease clinical trials with α-GalCer have shown suboptimal results. The majority of α-GalCer studies occur in pre-clinical mouse models with no standard protocol for clinical translation of experimental strategies (reviewed in [191]). Thus, 7DW8-5 may face similar translational challenges. However, pre-clinical studies in mice have shown that 7DW8-5 binds CD1d with a higher affinity and has a 100-fold higher dose-sparing effect than α-GalCer [199]. This data and promising studies in humanized mice [197] and NHPs [190] suggest that 7DW8-5 is beneficial as a malaria vaccine adjuvant in larger animal models and potentially even humans.

This optimized prime-and-trap strategy was designed to improve translation of the existing strategy, but still has several shortcomings. First, this vaccine strategy contains two unique components each with separate regulatory, GMP, storage, and administration requirements. There are no licensed human vaccines utilizing truly heterologous prime/boost doses, even though heterologous prime/boost strategies are widely understood to maximize immunogenicity in pre-clinical models [217,219,261] and human clinical trials [210,212]. Second, although widely used in veterinary medicine, DNA vaccines have not yet been licensed for any disease in humans [262,263]. Numerous clinical trials have been completed and are ongoing demonstrating efficacy and safety of DNA (and mRNA) vaccines for various pathogens including *P. falciparum*, SARS-CoV-2, Zika virus, and Ebola virus [264-267]. Clinical trials involving gene gun-administered DNA have shown good tolerability and immunogenicity for other pathogens [268,269], in agreement with preclinical studies in small and large animal models [246,270-272]. Specifically, gene gun-administered vaccines have been shown to increase immunogenicity [121,273], be highly reproducible [274], painless, needle-free, and dose-sparing in mice and NHPs [275,276]. Lastly, the use of cryo-RAS for prime-and-trap is an improvement over the original fresh-RAS-dependent strategy but faces some implementation hurdles. Cryo-RAS, like any eukaryotic cells, require liquid nitrogen vapor phase storage and currently require IV administration. Ongoing clinical trials involving PfSPZ vaccines have demonstrated that large-scale implementation in endemic regions is achievable [177,231] and could be easier than distribution of products requiring -80°C storage because liquid nitrogen does not require electricity in the cold chain. Coupled with these PfSPZ manufacturing, storage, and administration successes, the fact that prime-and-trap requires only a single dose of spz further simplifies their use here, which may make it easier to translate these findings to the clinic. To further aid this translation to clinical candidates, additional studies are also exploring alternative priming strategies as well as non-IV routes of cryo-RAS trapping (discussed in **Chapter 3**). Beyond DNA vaccination, mRNA vaccines for malaria are also under consideration here given the recent successes with safe and efficacious mRNA vaccines to combat SARS-CoV-2 [277].

In summary, prime-and-trap with cryo-RAS is a vaccine strategy with considerable clinical potential. For priming, nucleic acid vaccines are now being widely used to fight SARS-CoV-2, and there are many other DNA and mRNA vaccines in clinical development. For trapping, GMP-grade, aseptic, purified cryopreserved PfSPZ have been used in clinical trials in thousands of subjects in seven countries in Africa [177,230], five countries in Europe [278], and at multiple sites in the USA [92,155] and are known to be safe and efficacious (reviewed in [158]). The one-time use of cryo-RAS in prime-and-trap vaccination here greatly simplifies the immunization schedule, which will aid manufacturing and likely improve adherence as well [279]. Lastly, the adjuvant 7DW8-5 was dose-sparing for cryo-RAS, which could also simplify manufacturing. Overall, the data suggest that prime-and-trap with cryo-RAS and the glycolipid adjuvant 7DW8-5 should be further investigated in pre-clinical vaccine studies in NHP and in human clinical trials.

2.6 ACKNOWLEDGMENTS

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2.7 SUPPLEMENTAL FIGURES

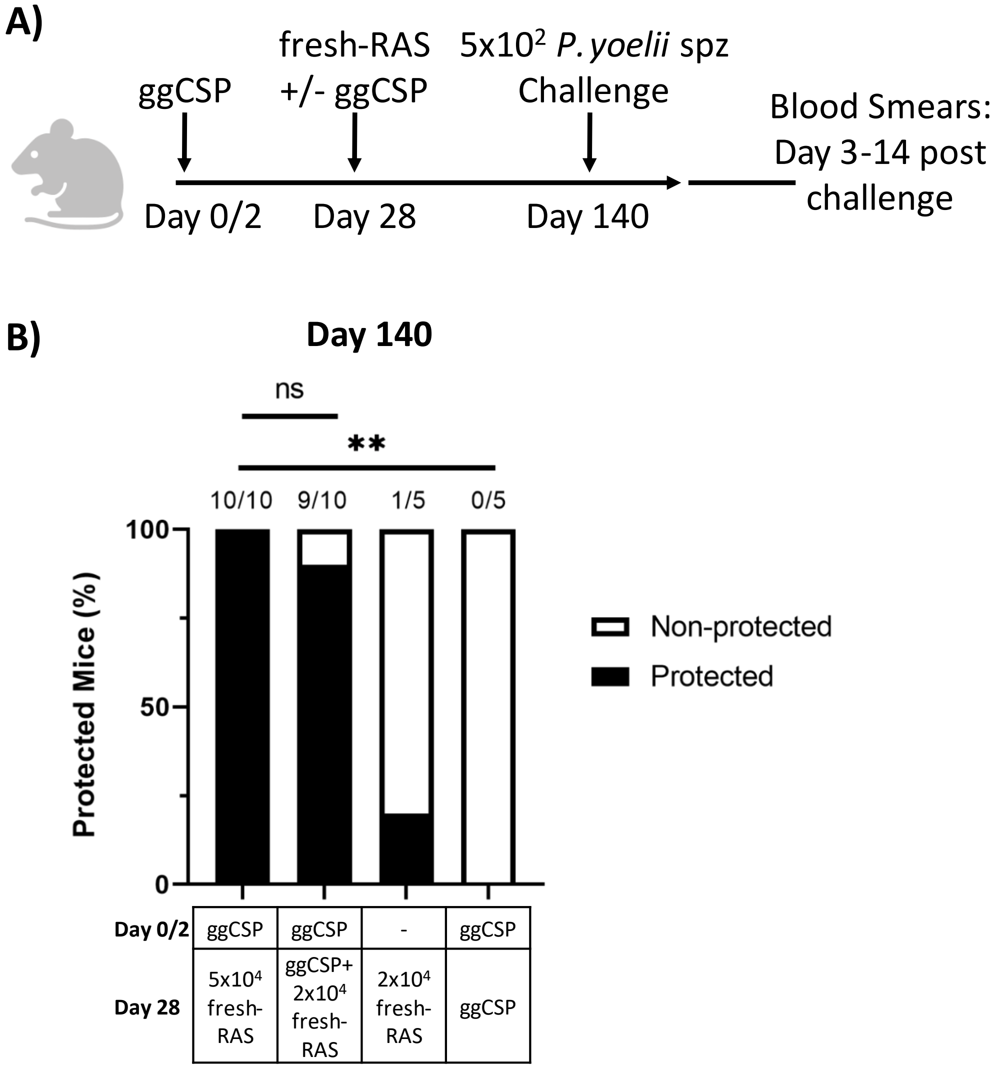


Figure S2.8. Prime-and- fresh-RAS trap protects mice from spz challenge at four months. **A)** Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with $5x10^2$ WT purified *P. yoelii* spz administered four months (Day 140) after trapping. N=5-10 mice from one experiment. Fractions above bars indicate number of animals protected out of total group size. Protection was assessed with thin blood smears days 3-14 post challenge. Fisher Exact Test: fresh-RAS vs all other groups. ns=p>0.05. **p<0.001.

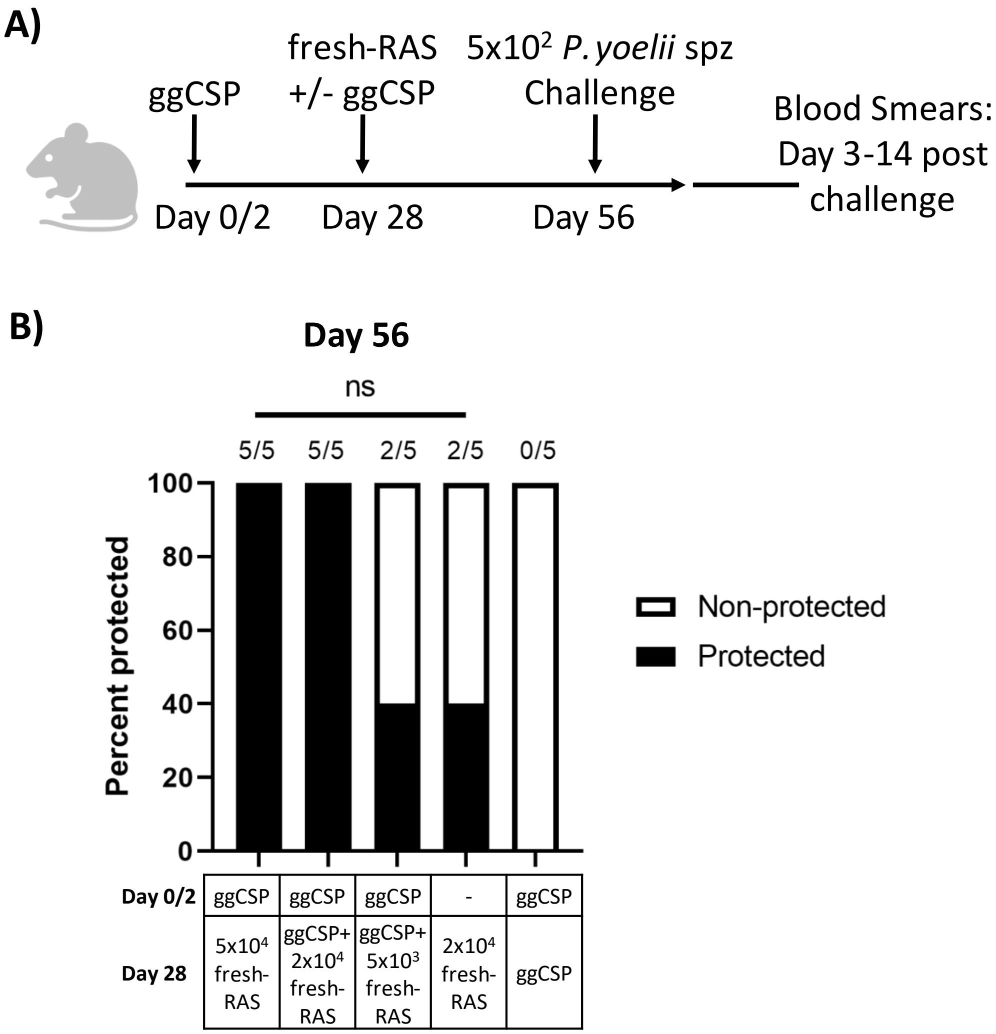


Figure S2.9. Dose-dependent protection of prime-and- fresh-RAS trap vaccination.
A) Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with $5x10^2$ WT purified *P. yoelii* spz administered four weeks after trapping. N=5 mice from one experiment. Fractions above bars indicate number of animals protected out of total group size. Protection was assessed with thin blood smears days 3-14 post challenge. Fisher Exact Test: fresh-RAS vs all other groups. ns=p>0.05.

Chapter 3. ULTRA-LOW VOLUME INTRADERMAL ADMINISTRATION OF RADIATION-ATTENUATED SPOROZOITES WITH THE GLYCOLIPID ADJUVANT 7DW8-5 COMPLETELY PROTECTS MICE AGAINST MALARIA

3.1 CHAPTER SUMMARY

Malaria is caused by *Plasmodium* parasites and was responsible for over 247 million infections and 619,000 deaths in 2021. Pre-erythrocytic vaccines including radiation-attenuated sporozoite (RAS) vaccines can induce invasion blocking antibodies and liver-resident memory $CD8^+$ T (Trm) cells that can completely protect against malaria. Trm cells can also be efficiently induced in the *Plasmodium yoelii* rodent model by ‘prime-and-trap’ vaccination, which here combines DNA priming against the *P. yoelii* circumsporozoite protein (CSP) with a single subsequent intravenous (IV) dose of liver-homing RAS to direct and “trap” activated and expanded $CD8^+$ T cells in the liver. Although prime-and-IV trap confers reliable and durable protection in mice, it is unclear whether the RAS dose must be administered IV. This study investigates if intradermal (ID) RAS administration could be a feasible alternative strategy in prime-and-trap vaccination. Considering the unique biology of the sporozoite, the study sought to investigate if reducing the ID injection to ultra-low volumes could overcome the historically poor protection outcomes reported following ID-RAS administration. The ID injection volume was indeed found to be a key factor in determining the success of ID-RAS administration, with ultra-low volumes (2.5 µL) significantly increasing the number of parasites that successfully invade the liver compared to standard ID volumes of 10-50 µL. The co-administration of prime-and-ID-RAS trap with the glycolipid adjuvant 7DW8-5 in an ultra-low volume was dose-sparing and completely protective against *P. yoelii* wild-type sporozoite challenge. Finally, this study demonstrated that equivalent numbers of

P. yoelii CSP-specific liver CD8$^+$ Trm cells are induced by trapping with IV-RAS or ID-RAS with 7DW8-5. Together, these findings demonstrate that DNA prime and ID-RAS trap with 7DW8-5 is a highly effective preclinical malaria vaccine candidate. The ID route may offer significant translational advantages over the IV route and could help advance the prime-and-trap vaccine toward the clinic.

3.2 INTRODUCTION

The global burden of malaria remains unacceptably high with an estimated 247 million infections and 619,000 deaths in 2021 [2]. Many clinical malaria cases are concentrated in sub-Saharan Africa and are caused by *P. falciparum* (Pf), which is transmitted through the bites of infectious female *Anopheles* mosquitoes. Several pre-erythrocytic and erythrocytic vaccines target Pf and can provide varying degrees of protection against infection, clinical disease, and death (reviewed in [130,152]). However, the only vialed vaccines to routinely induce sterile protection against Pf challenge in humans are live-attenuated whole sporozoite (spz) vaccines (*i.e.*, Sanaria, Inc. PfSPZ Vaccine and PfSPZ-CVac [92,154,155,158,176,177,230-232]). These are aseptic, purified, cryopreserved spz vaccines that induce both humoral and cellular immune responses [92]. Antibodies are mainly induced by the immunodominant circumsporozoite protein (CSP) antigen, and these antibodies can bind to spz to block hepatocyte invasion [155,165,234]. Although high titers of CSP-binding antibodies alone can confer high levels of protection [94,95], induction of CD8$^+$ T cells, specifically liver-resident memory CD8$^+$ T (Trm) cells, appears to be critical for reliable and durable sterile protection [82,235].

To simplify and improve whole spz vaccination, a two-step heterologous vaccine strategy called prime-and-trap was developed [121,173]. Prime-and-trap combines priming with a nucleic acid-based vaccine in the periphery (*e.g.*, skin) followed by expression of the cognate antigen in the liver through spz- or other vehicle-mediated delivery. In its first generation, prime-and-trap was based on skin priming using plasmid DNA encoding the *P. yoelii* (Py) rodent malaria CSP antigen followed by a single intravenous (IV) dose of cryopreserved radiation attenuated spz (cryo-RAS) to direct and “trap” the activated and expanded $CD8^+$ T cells in the liver. This strategy induced robust CSP-specific $CD8^+$ Trm responses in the liver and conferred durable sterile protection in this rodent malaria model for at least four months [173]. However, it was unclear whether the RAS dose must be strictly administered IV. This question is of substantial interest since success with non-IV administration routes could simplify the translational feasibility of spz vaccines.

Intradermal (ID) administration of RAS is an attractive alternative to IV administration since it attempts to mimic the natural route of exposure via mosquito bite. Moreover, the skin is accessible, patrolled by antigen presenting cells (APCs), and compared to other routes, can be dose-sparing [280-282]. Unfortunately, previous attempts at ID RAS administration (ID-RAS) in mice or humans were ineffective – there was substantially higher vaccine efficacy following IV RAS administration (IV-RAS) than after ID [92,278,283-285]. In prior studies, the amount of vaccine spz delivered to the liver as measured by total liver parasite burden was reduced after ID as compared to IV administration, and this difference was implicated as a primary reason for the failure of the ID route [286,287]. Other studies have also suggested that the reason for ID spz administration failure may be due to the more tolerogenic environment of the skin, which may ultimately lead to more regulatory immune responses in the liver [77,283]. However, most of these studies used standard ID injection volumes (10-50 µL), which do not mimic the ultra-low volumes delivered by probing mosquitoes [288,289], nor facilitate efficient exit of spz from the skin, since spz must move by contact-dependent motility [32,290]. Based on the available data, and recognition of this unique biology and motility requirements of spz, two key aspects of RAS administration were hypothesized to be critical for effective ID vaccination: 1) the injection volume must be compatible with the contact-dependent motility of the spz, and 2) the tolerogenic skin immune environment must be overcome.

In this study, the Py rodent malaria model was used to determine if ID-RAS can replace IV-RAS as the trapping component of the prime-and-trap vaccine. As ID-RAS are known to be less immunogenic and protective than IV-RAS, the study investigates if the efficacy of ID-RAS trapping can be improved by reducing the volume and/or co-administering RAS with the glycolipid adjuvant, 7DW8-5. 7DW8-5 is a synthetic glycolipid adjuvant that was selected for this vaccine approach because it potently activates *i*NKT cells to preferentially induce Th1 cytokines (e.g., IFN-γ), inducing a cascade of immune cell activation including CD8$^+$ T cells (reviewed in [199]). Mice primed with DNA encoding the PyCSP antigen administered via gene gun followed by trapping with 7DW8-5-adjuvanted ID-RAS (ID-RAS+7DW8-5) were found to be highly protected against Py spz challenge. Additionally, reducing the volume used for ID-RAS to an ultra-low volume of 2.5 μL was dose-sparing and provided sterile protection for at least four months. Furthermore, these modifications have the potential to improve RAS-only vaccination in addition to prime-and-trap vaccination. Overall, these findings demonstrate that ID-RAS is as protective as IV-RAS when co-administered with a potent adjuvant in an ultra-low volume and provide an alternative non-IV route for spz vaccination.

3.3 METHODS AND MATERIALS

3.3.1 *Mice*

Female 4-6 week-old BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the University of Washington in an Institutional Animal Care and Use Committee (IACUC)-approved animal facility. All mice were used under IACUC protocol 4317-01 to SCM.

3.3.2 *Freshly-dissected spz production and challenge*

Female *Anopheles stephensi* mosquitoes infected with wild-type *P. yoelii* 17XNL (Py WT) were reared at Seattle Children's Research Institute (Seattle, WA). Fresh spz were obtained by salivary gland dissection 14-18 days post-infection followed by Accudenz gradient purification as described [242]. Heat-killed spz (HK-spz) were generated by incubating freshly-dissected Py WT spz in a 55°C water bath for 30 minutes. All spz were diluted in Schneider's insect media for administration. Figure legends specify the dose, volume, route, and number of injections for each experiment. For all spz challenge administrations, $1x10^3$ freshly dissected Py WT spz in 100 µL were injected retro-orbitally (RO) IV. Blood stage protection after spz challenge was assessed by Giemsa-stained thin blood smear microscopy on Days 3-14 post-challenge. Mice were deemed protected if blood smears remained negative for parasites up to Day 14.

3.3.3 *DNA vaccination by gene gun*

The Py circumsporozoite protein (CSP) DNA vaccine plasmids were constructed in the pUb.3 vector and co-administered with *Escherichia coli* heat-labile toxin (LT)-encoding plasmid adjuvant as described [121,243-245]. The PyCSP-minigene encodes the SYVPSAEQI epitope and the PyCSP plasmid encodes the full-length CSP protein without the major repeat region. **Supplementary Figure S3.7** details amino acid sequences and agarose gel restriction digest plasmid validation for all PyCSP vaccines. All plasmid stocks were Sanger sequenced (GeneWiz Inc.) before use. Gene gun DNA vaccine cartridges were constructed as previously described [173,244]. Mice were vaccinated on a shaved abdomen using a PowderJect-style gene gun [246] by priming using two cartridges per day on Days 0 and 2 (0.5 µg DNA per cartridge). This method of priming with PyCSP/LT-encoding plasmids via gene gun is referred to as ggCSP.

3.3.4 *Cryopreserved irradiated spz vaccination*

Cryopreserved Py wild type (WT) 17XNL (cryo-RAS) were radiation-attenuated (100 Gy by C0-60), purified, vialed, and produced by Sanaria, Inc. (Rockville, MD) [92,247]. The vials were shipped to Seattle and stored in vapor phase liquid nitrogen per manufacturer recommendations. Cryo-RAS were thawed in a 37°C water bath for 30 seconds, diluted in Schneider's insect media (Gibco, Thermo Fisher Scientific), and administered within 30 minutes of thawing. Spz counts were confirmed on a hemocytometer within one hour of injection. Figure legends specify the dose, volume, route, and number of injections for each experiment.

3.3.5 *Glycolipid adjuvant preparation*

7DW8-5 powder previously made under Good Manufacturing Practice (GMP) conditions was reconstituted in DMSO and prepared for injection as described [173]. 7DW8-5 or DMSO vehicle control was mixed with the cryo-RAS vaccines immediately before administration. All mice received 2 µg of 7DW8-5 adjuvant per immunization.

3.3.6 *Intradermal and intravenous spz injections*

ID injections in standard volumes (STV) of 10-50 µL were administered with a BD Veo Insulin Syringe with Ultra-Fine needle 6mm x 31G 3/10 mL/cc (#324909). STV injections were administered in two ID injections per dose on the lower back near the base of the tail. Ultra-low volume (ULV) ID injections of 2.5 µL were administered with a 10 µl Sub-microliter injection syringe (World Precision Instruments, Inc #NANOFIL) and a 36G Beveled needle (World

Precision Instruments, Inc #NF36BV). ULV injections were administered in two ID injections per dose on the left rear footpad. IV injections were all administered RO in 100 µL with an Exel International Insulin Syringe with a 29G permanently-attached needle. **Supplementary Figure S3.8** diagrams the locations of all ID and IV injections.

3.3.7 *Parasite burden reverse transcription polymerase chain reaction (RT-PCR)*

To quantify liver burden, half of the liver was excised, pulverized by bead beating (1.0mm zirconia beads, BioSpec) into NucliSENS lysis buffer (bioMérieux), and nucleic acid was extracted as previously described [173,248]. RNA was subjected to RT-PCR with the SensiFAST™ Probe Lo-ROX Kit (Bioline, London, UK) using a mouse GAPDH RT-PCR assay (IDT Inc, Coralville, IA) multiplexed with a Pan-*Plasmodium* 18S rRNA assay on a QuantStudio 5 real-time PCR machine (Thermo Fisher Scientific) as described [253]. *Plasmodium* 18S rRNA copy numbers per reaction were determined using a custom lot of quantified Armored RNA encoding full-length *Plasmodium* 18S rRNA (Asuragen, Austin, TX). To quantify popliteal draining lymph node (PO dLN) burden, the left PO dLNs were excised and pooled with alike PO dLN from the same group. Pooled PO dLNs were pulverized by bead beating in NucliSENS lysis buffer and processed for RT-PCR as described above.

3.3.8 *ELISA*

IFN-γ or IL-4 cytokine levels were determined by commercial ELISA kit according to manufacturer’s instructions (BioLegend, San Diego; #430801 and #431104). Blood was collected into tubes containing EDTA and then plasma was isolated and frozen. For liver tissue, half of the liver was excised, weighed, and pulverized by bead beating in 3 mL lysis buffer (phosphate-buffered saline (PBS), 1:100 Pierce protease inhibitor (Thermo Fisher, # A32953), 0.05% Triton

X-100). Homogenized samples were centrifuged at 16,000 x g for 10 min at 4°C. Supernatant was collected and frozen. All samples were diluted in the kit diluent, and absorbance was read on the CLARIOstar Plus plate reader (BMG Labtech, Germany) according to kit instructions. Standard curves and concentrations were calculated in Microsoft Excel.

