Investigating sex differences in mouse models of malaria vaccination using acute, reversible hormone manipulation

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Abstract

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In many species, including mice and humans, biological sex impacts immunity. In general, females mount greater innate and adaptive immune responses, leading to sex differential vaccine outcomes. Prior research with mouse malaria models has shown female mice are better protected after vaccination and challenge than males. Prior studies further showed that castration significantly improved protection in males, suggesting that testosterone in intact males may be immunosuppressive. Here, we investigated the extent to which testosterone suppresses the formation of productive immune responses at vaccination compared to challenge in the context of malaria vaccination. We used a well-known GnRH antagonist, acyline, to suppress testosterone in BALB/cJ males. After dose optimization studies, mice were vaccinated with a two-dose vaccine and then challenged with wild type *P. yoelii* mouse malaria parasite. Acyline was injected subcutaneously on days 4 and 5 at a

dose of 300 μg before either vaccination or challenge. Protection was measured using our standard endpoint assay, quantitative *Plasmodium* 18S rRNA RT-PCR. Male mice that had testosterone suppressed at challenge were better protected than those who had testosterone suppressed at vaccination. Adding exogenous testosterone abolished the protective effect of acyline. Taken together, this suggests that testosterone specific immunosuppression decreases protection to a greater extent during challenge compared to vaccination. Additionally, we also correlated suppressed peripheral testosterone levels with decreased androgen receptor expression in the liver as a surrogate measure for the liver hormone environment. Finally, the protective effect of suppressed testosterone during challenge was validated in another mouse and parasite model, C57BL/6 and *P. berghei*. Insight into the role of testosterone in vaccine response could help to engineer a better malaria vaccine, and further our understanding of sex differential outcomes in immunity.

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1. Introduction

1.1 The ever-present threat of malaria

Malaria is a mosquito-borne parasitic disease caused by single celled protozoans of the genus *Plasmodium*. In uncomplicated cases, it is marked by non-specific symptoms such as fever, chills, malaise, headache, and nausea. Severe or complicated cases of malaria have varying symptoms based on age and pregnancy status, but may include impaired consciousness, pulmonary edema, shock (compensated or decompensated), hypoglycemia, anemia, prostration, and severe kidney injury[1]. Human malaria is primarily caused by six species of *Plasmodium*, of which *Plasmodium falciparum* and *P. vivax* account for the greatest number of cases. Of the others, *P. malariae, P. ovale curtisi* and *P. ovale wallikeri* are human-only species. The natural host of *P. knowlesi* are macaque monkeys in Southeast Asia but it can also cause fatal zoonotic cases in humans[1].

In 2022, malaria caused an estimated 249 million cases and 608,000 deaths worldwide. Most of the global burden of disease and mortality due to malaria was shared by just 29 countries, the most severely affected of which was sub-Saharan African region[1,2]. In areas of endemicity, individuals often develop premunition against the disease after lifelong exposure to infected mosquito bites. This results in a disproportionately high incidence of malaria in the immunologically naive population of children of 5 years or younger[1,3]. Pregnant women are also at a significantly higher risk of developing severe symptoms such as anemia upon infection due to their immunocompromised status during pregnancy and placental sequestration of infected erythrocytes. Pregnancy associated malaria can also

result in fetal complications, including miscarriages, low birth weight, neonatal malaria, or even death of the infant and/or mother[4].

1.2 Biology

The cause of the severe symptoms and mortality associated with malaria is rooted in the *Plasmodium* parasite's biology. Malaria infection begins with the bite of a female *Anopheles* mosquito during a blood meal. *Plasmodium* sporozoites (spz) enter the host's bloodstream and migrate to the liver, initiating the asymptomatic pre-erythrocytic (PE), or liver stage. The liver stage lasts for approximately two days in mice and 5-8 days in humans, during which spz differentiate into trophozoites in the hepatocytes. The trophozoites replicate to form schizonts, and then eventually merozoites. At this stage, the hepatocytes rupture and release the merozoites in the bloodstream, which then go on to infect erythrocytes and start the symptomatic erythrocytic, or blood stage of infection. Here, additional rounds of asexual parasite replication produce more merozoites that are released into the bloodstream to invade surrounding erythrocytes and start the cycle anew[1]. At the same time, sexually committed parasites go on to form circulating gametocytes, which are taken up by mosquitos feeding on the infected host. Inside the mosquito, further developmental stages give rise to spz in 2-3 weeks. The spz migrate to the salivary glands of the mosquito, ready to be introduced into a new host during the next blood meal[1].

1.3 Vaccines

While each stage of a malaria illness presents a novel opportunity for vaccine development, the pre-erythrocytic stage is particularly attractive for a few different reasons[5]: (1) it acts as a bottleneck for the infection when the number of parasites is relatively low (averaging around 100 spz per bite)[6–8] (**Figure 1**); (2) unlike erythrocytes, hepatocytes express antigen presenting MHC class I molecules that allow immune cells to target infected cells[9,10]; (3) parasites are localized in the human liver for up to a week during development[1] compared to the few seconds merozoites spend traveling between erythrocytes during the blood stage[11].

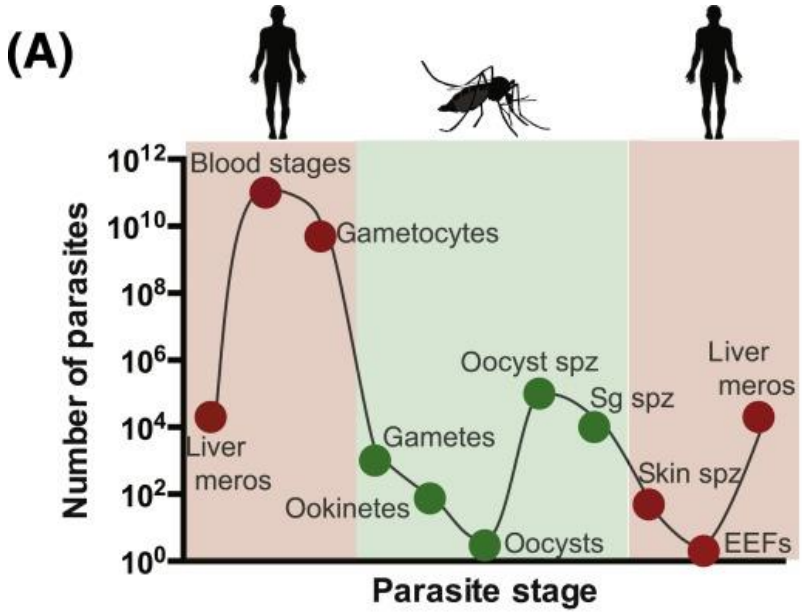

Figure 1: *Liver stage bottleneck with relatively low parasite density.*

Adapted from Graumans et al., 2020[7].