PyCSP binding antibodies in mouse serum were determined by direct ELISA as previously described [93]. Blood was collected via submental bleed; serum was isolated and frozen. All serum samples were heat inactivated for 30 min at 56°C and centrifuged at 17,000 x g for 10 minutes prior to ELISA analysis. 50 ng per well recombinant PyCSP was plated in in 0.1M $NaHCO_3$, pH 9.5, and incubated overnight at room temperature. Serum was diluted over a range of 1:50 to 1:109,350, and binding was detected with goat anti-mouse IgG Fc-HRP (Southern Biotech, #1013-05). Absorbance at 450 nm was determined with the BioTek ELx800 reader.

3.3.9 *Depletion/Blocking antibodies*

For CD1d and CD8 depletion/blocking studies, mice were injected intraperitoneally (IP) with 100 μg of anti-mouse CD1d (BioXcell, Lebanon; #BE0000) or 500 μg of anti-mouse CD8 (BioXcell, #BE0061) 24 hours before challenge. Matched isotype controls were used at the same concentration respectively (BioXcell, #BE0088 (CD1d) or #BE0090 (CD8)). Additional animals were used to validate the depletion doses and schedule used for these studies (**Supplementary Figure S3.12**). The depletion dose for CD8 was validated by whole blood leukocyte flow cytometry and CD1d dose was validated by plasma IFN-γ ELISA, as described below.

For CD8 depletion confirmation by flow cytometry, blood was collected via submental bleed into tubes containing EDTA 24 hours post CD8 depletion antibody or isotype injection. Whole blood was then resuspended in ammonium-chloride-potassium lysis buffer for 2-3 min to lyse red cells.

The reaction was quenched with MACS buffer (1X PBS, 1 mM EDTA, 0.5% fetal bovine serum (FBS)). The final cell pellet containing whole blood leukocytes was resuspended in MACS buffer, blocked, stained, and fixed for flow cytometry as described below. The following antibodies were used to assess CD8 cell depletion validation: live/dead dye-NIR, CD3e-BUV395, B220-BV711, CD4-Alexa Fluor 700, CD8a-BV421. Detailed information on flow reagents in **Appendix A**. Cell count per 100 μL blood was calculated based on known starting volume of mouse blood to normalize data. Flow cytometry was conducted on the LSRII instrument (BD Biosciences), and data were analyzed with FlowJo version 10.7.1 (BD Biosciences). For CD1d blocking confirmation, IFN-γ induced by 7DW8-5 was measured by ELISA. At 24 hours post CD1d or isotype depletion, 7DW8-5 was injected by the IV route. Six hours later, blood was collected, plasma was isolated, and IFN-γ cytokine levels were analyzed by ELISA (as described above).

3.3.10 *RAM2 spz-invasion blocking antibodies*

Py RAM2 monoclonal antibodies were kindly provided by Noah Sather at Seattle Children's Research Institute. RAM2 antibodies were produced as described [93]. For spz-invasion studies, mice were injected IP with 150 μg of RAM2 or matched isotype control 24 hours before RAS immunization. Two hours post immunization, blood was collected via submental bleed and serum was isolated to quantify the amount of antibody circulating via ELISA, using RAM2 as a standard curve as previously described [291]. Serum was serially diluted over a range of 1:25 to 1:1,476,225 and binding was determined as described above with goat anti-mouse IgG-HRP (Southern Biotech, #1015-05). Standard curves for RAM2 were generated by nonlinear regression (log[agonist] vs response[three parameters]). Serum antibody concentrations were quantified by interpolating the average values from three different dilutions along the sample binding curve to the corresponding standard curves and multiplying by the dilution factor to determine the final concentration.

3.3.11 *Liver lymphocyte isolation and flow cytometry*

Liver lymphocytes were isolated by mechanical dissociation and Percoll density gradient as previously described [121,250]. Briefly, livers were excised, mashed into a single cell suspension, and intrahepatic lymphocytes were isolated. Final liver lymphocyte pellets were transferred to a V-bottom 96-well plate for blocking, staining, and fixing for flow cytometry. All antibodies and staining conditions were previously described [121,173] and reagents and a representative gating strategy are detailed in **Appendix A**. Flow cytometry was conducted on the LSRII instrument (BD Biosciences), and data were analyzed with FlowJo version 10.7.1 (BD Biosciences).

3.3.12 *Ex vivo IFN-γ ELISPOT*

PyCSP peptide (SYVPSAEQI) was synthesized by Genemed Synthesis and reconstituted in DMSO. Mouse IFN-γ ELISPOT (eBioscience) was conducted by stimulating 5×10^5 splenocytes with CSP peptide (or DMSO vehicle control) at 1 μg/ml for 18 hr at 37°C and developed following manufacturer guidelines as reported previously [121,251]. The number of spot-forming units (SFU) in each well was calculated using an ImmunoSpot 5.1 Analyzer (Cellular Technology Limited, OH). SFU were normalized to DMSO and SFU per million splenocytes were reported.

3.3.13 *Gene expression by NanoString nCounter®*

Gene expression analysis was performed using the NanoString nCounter® Mouse Host Response Panel. Liver samples were prepared as described above for RT-PCR with N=3 mice per group. Total RNA was extracted on the EasyMag system (bioMérieux) and the concentration was estimated with Nanodrop (Thermo Fisher Scientific). RNA (100 ng) was prepared for gene expression analysis at the Fred Hutchinson Cancer Research Center Genomics & Bioinformatics Core (Seattle, WA). Briefly, RNA samples were mixed with biotinylated capture and florescent reporter probes that were hybridized at 65°C for 12-16 hours. Hybridized samples were run on the NanoString nCounter® Mouse Host Response Panel using the recommended manufacturer protocol. After data collection, the nCounter® .RCC files were imported into nSolver Analysis Software 4.0 for review of quality control metrics, and the panel of housekeeping genes and positive controls was used to compute the normalization factor. Further data analysis was performed in RStudio version 2022.02.01+461 with R version 4.1.3. The normalized count matrix was evaluated for outliers using principal component analysis and no outliers were identified. Log_2 transformed normalized counts per million were assessed for differential expression for ~0 + vaccine using limma version 3.50.3 [292]. Pairwise contrasts were performed for each vaccine group (IV-RAS, ID-RAS, ID-RAS+7DW8-5) and control (ggCSP only). Significant genes were defined at FDR <0.05 with BH correction and an absolute $\log_2$ fold change >1 (**Supplementary Figure S3.13** and **Appendix B**). Selected pathways from MSigDB hallmark and KEGG collections [293,294] were utilized to visualize differentially expressed genes.

3.3.14 *Statistics*

Comparisons of parasite burden RT-PCR, flow cytometry, and ELISPOT groups were done using non-parametric Kruskal-Wallis one-way analysis of variance with Dunn’s multiple comparisons test. ELISA data was analyzed with non-parametric Mann-Whitney test unless otherwise specified in the figure legend. Protection data was evaluated using Fisher’s Exact test. All groups were compared against the ggCSP prime and $2x10^4$ IV-RAS trap positive control as a benchmark. Error bars in figures are reported as standard deviation (SD) of the mean with individual mouse samples shown if applicable. All p-values and individual experiment statistics are listed in corresponding figure legends. Statistical significance was defined as $p<0.05$. Prism GraphPad 9.1.2 Software (San Diego, CA) was used for all calculations, unless noted otherwise.

3.4 RESULTS

3.4.1 *Glycolipid adjuvant 7DW8-5 potentiates prime-and-ID RAS trap vaccination*

Consistent with previous reports [92], ggCSP prime-and-ID RAS trap using standard ID injection volumes (STV) did not protect BALB/cJ mice against Py spz challenge (**Figure 3.1**). Previous studies demonstrated that ggCSP prime and IV-RAS trap with or without 7DW8-5 completely protected BALB/cJ mice when challenged with Py spz at four months post-vaccination [173]. Although 7DW8-5 appears to be dispensable for IV-RAS in prime-and-trap, the adjuvant was hypothesized to improve the efficacy of ID-RAS by possibly helping to overcome the tolerogenic environment of the skin [77,283,295]. To investigate this, mice were ggCSP primed and trapped four weeks later with 2×10^4 ID-RAS with 7DW8-5. Four weeks thereafter, mice were challenged with 1×10^3 IV-administered freshly-dissected WT Py spz (IV-spz), and protection was assessed. Protection induced by ID-RAS was only 10% without adjuvant but was significantly improved to 50% by the addition of 7DW8-5 (**Figure 3.1**). Additionally, the efficacy of ggCSP prime and ID-RAS+7DW8-5 trap was improved to 80% by decreasing the administration volume from 50 µL to 10 µL. Thus, co-administration of ID-RAS and 7DW8-5 in 10 µL achieved 80% sterile protection against IV-spz challenge, which was not significantly different from the 100% protection achieved by IV-RAS trap. This suggested that ID-RAS trapping could be effective in the prime-and-trap vaccine strategy when combined with the potent adjuvant 7DW8-5.

In prime-and-IV trap, the dose of IV-RAS could be reduced four-fold by co-administration with 7DW8-5 while still maintaining ≥90% sterile protection [173]. Therefore, the next step was to determine if the ID-RAS dose could be de-escalated while maintaining high levels of sterile protection. Mice were ggCSP primed and trapped with 5×10^3 or 5×10^2 ID-RAS+7DW8-5 in 10 µL. However, reducing the dose of ID-RAS completely abrogated protection, despite the presence of the adjuvant (**Figure 3.1**). Taken together, this data demonstrates that prime-and-ID-trap vaccination is significantly improved by the addition of 7DW8-5 and by decreasing the ID injection volume to 10 µL, but that these changes alone were insufficient to also allow for a reduction in the ID-RAS dose.

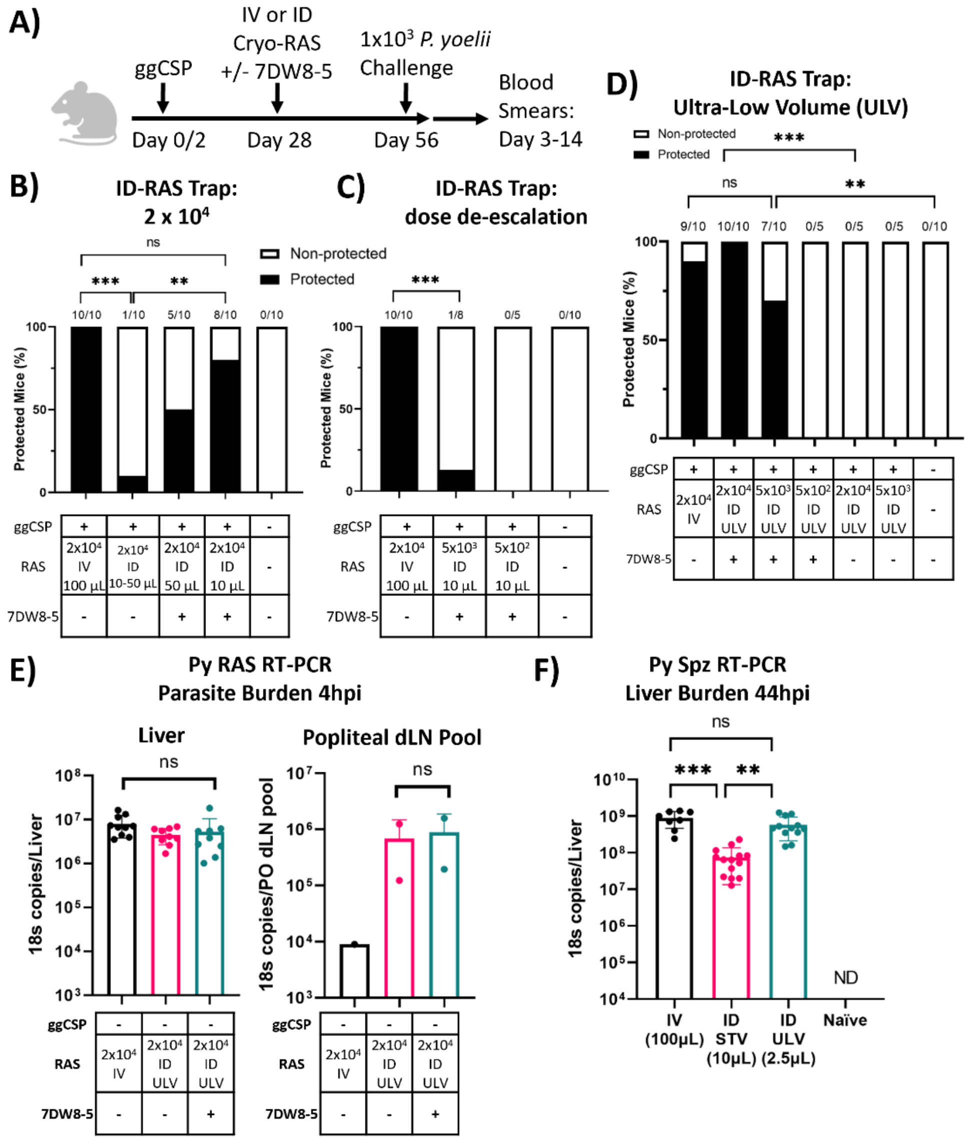


Figure 3.1. Prime-and-ultra-low volume 7DW8-5-adjuvanted ID-RAS trap completely protects mice against Py spz challenge.

A) Experimental design of prime-and-trap protection studies. **B-C)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks after trapping with $2x10^4$ RAS +/- 7DW8-5 **(B)** or a dose de-escalation of RAS+7DW8-5 **(C)**. Protection data from N=8-10 mice across two independent experiments (N=5 from one experiment for the $5x10^2$ ID-RAS group in **(C)**). **D)** Results of protection studies after challenge with $1x10^3$ WT purified Py spz administered four weeks after trapping with RAS +/- 7DW8-5 administered IV or ID ULV (ultra-low volume, 2.5 µL, X2 injections). Protection data from N=10 mice across two independent experiments (N=5 from one experiment for no adjuvant and $5x10^2$ ID-RAS+7DW8-5 groups). Protection data was analyzed with Fisher Exact test, ***p<0.001, **p<0.01, ns=p>0.05. **E)** Naïve mice were immunized with cryo-RAS IV (100 µL) or ID ULV (2.5 µL, X2 injections) +/- 7DW8-5. Four hours post injection livers **(left)**, and PO dLN **(right)** were excised and processed for real-time reverse transcription polymerase chain reaction (RT-PCR) to measure parasite burden with18S pan *Plasmodium* primers. Error bars represent the SD of the mean of N=10 mice from two experiments. PO dLN samples were collected from the injected side and samples were processed in two pools of N=5 alike dLN (one pool for IV group). **F)** Naïve mice were challenged with infectious Py spz IV (100 µL), ID ULV (2.5 µL, X2 injections) or ID STV (standard volume,10 µL, X2 injections). 44 hours post Py challenge livers were excised and processed for RT-PCR to measure liver stage parasite burden with 18S pan *Plasmodium* primers. Error bars represent the SD of the mean of N=8-14 mice across two independent experiments (N=3 mice for Naïve group). RT-PCR data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, ***p<0.001, **p<0.01, ns=p>0.05. ND=Not Detected. RT-PCR data are shown as absolute 18S rRNA copy numbers based on absolute calibrators.

3.4.2 *Prime-and-ultra-low volume 7DW8-5-adjuvanted ID-RAS trap completely protects mice against Py spz challenge*

Invasion of hepatocytes by RAS is critical for protection in mice [173]. Previous studies found that fewer ID-RAS administered in STV homed to the liver compared to IV-RAS, and this was suggested to be a primary reason why ID-RAS vaccination was less effective [286,287]. Differential parasite liver burdens after RAS administration were hypothesized to be responsible for the difference in protection observed when trapping with ID-RAS in 50 µL versus 10 µL. Moreover, since spz are known to migrate out of the skin in a process that requires surface contact [32], further reducing the volume used for ID-RAS was reasoned to improve the motility of the spz to allow them to more effectively migrate out of the skin and home to the liver.

To investigate the impact of injection volume on ID-spz liver burden, protection following ggCSP prime and 2.5 µL ultra-low volume (ULV) ID-RAS+7DW8-5 trap vaccination was investigated.

Mice were ggCSP primed and trapped with ULV ID-RAS+7DW8-5 with the RAS doses de-escalating from $2x10^4$ to $5x10^3$ or $5x10^2$. 100% of the mice trapped with $2x10^4$ ULV ID-RAS+7DW8-5 were protected against spz challenge (**Figure 3.1**). Additionally, the dose of ULV ID-RAS could be reduced four-fold to $5x10^3$ RAS with only a modest loss of protection. However, protection was completely lost when the dose was reduced forty-fold to $5x10^2$ RAS, which suggests that the number of ID-RAS trap required for protection in this model is between $5x10^2$ and $5x10^3$ parasites. This data demonstrates that prime-and-ULV ID-RAS+7DW8-5 trap vaccination is equivalently protective at four weeks to the previously established prime-and-IV-RAS trap strategy [173].

To confirm that 7DW8-5 was not detrimental to spz viability, the study examined if the co-administration of ULV ID-RAS+7DW8-5 impacted the number of spz that reached the liver. Previous ID-spz studies demonstrated that ID-spz travel to the liver via lymphatic and vascular systems, with a significant portion detectable in the draining lymph node [29]. To investigate these relevant tissue sites, naïve mice were immunized with $2x10^4$ ULV ID-RAS with or without 7DW8-5. Four hours later, livers and the ipsilateral PO dLNs were harvested for RT-PCR. The parasite liver burden was found to be similar across all groups (**Figure 3.1**). As expected, both ULV ID-RAS groups had a substantially higher parasite load in the PO dLN compared to the IV-RAS group. This data suggests that 7DW8-5 does not impact spz homing or liver invasion and that equivalent numbers of parasites invade the liver following $2x10^4$ IV-RAS or ULV ID-RAS.

In this model, a completely protective ULV ID-RAS dose was found to be between $5x10^2$ and $5x10^3$ parasites. To estimate the minimum vaccine liver burden needed to protect animals, the study compared parasite liver burdens in mice immunized with $5x10^3$ or $5x10^2$ ULV ID-RAS and found a minimum protective threshold of ~$3x10^5$ *Plasmodium* 18S rRNA copies per liver

(**Supplementary Figure S3.9**). Additionally, 80% of the samples from the $5x10^2$ ULV ID-RAS group were below the limit of detection in the RT-PCR assay, which aligns with our finding that trapping with an ID-RAS dose of $5x10^2$ is insufficient for protection. Next, the parasite burden in the liver at 44 hours post IV- or ID-administered Py spz challenge in a STV of 10 μL or the ULV of 2.5 μL was compared. Both IV-spz and ULV ID-spz yielded similar numbers of parasites in the liver, but the STV ID-spz parasite load was significantly lower (**Figure 3.1**). Together, this data validates that ID-spz utilize lymphatics and vascular systems to home to the liver and that when injected in an ULV, ID-RAS reach the liver in equivalent numbers as IV-RAS.

Finally, the study sought to determine if active spz motility in the skin and during liver invasion were critical for protection using ULV ID-RAS+7DW8-5. Non-motile heat-killed spz (HK-spz) cannot actively migrate, do not invade hepatocytes, and do not achieve sterile protection against IV-spz challenge in mice [173,296]. Consistent with the previous studies, ggCSP prime-and-trap with IV- or ID- HK-spz administered either with or without 7DW8-5 did not provide significant protection against spz challenge (**Supplementary Figure S3.10**). Thus, this data confirms the critical importance of spz motility in the dermis for immunogenicity and protection when ID-RAS is used for prime-and-trap vaccination.

3.4.3 *7DW8-5 potentiates ULV repeated ID-RAS only vaccination*

To confirm that our findings were not unique to prime-and-trap malaria vaccination, the study was expanded to investigate another vaccine strategy. RAS-only vaccines administered by direct venous inoculation 3-5 times are a benchmark malaria vaccination strategy that achieves sterile protection in mice and humans (reviewed in [158,297]). Thus, next the study investigated if ULV ID-RAS was compatible with repeated RAS-only vaccination. To assess this, mice were immunized with $2x10^4$ ULV ID-RAS with or without 7DW8-5 three times at one-month intervals. Repeated IV-RAS routinely achieves 100% sterile protection in the BALB/cJ mouse model and was used as the benchmark in this experiment [297]. Here repeated dosing of ULV ID-RAS+7DW8-5 was as protective as repeated IV-RAS (**Supplementary Figure S3.11**). Taken together, this data demonstrates that using the same spz dose, ID-RAS+7DW8-5 is equivalently protective as IV-RAS in both prime-and-trap and repeated RAS-only vaccination strategies.

3.4.4 *Prime-and-ULV 7DW8-5 adjuvanted ID-RAS trap induces high levels of CSP-specific liver CD8$^+$ Trm cells*

Returning to the prime-and-trap vaccine, the study next investigated the magnitude of the induced liver CD8$^+$ T cell responses. Mice were prime-and-trap immunized with IV-RAS or ULV ID-RAS+7DW8-5 trap as before, and four weeks post-trapping, spleens and livers were harvested for CD8$^+$ T cell analysis. CSP-specific CD8$^+$ Trm cells in the liver were defined as either CD69$^+$/KLRG1^{lo}/CSP-tet$^+$ or CD69$^+$/CXCR6^{hi}/CSP-tet$^+$ as previously described [82,121]. The number of CD69$^+$/KLRG1^{lo}/CSP-tet$^+$ Trm cells were similar in all the immunized groups, but the number of CD69$^+$/CXCR6^{hi}/CSP-tet$^+$ Trm cells were significantly reduced in the ULV ID-RAS group compared to the ULV ID-RAS group with 7DW8-5 (**Figure 3.2**). Additionally, the total number of CD44^{hi}/CD62L^{lo} activated CD8$^+$ T cells in the liver were significantly reduced in the ID-RAS group compared to the ID-RAS+7DW8-5 group (**Figure 3.2**). This data suggests that the high parasite burden observed following IV-RAS or ULV ID-RAS +/- 7DW8-5 induces a high-frequency of CSP-specific liver CD8$^+$ T cells. However, since similarly high numbers of CD69$^+$/KLRG1^{lo}/CSP-tet$^+$ Trm cells were observed for treatments that differed in protection outcomes in the challenge experiments above, this suggests that CD69$^+$/KLRG1^{lo}/CSP-tet$^+$ Trm cells defined by phenotypic surface markers alone may not be sufficient to explain protection. Consistent with other malaria vaccination studies in rodents, in this model CD69^{+}/CXCR6^{hi}/CSP-tet^{+} defined Trm cells may be especially critical for protection [82,298].

This study also determined if the different administration routes impacted CD8^{+} T cell responses in the spleen. Previous research suggests that unlike CD8^{+} T cell responses in the liver, splenic cellular responses do not correlate with protection from IV-spz challenge [285]. Here, CSP-specific splenocyte responses were investigated to determine the similarity of IV-RAS and ULV ID-RAS +/- 7DW8-5. CSP-specific responses measured by IFN-γ ELISPOT were detectable in all the vaccinated groups, but there was a significant decrease in responses observed in the ULV ID-RAS+7DW8-5 group (**Figure 3.2**). Taken together, these findings corroborate previous work suggesting liver CSP-specific CD8^{+} Trm cells are induced by RAS vaccination and are likely the most important immune cell populations for protection in mice.

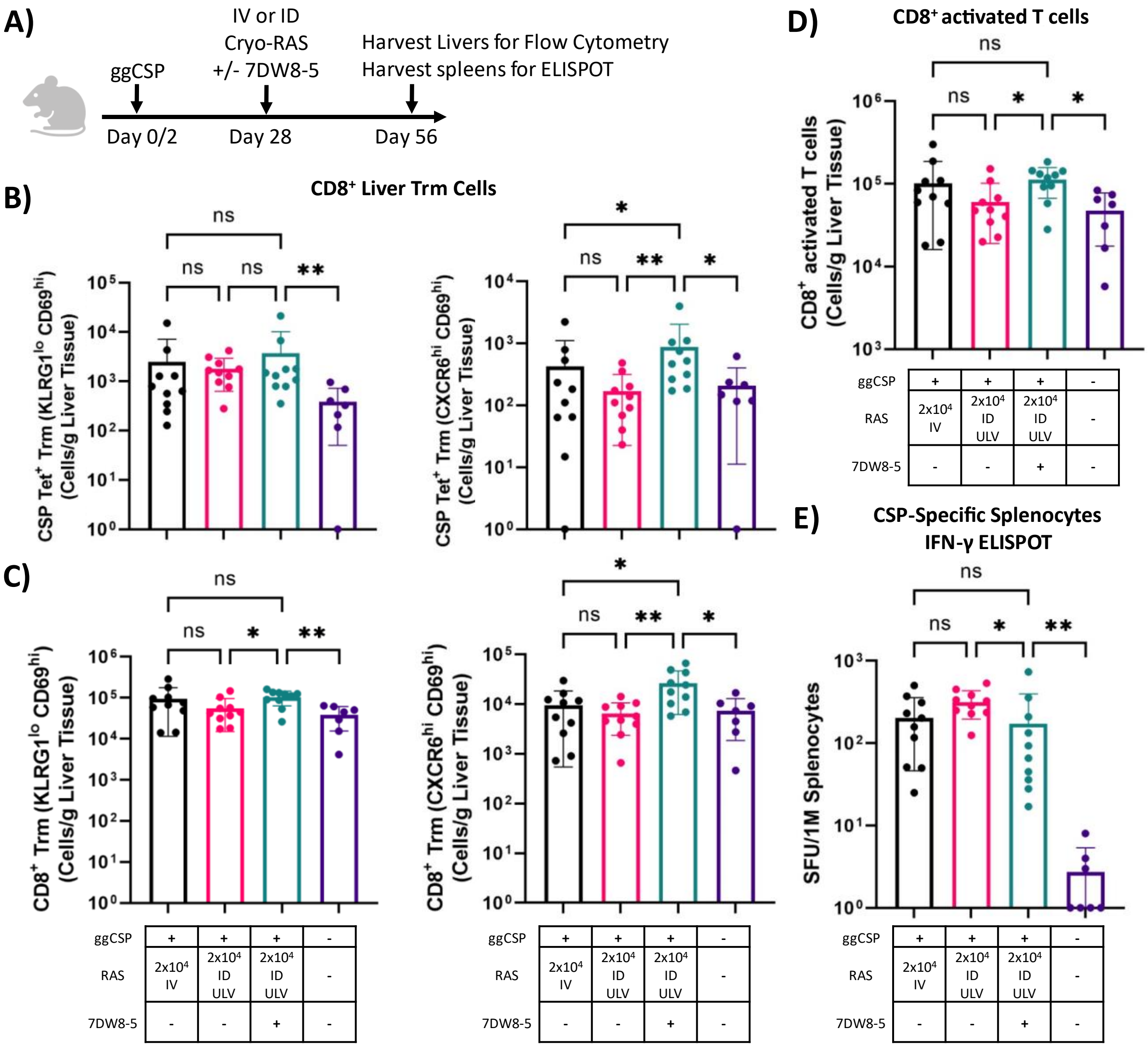


Figure 3.2. Prime-and-ULV 7DW8-5 adjuvanted ID-RAS trap induces high levels of CSP-specific liver CD8$^+$ Trm cells.

A) Experimental design of prime-and-trap studies. **B)** Flow cytometry of CD69^{hi}/KLRG1^{lo}/CSP-tetramer$^+$ **(left)** or CD69^{hi}/CXCR6^{hi}/CSP-tetramer$^+$ **(right)** CD8$^+$ liver Trm cells from **(A)** livers. **C)** Flow cytometry of CD69^{hi}/KLRG1^{lo} **(left)** or CD69^{hi}/CXCR6^{hi} **(right)** CD8$^+$ liver Trm cells from **(A)** livers. **D)** Flow cytometry of CD44^{hi}/CD6L^{lo} total activated CD8$^+$ T cells from **(A)** livers. **E)** IFN-γ ELISPOT from **(A)** splenocytes stimulated with CSP peptide (SYVPSAEQI) or DMSO vehicle control. Data was normalized to vehicle control. Error bars represent SD of the mean from N=10 mice across two independent experiments (N=7 for Naïve group). Data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, **p<0.01, *p<0.05, ns=p>0.05. All ULV ID-RAS injections were 2.5 μL, X2 injections.

3.4.5 *Prime-and-7DW8-5 adjuvanted ID-RAS trap induces inflammatory innate immune responses in the liver*

$CD8^+$ T cells but not *i*NKT cells are critical for protection from spz challenge following RAS vaccination in mice [92,112]. However, the immunostimulatory mechanism by which 7DW8-5 acts is through binding CD1d-expressing APCs and activating *i*NKT cells, so the study investigated if *i*NKT cells at the time of challenge were required for protection [195]. One day before spz challenge, prime-and-ULV ID-RAS+7DW8-5 trap vaccinated mice were injected with anti-CD8, anti-CD1d, or matched isotype control antibodies to deplete or block each cell type before challenge. As expected, protection was completely lost when $CD8^+$ cells were depleted but was not impacted by the significant reduction of CD1d cells (**Figure 3.3**, **Supplementary Figure S3.12**). Thus, prime-and-ID trap protection is likely driven primarily by $CD8^+$ cells.

Previous studies have shown that IV-administered 7DW8-5 induced a potent and transient spike of systemic IFN-γ (and to a lesser extent IL-4) in mouse blood [173], but intramuscular administration of 7DW8-5 did not [189]. Next, the study measured levels of these cytokines in mouse plasma after 7DW8-5 was administered by the STV ID route. Consistent with prior data, ID administration of 7DW8-5 did not induce systemic IFN-γ or IL-4 in mouse blood (**Figure 3.3**). To further dissect the effects of the 7DW8-5 adjuvant when co-administered with spz vaccines, IFN-γ was measured in the livers of prime-and-ULV ID-RAS+7DW8-5 immunized mice at four hours post trapping. Liver IFN-γ concentrations were significantly increased after prime-and-ULV ID-RAS+7DW8-5 compared to the unadjuvanted IV-RAS or ULV ID-RAS controls (**Figure 3.3**). This finding suggests that although ID-7DW8-5 does not induce systemic cytokine expression, it likely impacts local tissue cytokine expression.

Next, the study explored the key factors in the liver responsible for the differential protection outcomes. The above flow cytometry data revealed that ULV ID-RAS +/-7DW8-5 induced similarly high numbers of $CD69^+$/$KLRG1^{lo}$/CSP-tet^+ Trm cells in the liver (**Figure 3.2**). However, sterile protection was only achieved with the addition of 7DW5-8. Based on the IFN-γ cytokine data above, 7DW8-5 was hypothesized to influence the innate immune responses in the liver, which subsequently influences the quality and polyfunctionality of the induced $CD8^+$ memory T cell responses. To evaluate this, livers were harvested from prime-and-ULV ID-RAS+7DW8-5 trap vaccinated animals 44 hours post-trapping for gene expression evaluation. The NanoString nCounter® Mouse Host Response Panel of ~800 target genes was used to explore expression changes induced by 7DW8-5 in the liver. Unadjuvanted RAS immunization (IV-RAS or ULV ID-RAS) was the least immunogenic and showed no differentially expressed genes compared to the ggCSP only control animals (**Supplementary Figure S3.13**). However, in the ULV ID-RAS+7DW8-5 group, 119 and 154 differentially expressed genes were found (FDR Adj. $P \leq 0.05$ and log2 fold change of ±1) compared to ggCSP only and ULV ID-RAS groups respectively (**Figure 3.3**). Most notably, genes associated with interferon signaling, natural killer cytotoxicity, and antigen processing were significantly upregulated in the 7DW8-5 groups (**Figure 3.3**). Parasite liver burden was also measured at the time of transcriptomic analysis sampling and showed a significant decrease of *Plasmodium* 18S rRNA copies in the 7DW8-5 adjuvated ULV ID-RAS group compared to IV-RAS (**Supplementary Figure S3.13**). This data suggests that the kinetics of parasite clearance in the liver differ between ULV ID-RAS+7DW8-5 and IV-RAS, which is likely driven by the immunoinflammatory adjuvant effects. Taken together, this data indicates that co-administration of RAS+7DW8-5 drives the immune environment in the liver toward a pro-inflammatory state that is hypothesized to be favorable for memory $CD8^+$ T cell formation.

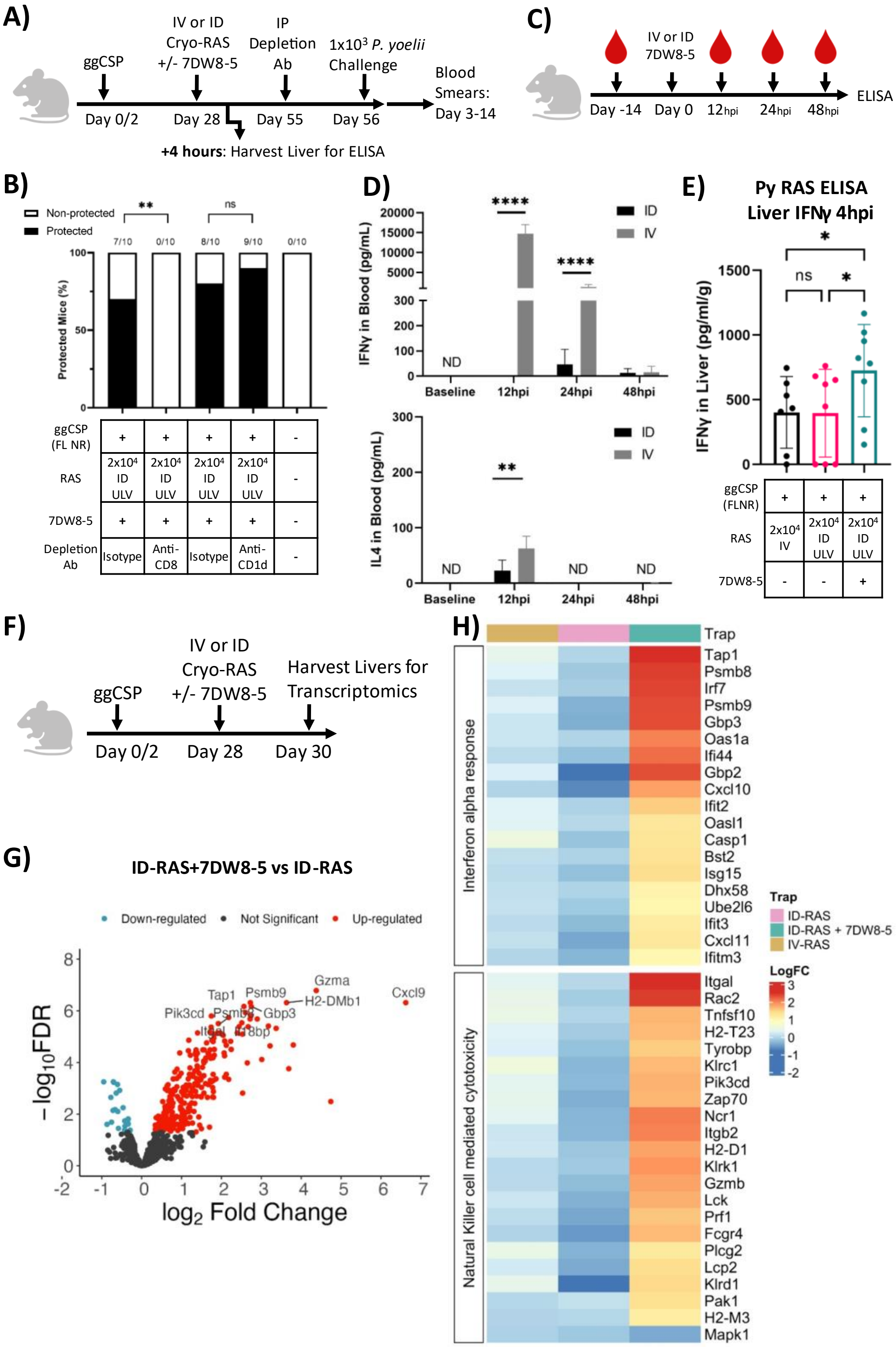


Figure 3.3. Prime-and-7DW8-5 adjuvanted ID-RAS trap induces inflammatory innate immune responses in the liver.

A) Experimental design of prime-and-trap studies in **(B)** and **(E)**. **B)** Results of protection studies after challenge with $1x10^3$ WT purified Py spz administered four weeks after trapping with RAS +/- 7DW8-5 administered ID ULV (2.5 μL, X2 injections). Depletion antibodies were injected IP into all animals 24 hours before challenge as indicated. Protection data from N=10 mice across two independent experiments was analyzed with Fisher Exact test, **p<0.01, ns=p>0.05. **C)** Experimental design of blood plasma ELISA studies in **(D)**. **D)** Cytokine levels, IFN-γ **(top)** and IL-4 **(bottom)**, in mouse blood plasma following IV (100 μL) or ID (10 μL, X2 injections) administration of 7DW8-5. IV data reproduced from Watson et al for comparison [173]. Error bars represent the SD of the mean of N=10 mice across two independent experiments. ELISA data was analyzed with Mann-Whitney tests, ****p<0.0001, **p<0.01. ND=Not Detected. **E)** Four hours after trapping with RAS IV (100 μL) or ID ULV (2.5 μL, X2 injections) +/- 7DW8-5 livers were excised and processed for ELISA to measure IFN-γ. Error bars represent SD of N=7-8 mice across two independent experiments. Data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, **p<0.01, *p<0.05, ns=p>0.05. **F)** Experimental design of transcriptomics studies after trapping with RAS +/- 7DW8-5 administered IV (100 μL) or ID (2.5 μL, X2 injections). Transcriptomic data in **(G-H)** represents data from N=3 mice per group from one experiment. Group averages are displayed for all groups. **G)** Volcano plot of differentially expressed genes comparing ID-RAS versus ID-RAS+7DW8-5 from **(F)** livers. Genes in red have higher expression in the ID-RAS+7DW8-5 group and genes in blue have higher expression in the ID-RAS group. The top 10 most significant genes are labeled. **H)** Heatmap and hierarchal clustering of genes that contain at least one significant differentially expressed gene within the selected MSigDB hallmark interferon alpha pathway and KEGG natural killer cell mediated cytotoxicity pathway. Data are represented as logFC as compared to the control group receiving ggCSP only (no RAS Trap). Significance defined as FDR Adj. p≤0.05 and log2fold change of ±1.

3.4.6 *PyCSP antibodies induced by priming are not detrimental to ID-RAS trapping*

All experiments thus far used the well-characterized and immunogenic BALB/cJ epitope of CSP (SYVPSAEQI, presented on H2-K^d MHC) for ggCSP priming, but this priming vaccine does not induce anti-CSP IgG antibodies (**Supplementary Figure S3.14**). Vaccination with full-length CSP protein is important for increasing epitope diversity and will likely be required for translation of the prime-and-trap vaccine strategy. However, it was not yet clear if antibodies induced by full-length CSP priming would be detrimental to ID-RAS trap since anti-spz antibodies are known to be active in the dermis [76]. The major repeat region of CSP binds the majority of potent spz neutralizing antibodies [101], so the full-length CSP gene – without the major repeat region – was cloned into the plasmid backbone (ggCSP full-length no repeat (FL NR)) (**Supplementary Figure S3.7**). The intention of this construct was to maximize the antigenic landscape while eliminating the target of the most potent spz neutralizing antibodies. To evaluate antibody responses to priming, the study compared anti-CSP antibodies induced by ggCSP (epitope), ggCSP (FL NR), or the pUb.3 plasmid backbone without the CSP insert (control DNA) via ELISA. As expected, only the mice immunized with ggCSP (FL NR) produced anti-CSP antibodies on day 28 (**Supplementary Figure S3.14**), which due to the design of the ggCSP (FL NR) construct could be attributed to epitopes outside the repeat region.

Next, the study investigated if these priming-induced antibodies targeting epitopes outside the major repeat region could impact the number of ID-RAS that reached the liver in prime-and-ULV ID-RAS vaccination. To do this, mice were ggCSP (FL NR) primed and trapped with ULV ID-RAS +/- 7DW8-5 as above. Four hours post-trapping, the livers and PO dLNs were harvested for RT-PCR to compare the parasite burdens and evaluate spz exit from the skin and liver homing ability. Although the parasite liver burden was significantly reduced in the prime-and-ULV ID-RAS trap groups compared to prime-and-IV-RAS group, the levels were still relatively high and well above the defined protective threshold (**Figure 3.4**). Additionally, parasite burdens in the PO dLNs were still significantly higher in the prime-and-ULV ID-RAS groups compared to the prime-and IV-RAS group. This data suggests that priming did indeed induce antibodies against the non-repeat regions of CSP that impacted ID-RAS liver homing, but the impact was relatively minor.

The study hypothesized that the minor reduction in liver burden would not impact protection from ggCSP (FL NR) prime and ULV ID-RAS+7DW8-5 trap vaccination. To investigate this, mice were primed with ggCSP (FL NR) and trapped with $2x10^4$, $5x10^3$, or $5x10^2$ ULV ID-RAS+7DW8-5. Similarly high levels of protection were achieved in ggCSP (FL NR) primed animals as observed in the ggCSP (epitope) primed mice despite trapping in the presence of anti-CSP antibodies and reduced liver burdens (**Figure 3.4**). Importantly, the trapping dose could still be reduced four-fold without a significant loss of protection (**Figure 3.4**). To evaluate the durability of protection, mice were similarly immunized, and protection was assessed four months post trapping. Strikingly, all mice were equivalently highly protected from spz challenge in both the high ($2x10^4$) and low ($5x10^3$) dose ULV ID-RAS+7DW8-5 groups (**Figure 3.4**). This data demonstrated that antibodies against the non-repeat regions of CSP induced by priming with ggCSP (FL NR) were not detrimental to IV-RAS or ULV ID-RAS +/- 7DW8-5 trapping.

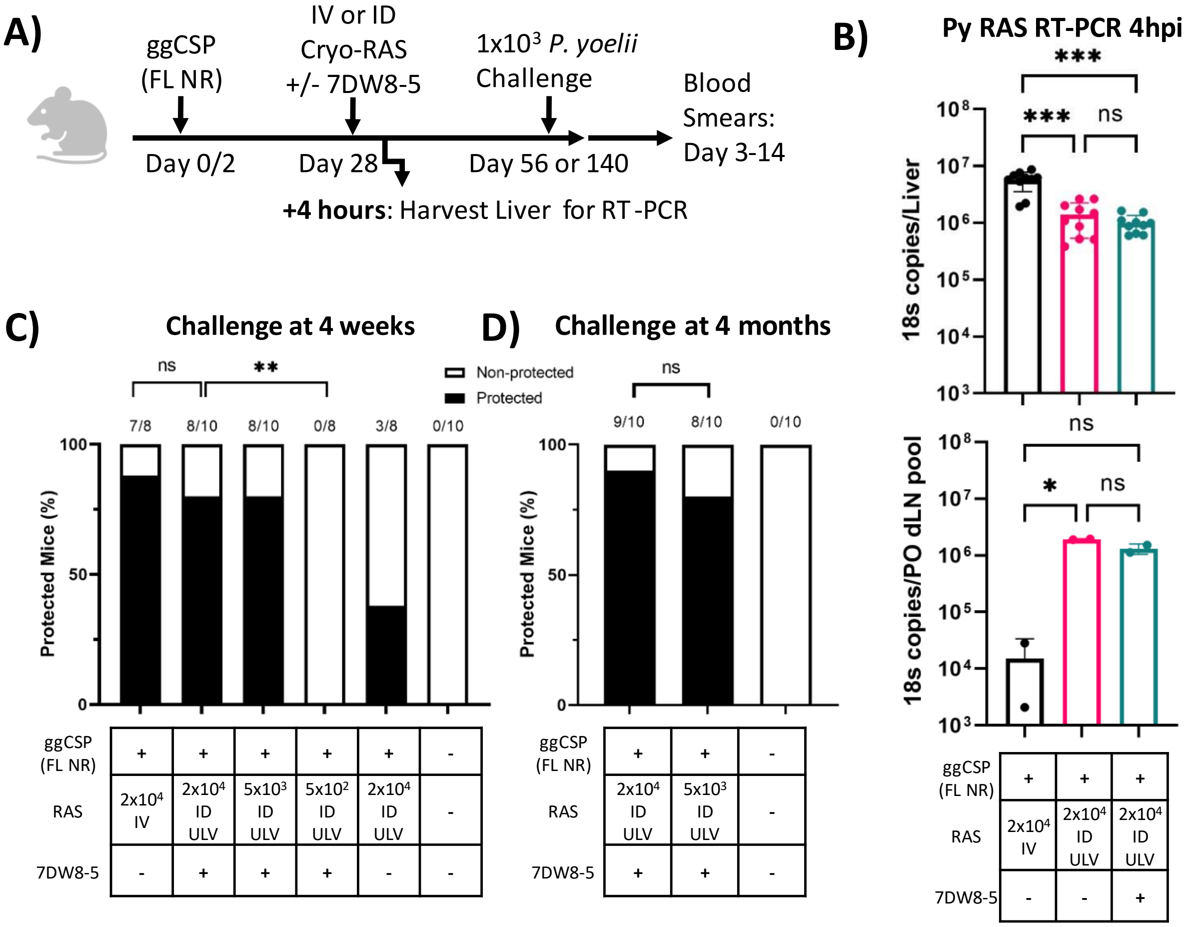


Figure 3.4. CSP antibodies induced by priming are not detrimental to ID-RAS trapping. A) Experimental design of prime-and-trap studies. **B)** Four hours after trapping with RAS IV (100 µL) or ID ULV (2.5 µL, X2 injections) +/- 7DW8-5 livers **(top)** and popliteal draining lymph nodes (PO dLN) **(bottom)** were excised and processed for RT-PCR to measure parasite burden with 18S pan *Plasmodium* primers. RT-PCR data are shown as absolute 18S rRNA copy numbers based on absolute calibrator. Error bars represent SD of the mean of N=10 mice across two independent experiments. PO dLN samples were collected from the injected side and samples were processed in two pools of N=5 alike dLN. RT-PCR data was analyzed with

Kruskal-Wallis test with Dunn's multiple comparisons, ***p<0.001, *p<0.05, ns=p>0.05. **C-D)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks **(C)** or four months **(D)** after trapping with RAS +/- 7DW8-5 administered IV or ID ULV (2.5 µL, X2 injections). Protection data from N=8-10 mice across two independent experiments was analyzed with Fisher Exact test, **p<0.01, ns=p>0.05.