Pre-erythrocytic vaccines target the PE stage of a malaria infection by either identifying and eliminating spz migrating to the liver, or through CD8+ T-cell mediated killing of infected hepatocytes[5]. They do so by eliciting immune responses against a surface antigen protein.

While there are a number of candidate genes, the most commonly used in vaccine development is the circumsporozoite protein (CSP), which is involved in various functions such as spz development and hepatic invasion[12]. As described below, PE vaccines have not only earned a place in current WHO recommendations for malaria prevention[13], but have also been a focus of the Murphy laboratory for new vaccine development.

1.3.1 Current vaccines

RTS,S/AS01- Mosquirix

In October 2021, RTS,S/AS01 made history by being the first anti-parasite vaccine to be recommended for use in humans by WHO[13,14]. RTS,S is a *P. falciparum* specific subunit vaccine intended to generate an immune response against the PfCSP antigen. It is comprised of the CSP central repeat region ("R") and T-lymphocyte epitopes ("T") genetically fused to the Hepatitis-B surface antigen ("S")[15,16]. The vaccine is administered along with liposome-based adjuvant AS01, which was found to have greater immunogenicity in phase II trials compared to RTS,S alone[17]. In phase III testing conducted in 7 different sub-Saharan African countries in 2009-2014, the vaccine had a 25.9% efficacy (VE) in infants of 6-12 weeks of age after a 4-dose regimen over a period of 4 years. In children of 5-17 months of age, a VE of 36.3% was observed over the same time period. VE has been correlated with serum levels of anti-CSP antibodies[18]. Despite the modest initial efficacy, WHO recommended the vaccine for malaria prevention in children through a pilot program in 3 African countries, Ghana, Kenya, and Malawi. Deployment for implementation in other countries is set to begin in 2024[13].

R21/Matrix M

Two years after RTS,S was recommended by WHO, R21/Matrix M became the second *P. falciparum* specific malaria vaccine to be recommended for clinical use in children of 5-36 months of age in Ghana in April 2023[19]. A subunit vaccine similar to RTS,S, R21 differs from its predecessor by having 5 times greater CSP density on its Hepatitis-B surface protein. Additionally, it is administered along with saponin-based adjuvant Matrix-M[20]. In phase III trials conducted in four African countries amongst children of 5-36 years, R21 had a 12-month VE of 75% at sites with seasonal vaccine administration, and a VE of 68% at sites that followed a standard age-based year-round vaccination regimen. Like RTS,S, VE correlated with the presence of antibodies against the central repeat region of CSP[21]. In October 2023, WHO recommended R21 for malaria prevention in children 5 months and older, administered through a series of 4 doses.

1.3.2 Vaccines in development

Radiation attenuated whole organisms

While subunit vaccines have made large strides towards malaria prevention, their modest efficacy can be owed to a few different factors: (1) the large genetic diversity of the *Plasmodium* genome reduces VE against mismatched CSP alleles[22]; (2) subunit vaccines that intend to block spz invasion require high titters of CSP antibodies for efficacy, which have proven difficult to sustain in human populations even with multi-dose regimens and boosters [18,23].

An alternative to subunit vaccines are radiation attenuated whole organism vaccines, or radiation attenuated spz (RAS). RAS vaccines employ radiation (commonly X-rays) to inactivate whole organisms containing a wide array of antigens to elicit an immune response[24]. These spz migrate to the liver and induce CD8+ T-cell responses[25] but are unable to replicate and cause blood-stage infection due to being attenuated. IV-administered attenuated spz have successfully induced sterile protection in mice[26,27] and humans[28,29]. While in humans RAS has been shown to induce both humoral and peripheral T-cell mediated protection[30,31], mouse studies have revealed the formation of vaccination induced liver specific resident memory T-cells (Trms)[32]. A subset of CD8+ T-cells, Trm's have been strongly correlated with sterile long-term protection from malaria[29,33].

However, widespread implementation of RAS as a vaccine comes with its own challenges. The sporozoites required for RAS can only be manufactured in mosquitoes, and multiple doses are required for protection[34]. In an effort to reduce the cost associated with the supply, maintenance, and administration of multiple RAS doses, an alternative prime-and-trap (P&T) model of vaccination has been suggested by the Murphy Laboratory[34].

Prime and trap

Prime and trap is a heterologous vaccine strategy that combines DNA vaccination with RAS in order to induce long term Trm mediated protection. A gene-gun is used to administer a DNA minigene encoding part or all of the CSP (ggCSP) into the skin, which primes CSP

specific CD8+ T-cells. 28 days later, a single dose of RAS is intravenously delivered to attract to and "trap" the primed CD8+ T-cells in the liver as liver-specific Trms. Protection in mice is assessed by infection with WT (wild type) spz challenge 4-6 weeks after vaccination. P&T has successfully been used to achieve sterile protection in mice[34,35]. Recognizing the excellent efficacy observed in mice, and the promise it holds for human malaria prevention, the Murphy Laboratory is currently in the process of optimizing a prime-and-trap regimen in non-human primates. As it is the focus of the Murphy Laboratory, this thesis will use the P&T model of vaccination to assess the effect of testosterone on vaccine efficacy and protection outcomes in mice.

Other vaccines

While multiple other vaccine candidates are currently in development, since they are not the focus of this thesis, they will not be discussed further. Readers are referred to other excellent reviews for more information[24,36].

1.4 Sex differences in malaria and beyond

Prior literature has established a strong correlation between biological sex and immunity. In many species and in the case of various diseases, females have been shown to mount stronger adaptive and innate immune responses compared to males. For example, females show higher antibody levels, B-cell numbers, and basal immunoglobulin levels compared to males[37]. Depending on the pathogen and mechanism of disease, this may manifest as differential responses to vaccination[38], differences in disease course and/or

severity[39], or an increased susceptibility to auto-immune diseases in females[40]. Sex-based differences may arise as an effect of sex chromosomes, sex hormones, and the environment[37].