3.4.7 *High titers of exogenously-administered spz neutralizing monoclonal antibodies inhibit prime-and-trap vaccination*

Attenuated spz vaccines are more effective in malaria naïve individuals (reviewed in [152]), which may in part be due to pre-existing antibodies in malaria-experienced individuals neutralizing vaccine spz before they can reach the liver. Thus, in addition to evaluating the effects of priming-induced antibodies, the study also evaluated an extreme scenario where high titers of pre-existing anti-spz antibodies were present prior to prime-and-ULV ID-RAS vaccination. As described above, the major repeat region of CSP is the target of the most potent spz neutralizing antibodies [101], and these antibodies can be found in varying levels in naturally exposed individuals [99,100]. The study therefore investigated if prime-and-trap would still be effective if the RAS were administered in the presence of high titers of potent pre-existing spz neutralizing antibodies. RAM2 is a spz neutralizing monoclonal antibody (mAb) that binds PyCSP with high affinity and induces high rates of sterile protection against mosquito bite challenge in mice [93]. Here, the impact of immunizing in the presence of high titers of RAM2 was investigated. Mice were primed with ggCSP (FL NR) and trapped with 2x10^4 IV-RAS or ULV ID-RAS+7DW8-5 (**Figure 3.5**). Importantly, 24 hours prior to trapping, 150 µg RAM2 or matched isotype control mAb were administered. Protection induced by prime-and-trap was completely abrogated by the presence of high titers of RAM2 antibodies regardless of whether the RAS trap was delivered by IV or ULV ID (**Figure 3.5**). Circulating anti-CSP mAb titers were confirmed to be ~40 ng/µL at the time of immunization by ELISA (**Figure 3.5**). To further elucidate the impact of RAM2 on vaccine spz, the parasite liver burden was measured 24 hours post RAM2 or isotype injection. Mice were immunized with 2x10^4 IV-RAS or ULV ID-RAS and four hours later livers and PO dLNs were harvested for RT-PCR. RAM2 did not significantly reduce *Plasmodium* 18S rRNA copies in the liver of the IV-RAS group, but significantly reduced the liver burden of the ULV ID-RAS group (**Supplementary Figure S3.14**). Taken together, this data suggests that high titers of spz invasion blocking antibodies may interfere with prime-and-trap or attenuated spz efficacy, but notably, sterile protection was similarly impacted in both IV- and ULV ID-RAS groups.

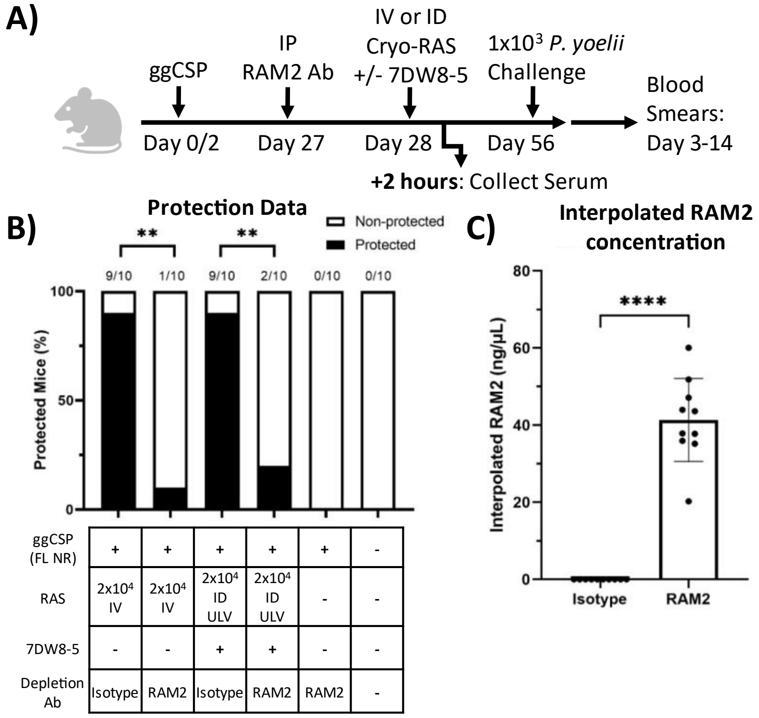
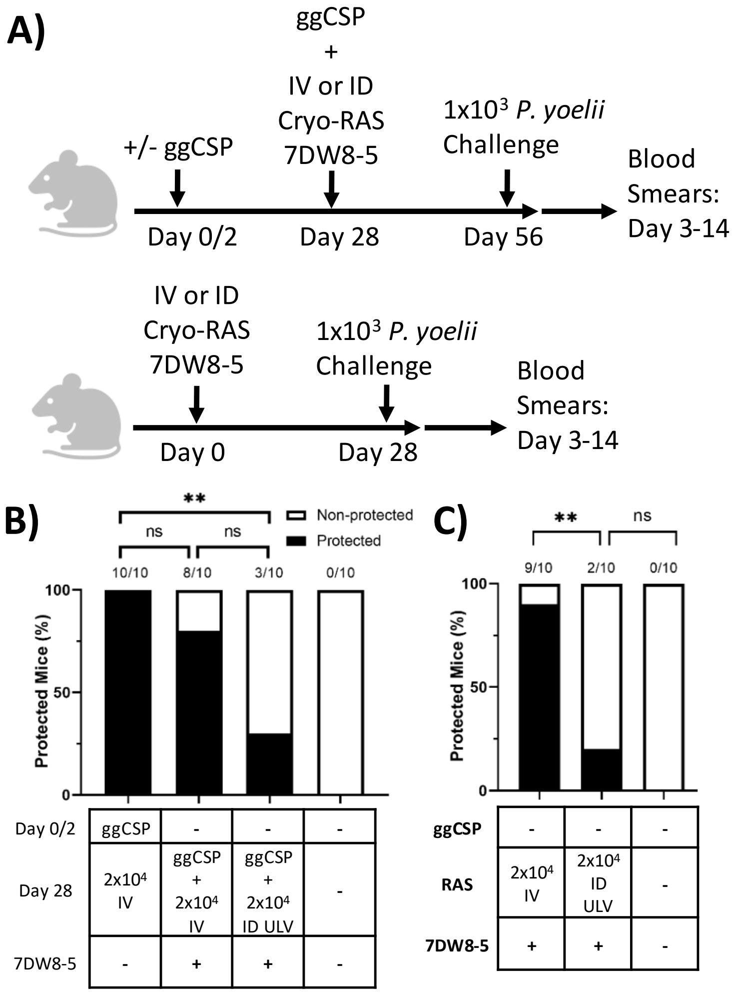


Figure 3.5. High titers of exogenously-administered spz neutralizing mAb inhibit prime-and-trap vaccination.

A) Experimental design of prime-and-trap studies. **B)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks after trapping with RAS +/- 7DW8-5 administered IV or ID ULV (2.5 µL, X2 injections). RAM2 or isotype control mAb was injected IP into mice 24 hours prior to trapping as indicated. Protection data from N=10 mice across two independent experiments was analyzed with Fisher Exact test, **p<0.01. **C)** Results of anti-RAM2 serum ELISA from a subset of the **(B)** mice that received RAM2 or Isotype control. Error bars represent the SD of the mean of N=10 mice from two experiments. ELISA data was analyzed with Mann-Whitney test, ****p<0.0001.

3.4.8 *Single dose vaccination*

The final set of experiments sought to investigate if a condensed single day prime-and-trap schedule could be effective. Another group reported that a single day prime-and-trap vaccine induced sterile protection from *P. berghei* spz challenge in C57BL/6 mice [82,123]. An accelerated vaccination regimen would further enhance and simplify the prime-and-trap vaccine. However, previously a condensed single day prime-and-trap (ggCSP and IV-RAS administered on the same day) induced significantly less CSP-specific CD8$^+$ Trm cells in the liver compared to the standard prime-and-trap (ggCSP and IV-RAS administered 4 weeks apart) [121]. Thus, the final experiments aimed to investigate if the addition of 7DW8-5 to the condensed single day prime-and-trap would improve the vaccine efficacy. Interestingly, IV-RAS+7DW8-5 administered on a single day with or without ggCSP priming was protective in mice (**Figure 3.6**). On the contrary, ULV ID-RAS+7DW8-5 administered on a single day with or without ggCSP priming was not protective in mice (**Figure 3.6**). This data suggests that the mechanism of protection induced by IV- or ID-RAS+7DW8-5 may differ, and future studies are warranted to uncover the correlates of protection. In summary, a single immunization of IV-RAS+7DW8-5 (but not ID-RAS+7DW8-5) is an effective and simple vaccine strategy with significant translational potential from mice to NHPs and humans.



Day 0/2	ggCSP	-	-	-
Day 28	2x10⁴ IV	ggCSP + 2x10⁴ IV	ggCSP + 2x10⁴ ID ULV	-
7DW8-5	-	+	+	-

ggCSP	-	-	-
RAS	2x10⁴ IV	2x10⁴ ID ULV	-
7DW8-5	+	+	-

Figure 3.6. Single-day condensed prime-and-trap is protective with IV RAS+7DW8-5. **A)** Experimental design of standard or single-day condensed prime-and-trap protection studies. For single-day condensed prime-and-trap groups, mice were gene gun primed with two cartridges (0.5 µg DNA per cartridge) immediately followed by RAS+7DW8-5 administration. **B-C)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered 4 weeks after RAS trapping. Protection data was analyzed with Fisher Exact test, **p<0.01, ns=p>0.05 from N=10 mice across two independent experiments.

3.5 DISCUSSION

Malaria cases increased by ~12% in 2020, highlighting the importance of a more effective vaccine that can prevent clinical manifestations and stop further transmission. Decades of pre-clinical and clinical studies of RAS vaccines have demonstrated the safety, feasibility, and efficacy of this vaccine strategy [92,154,155,176], but efforts to simplify and improve administration may further improve the impact of spz vaccines. ID vaccine administration is of growing interest due to the increased immunogenicity and dose sparing potential [299]. A systematic review and meta-analysis found that ID immunization is dose-sparing for many non-malaria infectious diseases as compared to intramuscular (IM) or subcutaneous (SC) administration (reviewed in [282]). However, IV administration of RAS is much more efficient than IM or SC administration and ID-RAS vaccination has previously required ~7X *higher* doses to reach equivalent protection as IV-RAS in mice [92]. This aim of the dissertation explored two methods to increase the efficacy of prime and ID-RAS vaccination: 1) reduction in the administration injection volume, and 2) use of a glycolipid adjuvant. These studies demonstrated that prime-and-trap vaccination with an equivalent dose of ID-RAS is as effective as IV-RAS when co-administered in an ultra-low volume with the glycolipid adjuvant 7DW8-5. Thus, both microvolumes and adjuvanting were critical for the success of prime and ID-RAS trap vaccination.

In human and mouse studies, ID-RAS vaccine failures were attributed to regulatory cellular responses [283] and low parasite burdens in the liver [286,287]. The data in this dissertation chapter supports both hypotheses. First, ggCSP priming followed by co-administration of ID-RAS and the 7DW8-5 adjuvant significantly improved protection from spz challenge. Glycolipid adjuvants, including 7DW8-5, bind CD1d expressing APCs and are known to induce a cascade of immune cell activation [199]. In this model, the 7DW8-5 adjuvant effects appeared to be necessary to modulate a favorable pro-inflammatory immune environment in the liver. Significant levels of protection were never achieved in these experiments after ID-RAS immunization without 7DW8-5. This finding is supported by the previous literature also showing that modulation of the immune environment with adjuvants or epidermal disruption improves non-IV RAS administration [295,300,301], and that adjuvants or other pro-inflammatory modulating factors are likely required to overcome tolerogenic skin responses and/or regulatory liver responses for efficacious ID-RAS vaccination [77,283].

Second, protection achieved from prime and ID-RAS+7DW8-5 trap vaccination was further improved by reducing the ID injection volume. Others have also noted that lower volumes may improve the migration capacity of spz out of the dermis [287,302]. In line with prior work, this study found that ULV ID-RAS significantly increased the number of parasites that reached the liver compared to STV ID-RAS. It further demonstrated that although the parasite liver burden alone is not sufficient for protection, protection was not observed when liver burden was below a certain threshold (~$3x10^5$ *Plasmodium* 18S rRNA copies in this model). Thus, the combination of high parasite liver burden and a pro-inflammatory liver immune environment is likely favorable for malaria vaccination.

Inducing high levels of malaria-specific $CD8^+$ Trm cells in the liver is required for RAS vaccine efficacy [82]. This data suggests that regardless of administration route or inclusion of 7DW8-5, all the tested prime-and-trap vaccine strategies induced $KLRG1^{lo}$-defined $CD8^+$ Trm cells in the liver. Yet, in challenge experiments, the same treatment groups did not show equivalent protection. However, and consistent with other studies, there were reduced numbers of $CXCR6^{hi}$-defined $CD8^+$ Trm cells in the livers of the unadjuvanted ID-RAS mice compared to adjuvanted mice [82,285], which were associated with protection outcomes. CXCR6 has been implicated as a key liver homing marker that may be critical for memory T cell maintenance in the liver [298]. Thus, $CXCR6^{hi}$-defined $CD8^+$ Trm cells may be more important for sterile protection in this model. It is tempting to speculate that the quality and functionality of the $CD8^+$ Trm cells is driving the protective differences, but the data cannot definitively address this question at this time. In addition to the number of CD8$^+$ Trm cells shown here, the findings warrant future exploration into polyfunctionality of vaccine-induced CD8$^+$ Trm cells in the liver.

Further evidence for the important role of CD8$^+$ T cells in conferring protection after prime-and-trap vaccination came from the antibody depletion experiments. At the time of challenge, CD8$^+$ cells but not CD1d-expressing cells, were critical for sterile protection. While a potential limitation of the study is that it did not achieve full CD1d cell neutralization, the data nonetheless agrees with several other studies in CD1d knockout mice that also concluded CD1d was dispensable at the time of challenge for RAS vaccine efficacy [74,82]. The data suggests that CD1d-expressing cells are critical for ID-RAS+7DW8-5 trapping to bind 7DW8-5 and induce a strong pro-inflammatory immune response to activate and form CD8$^+$ Trm cells. Then, if induced correctly, liver CD8$^+$ Trm cells may be sufficient for protection from spz challenge. In this model, the data suggests that CD1d-expressing cells are needed for trapping, but not for sensing parasites or activating CD8$^+$ T cells at the time of challenge.

Given the clear importance of CD8$^+$ T cells for conferring protection, the events during vaccination that gave rise to either protective or non-protective responses were investigated. Innate immune responses during vaccination are known to be critically important for shaping the subsequent adaptive response, including the quality and the durability of CD8$^+$ T cell responses [74,83,303]. The targeted gene expression studies using the NanoString platform provided helpful insight into the immune response in the liver after trapping. These studies revealed several key findings. First, despite the high RAS dose used for immunization, Sanaria-produced aseptic, cryo-RAS are highly purified and did not induce innate inflammatory responses in the liver at the 44-hour timepoint regardless of administration route. Second, the addition of 7DW8-5 completely altered the innate response to trapping in the liver, with interferon signaling and other pro-inflammatory associated pathways significantly upregulated in prime and ULV ID-RAS+7DW8-5 vaccinated mice in comparison to those administered ULV ID-RAS alone. Based on these data, interferon signaling and pro-inflammatory responses at the time of trapping likely result in a recruitment of leukocytes, an increase in antigen processing and presentation, and enhanced memory $CD8^+$ Trm cell formation. Other groups have studied adaptive regulatory cellular responses to ID-RAS and detected higher $CD4^+$ regulatory immune responses and lower $CD8^+$ T cell activation seven days post-spz administration [283]. Unfortunately, this study did not perform gene expression analysis at an equivalent timepoint, and future studies could address whether 7DW8-5 overcomes these adaptive regulatory responses at seven days. Such studies would enable a more complete picture of the full suite of events leading to protective immune responses to prime-and-trap vaccination in the liver.

Antibodies also play an important role in pre-erythrocytic vaccine protection. Previous studies suggested that the majority of antibodies act to inhibit spz in the skin [76], but increasingly the importance of anti-spz antibodies in mediating clearance of parasites outside of the skin are appreciated [91]. This study hypothesized that ID-RAS vaccines would be inhibited to a greater extent by anti-spz antibodies compared to IV-RAS, and it found that this was indeed the case. Using ggCSP (FL NR) priming, the liver burden of ID-RAS (but not IV-RAS) was significantly reduced by the anti-CSP antibodies induced by priming, but protection was not affected. However, regardless of the administration route, protection from prime-and-trap vaccination was significantly impacted by the presence of high titers of potent anti-CSP mAb exogenously administered prior to RAS trapping. This observation was unexpected as it was hypothesized that protection induced from ID-RAS would be more impacted by high titer mAb than IV-RAS. Nonetheless, this finding is supported by another research group that found mosquito bite (MB) administered spz are more infectious than IV-spz and that high titers of mAb blocked IV-spz but not MB-spz [174]. Therefore, more studies may be warranted to understand the impact of anti-spz antibody responses to both prime-and-IV trap and IV-RAS only vaccines. Such studies could provide important information about the levels of circulating pre-existing antibodies that inhibit successful spz vaccination.

Live-attenuated vaccines in the clinic are often not administered with adjuvants since such vaccines are generally considered to be self-adjuvanting. However, as described above, cryo-RAS used in these studies are aseptic and highly purified and do not induce strong inflammatory responses on their own. Live-attenuated bacterial or viral vaccines and live-attenuated parasite vaccines may differ significantly in their underlying inflammatory potential. An alternative administration method for attenuated spz vaccines in clinical trials has been via MB [156,304]. There may be a critical difference between ID-RAS in a syringe and RAS administered via MB in saliva that has been shown to introduce hundreds of proteins into the host that can impact the immune system [305,306]. Additionally, when humans are naturally exposed to malaria it is through the bite of an infectious mosquito, which differs from the IV-spz challenge route used here. To date, this is the first ID-RAS vaccination study to achieve equivalent protection as IV-RAS using equivalent RAS doses and an IV-spz challenge at a memory timepoint. The IV challenge route allows for a reliably uniform spz challenge, bypasses the spz skin stage where the majority of spz neutralizing Ab would play a role, and assesses the memory $CD8^+$ T cell responses in the liver. Quantitative studies in mice and humans comparing MB-, IV-, or ID- administration routes have demonstrated differences in vaccine efficacy and infectivity with the various pathogen inoculation routes [174,241,302,307,308]. Thus, it does merit more studies to determine if the protection induced by prime-and-trap would differ following MB- or ID-spz challenge.

Understanding the key factors that lead to the differences previously observed between MB- and ID- administered spz may help develop a further-improved spz-based vaccine.

Finally, one of the key findings in this study is that administration in an extremely low volume is critically important for successful ID-RAS vaccination. It is likely that such low volumes will still be necessary when scaling up ID-RAS to larger animal models or humans. Inoculation in such low volumes improves spz motility in the skin and allows spz to efficiently invade blood vessels and lymph to home to the liver and dLN, respectively [290]. In this report, 2.5 µL was selected as the smallest volume that could be reliably prepared in the research laboratory for pre-clinical mouse injections. This volume is very low compared to standard ID-administered vaccines (50-100 µL), but still higher than the estimated mosquito saliva injection of <1 µL [306]. Additionally reducing the injection volume to more closely mimic the volumes delivered during mosquito probing would likely further improve ID-RAS [290]. Studies with PfSPZ Vaccine and PfSPZ Challenge have shown that direct venous inoculation of 0.3-0.5 mL of PfSPZ through a 25-gauge needle is extremely well tolerated, simple, and reliable when administered by personnel after minimal training. Conceptually, ID administration appears easier, but reproducibly injecting even 50-100 µL ID at an accurate depth and volume with a standard single needle syringe can be challenging [309]. However, accurate and reliable ID injection may be possible through the development of a microarray needle patch or another as-yet-to-be-developed administration device. Moreover, without a dedicated administration device, ULV ID-RAS injections could further complicate administration for larger scale vaccine implementation. Thus, engineering innovations like microarray patches could revolutionize ID-RAS administration in the field and allow simple, quick, and pain-free administration of ULV ID-RAS.

In summary, the use of ultra-low volumes for ID-RAS administration significantly improves the number of vaccine parasites that home to and invade the liver. However, in the context of prime-and-trap vaccination, the combination of both 7DW8-5 and ULV ID-RAS at the trapping step is required for complete protection from spz challenge. Taken together, prime-and ULV ID-RAS+7DW8-5 trap is a highly effective vaccine in mice that has a significant translational potential. Combined with the recent report of *in vitro* production of *Plasmodium falciparum* sporozoites [310], the insights about lower administration volumes and adjuvants provide a potential path forward for simplifying attenuated PfSPZ vaccination.

3.6 ACKNOWLEDGMENTS

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Thank you to Tess Seltzer, Veronica Primavera, Cecilia Kalthoff, and Alexis Kaushansky (Seattle Children's Research Institute) for assistance and support of Py–infected mosquito production and the NIH Tetramer Core Facility (contract number75N93020D00005) for providing PyCSP monomers. Thank you to veterinary staff of the UW Department of Comparative Medicine, to Sanaria for the cryopreserved Py sporozoites, and to Moriya Tsuji for the 7DW8-5 adjuvant.

3.7 SUPPLEMENTAL FIGURES

A) **CSP FULL-LENGTH (289aa)**

MKKCTILVVASLLLVDSLLPGYGQNKSVQAQRNLNELCYNEENDNKLYHVLNSKNGKIYNRNIVNRLLGDALNGKPEEK
KDDPPKDGNKDDLPKEEKKDDLPKEEKKDDPPKDPKKDDPPKEAQNKLNQPVVADENVDQ | **GPGAPQGPGAPQG
PGAPQGPGAPQEPPQQPPQQPPQQPPQQ** | PRPQPDGNNNNNNNNGNNNED**SYVPSAEQI**LEFVKQISSQLTEE
WSQCSVTCGSGVRVRKRKNVNKQPENLTLEDIDTEICKMDKCSSIFNIVSNSLGFVILLVLVFFN

CSP FULL-LENGTH NO REPEAT (CSP FL NR) (248 aa)

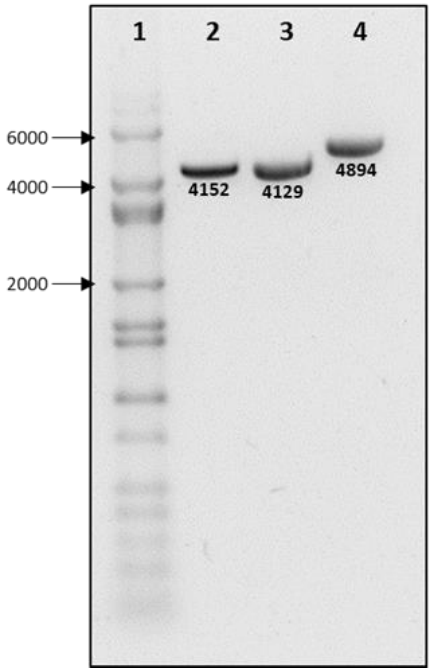
MKKCTILVVASLLLVDSLLPGYGQNKSVQAQRNLNELCYNEENDNKLYHVLNSKNGKIYNRNIVNRLLGDALNGKPEEK
KDDPPKDGNKDDLPKEEKKDDLPKEEKKDDPPKDPKKDDPPKEAQNKLNQPVVADENVDQ | PRPQPDGNNNNNN
NNGNNNED**SYVPSAEQI**LEFVKQISSQLTEEWSQCSVTCGSGVRVRKRKNVNKQPENLTLEDIDTEICKMDKCSSIFNI
VSNSLGFVILLVLVFFN

Major Repeat Region (41aa)
MHC I epitope (9aa)

CSP MINIGENE (25 aa)

NNNNNGNNNE**SYVPSAEQI**GLSERH

B) **Agarose restriction digest gel**



Lane 1: Bionexus Hi-Lo DNA Marker
Lane 2: pUb.3 Parent plasmid (4152 bp)
Lane 3: pUb.3 CSP minigene plasmid (4129 bp)
Lane 4: pUb.3 CSP full-length no repeat plasmid (4894 bp)

Figure S3.7. CSP DNA vaccines.
A) Py CSP DNA vaccine insert amino acid sequences**. B)** Agarose restriction digest gel of 100 ng plasmid digested and cut once with a restriction enzyme to linearize to visualize true sizes on the gel. A Bionexus Hi-Lo DNA Marker ladder was run in lane 1 for reference with key band sizes listed in base pairs (bp).

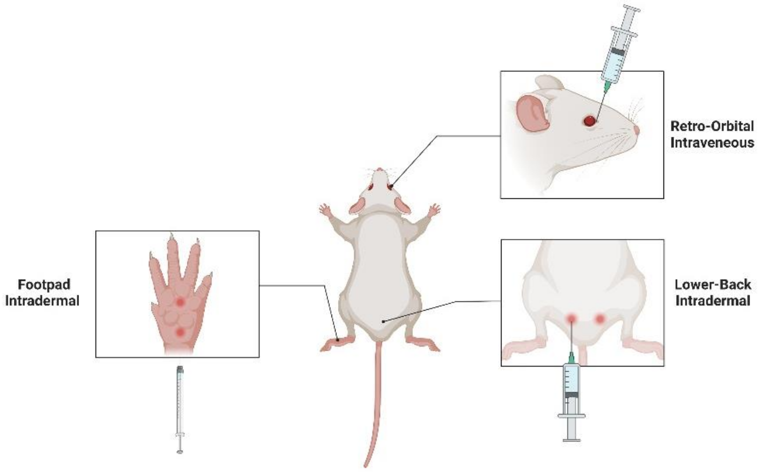


Figure S3.8. Mouse injection sites.
Image created in BioRender and adapted from an image template.

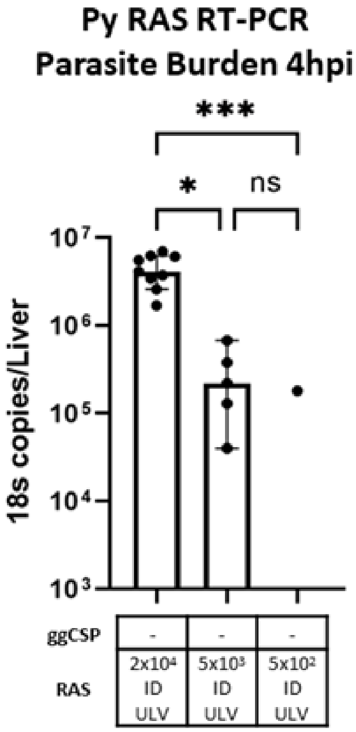
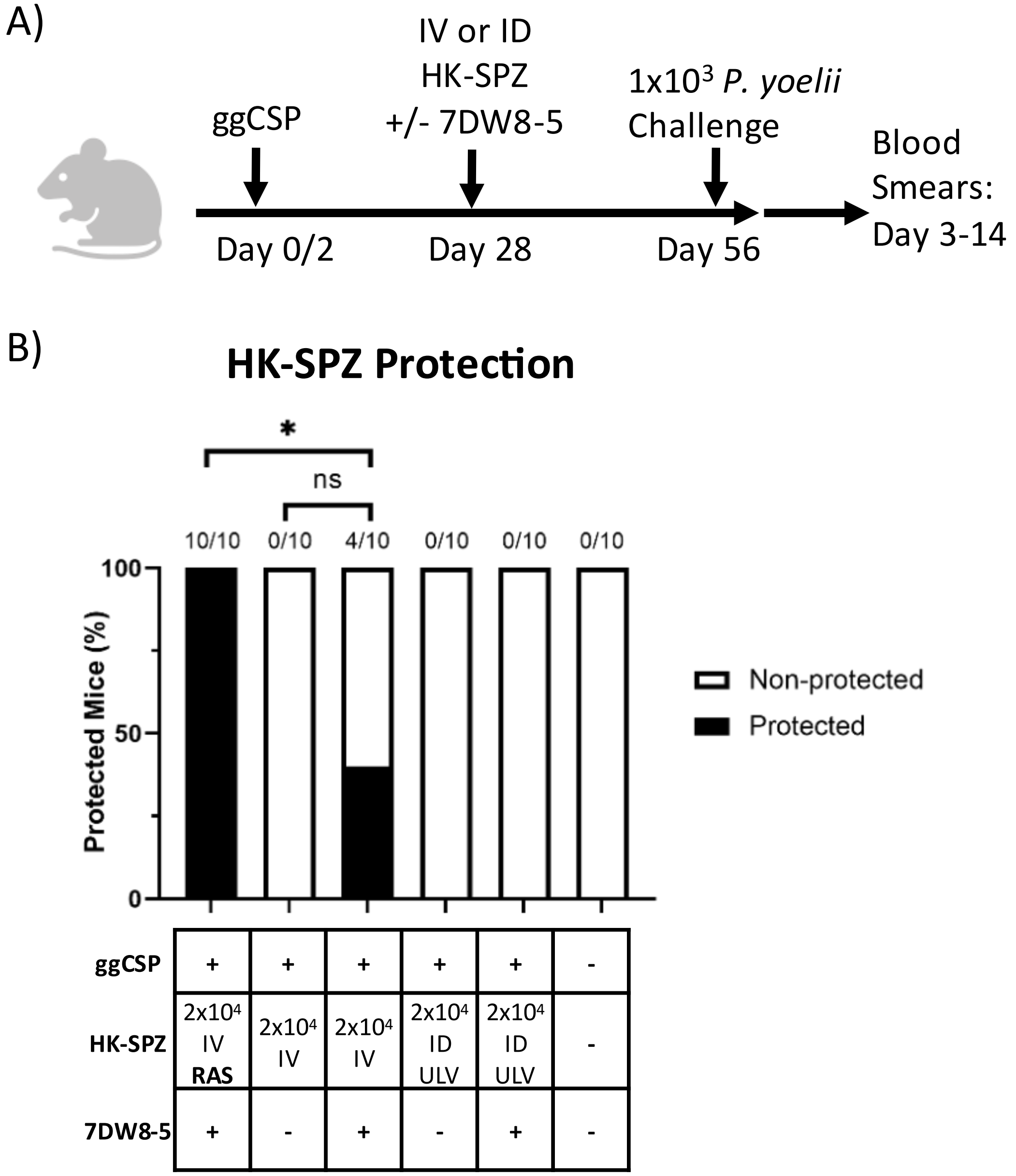
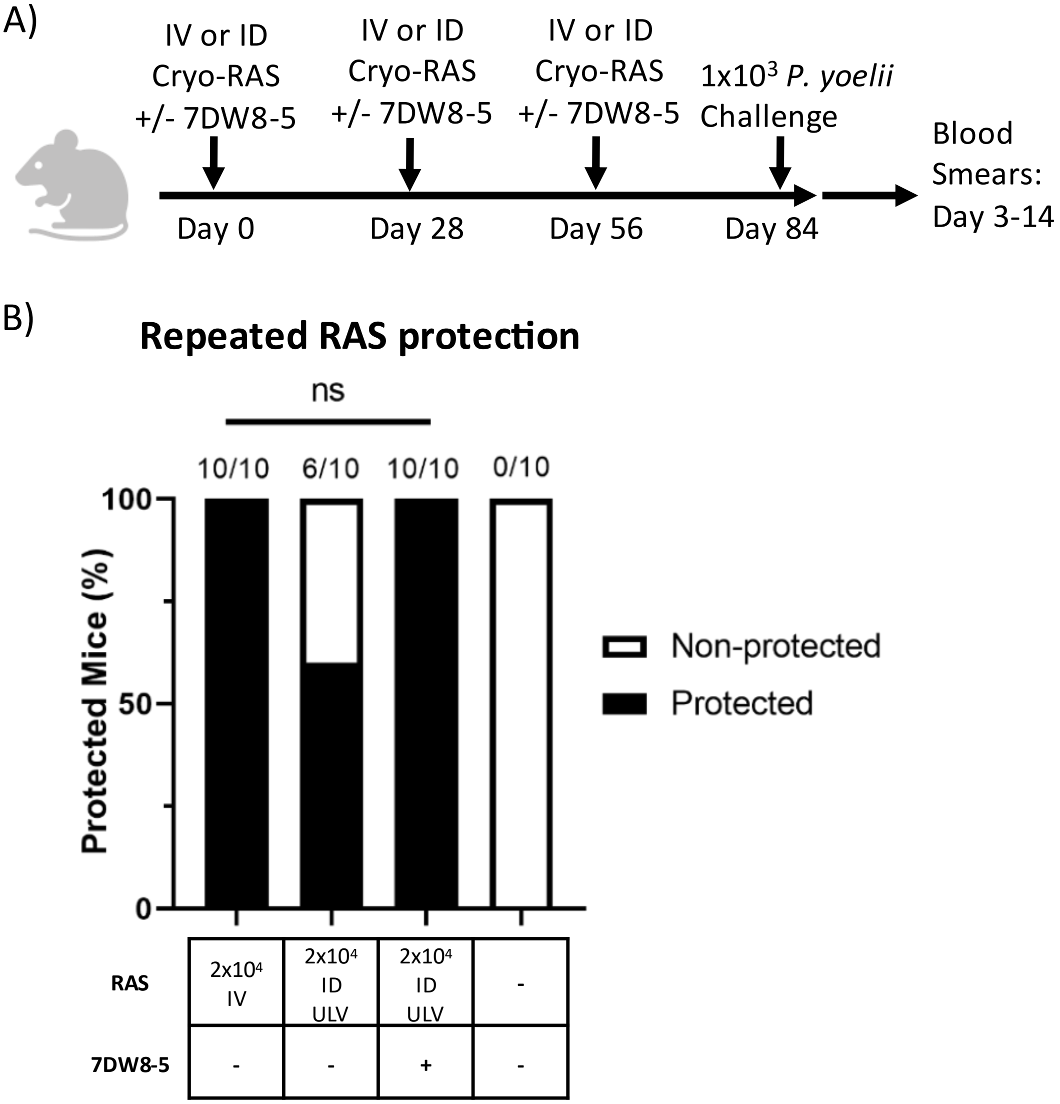


Figure S3.9. Ultra-low volume ID-RAS dose de-escalation parasite liver burden.
Naïve mice were immunized with cryo-RAS ID ULV (2.5 µL, X2 injections). Four hours post injection livers were excised and processed for real-time reverse transcription polymerase chain reaction (RT-PCR) to measure parasite burden with pan-*Plasmodium* 18S rRNA primers. Error bars represent the SD of the mean of N=5 mice from one experiment. RT-PCR data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, ***p<0.001, *p<0.05, ns=p>0.05. RT-PCR data are shown as absolute 18S rRNA copy numbers based on absolute calibrators. 2x10^4 ID ULV data (left column) reproduced from **Figure 3.1E** for comparison.



ggCSP	+	+	+	+	+	-
HK-SPZ	2×10^4 IV **RAS**	2×10^4 IV	2×10^4 IV	2×10^4 ID ULV	2×10^4 ID ULV	-
7DW8-5	+	-	+	-	+	-

Figure S3.10. HK-spz with or without 7DW8-5 are not protective in prime-and-trap. **A)** Experimental design of prime-and-trap protection studies. **B)** Results of protection studies after challenge with 1×10^3 WT purified Py spz administered four weeks after trapping with 2×10^4 HK-spz +/- 7DW8-5 administered IV or ID ULV (2.5 µL, X2 injections). 2×10^4 IV RAS+7DW8-5 was used as positive control. Protection data from N=10 mice across two independent experiments was analyzed with Fisher Exact test, $*p<0.05$, ns=$p>0.05$.



RAS	$2x10^4$ IV	$2x10^4$ ID ULV	$2x10^4$ ID ULV	-
7DW8-5	-	-	+	-

Figure S3.11. 7DW8-5 potentiates ultra-low volume repeated ID-RAS only vaccination. A) Experimental design of repeated RAS protection studies. **B)** Results of protection studies after challenge with $1x10^3$ WT purified Py spz administered four weeks after final RAS immunization. RAS was administered IV or ID ULV (2.5 µL, X2 injections) with or without 7DW8-5. Protection data from N=10 mice across two independent experiments was analyzed with Fisher Exact test, ns=p>0.05 (p-value=0.08).

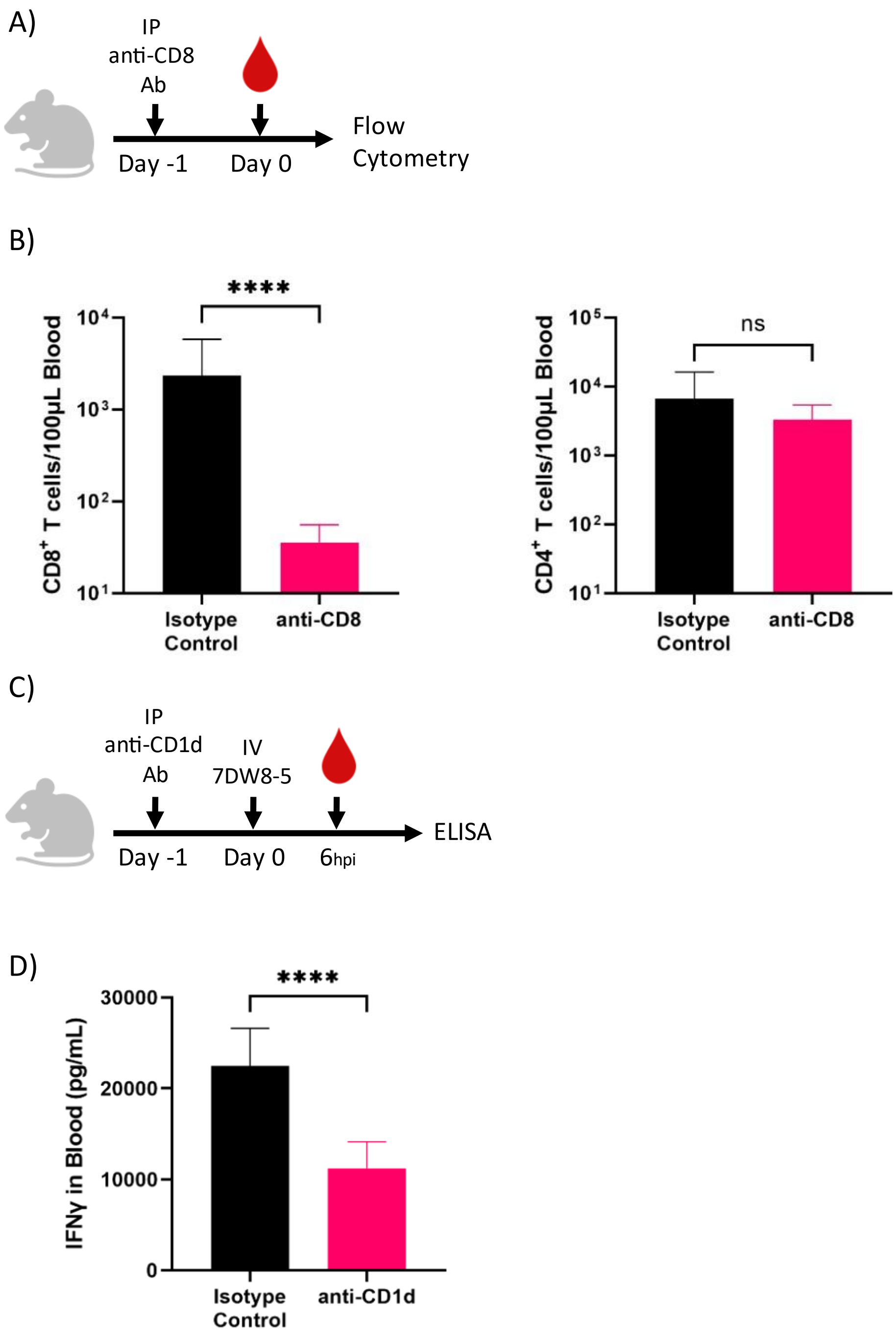
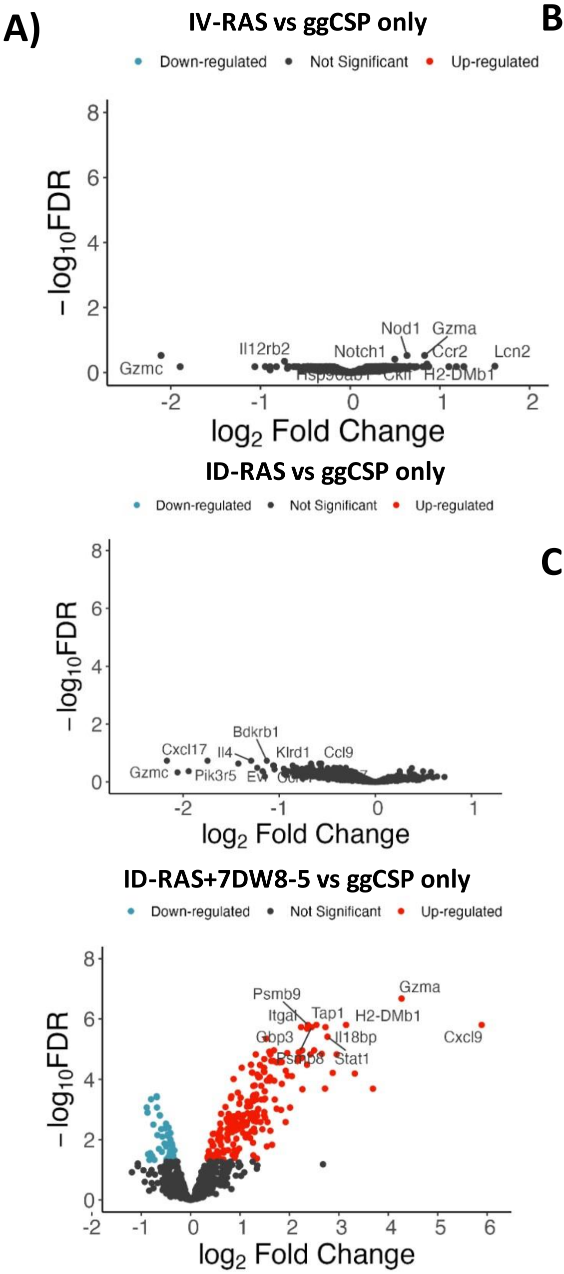


Figure S3.12. Cell depletion/blocking confirmation.
A) Experimental design of mouse whole blood leukocyte flow cytometry to confirm CD8 cell depletion at specified schedule and dose. **B)** Flow cytometry of leukocytes from **(A)** mouse blood. Error bars represent the SD of N=9 mice from one experiment. Mann–Whitney test, ****$p<0.0001$, ns=$p>0.05$. **C)** Experimental design of mouse blood plasma ELISA studies to confirm CD1d cell blocking at specified schedule and dose. **D)** IFN-γ cytokine levels in plasma from **(C)** mouse blood. Error bars represent the SD of N=10 mice from one experiment. Unpaired T test, ****$p<0.0001$.



B)

RAS Trap Group	up DEGs	down DEGs	Log_2FC	FDR P-value
IV-RAS vs ID-RAS	17	0	≥0.8	≤0.1
IV-RAS vs ggCSP	0	0	≥0.8	≤0.1
ID-RAS vs ggCSP	0	0	≥0.8	≤0.1
ID-RAS+7DW8-5 vs ggCSP	119	0	≥1	≤0.05
ID-RAS+7DW8-5 vs ID-RAS	154	0	≥1	≤0.05
ID-RAS+7DW8-5 vs IV-RAS	106	0	≥1	≤0.05

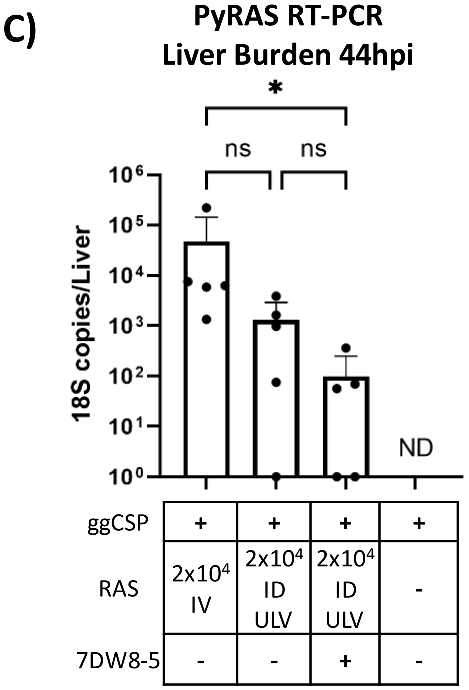


Figure S3.13. Nanostring differentially expressed genes and parasite liver burden.
A) Volcano plots of **Figure 3.3F** liver transcriptomic data. Only significance genes are highlighted, and the top 10 most significant genes are labeled. Significance is defined as FDR Adj. p≤0.05 and log2 fold change of ±1. **B)** Summary of differentially expressed genes (DEGs) for indicated groups. **C)** Livers from **Figure 3.3F** were harvested 44 hours after trapping with RAS IV (100 μL) or ID ULV (2.5 μL, X2 injections) with or without 7DW8-5 and subjected to Nanostring analysis (**Figure 3.3**) or RT-PCR liver burden analysis (shown here). 18S pan-*Plasmodium* primers were utilized to measure parasite liver burden. RT-PCR data are shown as absolute 18S rRNA copy numbers based on absolute calibrator. Error bars represent the SD of the mean of N=5 mice from one experiment. Data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, *p<0.05, ns=p>0.05. ND=Not Detected.

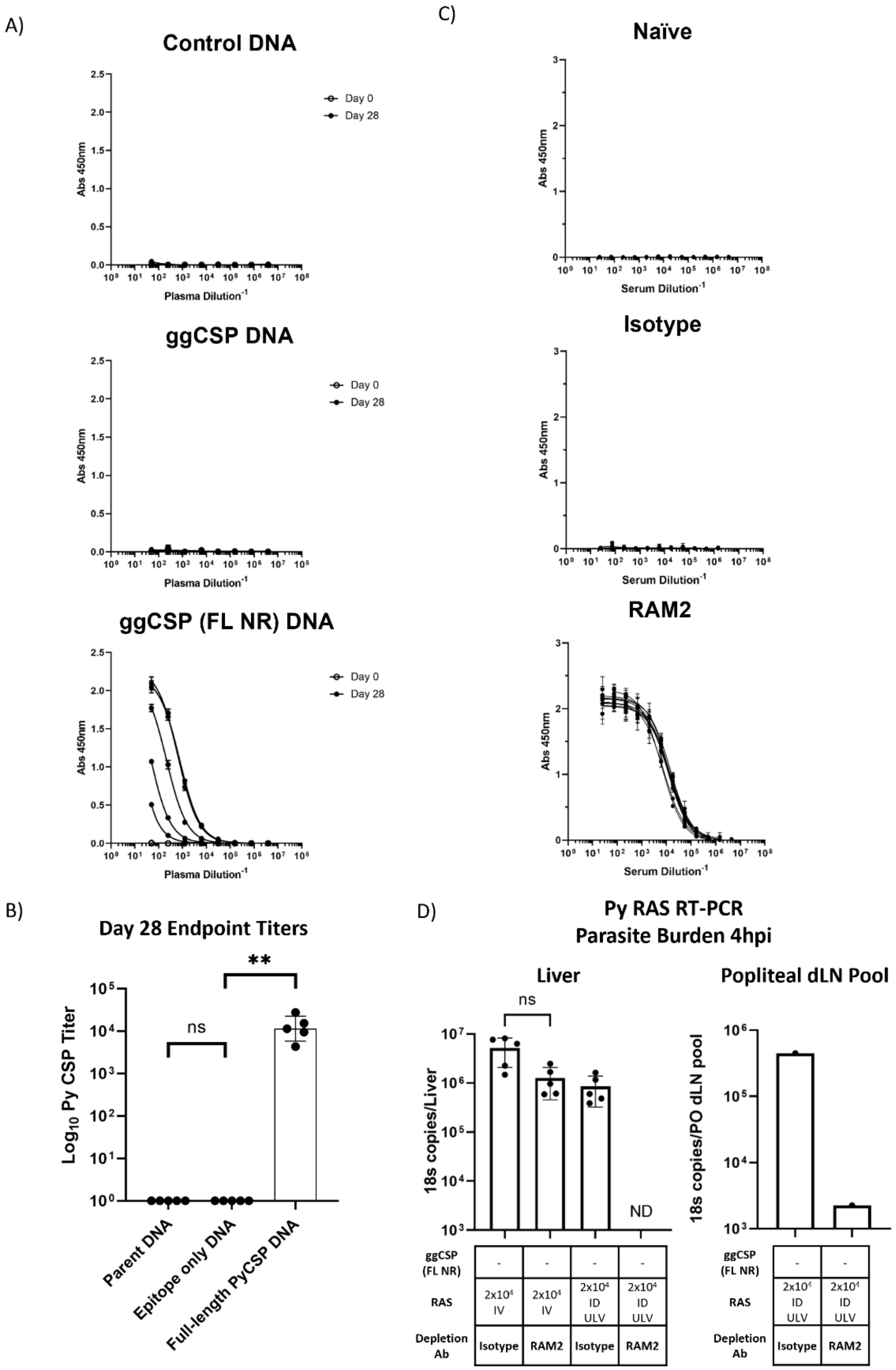


Figure S3.14. PyCSP antibodies impact spz vaccination.