In the case of malaria, data on sex-based differences in human infections is less conclusive. Multiple studies in endemic regions report a higher incidence in males[39,41–44], but higher levels of anti-malarial antibodies in females[44,45] and more lives lost due to disability (DALYs) in females[42]. However, other studies in regions of endemicity report no differences in the incidence[46] or prevalence[47] of malaria between sexes. A longitudinal study in Eastern Uganda found a lower prevalence of infection in females compared to males despite no differences in incidence rates. Further analysis revealed a faster clearance rate of asymptomatic infections in females[46].

Response to malaria vaccines also bring to attention sex-specific differences. Amongst children (6-12 weeks and 5-17 months) with severe malaria, girls vaccinated with RTS,S had a twofold higher mortality risk compared to girls who received the control vaccines. This trend was not seen in boys, prompting discussion of whether girls should receive a reduced or altered RTS,S dosing[48]. Another study examining sex-specific responses to radiation-attenuated *P. falciparum* whole sporozoite vaccines (PfSPZ) found that while post-pubertal (≥11 years) females produced significantly greater amounts of antibodies, this did not correlate to increased protection compared to males[49].

It is clear that sex-specific responses to malaria infection and malaria vaccines not yet well-understood. Part of what makes studying this phenomenon difficult in humans is the vast amount of genetic and environmental variability such as level of endemicity, vector exposure, and presentation/severity of clinical symptoms. Confounding factors such as these are inherent in any observational study and make it difficult to draw conclusive results or study the mechanism of effect. Hence, animal models provide a suitable alternative to investigate these findings in a controlled, experimental environment.

1.5 Mouse malaria models

Mice have long been used to model malaria pathogenesis[50] and in vaccine development[51]. They are small and inexpensive, making them more accessible compared to other animal models such as non-human primates[52]. In most of the studies outlined in this thesis, we have used BALB/cJ mice, with additional validation in C57BL/6 mice. These are both well-characterized in-bred strains that have stable strain-specific phenotypes, reducing variability between experiments[53,54]. They are pivotal in the research of PE stages of malaria as they allow the study of the liver, which is inaccessible in humans[52].

While rodents provide an accessible and reproducible model for studying malaria, they are not without their shortcomings. Human infecting species of *Plasmodium* are strongly species specific with high host-cell tropism and cannot establish effective *in vivo* infections in mice. As a result, mouse studies are usually conducted using rodent specific *Plasmodium* species, such as *P. yoelii, P. berghei* and *P. chabaudi*. While these share

many similarities with their human counterparts, differences in host biology and strain-specific pathogenesis pose challenges to translating findings in murine models to humans. For example, the asymptomatic pre-erythrocytic stage of an infection lasts for only 2-3 days in mice, compared to 5-8 days in humans[52,55]. Work by the Murphy laboratory has shown that using a two-dose challenge to increase the length of liver stage antigen exposure in mice to 4-5 days to more closely mimic human infection results in increased vaccine efficacy[56]. However, despite their limitations, mouse models are ideal for early phase preclinical vaccine development and for hypothesis driven mechanistic immunology studies.

1.6 Mouse models to study biological sex

Sex-specific differences in protection outcomes to malaria vaccination can be attributed to three factors: sex chromosomes, sex hormones, and the environment. Rodent models exist to study the contribution of each of these. The four core genotypes models is comprised of XX gonadal males and females, and XY gonadal males and females and helps to tease apart the impacts of sex chromosomes and sex hormones. XX vs XY differences in gene expression or disease susceptibility discovered through this model occur independent of the effect of sex hormones.[57] The impact of sex hormones has also been widely studied in mice through means such as orchiectomies (removal of gonads)[58–60] or addback experiments using exogenous sex steroids[60,61]. Finally, one way to study the impact of environment and physiological conditions is by modulating the microorganisms mice are exposed to. For example, axenic mice are free or all microorganisms, while

gnotobiotic mice are inoculated with specific non-pathogenic species. In contrast, specific pathogen free (SPF) mice are clear of a specific list of pathogens as determined by routine testing[62]. On the other hand, to simulate the diversity found in natural human populations, mice may also be physiologically introduced to the wide array of microbe strains found in pet store mice[63].

While each factor is no doubt important biologically, this thesis will focus on the effect of sex hormones as to narrow the question down to a scope appropriate for a master's thesis.

1.7 Sex differences in mouse models of malaria

Sex-based differences in malaria infection and vaccination in mice are well-characterized. As in humans, male mice have been shown to display increased susceptibility, severity, and mortality from malaria infections compared to females. This has been seen in *P. chabaudi* caused infections in C57BL/6[64] and H-2 congenic mouse strains[65]. C57BL/6 females also mount greater adaptive immune responses than males and recover faster from *P. chabaudi* infections[66]. Sex-specific outcomes of malaria vaccination have also been documented in literature. In humans, females have been documented to display higher antibody responses to vaccination without differences in efficacy compared to males[49]. However, C3H/HeNCr MTV female mice vaccinated with *P. berghei* RAS have been shown to display more immunogenic responses and are better protected from malaria infection compared to male vaccinated mice[67,68]. Improved protection in female

mice has also been observed by the Murphy Laboratory in a different mouse/parasite model, P&T vaccinated BALB/cJ and *P. yoelii* **(Figure 2).**