A) Mice were primed with ggCSP, ggCSP (FL NR), or control DNA (pUb.3 plasmid backbone without the CSP insert). Serum was isolated from mouse blood on Day 0 and Day 28 for CSP

ELISA analysis. Data reported as anti-CSP IgG titers. **B)** Day 28 CSP ELISA endpoint titers from **(A)** mice. Error bars represent the SD of the mean of N=5 mice from one experiment. ELISA data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, **$p<0.01$, ns=$p>0.05$. **C)** Anti-RAM2 serum direct ELISA dilutions from a subset of isotype, RAM2, or naïve mice from **Figure 3.5** mice. **D)** Naïve mice were injected with 150µg RAM2 or isotype control mAb IP. 24 hours later, mice were immunized with cryo-RAS IV (100 µL) or ID ULV (2.5 µL, X2 injections). Four hours post injection livers (**left**), and popliteal draining lymph nodes (PO dLN) (**right**) were excised and processed for real-time reverse transcription polymerase chain reaction (RT-PCR) to measure parasite burden with18S pan-*Plasmodium* primers. Error bars represent the SD of the mean of N=5 mice from one experiment. PO dLN samples were collected from the injected side of ID-RAS animals and samples were processed in a pool of N=5 mice. Data was analyzed with Kruskal-Wallis test with Dunn's multiple comparisons, ns=$p>0.05$. ND=Not Detected.

Chapter 4. SEX-SPECIFIC DIFFERENCES IN CYTOKINE INDUCTION BY THE GLYCOLIPID ADJUVANT 7DW8-5

This chapter was previously published in "Biomolecules" in 2022 and has been reproduced and adapted here with permission [311].

4.1 CHAPTER SUMMARY

7DW8-5 is a potent glycolipid adjuvant that improves malaria vaccine efficacy in mice by inducing IFN-γ and increasing protective CD8$^+$ T cell responses. The addition of 7DW8-5 was previously shown to improve the efficacy of a CD8$^+$ T cell-mediated heterologous 'prime-and-trap' malaria vaccine against *Plasmodium yoelii* sporozoite challenge in inbred female mice. This study reports significant differential sex-specific responses to 7DW8-5 in inbred and outbred mice. Male mice express significantly less IFN-γ and IL-4 compared to females following intravenous 7DW8-5 administration. Additionally, unlike in female mice, 7DW8-5 did not improve the vaccine efficacy against sporozoite challenge in prime-and-trap vaccinated male mice. These findings highlight the importance of including both female and male sexes in experimental adjuvant studies.

4.2 INTRODUCTION

Adjuvants are compounds that are added to vaccines to enhance immune responses. Currently very few adjuvants have been licensed for clinical vaccination and the majority of these have a bias toward enhancing humoral and helper T cell immune responses (reviewed in [179]). There is a critical lack of approved adjuvants that enhance cytotoxic CD8$^+$ T cell responses to vaccination [179]. Importantly, many pathogens, including the *Plasmodium* parasites that cause malaria, are best controlled by the host through mechanisms that include cytotoxic CD8$^+$ cell responses [112]. Thus, more adjuvants, and especially those that improve cytotoxic CD8$^+$ T cell responses, will likely be needed to improve the efficacy of vaccines targeting these complex pathogens.

Glycolipid adjuvants that stimulate *i*NKT cells are of increasing interest due to their ability to activate not only humoral immunity, but also cytotoxic CD8$^+$ T cells (reviewed in [312]). α-Galactosylceramide (α-GalCer) is a glycolipid adjuvant that binds CD1d on antigen presenting cells (APCs), activates *i*NKT cells, and induces a cascade of immune cell activation that improves vaccine-induced immune responses [184-186]. α-GalCer has been studied for many years and clinical trials with this compound demonstrated safety and potent enhancement of immune responses in humans (reviewed in [191]). However, several clinical trials with α-GalCer have shown suboptimal results, which inspired the development of synthetic α-GalCer analogs. There are now hundreds of α-GalCer analogs that have been developed, which have different toxicological profiles and/or immunostimulatory effects [194].

7DW8-5 is a synthetic analog of α-GalCer that has significant translational potential. 7DW8-5 has been tested as an adjuvant and therapy for a variety of infectious diseases and cancer (reviewed in [199]). 7DW8-5 binds CD1d with higher affinity than α-GalCer and has shown higher efficacy at a 100-fold lower dose compared to α-GalCer in rodent and *in vitro* human cell models [195]. Importantly, 7DW8-5 increases the efficacy of live-attenuated malaria sporozoite (spz) vaccines by increasing CD8$^+$ T cell responses [173,189]. However, the majority of pre-clinical rodent studies involving 7DW8-5 were completed in female inbred mice. Since substantial sex-specific differences in immune responses to adjuvants including α-GalCer have been noted [313,314], this study sought to directly compare the impact of biological sex on 7DW8-5 adjuvant effects.

4.3 METHODS AND MATERIALS

4.3.1 *Mice*

Male and female inbred BALB/cJ or Swiss outbred J:ARC(S) mice were purchased at 4-6 weeks of age from Jackson Laboratories (Barr Harbor, ME, USA). All animals were housed at the University of Washington Institutional Animal Care and Use Committee (IACUC)-approved animal facility and were used under an approved IACUC protocol (4317-01 to S.C.M.).

4.3.2 *7DW8-5*

7DW8-5 was gifted from Moriya Tsuji and prepared as described [173]. All mice received 2 μg 7DW8-5 administered via intravenous (IV) or intradermal (ID) route. IV injections were administered retro-orbitally in 100 µL with an Exel International Insulin Syringe with a 29G permanently attached needle (product #26018). ID injections were administered on the lower back near the base of the tail in two 10 µL injections with a BD Veo Insulin Syringe with Ultra-Fine needle 6mm x 31G 3/10 mL/cc (product #324909).

4.3.3 *ELISA*

Plasma was isolated from mice and IFN-γ and IL-4 cytokines were measured with commercially available ELISA kits (BioLegend, San Diego, CA, USA) as previously described [173]. All plasma was frozen at -80°C in single-use aliquots.

4.3.4 *Plasmodium yoelii DNA prime-and-RAS trap immunization and challenge*

Prime-and-trap vaccines were prepared as previously described with minor modifications described below [173]. Briefly, a DNA insert encoding the full-length *Plasmodium yoelii* (Py) circumsporozoite protein (CSP) without the major repeat region (248aa: MKKCTILVVASLLLVDSLLPGYGQNKSVQAQRNLNELCYNEENDNKLYHVLNSKNGKI YNRNIVNRLLGDALNGKPEEKKDDPPKDGNKDDLPKEEKKDDLPKEEKKDDPPKDPKK DDPPKEAQNKLNQPVVADENVDQ|PRPQPDGNNNNNNNNGNNNEDSYVPSAEQILEFV

KQISSQLTEEWSQCSVTCGSGVRVRKRKNVNKQPENLTLEDIDTEICKMDKCSSIFNIVSNSLGFVILLVLVFFN) was cloned into the pUb.3 vector, loaded onto gold beads, and administered to mice via gene gun (ggCSP). One month later, 2x10^4 total cryopreserved Py radiation attenuated sporozoites (cryo-RAS, Sanaria, Inc., Rockville, MD, USA) with or without 7DW8-5 were administered IV (as described above) or ID into the rear left footpad in two 2.5 µL injections with a NanoFil syringe (World Precision Instruments, LLC., Sarasota, FL). Cryo-RAS and 7DW8-5 were mixed immediately before injection. Animals were challenged IV via the retro-orbital route with 1x10^3 freshly dissected wild-type infectious Py spz (obtained from Seattle Children's Research Institute, Seattle, WA) and monitored for the presence of blood stage parasites by Giemsa-stained thin blood smear microscopy for 14 days.

4.3.5 *Statistics*

ELISA data was analyzed with non-parametric Mann-Whitney tests. All error bars represent the standard deviation (SD) of the mean with individual mouse samples shown, if applicable. Protection data was analyzed with Fisher Exact test. P values above 0.05 were considered not significant (ns). GraphPad Prism 9.1.2 Software (San Diego, CA) was used for all calculations.

4.4 RESULTS

As a first step towards evaluating the impact of biological sex on 7DW8-5 adjuvant effects, a review of prior literature on 7DW8-5 was conducted to search for information on research subject sex. A total of 17 papers were identified by searching "7DW8-5" on PubMed®. Two papers were excluded - one contained no animal experiments [315] and one was a review article with limited primary research data [316]. Of the 15 remaining papers, 13 used 7DW8-5 in rodent models, one used 7DW8-5 in non-human primates (NHP), and one used 7DW8-5 with human *ex vivo* samples

(**Table 4.3**). The single study in NHPs used only male animals, while only 9 of the 13 papers in rodent models listed the sex of the animals, and all of these used female mice (**Table 4.3**). Additionally, the studies of 7DW8-5 in rodent models were also exclusively performed in inbred mouse models. Thus, there was a need for further evaluation of the impact of biological sex on 7DW8-5 adjuvant effects in pre-clinical research models.

Table 4.3. Biological sex of research subjects in published articles with 7DW8-5.

	Female	**Male**	**Sex matched**	**Not listed**	**TOTAL**	**References**
Mouse	9	0	0	4	13	[173,189,195,197,198,317-324]
NHP	0	1	0	0	1	[190]
Human	0	0	1	0	1	[325]

It was previously reported that IV administration of 2 μg 7DW8-5 induced potent transient levels of IFN-γ cytokines in female inbred BALB/cJ mouse blood with strong responses peaking at 12 hr and declining by 24 hr [173]. To assess the impact of biological sex on this phenomenon, the same experiment was repeated in male BALB/cJ mice and found a ~6-fold reduction in IFN-γ induced by IV-7DW8-5 administration in male mice compared to female mice at the 12 hr timepoint (**Figure 4.1**). 7DW8-5 is known to preferentially induce Th1 cytokines (*e.g.*, IFN-γ), but detectable levels of Th2 cytokines (*e.g.*, IL-4) are also induced by IV 7DW8-5 administration with similar expression kinetics [173]. Since 7DW8-5 is also known to induce IL-4 production, the study also assessed IL-4 levels in the same mice. There was no detectable IL-4 induced by IV-7DW8-5 administration in male mice, but IL-4 was detected in female mice at the 12 hr timepoint (**Figure 4.1**). Thus, male and female BALB/cJ mice show striking sex-specific differences in the levels of these plasma cytokines in response to IV 7DW8-5 administration.

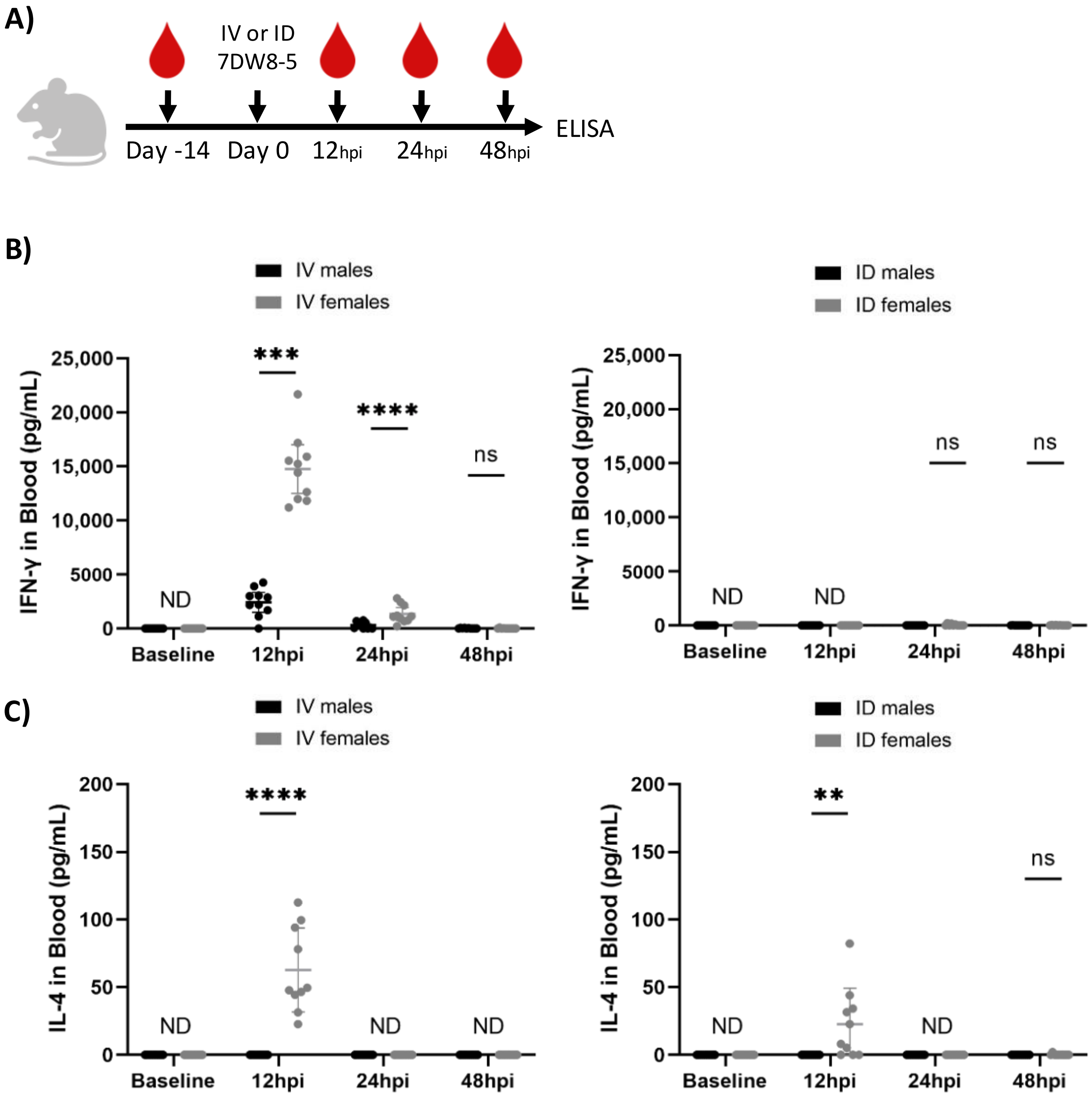


Figure 4.1. 7DW8-5 induces higher levels of systemic IFN-γ and IL-4 in female vs. male BALB/cJ mice.

A) Experimental design of blood plasma ELISA studies. IFN-γ (**B**) or IL-4 (**C**) cytokine levels induced by IV-7DW8-5 (**left**) or ID-7DW8-5 (**right**) in mouse blood plasma. Female IV data adapted from Watson et al. for comparison [173]. Error bars represent the SD of the mean of N=10 BALB/cJ mice across two independent experiments. ELISA data analyzed with Mann–Whitney tests, ****$p<0.0001$, ***$p<0.001$, **$p < 0.01$, ns=$p>0.05$. ND=Not detected. ID=two 10 µL injections for all.

Non-systemic administration routes are also of interest. This is because non-IV administration of 7DW8-5 induced little to no systemic cytokine responses in female mice [189], which may be expected to translate to a better safety profile for clinical use. Therefore, the study next evaluated the impact of sex on ID-7DW8-5 administration in mice. 2 µg of 7DW8-5 were ID administered to male and female BALB/cJ mice and IFN-γ and IL-4 were assessed by ELISA as above. As expected, the ID route induced significantly lower levels of these cytokines, but female mice still had higher levels than males at the 12 hr timepoint (**Figure 4.1**). Thus, male and female BALB/cJ mice also show sex-specific differences in the levels of these plasma cytokines in response to 7DW8-5 non-systemic ID administration.

As it was possible that these findings were a phenomenon of the BALB/cJ mouse model, the study investigated if this sex-specific phenotype was apparent in another mouse model. To assess whether these findings were generalizable to outbred mouse strains, IFN-γ induction was compared following systemic IV or local ID administration of 7DW8-5 in Swiss outbred mice. As seen in the inbred BALB/cJ mice, higher levels of IFN-γ cytokine was detected in females compared to male mice following IV-7DW8-5 administration at 6 hr post-injection (**Figure 4.2**). As expected, only low levels of IFN-γ were induced by ID-7DW8-5 administration in these mice. Thus, male and female genetically-diverse Swiss outbred mice also showed sex-specific differences in the levels of plasma IFN-γ in response to 7DW8-5.

Lastly, this study determined if 7DW8-5 would be an effective adjuvant in male BALB/cJ mice. Previous literature has demonstrated that live RAS malaria vaccines are improved by the co-administration of 7DW8-5 in female mice [189]. Additionally, 7DW8-5 improved a heterologous two-step DNA-prime-and-RAS trap malaria vaccine strategy in female BALB/cJ mice [173]. Moreover, experiments from **Chapter 3** found that 7DW8-5 co-administration with ID-RAS was required to achieve 100% protection in female BALB/cJ mice (**Chapter 3** and [326]). This final study evaluated the efficacy of the same DNA-prime and 7DW8-5-adjuvanted IV- or ID-RAS trap vaccine in male BALB/cJ mice. Strikingly, 7DW8-5 did not significantly improve vaccine efficacy, with poor protection seen following DNA-prime and IV- or ID-RAS trap groups both with and without 7DW8-5 (**Figure 4.3**). Thus, the adjuvant effects of 7DW8-5 are drastically different in male and female mice when used in this malaria vaccine strategy.

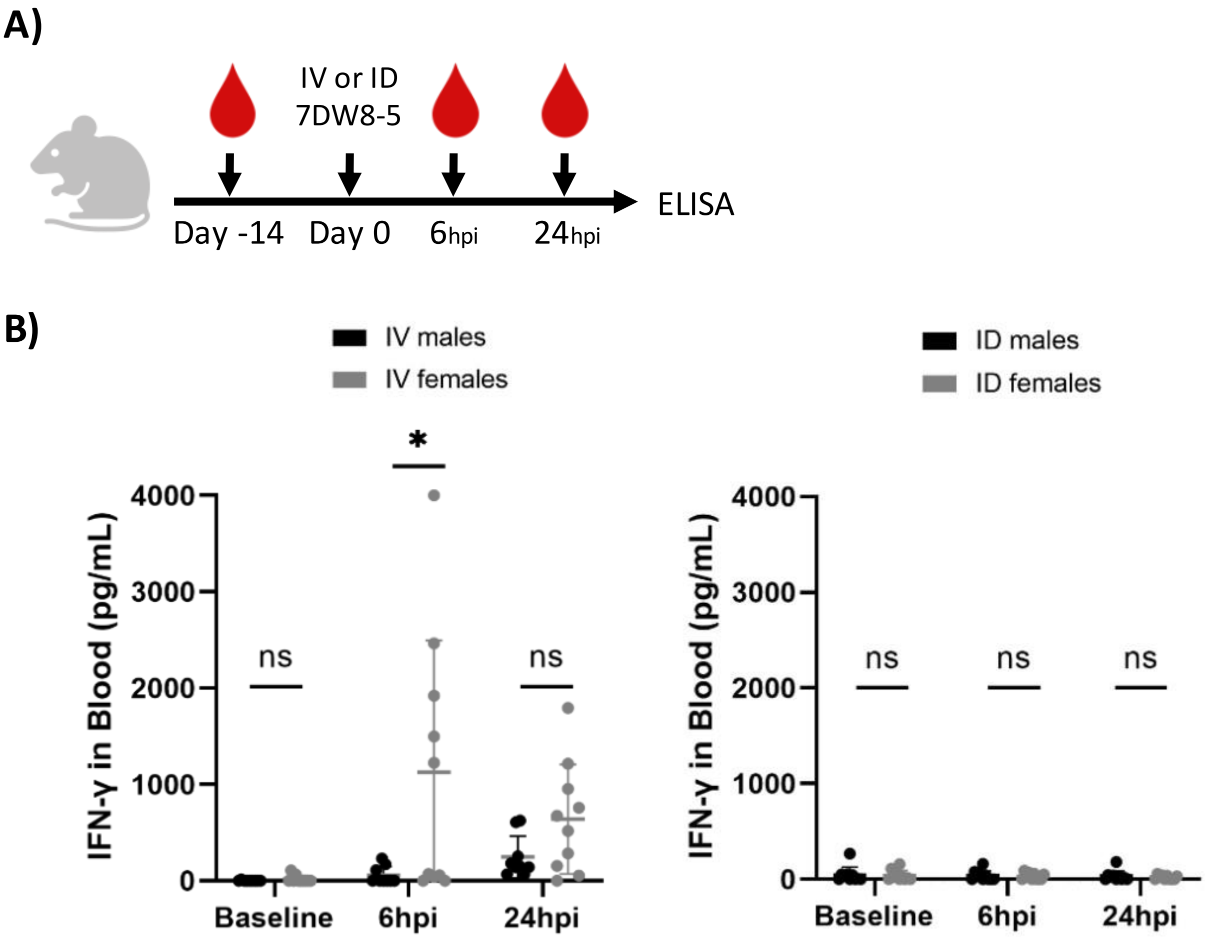


Figure 4.2. 7DW8-5 induces higher levels of systemic IFN-γ in female vs. male Swiss outbred mice.

A) Experimental design of blood plasma ELISA studies. **B**) IFN-γ cytokine levels induced by IV-7DW8-5 (**left**) or ID-7DW8-5 (**right**) in mouse blood plasma. Error bars represent the SD of the mean of N=9–10 Swiss outbred mice across two independent experiments. ELISA data analyzed with Mann–Whitney tests, *$p<0.05$, ns=$p>0.05$. ID=two 10 μL injections for all.

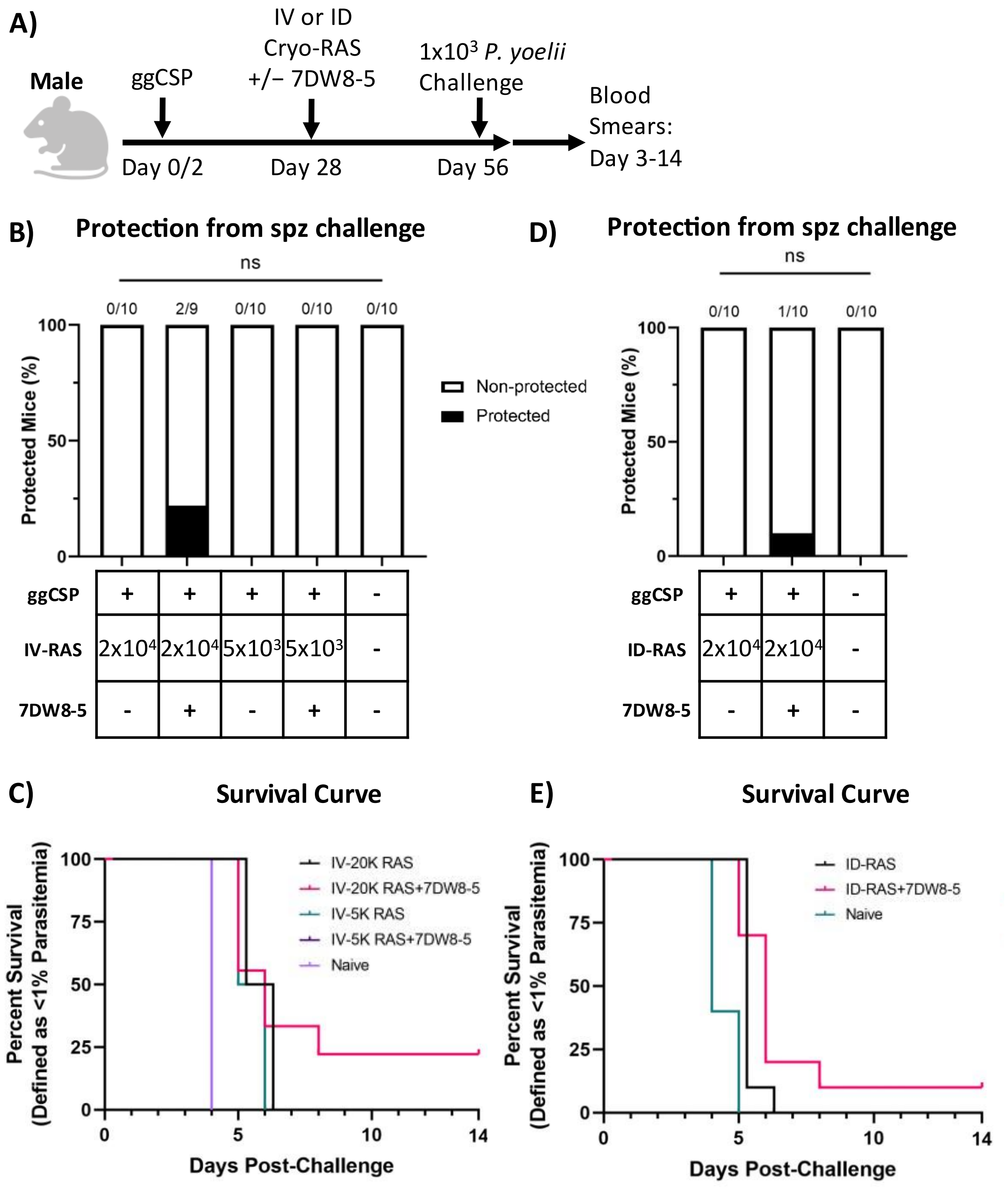


Figure 4.3. Prime-and-trap vaccination is not protective in male BALB/cJ mice.
A) Experimental design of prime-and-trap protection studies. Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks after trapping with 2x10^4 or 5x10^3 IV **(B)** or ID **(D)** administered cryo-RAS +/- 7DW8-5. Protection data was from N=9-10 male BALB/cJ mice across two independent experiments and analyzed with Fisher Exact test, ns=p>0.05. ID=two 2.5 μL injections for all. **C**) Survival curve of mice from (**B**). **E**) Survival curve of mice from (**D**).

4.5 DISCUSSION

Novel adjuvants are critically needed for the clinic. Glycolipids are potent adjuvants that have been shown to have potent immunostimulatory effects, including the ability to induce cytotoxic CD8$^+$ T cell responses [312]. As such, they are of increasing interest for use in vaccines targeting complex pathogens like *Plasmodium*. 7DW8-5 is of particular interest due its ability to activate *i*NKT cells and induce Th1-like responses, including the activation of CD8$^+$ T cells, and to do so at lower doses than α-GalCer [195]. Previous reports in rodent models only examined adjuvant effects of 7DW8-5 in female inbred mice. However, since significant biological sex-based differences have been noted for α-GalCer [313,314], here this study sought to investigate if 7DW8-5 similarly induced different adjuvant effects in male and female mice. IFN-γ was induced by IV-7DW8-5 administration in both inbred and outbred mice, but the cytokine levels were significantly lower in males compared to females. Additionally, unlike in females, 7DW8-5 did not improve the efficacy of a DNA prime-and-RAS trap malaria vaccine in male BALB/cJ mice. Taken together, these data demonstrate that 7DW8-5 adjuvant effects can be drastically different in male and female mice.

These findings are supported by other studies that found significant sex-specific differences in the *i*NKT immune responses of inbred C57BL/6 mice [313,314]. Although both reports concluded that IFN-γ cytokine levels induced by glycolipids were higher in female mice than male mice, these prior studies proposed different mechanisms and contributions from sex hormones. Gourdy et al. suggested that estrogens but not testosterone were responsible for the differential immune responses [313], while Lotter et al. suggested that testosterone moderated cytokine production of *i*NKT cells in mice [314]. Based on this data, the hypothesis for this model is that high testosterone in male mice inhibits the induction of IFN-γ following 7DW8-5 administration. Future efforts in the laboratory will seek to investigate the role of estrogens and androgens in mouse models of prime-and-trap malaria vaccination. A limitation of this study is that it did not account for weight differences between male and female mice. On average, six week old BALB/cJ male mice weigh more than age matched female mice (male: 23.7g SD 1.4 vs female: 19.1g SD 1.2) [327]. Thus, female mice are receiving a higher dose (mg/kg) of adjuvant. It is possible that a weight normalized adjuvant dose would minimize the differences in cytokine induction that was observed. Additionally, the experiments in Swiss outbred mice resulted in greater variation of the data, which was not unexpected since outbred mice are more genetically diverse and may better represent how diverse human populations may respond to adjuvants. As a future pre-clinical step, our understanding of 7DW8-5 will also greatly benefit from studies using NHP models of malaria RAS vaccination and challenge.

In 2016, the NIH introduced a policy mandating inclusion of sex as a biological variable in all vertebrate animals and human studies [328-330]. The findings here indeed highlight the importance of including both biological sexes in experimental adjuvant studies, documenting sex-specific data in publications, and considering how biological sex may impact pre-clinical and clinical outcomes. Strengthened efforts to investigate sex as a biological variable in preclinical research studies may be especially important for translating research findings from rodent models to larger NHP models and ultimately to humans.

4.6 ACKNOWLEDGMENTS

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Chapter 5. CONCLUSION

5.1 CONCLUDING REMARKS AND IMPLICATIONS

A more effective malaria vaccine is required to reduce the global burden of disease. Currently no vaccine achieves complete protection from malaria, but two promising vaccines, RTS,S (*i.e.,* an adjuvanted subunit vaccine) and PfSPZ (*i.e.,* a WSV), can reduce malaria mortality after 3-4 booster shots [128]. RTS,S prevents severe malaria disease and death, but has not markedly prevented infection or further transmission in field studies in malaria endemic regions [98,146]. PfSPZ can completely prevent infection in malaria-naïve populations but requires a specialized IV administration route [92,155]. As most vaccines are delivered IM into the upper arm, the IV route poses a hurdle for widespread use of PfSPZ. The heterologous prime-and-trap vaccine was designed to combine the benefits of these two suboptimal malaria vaccines into a single highly effective next-generation vaccine. Although prime-and-trap induces robust immune responses and confers durable protection in mice [121], further optimization was required to improve the dose and administration route of the RAS. The hypothesis of this dissertation was that decreasing the RAS trapping dose and/or removing the requirement for IV administration would significantly improve the translational potential of the prime-and-trap vaccine for use in NHPs or humans. This project demonstrated that the cryo-RAS trapping dose could be reduced by co-administration with the glycolipid adjuvant 7DW8-5 (**Chapter 2**) and sought to understand why ID-RAS administration has historically been less protective than IV-RAS (**Chapter 3**). Finally, this dissertation explored the significant sex-specific differences induced by 7DW8-5 in male vs female mice (**Chapter 4**).

The first aim of this study found that the co-administration of IV cryo-RAS and 7DW8-5 increased the immunogenicity of an incompletely protective RAS dose and protected mice against malaria spz challenge. Cryo-RAS and fresh-RAS both induced high levels of CSP-specific CD8$^+$ Trm cells in the liver that achieved equivalent levels of protection against spz challenge in female BALB/c mice. This finding was unexpected given the significant reduction in parasite liver burden between cryo-RAS vs Fresh-RAS and suggests that the liver burden may not always correlate with protection in mice (**Figure 5.1**). Additionally, HK-SPZ were not detectable in the liver of any vaccinated mice, suggesting these non-viable and non-motile spz do not invade the liver parenchyma and thus do not provide any protection and are not suitable for prime-and-trap. The 7DW8-5 glycolipid adjuvant induced transient levels of IFN-γ that may be beneficial for LS protection. The co-administration of IV RAS+7DW8-5 was found to be dose sparing in mice, but adjuvant was not required for durable protection up to 4 months. Taken together, these findings suggest that IV cryo-RAS with or without 7DW8-5 is efficacious in mice and is simple enough for translation to NHPs or humans.

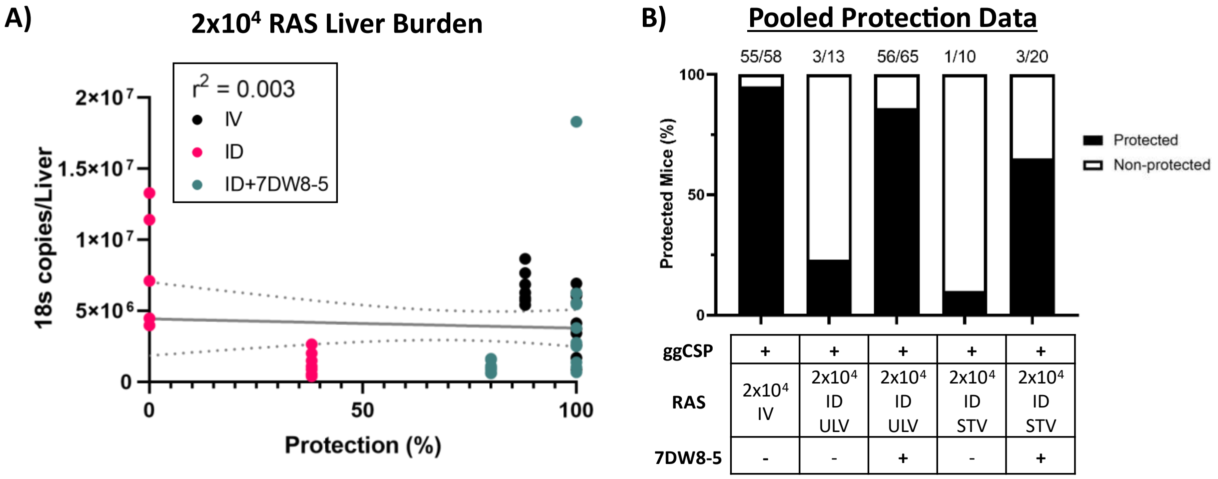

Figure 5.1.Pooled and summarized liver burden and protection data.
A) Simple linear regression analysis of parasite liver burden vs percent of animals protected from spz challenge. The dotted line indicates the 95% confidence interval. Liver burden RT-PCR and protection data was pooled from female BALB/cJ mice across >2 independent experiments. ID STV=two 10-50 µL and ID ULV=two 2.5 µL injections. **B)** Results of protection studies after challenge with 1x10^3 WT purified Py spz administered four weeks after trapping with 2x10^4 IV or ID administered cryo-RAS +/- 7DW8-5.

Despite the improvements to IV cryo-RAS trapping, the translation of IV injections may face many burdensome technical challenges and no other approved vaccine uses IV administration [331]. The ability to deliver the vaccine by standard administration routes (*e.g.*, ID or IM) would greatly improve the vaccine. ID vaccination is attractive because it is simple, it mimics the natural route of malaria exposure by mosquito bite, and immune cells are abundant in the skin. The second aim of this dissertation discovered that the key to successful ID administration was to mimic a mosquito bite by injecting RAS in an ULV in combination with 7DW8-5. This vaccine strategy completely protected mice against spz challenge and reduced the required dose by 4-fold. The inoculation volume of ID-RAS significantly impacted the parasite liver burden, with lower volumes associated with higher numbers of parasites in the liver. The ULV ID-RAS inoculation volume (2.5 µL) achieved equivalent liver burden levels as IV-RAS but was not protective in the absence of 7DW8-5 adjuvant (**Figure 5.1**). This finding suggested that a potent adjuvant like 7DW8-5 is critical to overcome the known tolerogenic immune environment induced by the spz in the skin [77,283]. ID-RAS+7DW8-5 induced pro-inflammatory genes and associated pathways in the liver that were immunomodulatory and advantageous for vaccination. The final studies of the second aim investigated an accelerated prime-and-trap schedule in which priming and trapping occurred on the same day. This strategy has been effective for other prime-and-trap malaria vaccine strategies and was hypothesized as a method to further simplify this prime-and-trap vaccine schedule [82,123]. Interestingly, a single dose of IV-RAS+7DW8-5 trap was highly effective with or without the ggCSP priming on the same day. However, in contrast to the IV route, a single dose of ID-RAS+7DW8-5 trapping with or without ggCSP on the same day was found to be not protective in mice. This finding was unexpected but opens new questions about possible different mechanisms of protection for IV-RAS+7DW8-5 vs ID-RAS+7DW8-5. A previous study demonstrated that immune responses differed after a single RAS injection administered via different routes [189]. IM-RAS induced the activation of DC and *i*NKT cells in the dLN but not the spleen, while IV-RAS induced the activation of DC and *i*NKT cells in the spleen but not the dLN [189]. Thus, for single dose RAS immunization, the spleen is likely a critical component of the protective immune response. Future studies could investigate protection after a single dose of IV-RAS +/− 7DW8-5 in splenectomized or sham mice to further explore the role of the spleen in protection. Consistent with this hypothesis, ggCSP priming induced CSP-specific $CD8^+$ T cells in the spleen that likely contribute to the protective immune responses observed in the standard two step prime-and-ID-RAS trapped animals. Taken together, data from this second aim suggests that prime-and-ID-RAS trap can be as effective as IV-RAS if the ID-RAS are co-administered with 7DW8-5 in an ULV.

The final aim of this study explored the differential sex-specific adjuvant effects of 7DW8-5 in male and female mice. Previous prime-and-trap vaccination studies in male and female inbred mice found that only female mice were reliably protected against spz challenge (unpublished). The third aim of this dissertation sought to improve vaccine efficacy in male mice by the co-administration of 7DW8-5. However, these studies uncovered significant sex-specific differences in the expression of cytokines induced by 7DW8-5 in both inbred and outbred mice. Female mice consistently showed higher levels of IFN-γ and IL-4 and were the only mice protected from spz challenge with both IV- and ID-RAS+7DW8-5 administration routes. Additionally, the adjuvant only significantly improved low dose ID-RAS trapping in female mice but not male mice. This finding suggests that 7DW8-5 adjuvant dose or route may require optimization for use in different sexes, which is important as the adjuvant is under investigation for use in NHP or human trials. However, as all these studies were conducted in laboratory mice, the relevance and translation of this data for NHPs or humans is not clear, but biological sex should continue to be considered as a biological variable. Previous RAS vaccination studies have also observed significant differences in protective efficacy in male and female mice, implicating testosterone as negatively influencing the adaptive immune responses in the liver [332]. Consistent with this finding, another group found that in mice, testosterone inhibited α-GalCer induced cytokine production by *i*NKT cells [314]. Based on this data, the high testosterone levels in male mice likely inhibit the induction of IFN-γ following 7DW8-5 administration, which then reduces the activation of $CD8^+$ T cells in the liver. To test this hypothesis, future studies could investigate protection after prime-and-trap +/− 7DW8-5 in gonadectomized or sham male mice to explore the role of testosterone in protection. Additionally, testosterone could be added back to the gonadectomized male mice or to female mice to further explore the hypothesis that high testosterone levels inhibit IFN-γ induced by 7DW8-5 administration. Finally, if testosterone is found to significantly impact the adjuvant effect of 7DW8-5, additional studies could investigate the protective efficacy of prepubescent vaccine regimens.

In summary, a more effective malaria vaccine is urgently needed to reduce global malaria deaths. This dissertation identified several methods to improve the translational potential of a promising two-step next-generation malaria vaccine. In mice, the optimized heterologous prime-and-trap vaccine is highly versatile, simpler to administer, and just as effective as the currently clinical gold standard, repeated PfSPZ-only IV immunizations. These studies identified key requirements for protective PE malaria vaccines and optimized a prime-and-trap vaccine strategy that could be translated to NHPs or humans.

5.2 LIMITATIONS AND FUTURE DIRECTIONS

There are several limitations of this research that could be addressed in future studies and are outlined below.

5.2.1 *Malaria mouse model*

Mouse models of malaria vaccination are critical, especially for studying the LS, but also face several limitations and do not always predict vaccine efficacy in humans [333]. First, inbred rodent models do not represent the genetic differences of diverse populations. The Py in BALB/cJ mouse model is a great tool for preclinical studies as the immunodominant CSP epitope for MHC-I is well characterized. However, studies in additional inbred and outbred mouse strains would strengthen and further validate the data. Second, rodent infecting *Plasmodium* spp. are highly divergent from human-infecting species [334,335]. Some critical differences include the duration of the LS (Pf takes up to 7-10 days and Py or Pb completes in 2-3 days), and the fact that the natural hosts of Pb and Py are thicket rats (*Grammomys surdaster* and *Thamnomys rutilans*, respectively) and not laboratory mice (*Mus musculus*) [333,336,337]. Previous studies indicate that the natural host-parasite combination is more difficult to protect than the mismatched experimental mouse model [163,337,338]. Thus, these major caveats are important considerations when translating the findings from this dissertation to NHPs or humans. Future studies should examine the efficacy of prime-and-trap with and without 7DW8-5 in natural host-parasite combinations, such as Pk in pigtail macaques (*Macaca nemestrina*) [339]. As the vaccine is translated from mice to NHPs, the adjuvant and vaccine doses will need to be scaled-up. Since the natural host-parasite combination is known to be more difficult to protect against challenge, the full weight-normalized IV-RAS dose will likely be required for sterile protection. Prime-and-full dose IV-RAS trap was protective in mice with or without 7DW8-5. However, in NHPs the addition of 7DW8-5 will likely be required for sterile protection. Additionally, the use of the ultra-low inoculum volume for ID-RAS mouse administration will likely still be required for NHPs as the spz motility in the dermis is expected to be similar in rodents and NHPs. Thus, the NHP ID-RAS vaccine will either need to be more concentrated than the mouse vaccine (to account for the scaled-up dose while maintaining the ULV) or will require more injections. Future studies could investigate sterile protection following IV-RAS or ULV ID-RAS +/− 7DW8-5 trapping in NHPs to determine if the concentrated vaccine or number of injections impact the vaccine efficacy.

5.2.2 *Priming optimization*

This dissertation focused on optimizing the RAS trapping component of prime-and-trap. However, the priming portion could also be optimized to improve the vaccine efficacy and clinical translational potential. As discussed in **Chapter 2**, few DNA vaccines have been licensed for human use [262,263], despite many promising clinical trials [264-267]. In 2021, India granted emergency approval for world's first DNA vaccine for COVID-19 called ZyCoV-D [340]. Additionally, although gene-gun administered vaccines have been reported to increase vaccine immunogenicity in mice and NHPs [121,273-276], there is currently no FDA approved gene-gun for the clinical use (although a clinical gene-gun is in development [341]). Thus, next steps for this project include further investigation of alternative DNA priming methods (*i.e.,* electroporation or lipid nanoparticles) and exploring alternatives like mRNA lipid nanoparticles that form the basis of the recently approved COVID-19 vaccines from Pfizer/BioNTech and Moderna [342,343]. Preliminary alternative priming data suggests that the priming step in prime-and-trap is versatile and ggCSP can be substituted for other nucleic acid priming methods without a loss in protection (**Figure 2.7** and unpublished data). However, Masopust et al 2006 reported that some priming methods and routes can significantly impact memory CD8$^+$ T cells regardless of the booster dose [205]. The study found that in general, prime-boost vaccination increased the total number of memory CD8$^+$ T cells and increased the ratio of Tem to Tcm cells compared to single dose immunization strategies [205]. Further, the type of the primary immunization was found to drastically impact the memory CD8$^+$ T cell response after the booster immunization [205]. Based on this data, substituting ggCSP priming with another priming strategy could impact the quality or quantity of the liver CD8$^+$ Trm cells induced by prime-and-trap. Preliminary studies suggest that although similar protection can be achieved with different nucleic acid priming methods in mice, the immunogenicity of the priming vaccine can be drastically different (unpublished). Thus, as prime-and-trap is translated from mice to NHPs, differences in the protective efficacy following different priming methods may become apparent. Therefore, further work in this area is needed to understand the requirements for priming.

5.2.3 *Trapping optimization*

The studies in this dissertation only utilized the EARD parasite, RAS. However, as described in **Chapter 1**, there are other promising WSV types including LARC parasites. LARC spz are more advantageous than RAS as they contain a larger and more diverse antigen load and induce more inflammation in the liver [160,344]. Future studies should investigate trapping with LARC parasites administered IV or ID with or without 7DW8-5. It is possible that the dose required for effective LARC spz trapping could be reduced without the need for an adjuvant as the replication capacity of the vaccine increases the antigen load and endogenous adjuvant capacity of the WSV. In addition, a promising new GAP vaccine called PfSPZ-LARC2 [160] will undergo clinical trials soon and if it is safe and efficacious, LARC GAP vaccines may begin to replace RAS as the gold-standard WSV for malaria. Future prime-and-trap vaccination studies with LARC parasites will involve priming with multiple PE antigens, including both spz or early LS antigens like CSP, and later LS antigens like RPL6. Priming with multiple LS antigens will increase the breadth of CD8$^+$ T cell priming in the periphery, which could lead to increased diversity and recruitment of antigen-specific CD8$^+$ Trm cells in the liver induced by the LARC parasites.

5.2.4 *Spz challenge model*

Field studies utilizing naturally reared mosquitoes found that a single infected mosquito injects <100 spz [345,346] and that laboratory reared mosquito spz inoculum per feed is ~125 spz [347]. However, the experiments in this study utilized a challenge dose of 1x10^3 IV administered Py spz, which is significantly higher than a mosquito bite (MB). Additionally, the challenge route is also an important consideration. Spz are naturally transmitted via MB and are subject to antibody neutralization in the dermis or migration out of the skin via lymphatics or vascular systems [26-28]. By utilizing an IV challenge route, the spz used herein by-passed the skin and were directly shuttled to the liver. The IV approach was selected as a more stringent and reliable spz challenge method to investigate the T cell-mediated efficacy of prime-and-trap, and this approach is more often than not the norm in such preclinical studies in the malaria research community. Indeed, throughout the study, 100% of the naïve infectivity control animals were successfully infected. The effectiveness of the IV challenge route was also validated in humans via a head-to-head comparison of IM, IV, and MB spz challenge [348], and is now readily utilized in CHMI clinical trials [230]. However, as previous studies have reported differences in vaccine efficacy and infectivity with different pathogen inoculation routes, investigating the efficacy of prime-and-trap with a MB challenge is warranted [174,241,302,307,308].

5.2.5 *Mechanism of protection*

A major limitation of this dissertation is that the data does not uncover a clear mechanism of protection as it relies heavily on spz challenge and protection outcomes and does not include many functional immunology studies. Analysis of the polyfunctionality of CD8$^+$ Trm cells in the liver after RAS vaccination with or without 7DW8-5 will likely uncover possible correlates of protection. The functional immunological readouts from IV-RAS and ID-RAS+7DW8-5 protected mice can then be compared to determine if the mechanism of protection is the same for both vaccination routes. Although the mechanism of action for 7DW8-5 has been thoroughly investigated [189,195], more studies are warranted to explore the mechanisms of protection induced by the adjuvant via different immunization routes (IV vs ID). Future studies could compare the kinetics and expression levels of cytokines induced by IV- or ID-7DW8-5 in the blood and liver using a multiplex cytokine analysis assay, such as Luminex. In addition, future studies should also dose de-escalate 7DW8-5 to determine the optimal RAS/adjuvant concentration to induce durable CD8$^+$ T cells in the liver.