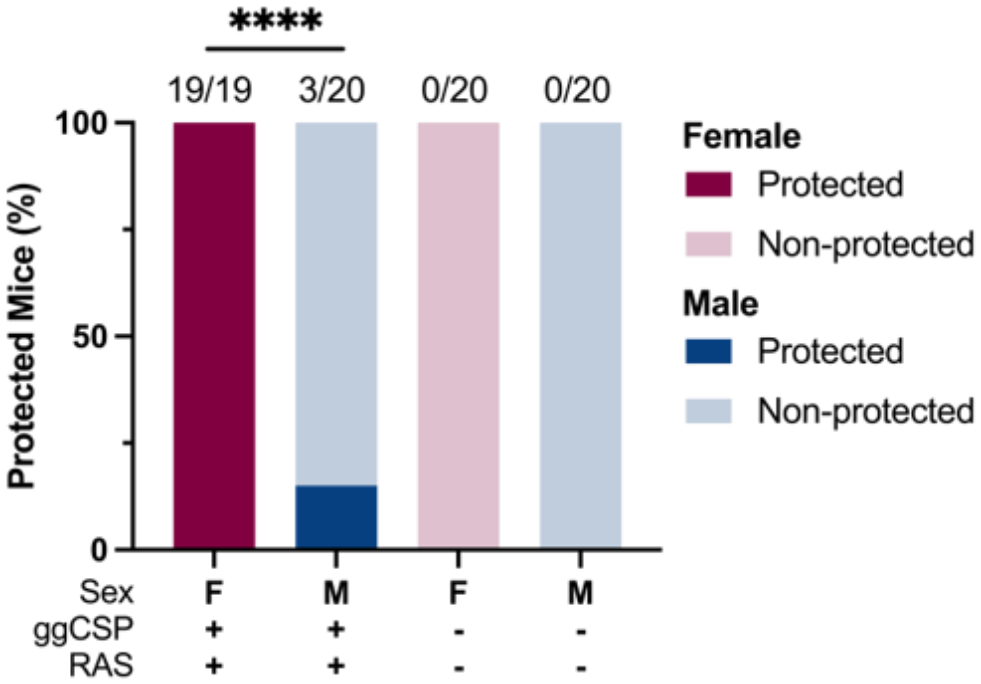

***Figure 2**: Sex differences in protection from malaria in prime-and-trap vaccinated male and female BALB/cJ mice.*

Unpublished data from co-author Caroline J. Duncombe, PhD candidate in Murphy laboratory.

Given the prior data on the impact of sex on malaria infection and vaccination described above, Murphy Laboratory PhD Candidate Duncombe began investigating the effect of sex on P&T using ORX. She found that testosterone depletion through castration or orchiectomy (ORX) improves protection outcome in vaccinated males **(Figure 3)**. Prior literature lends credence to this observation and also shows that exogenous testosterone add-back eliminates this protective effect[67–70]. Taken together, this suggests that testosterone has immunosuppressive effects that decreases protection against malaria,

as reported in numerous other disease models[37,39]. However, there have been relatively few mechanistic studies investigating how and when during a vaccination and challenge regimen do the immunosuppressive effects of testosterone act to decrease protection in male mice. The two events entail different immune processes. While vaccination primes antigen specific CD8+ T-cells and pre-positions them in the liver as Trms[34], a malaria challenge recruits patrolling memory CD8+ T-cells and activates CD4+ effector T-cells for immunoregulation[71]. Understanding the mechanisms at play that mediate sex-based differences in immunity is integral in the context of malaria, a disease that has been well-documented to have sex-specific differences.

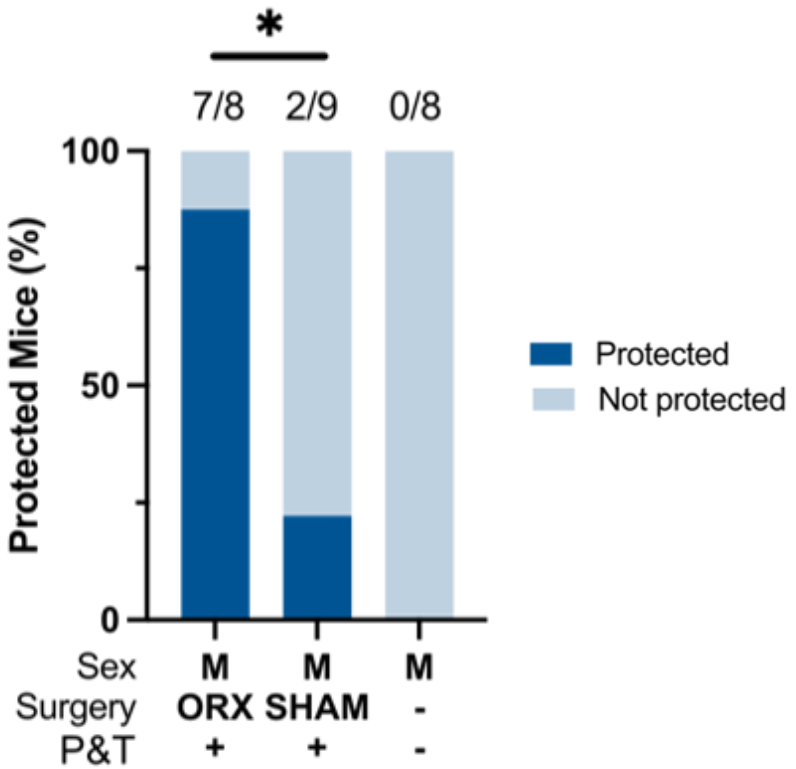

***Figure 3**: Castrated (ORX) vaccinated male mice are better protected from malaria infection compared to shame operated and vaccinated males.*

Unpublished data from co-author Caroline J. Duncombe, PhD candidate in Murphy laboratory.

To address the question of whether testosterone played a greater role in protection outcomes during vaccination or challenge, we needed to isolate its effects at each timepoint. Our goal was to suppress testosterone at either vaccination or at challenge and compare protection outcomes between the two groups. Unfortunately, prior studies looking at the effect of sex steroids in mouse models of malaria have relied on castration to deplete males of testosterone[66–68,70]. Being irreversible, castration makes it impossible to isolate suppression to just vaccination. Instead, we required an agent that would acutely and reversibly suppress testosterone.

1.8 Acyline, an acute sex steroid inhibitor

Acyline is a gonadotropin releasing hormone (GnRH) antagonist that reversibly suppresses peripheral levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone in a dose-dependent manner. Normally, testosterone secretion from the testes is governed by the hypothalamic-pituitary-gonadal axis (HPG) **(Figure 4)**. GnRH neurons in the hypothalamus secrete GnRH that binds to receptors on the anterior pituitary gland. This stimulates production of gonadotropins, LH and FSH, which in turn act on the testes to produce testosterone. Acyline competitively binds to GnRH neurons in the anterior pituitary, thus, suppressing downstream production of testosterone and gonadotropins[72–74].