Furthermore, this dissertation focused on the single adjuvant, 7DW8-5. Recently, a preclinical malaria vaccine co-administered with CpG adjuvants was shown to be highly effective in inducing malaria-specific CD8$^+$ Trm cells in the liver [183]. Thus, next steps include exploring the co-administration of RAS and different adjuvant classes, including CpG. These data would help to uncover if specific adjuvant types or pattern recognition receptors are more beneficial for LS vaccination than others. It is possible that the inclusion of different types of adjuvants will impact the durability and function of CD8$^+$ Trm cells in the liver. Thus, investigating the frequency and longevity of these cells is warranted. In addition, it is possible that other adjuvant classes may not show drastic sex differences as was observed with 7DW8-5. Future studies could investigate if prime-and-trap with other adjuvants could improve protection in male BALB/cJ mice.

5.2.6 *The need for more priming antigens beyond CSP*

Another limitation of this study is that all priming vaccines tested herein only target a single spz antigen, PyCSP. Preclinical and clinical studies suggest that PfCSP-specific CD8$^+$ T cells alone are insufficient for achieving sterile protection in all populations [136-141]. Variable protective efficacy is likely because CSP is poorly conserved across *Plasmodium* strains and species, which can lead to reduced protection in endemic areas where parasite genetic diversity is high [137-139]. Thus, more protective antigens that are conserved across *Plasmodia* are likely needed to improve priming. The search for protective PE antigens is an ongoing challenge in the malaria field; as of 2020, <15 spz or LS antigens demonstrated partial protective efficacy in clinical trials (reviewed in [131,134]). Future studies should identify novel, protective, and highly conserved LS antigens that can be incorporated into prime-and-trap with the goal of ensuring the vaccine retains high-levels of vaccine efficacy against diverse parasite strains and populations.

Identification of protective LS antigens has been challenging, but protective CD8$^+$ T cell antigens theoretically need to be processed in the host cell cytosol for presentation via MHC I molecules. Thus, exported, or secreted *Plasmodium* proteins are studied as likely CD8$^+$ T cell LS antigen candidates. A recent study identified a ribosomal protein (RP), RPL6, which protects mice against spz challenge and induces liver CD8$^+$ Trm cells [123]. RPL6 is an ideal LS vaccine candidate as it is abundant in LS and BS parasites and it is highly conserved across field isolates [123]. Another study identified a similar conserved RP, RPL3, that was found to be immunogenic and effectively primed CD8$^+$ T cells but was not protective in mice [251]. Investigation of characteristics that make certain RPs protective but not others will yield important information about LS antigen discovery selection criteria. The fact that RP make immunogenic CD8$^+$ T cell antigens was somewhat surprising as RPs were not previously suspected of exportation. Given this data, it is possible that RPs (and other abundant *Plasmodium* 'housekeeping' proteins such as heat-shock proteins) that are often identified in high-throughput screening assays could make protective vaccine targets. Future studies should investigate RPs and other conserved proteins as T cell vaccine antigens in prime-and-trap. Preliminary studies demonstrated that gg priming with RPL6-encoded DNA vaccines followed by IV-RAS trapping was completely protective in mice (unpublished). This data suggests that the prime-and-trap strategy can be used to screen and identify novel protective LS antigens. Then, the novel vaccine targets can be combined with existing protective antigens (like CSP and TRAP) to develop a more protective priming vaccine cocktail [135].

5.2.7 *Translational hurdles for RAS*

Although removing the IV requirement for RAS vaccination eliminated a major hurdle for vaccine accessibility, the WSV are still complicated by unique manufacturing constraints. Currently, WSV must be manufactured in mosquitoes, aseptically hand dissected by humans, and then purified, vialed, and cryopreserved in liquid nitrogen vapor [157]. Sanaria has developed and patented strategies to reliably produce GMP quality WSV for vaccination and challenge, but these methods are expensive and laborious, and the technology transfer required to scale out or scale up manufacturing is not readily accessible [157,158,349]. Fortunately, *in vitro* production of WSV in bioreactors is underway and has the potential to significantly improve WSV manufacturing and reduce costs [310]. Thus, future studies should continue to study WSV to identify protective antigens and key immune signatures that correlate with LS protection. This information can be used to design a simpler fully synthetic liver targeted non-spz trapping alternative vaccine.

5.3 FINAL THOUGHTS

WSV malaria vaccines are promising tools to reduce the global burden of malaria. This dissertation uncovered important methods to simplify WSV by adjuvanting to reduce the required dose and eliminating IV administration routes. The combination of subunit and WSV in a heterologous prime-and-trap further simplified vaccination and achieved synergy of immune responses in mice [173]. Although decades of research have studied WSV in mice, NHPs, and humans (reviewed in [158]), alternations in the schedule, dose, or administration route can drastically impact the protective nature of the vaccine. This project identified requirements for protection induced by a heterologous prime-and-trap strategy that includes WSV in mice, which aimed to understand why alterations in dose and administration routes impacted protection.

The research in this dissertation found that contrary to previously held notions, ID-RAS is as effective as IV-RAS if spz are administered in an ultra-low volume with an adjuvant. The initial volume hypothesis for these studies was not completely innovative but was inspired by the identification of inconsistencies of ID inoculum volumes in the literature. Several publications previously reported an association between protection or liver burden and ID spz inoculum volume [287,295,350], and many publications on spz biology in the dermis utilized microvolumes (<0.5 µL) for ID injections to mimic mosquito bites [26,29,30,351,352]. However, the importance of spz inoculum volume for effective spz motility and subsequent liver burden was not translated to most ID spz vaccine studies [92,283,285,286,353]. Furthermore, a recent paper reported that MB and syringe injected ID spz have drastically different migration patterns in the skin and suggested that microvolumes could improve ID spz injections [290]. Therefore, the findings in these studies are consistent with previous reports that ID spz are more effective when administered in ULV. ID-RAS administration has the potential to be translated to NHPs or humans. Currently in the clinic, accurate and reproduceable ID injections with standard syringes and volumes (50-100 µL) can be challenging [309]. However, ID-specific administration devices (*e.g.,* ID syringe adaptors) are now clinically available and can achieve reproduceable ID injections. In addition, next-generation vaccine administration devices such as microneedle patches could transform ID-RAS administration into a simple, quick, and pain-free procedure with minimal training for healthcare workers. Furthermore, microneedle patches may be more compatible with RAS than current ID devices as they can utilize microvolumes and can be cryopreserved.

In addition to the ultra-low inoculation volumes, the inclusion of a potent adjuvant was also required for successful ID-RAS vaccination. This finding was not unexpected as other groups found that adjuvating ID immunizations was well tolerated and improved vaccine efficacy [295,354-356]. Indeed, many ID spz studies implicated the tolerogenic environment that the malaria spz induces as a possible explanation for reduced efficacy following ID-RAS administration and suggested adjuvants to overcome this problem [77,283,357]. A previous study found that there was an increase in IL-10 producing lymphocytes in the skin dLN following ID-RAS and a decrease in protection compared to IV-RAS immunization [283]. The group further speculated that adjuvants in the skin could modulate the dermal immune environment and increase protection [283]. The studies in this dissertation built upon these previous findings and combined the two key requirements for ID-RAS vaccination: ULV and adjuvants, demonstrating that both are required. Taken together, these studies strengthened the WSV field’s understanding of requirements for successful IV- or ID-RAS vaccination.

A final significant finding in this dissertation is the difference observed between protection of female and male BALB/cJ mice following spz challenge. Protection was not improved by the addition of 7DW8-5 by either IV or ID routes. Interestingly, a recent study analyzed PfSPZ vaccine responses from human clinical trials and found that biological sex did not correlate with protection but did correlate with antibody titers [358]. The study analyzed multiple IV-RAS clinical trials and found that both male and female humans can be sterilely protective following IV-RAS vaccination [358]. Additionally, a heterologous prime-and-trap vaccine induced sterile protection in some male NHPs (Shears et al, unpublished). Taken together, these data suggest that the dramatic phenotype that is observed in male vs female BALB/cJ mice may not directly translate to NHPs or humans. However, the biological sex differences uncovered in these studies highlight the importance of including biological sex as a variable, but also remembering that the findings in the mouse model do not always translate into NHPs or humans.

Given the high burden of malaria disease, the introduction of novel and more effective vaccine strategies are urgently needed. In addition to the growing concern of parasite drug resistance [23], climate change is also impacting the range of the *Anopheles* mosquitoes and shifting malaria endemic regions [359]. This means that malaria could be introduced to areas that have less or no previous malaria immunity in the population. Thus, research efforts remain critical to improve existing malaria prevention tools and develop novel next-generation vaccines for the clinic. The findings from this dissertation can immediately be translated to NHP or human clinical trials, with the goal of improving next-generation malaria vaccination.

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APPENDIX A

Flow Cytometry Information

Table A.1. Flow cytometry reagents & antibodies to assess liver resident memory cells.

Specificity	Clone	Fluorochrome	Vendor	Catalog
CD3e	145-2C11	BUV 395	BD	563565
B220	RA3-6B2	BV711	BioLegend	103255
CD4	GK1.5	Alexa Fluor 700	BioLegend	100429
CD8a	53-6.7	BV421	BD	563898
CD69	H1.2F3	BV510	BD	563030
CD44	IM7	Alexa Fluor 488	BioLegend	103015
CD62L	MEL-14	PE-Cy7	BD	560516
KLRG1	2F1	PerCP-Cy5.5	BioLegend	138418
CXCR6	221002	PE	Fisher Scientific	FAB2145P100
CSP-tetramer	-	APC	NIH	-
APC	-	Streptavidin-APC	Prozyme	PJ27S
Zombie NIR™ Fixable Viability	-	NIR	BioLegend	423105
Fc CD16/32	2.4G2	-	BD	553141
Counting Beads	-	-	Polyscience	18328-5

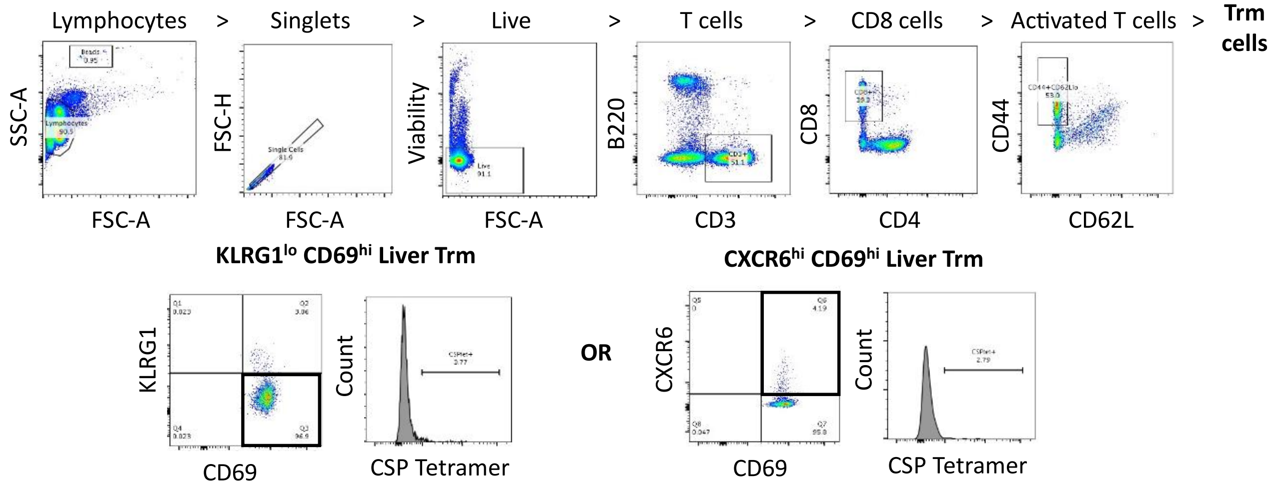

Figure A.1. Flow Cytometry gating strategy for mouse liver Trm cells. Representative gating strategy and identification of CSP-specific Trm cells in the liver of a prime-and-trap vaccinated animal. Cells were gated for CD8$^+$ T cells (CD3e$^+$, B220$^-$, CD4$^-$), CD44^{hi} by CD62L^{lo}, then assessed by either KLRG1^{lo} by CD69^{hi} or by CXCR6^{hi} by CD69^{hi}. Antigen specificity was then assessed by CSP-tetramer. Cell count per gram of tissue was calculated based on a known concentration of counting beads per samples to normalize data.

APPENDIX B

Table B.1. Differentially expressed genes (DEG) in ID-RAS+7DW8-5 compared to ID-RAS. List of DEG from prime-and-trap vaccinated mouse livers 2 days post trapping from **Figure 3.3**. DEG defined as log2FC>0.5 and FDR Adj. p<0.01.

Probe Name	Log2FC	FDR adj.P.Value	P.Value
Cxcl9	6.62	4.83E-07	2.43E-09
Batf	4.74	3.26E-03	6.71E-04
Gzma	4.38	1.64E-07	2.12E-10
H2-Eb1	3.80	2.10E-05	1.01E-06
Lcn2	3.69	1.75E-04	1.60E-05
H2-DMb1	3.63	4.83E-07	2.31E-09
H2-Ab1	3.37	4.77E-06	1.17E-07
Ccl5	3.22	2.28E-05	1.15E-06
Gbp2	3.18	3.89E-06	7.55E-08
Gbp5	3.01	7.66E-05	6.15E-06
Stat1	2.90	2.07E-06	3.21E-08
Gbp3	2.75	6.77E-07	5.26E-09
Il18bp	2.74	1.59E-06	1.85E-08
Psmb9	2.72	4.83E-07	2.50E-09
Zbp1	2.72	2.07E-06	3.08E-08
H2-DMa	2.67	4.13E-06	9.03E-08
Nkg7	2.65	1.03E-04	8.97E-06
Psmb8	2.60	1.17E-06	1.06E-08
Tap1	2.56	6.77E-07	5.04E-09
Gzmc	2.53	1.53E-03	2.75E-04
Irf7	2.52	7.99E-06	2.86E-07
Cxcl10	2.51	2.90E-06	4.87E-08
Il2rg	2.44	4.13E-06	9.08E-08
Ifi44	2.40	7.17E-06	2.23E-07
Ptprc	2.24	3.11E-05	1.63E-06
Ccr2	2.24	3.12E-05	1.69E-06
Rac2	2.23	1.51E-05	6.66E-07
Itgal	2.18	1.81E-06	2.34E-08
Pik3r5	2.18	4.49E-04	5.47E-05
Il2rb	2.12	2.16E-05	1.06E-06
Itgax	2.12	2.88E-04	3.01E-05
Fcgr1	2.08	1.48E-05	6.31E-07
Ncr1	2.08	3.72E-04	4.14E-05
Oas1a	2.08	5.51E-05	4.13E-06
Cd274	2.07	4.27E-05	2.72E-06
Itgb2	2.04	8.86E-06	3.32E-07

Hk3	2.00	7.99E-06	2.84E-07
Klrd1	1.97	7.17E-06	2.20E-07
Fcgr4	1.92	3.12E-06	5.65E-08
Cxcr3	1.90	7.19E-06	2.33E-07
Evl	1.86	4.31E-04	5.02E-05
Aif1	1.82	7.99E-06	2.89E-07
Lck	1.82	3.11E-05	1.65E-06
Cd3g	1.82	5.25E-05	3.86E-06
Ncf4	1.81	1.12E-05	4.34E-07
Gzmb	1.80	2.25E-03	4.39E-04
Cd3d	1.80	1.34E-03	2.29E-04
Zap70	1.79	5.02E-04	6.30E-05
Lgals3	1.79	1.55E-05	7.03E-07
Klrk1	1.79	2.88E-04	2.98E-05
Xaf1	1.79	6.60E-05	5.04E-06
Itgb7	1.78	9.41E-04	1.42E-04
Xcl1	1.78	2.17E-03	4.22E-04
Ccr5	1.76	1.37E-05	5.69E-07
Pik3cd	1.75	1.59E-06	1.67E-08
Prf1	1.75	5.63E-04	7.29E-05
Samhd1	1.74	4.28E-06	9.96E-08
Ctss	1.73	6.90E-05	5.44E-06
H2-D1	1.73	7.17E-06	1.99E-07
Klrc1	1.72	4.91E-04	6.10E-05
Cxcl11	1.71	3.26E-05	1.91E-06
Cd44	1.71	6.72E-06	1.74E-07
Pycard	1.69	5.00E-05	3.43E-06
Vcam1	1.68	5.25E-05	3.66E-06
Pdcd1lg2	1.67	7.22E-04	9.91E-05
Isg15	1.66	4.46E-05	3.00E-06
Relb	1.65	2.25E-03	4.46E-04
Tap2	1.63	1.74E-05	8.12E-07
Ifit2	1.60	6.75E-05	5.24E-06
Cd3e	1.60	6.58E-04	8.77E-05
Lcp2	1.59	8.85E-05	7.37E-06
H2-T23	1.57	3.26E-05	1.83E-06
Psmb10	1.56	1.02E-04	8.71E-06
Ltb	1.56	3.24E-04	3.44E-05
Rasgrp1	1.56	1.53E-03	2.74E-04
Ccl2	1.55	8.85E-05	7.45E-06
Tyrobp	1.53	3.26E-05	1.88E-06
Casp1	1.52	1.54E-04	1.38E-05

Ccl24	1.52	3.46E-04	3.80E-05
Csf2rb	1.50	9.24E-04	1.39E-04
Ifit3	1.49	2.17E-03	4.18E-04
Tnfsf10	1.48	4.13E-04	4.65E-05
Entpd1	1.48	4.71E-04	5.78E-05
Was	1.48	2.11E-03	4.04E-04
Ncf2	1.44	2.63E-04	2.59E-05
Il2ra	1.41	7.31E-04	1.02E-04
Apbb1ip	1.41	7.65E-04	1.11E-04
Tbxas1	1.40	5.95E-04	7.86E-05
H2-K1	1.40	7.17E-06	2.13E-07
Bst2	1.40	4.37E-05	2.88E-06
Slc11a1	1.39	1.84E-04	1.74E-05
Irf1	1.39	1.36E-04	1.19E-05
Plcg2	1.38	7.44E-04	1.06E-04
Tifa	1.38	2.01E-03	3.75E-04
Ctsw	1.37	7.66E-05	6.24E-06
Syk	1.37	4.21E-04	4.80E-05
Ripk3	1.36	3.26E-05	1.94E-06
Hck	1.36	5.25E-05	3.87E-06
Cxcr6	1.35	1.31E-03	2.23E-04
Ifitm3	1.35	4.27E-05	2.76E-06
Mx1	1.33	2.10E-04	2.01E-05
Fpr2	1.33	6.67E-04	8.97E-05
Tlr9	1.33	3.86E-03	8.20E-04
Lat2	1.31	7.85E-03	1.89E-03
Ifi27	1.28	2.86E-04	2.89E-05
Ddit3	1.28	3.44E-04	3.72E-05
Spi1	1.28	1.03E-03	1.60E-04
Oasl1	1.27	2.08E-03	3.91E-04
Plau	1.26	9.95E-04	1.53E-04
Sting1	1.25	3.68E-03	7.66E-04
Cd4	1.23	1.46E-03	2.55E-04
Tlr2	1.23	1.53E-03	2.75E-04
Ifi203	1.22	5.25E-05	3.85E-06
Csf2ra	1.22	1.73E-03	3.14E-04
Mlkl	1.21	1.63E-04	1.47E-05
Ccl22	1.20	4.38E-03	9.34E-04
Cd86	1.20	5.99E-03	1.36E-03
Cmklr1	1.20	2.86E-04	2.92E-05
P2rx7	1.19	7.43E-04	1.05E-04
Cd6	1.19	7.81E-03	1.87E-03

Ube2l6	1.18	1.37E-05	5.56E-07
Alox5ap	1.17	5.82E-03	1.31E-03
Sp100	1.17	3.51E-05	2.13E-06
Ccl19	1.17	4.33E-04	5.20E-05
Tlr1	1.13	2.00E-03	3.70E-04
Dhx58	1.12	9.69E-04	1.48E-04
Fgr	1.12	7.50E-04	1.08E-04
Pak1	1.12	8.47E-03	2.09E-03
Sell	1.11	1.21E-03	1.97E-04
Nlrc5	1.07	3.76E-03	7.93E-04
Il12rb2	1.06	1.21E-03	2.01E-04
Ccl7	1.05	1.83E-03	3.37E-04
Cd68	1.04	9.18E-04	1.37E-04
Ifit1	1.03	8.24E-03	2.00E-03
H2-M3	1.02	6.91E-03	1.61E-03
Il1b	1.02	9.20E-03	2.31E-03
Aim2	1.00	1.36E-03	2.35E-04
Nod1	0.99	2.63E-04	2.62E-05
Trim21	0.98	2.78E-03	5.61E-04
Oas2	0.98	3.22E-03	6.59E-04
Vsir	0.98	6.58E-03	1.52E-03
Socs3	0.97	5.28E-03	1.17E-03
Cd84	0.95	4.27E-05	2.70E-06
Adgre5	0.94	3.44E-04	3.73E-05
Marcks	0.94	2.64E-03	5.27E-04
Casp4	0.90	2.09E-03	3.94E-04
C2	0.89	1.81E-04	1.69E-05
Plscr1	0.88	8.73E-04	1.29E-04
Dtx3l	0.88	4.21E-04	4.84E-05
Prkcd	0.84	1.15E-03	1.82E-04
Tcirg1	0.84	1.21E-03	1.95E-04
Tgfb1	0.83	2.36E-04	2.29E-05
Cxcl16	0.81	1.09E-03	1.72E-04
Sirpa	0.79	4.33E-04	5.17E-05
Rhog	0.79	4.33E-04	5.21E-05
Akt3	0.78	3.71E-03	7.78E-04
Adar	0.77	1.75E-03	3.19E-04
Ap1s2	0.77	8.44E-03	2.06E-03
Cd38	0.77	5.04E-03	1.10E-03
Irf9	0.76	1.25E-03	2.09E-04
Il10ra	0.75	5.31E-03	1.18E-03
Hmox1	0.74	2.74E-03	5.50E-04

Lcp1	0.72	1.38E-03	2.40E-04
Ifitm1	0.71	2.11E-03	4.03E-04
Rps6ka1	0.69	1.21E-03	2.01E-04
Arrb2	0.68	4.38E-03	9.41E-04
Stat2	0.68	3.03E-03	6.16E-04
Ackr1	0.67	7.23E-03	1.70E-03
Psap	0.66	7.28E-04	1.01E-04
Pxn	0.66	7.43E-03	1.77E-03
Ptpn6	0.64	8.01E-04	1.17E-04
Il16	0.64	1.21E-03	1.98E-04
Rsad2	0.63	4.73E-03	1.02E-03
Cd247	0.63	6.20E-03	1.42E-03
Ccrl2	0.63	8.47E-03	2.09E-03
Fyn	0.61	5.28E-03	1.16E-03
Npc2	0.59	1.06E-03	1.66E-04
Ctsz	0.59	5.99E-03	1.36E-03
Stat3	0.59	7.23E-03	1.71E-03
Rnf135	0.58	5.15E-03	1.13E-03
Tlr3	0.58	9.05E-03	2.26E-03
Mvp	0.56	9.05E-03	2.25E-03
Calm1	0.53	3.26E-03	6.76E-04

VITA

Felicia Watson was born in 1994 in Eureka, CA in Humboldt County, where she attended Arcata High School in Arcata, CA. In 2016, she graduated from the University of California, Berkeley with a B.S. in Molecular Environmental Biology with a concentration in Ecology. She studied pathogen-vector-plant interactions of emerging plant diseases in California in the Laboratory of Dr. Rodrigo Almeida. Felicia then joined the Laboratory of Dr. Richard Klemke at the University of California, San Diego where she studied the bioengineering and enucleation of mesenchymal stromal cells as a cancer therapy. Felicia then joined the University of Washington Pathobiology Program in 2019 to pursue a PhD. Felicia completed her dissertation research in the Laboratory of Dr. Sean C. Murphy where she studied live-attenuated vaccine development for malaria.