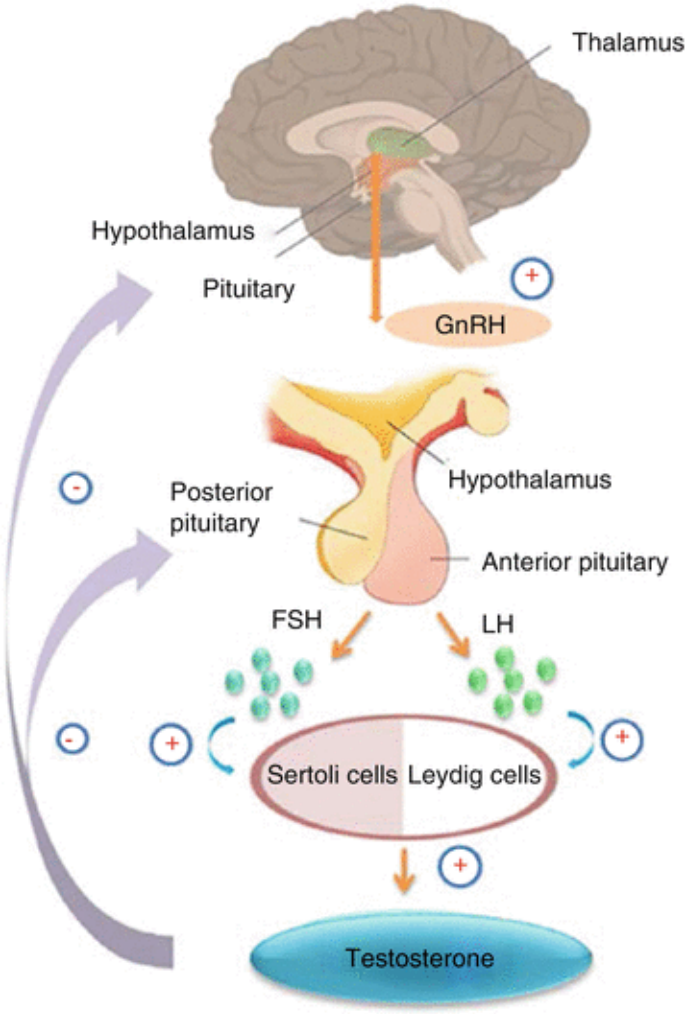

Figure 4: *Hypothalamus-pituitary-gonadal axis.*

Image adapted from Singh V. et al, 2017[74]

Acyline has been previously used in canines[75] and humans[72,73] to suppress peripheral testosterone levels. Unlike typical methods of hormone manipulation such as castration, its effects are documented to be acute and reversible. Moreover, not only is the administration of acyline via subcutaneous injection easier and more resource efficient

than performing a surgery, but it also avoids the risk of post-surgical mouse mortality associated with castration.

In this thesis, we aim to address the temporal significance of the immunosuppressive effects of testosterone on malaria vaccination via a two-step approach. First, dose-optimization studies are performed to assess the efficacy and reversibility of acyline in a BALB/cJ mouse model and to determine an appropriate dose. Peripheral testosterone levels are monitored at regular intervals through serum ELISAs. Next, acyline is used to suppress testosterone levels before either P&T vaccination or challenge with *P. yoelii* and protection outcomes are measured through qRT-PCR quantified18S rRNA burden in the liver. To see if the protection afforded by suppression can be reversed, testosterone is exogenously reintroduced after acyline in add-back experiments. Finally, we also validate the effects of acyline in another mouse and parasite strain model, C57BL/6 and *P. berghei*. The overall goal is to assess the effect of testosterone on our immune processes, especially in the context of prevention of infectious diseases through vaccination.

2. Methods

2.1 Mice

Male and female BALB/cJ and C57BL/6 mice (4–6 week old) were obtained from Jackson Laboratories (Bar Harbor, ME). They were housed in an Institutional Animal Care and Use Committee (IACUC)-approved vivarium at the University of Washington and used under approved IACUC protocol (4317-01 to S. C. M.).

2.2 Acyline injections

Acyline (lot # RDZ001) was provided by John K. Amory, MD, MPH at the University of Washington. The lyophilized vials were stored at -20°C until resuspension. At the time of use, 2.2 mL of Molecular Biology Grade Water (Cytiva) was added and the vial vortexed to resuspend the acyline. Resuspended vials were stored for a maximum of 1 week at 4°C. Mice were subcutaneously injected one or more time with the desired amount of acyline solution as specified in the given experiment.

2.3 Testosterone serum ELISA

Enzyme-linked immunosorbent assays (ELISAs) have been identified in literature as a reliable and accurate way of quantifying serum testosterone[76]. A testosterone ELISA kit was thus used to detect and quantify testosterone concentration in serum. At the timepoints specified in each given experiment, approximately 0.1-0.2 mL of blood was obtained in 1.5

mL Eppendorf tubes from mice through submental bleeds with a 5 mm Goldenrod™ lancet. After incubation for 1 hour at room temperature, the blood was centrifuged at 3500 RPM for 10 minutes at 4°C. The serum supernatant was collected and stored in -20°C until testing. Testosterone ELISA kits from Crystal Chem (Catalog #80552) were then used per manufacturer guidelines. Singlet absorbance values of each sample were measured with CLARIOstar Plus Microplate Reader (BMG Labtech) and analyzed using MARS data analysis interface. A calibration curve was generated using manufacturer provided standards (0, 0.1, 0.4, 1.5, 6.0, and 25 ng/mL) and a four parametric logistic (4-PL) curve fit was used to calculate sample values.

2.4 Prime and trap vaccination and challenge

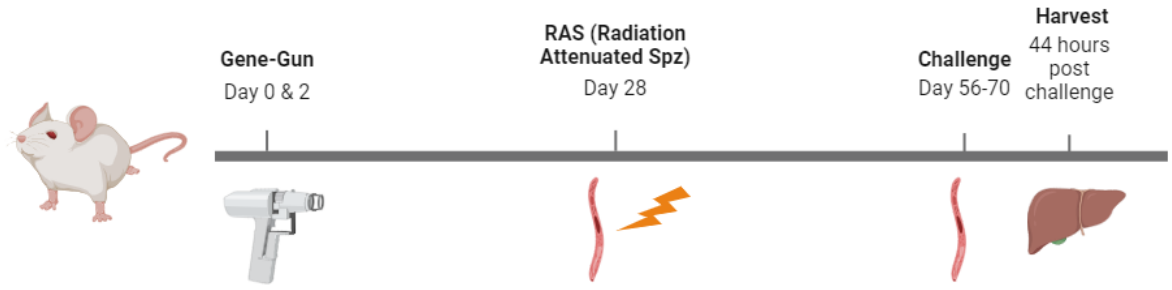

***Figure 5**: P&T vaccination and challenge timeline.*

Created with BioRender.com

<u>Gene-gun priming</u>

For priming, minigene DNA vaccines encoding the CSP (to vaccinate against *P. yoelii*) or ribosomal protein RPL6[77] and adhesive protein TRAP (to vaccinate against *P. berghei*) were prepared as described[34]. An endotoxin-free purification kit (Qiagen) was used to purify the DNA, which was then loaded onto gold beads (1–2-μm diameter; InBio Gold) and coated on tubing as cartridges[78]. Plasmids encoding the *Escherichia coli* heat-labile lymphotoxin (LT)[79] were used adjuvants in a 1:10 ratio with the minigene vectors.

Vaccine cartridges were administered to mice using a Powderject style gene-gun. Mice had their abdominal fur shaved and each received 2 cartridges two days apart for a total of 4 cartridges per mouse **(Figure 5)**. This is known as cluster priming[34].

Radiation attenuated sporozoite (RAS) trapping and infectious sporozoite challenge

Female *Anopheles stephensi* mosquitoes were reared at the Brotman building insectary (University of Washington) or the Seattle Children's Research Institute (Seattle, WA) and infected with wild-type (WT) *P. yoelii* or *P. berghei*. At 14-18 days (*P. yoelii)* or 21-24 days (*P. berghei*) post infection, salivary glands were microdissected from the mosquitoes and purified using an Accudenz gradient as described[80]. Since a significant amount of spz are lost during purification, this step was only performed if a preliminary count showed sufficiently high amounts of spz (>50% of the required spz quantity). To generate the RAS, the spz were irradiated by X-ray exposure (10,000 rads; Rad Source, Buford, GA).

For trapping vaccination, mice were retro-orbitally injected 20,000 RAS resuspended in 100 µL of Schneider's insect media (Gibco, Thermo Fisher Scientific, Waltham, MA). For challenge, mouse were retro-orbitally injected with 1000 WT spz in 100 µL of media.

2.5 Testosterone injections

Testosterone propionate (Sigma Aldrich) was dissolved in sesame oil (Sigma Aldrich) at a concentration of 20 µg/µL. Mice were weighed to determine average weight per group, and then subcutaneously injected with 100 µg testosterone/g body weight. The following formula was used to calculate the amount of testosterone to be injected:

$$Amount\ of\ testosterone\ solution\ per\ mouse\ (\mu L) = \frac{[(Average\ weight)x\ 100]\mu g}{20\frac{\mu g}{\mu L}testosterone}$$

2.6 Liver harvest and nucleic acid extraction

Mice were humanely euthanized by the carbon dioxide method 44 hours post challenge, and approximately half of the liver was excised and placed into 4 mL of NucliSENS Lysis Buffer (bioMérieux, Durham, NC). NucliSENS tubes were weighed before and after addition of tissue to determine net liver weights. Liver tissue was then bead-beaten until homogenized, and 100 µl of the homogenate was diluted in 900 µl of NucliSENS Lysis Buffer in a 1:10 ratio. Homogenates were stored at -80°C until total nucleic acid (TNA)

extraction on the EasyMag system (bioMérieux, Marcy-l'Étoile, France) as described[81]. Each extraction included an 18S high (9.47 log18S copies/mL whole blood), low (7.17 log18S copies/mL whole blood), and negative control, which were subsequently subjected to qRT-PCR in parallel with the samples.

2.7 *Plasmodium* 18S qRT-PCR

Reverse transcription polymerase chain reaction (qRT-PCR) is an assay that determines the amount of a specific sequence of messenger RNA (mRNA) present in a sample, which can then be used to characterize gene expression[82]. Here, it has been used to quantify parasite burden in mouse livers as described in prior Murphy laboratory papers[35,81].

Prior extracted total nucleic acid eluates were subjected to qRT-PCR on the QuantStudio 5 real-time PCR machine (ThermoFisher, Waltham, MA). The SensiFAST™ Probe Lo-ROX Kit (Bioline, London, United Kingdom) was used along with a predesigned HEX-labeled mouse GAPDH RT-PCR assay (IDT Inc, Coralville, IA) multiplexed with a Pan-*Plasmodium* 18S rRNA assay as described[81]. The primers and probes used are as follows:

Pan-*Plasmodium* 18S primers: Synthesized by BioSearch Technologies, Inc.

PanDDT1043F19: 5'- AAAGTTAAGGGAGTGAAGA -3'

PanDDT1197R22: 5'- AAGACTTTGATTTCTCATAAGG -3'

Pan-*Plasmodium* 18S probe: Synthesized by BioSearch Technologies, Inc., Navato, CA

5'-[CAL Fluor Orange 560]- ACCGTCGTAATCTTAACCATAAACTA[T(Black Hole Quencher-1)]GCCGACTAG-3'[Spacer C3]

Mouse GAPDH primers: Synthesized by Integrated DNA Technologies Inc., Coralville, IA

Forward Primer: 5' - CTCCACGACATACTCAGCAC - 3'

Reverse Primer: 5' – CCACTCACGGCAAATTCAAC - 3'

Mouse GAPDH probe: Synthesized by Integrated DNA Technologies Inc., Coralville, IA

5' – [56-FAM]- AGGAGCGAG[ZEN]ACCCCACTAACATCA[3IABkFQ] - 3'

A quantified Armored RNA calibrator encoding full-length *Plasmodium* 18S rRNA (Asuragen, Austin, TX) was included in each run. PCR conditions were set to 45°C for 10 minutes, 95°C for 2 minutes, and 45 cycles of 95°C for 5 seconds, 50°C for 35 seconds. Linear 18S copies per reaction were calculated using a 4 or 3-point RNA standard curve generated from the Armored RNA calibrator and log-transformed. 18S copies corresponding to a CT value of 35 was considered the protection threshold, below which a mouse was considered infected or unprotected.

2.8 Mouse androgen receptor (AR) QRT-PCR

Total nucleic acid concentrations in the eluates were determined using the NanoDrop™ 2000 from Fisher Scientific. Nucleic acid concentrations were standardized amongst samples using NucliSENS EasyMag Extraction Buffer 3 (bioMérieux, catalog #280132).

qRT-PCR was performed on the standardized TNA eluates using Ag-PATH one-time kits (Catalog #4387391) on the Bio-rad CFX96 Real Time machine. Mouse GAPDH was used as an endogenous control. Each sample was run in duplicates for each target gene. The AR primer and probes (PrimeTime) used were:

AR primers: Synthesized by Integrated DNA Technologies Inc., Coralville, IA

Forward primer: 5'CTGCCTTGTTATCTAGCCTCA-3'

Reverse primer: 5'-AAATACCATCAGTCCCATCCAG-3'

AR probe: Synthesized by Integrated DNA Technologies Inc., Coralville, IA

5'-[56-FAM]- TGCACAAGC[ZEN]TGCCTCTCTCCAA[3IABkFQ]-3'

Data was analyzed for fold change information as follows:

1) Calculate mean CT value of each sample for each target gene (test and endogenous control)

2) Calculate ΔCT = Mean CT of AR – Mean CT of GAPDH

3) Calculate ΔΔCT = ΔCT value of sample – Average ΔCT value of control group

4) Fold change calculation:

$$(FC) = 2^{(-\Delta\Delta CT)}$$

2.9 Statistical analysis

All statical tests were performed on GraphPad Prism 9. Percent protection data between groups was analyzed using Fisher's exact test, while all other analyses were performed using Kruskal-Wallis test.

2.10 Figures

All experimental layout diagrams were created with BioRender.com, and all graphs were created with GraphPad Prism 9.

3. Results

3.1 Acyline dose optimization

Prior data from literature shows that females of multiple species demonstrate stronger innate and adaptive immune responses compared to males[37]. Work by the Murphy laboratory has shown that male vaccinated mice show poorer protection outcomes compared to vaccinated females **(Figure 2)**, and that castration significantly improves male protection **(Figure 3)**. The overall aim of this thesis is to understand the effect of testosterone on the murine immune system, specifically during vaccination and infection. Since testosterone may have varying effects on these different immunological events, we want to investigate the extent to which testosterone suppresses the formation of productive immune responses at vaccination compared to recall of effective immune responses at challenge. We decided to approach this question by suppressing testosterone at either vaccination or at challenge and comparing protection outcomes between the two groups, and also relative to naïve males or other appropriate controls.

Historically, irreversible surgeries such as castration, also called orchiectomy (ORX), has been the method of choice to suppress levels of sex steroids in mice[66–68,70]. However, since we wanted to isolate the effects of testosterone suppression to a particular timepoint in our experiment, we needed an acute and reversible method. The GnRH antagonist acyline has previously been used in various mammals, including humans,[72,73] to acutely suppress peripheral levels of testosterone, but to our knowledge, its effects are yet to be studied in

mice. Thus, our first experiment was an acyline dose optimization experiment in naïve male mice. Here, we sought to establish that the effects of acyline on peripheral testosterone levels were acute and reversible in mice, and to determine an appropriate dosage for further use. Our ideal period of testosterone suppression was envisaged to be ~7 days, as informed by two criteria: (1) to allow enough time for the liver environment to respond to altered levels of testosterone prior to prime, trap, or challenge, and (2) for subsequent immune processes to take place in a testosterone depleted state.

3.1.1 Experimental layout

Male BALB/cJ mice (4-6 weeks old) were divided into six groups **(Figure 6)**, with five per group. The groups each received subcutaneous injections of acyline resuspended in molecular biology grade water varying in both administration frequency and dosage **(Table 1).** A total of one, two, or three injections were administered on consecutive days. Injections were either 300 µg (150 µL) or 100 µg (50 µL) of acyline.

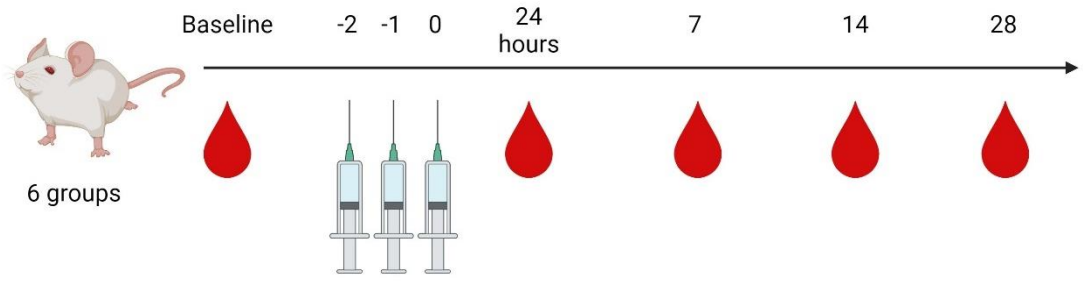

Figure 6: *Experimental layout for acyline dose optimization experiments.*

	Dosage and Course
Group 1	**1 x 300 µg (Day 0)**
Group 2	**2 x 300 µg (Days -1 and 0)**
Group 3	**3 x 300 µg (Days -2, -1, and 0)**
Group 4	**1 x 100 µg (Day 0)**
Group 5	**2 x 100 µg (Days -1 and 0)**
Group 6	**3 x 100 µg (Days -2, -1, and 0)**

Table 1: *Frequency and dosage of acyline administered to each group of mice.*

Serum was collected via submental bleeds at specific timepoints: baseline measurements before acyline injections, and then 24 hours, 7 days, 14 days, and 28 days post injections. Testosterone was quantified by ELISA, which has an upper limit of detection (LOD) at 25 ng/mL.

3.1.2 Acyline acutely and reversibly suppresses peripheral testosterone in male mice

The collected testosterone measurements for each group were averaged and graphed over time with line charts **(Figure 7).**

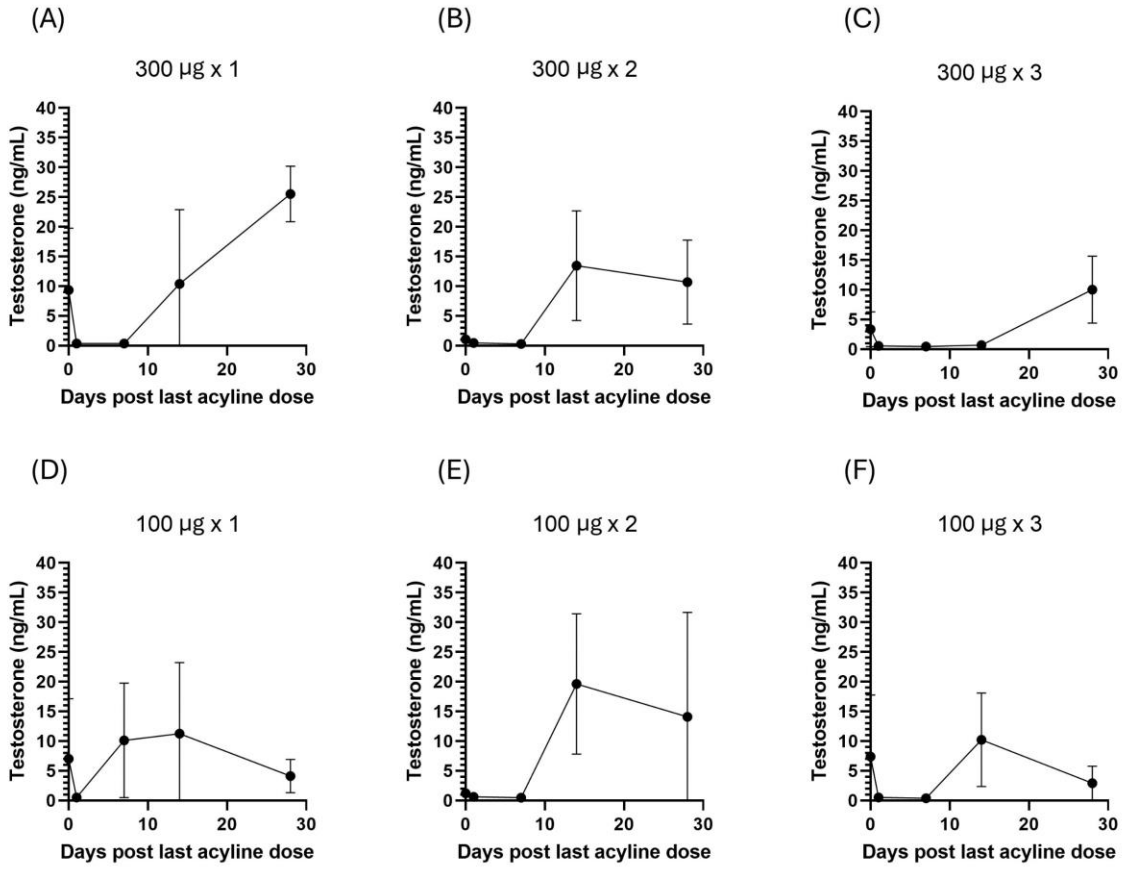

Figure 7: *Acute and reversible peripheral testosterone suppression after acyline injections.* Each graph shows data from 5 animals in a single experiment; error bars show mean +/- standard deviation.

Baseline testosterone measurements ranged from 0.61 ng/mL to 25 ng/mL (upper LOD), with a mean of 4.9 ng/mL. Acute and reversible peripheral testosterone suppression was observed for each group in a dose dependent manner, i.e., larger doses of acyline resulted in longer periods of suppression or near undetectable levels of testosterone. In each instance, a temporary but noticeable testosterone spike was observed where levels temporarily overshot baseline measurements before decreasing again.

For high doses, such as 3 x 300 µg **(Figure 7C)**, testosterone suppression was observed for around 14 days. The long period of suppression raised concerns about lingering effects of testosterone suppression at unintentional timepoints, i.e., mice injected with acyline before RAS vaccination might still have low levels of testosterone at challenge. In support of this potential concern, 1 of the 5 mice in that group had a baseline measurement of 7.4 ng/mL and a 28-day measurement of 4.6 ng/mL. This dose was deemed too high for our purposes.

For low doses, such as 1 x 100 µg **(Figure 7D)**, we were only able to capture near undetectable levels of testosterone at 24 hours post injection. This was suspected to be insufficient time to allow the liver environment to respond to depleted levels of testosterone prior to vaccination or challenge, and for subsequent immune processes to occur in a state of suppressed hormone levels. This dose was deemed too low for our purposes.

Of all the tested frequencies and doses, we opted for a moderate dose of 2 x 300 µg **(Figure 7B)**. This dose suppressed peripheral levels of testosterone for around 7 days. We speculated this would allow us to administer acyline 4 to 5 days **prior to** vaccination or challenge and allow adequate time for the liver environment to respond to depleted levels hormones prior to challenge. Additionally, testosterone suppression would then continue for at least 3 days **after** vaccination or challenge. Moreover, all 5 mice in the group had

higher than baseline levels of testosterone on day 28, minimizing the risk of persisting low levels of hormone at unintentional timepoints.

In conclusion, based on this experiment, we opted for an acyline dose of 300 µg administered on days 4 and 5 before prime, trap, or challenge.

3.2 Acyline P&T optimization

The aim of this experiment was to identify the extent of the immunosuppressive effects of testosterone on vaccination and challenge. To answer this question, we used the previously determined acyline dose to suppress testosterone during either vaccination or challenge and compared protection data between the two groups, and relevant controls. Prior data has shown that P&T vaccination confers as a low as 15% protection in intact males, compared to 90-100% protection in intact females **(Figure 2)**. The rationale was that depleting testosterone at the timepoint when it impacts immune processes the most would result in greater protection in that group. Alternatively, if testosterone was equally suppressive at both timepoints, we would see equal protection in both groups.

3.2.1 Experimental layout

Male BALB/cJ mice (4-6 weeks old) were divided into 5 groups, with 5 per group **(Figure 8)**. The groups that were vaccinated (Groups 1-4) were gene-gunned with ggCSP, then trapped with a dose of purified or unpurified fresh *P. yoelii* RAS 28 days later. A final group (Group 5)

was not vaccinated and used as a naïve control. At 4-6 weeks after trapping, all 5 groups were challenged with a dose of WT *P. yoelii* sporozoites and had their livers harvested 44 hours post infection for 18S qRT-PCR quantification. A qRT-PCR cycle threshold (CT) value of 35 was assumed to indicate sterile protection, below which mice were considered unprotected. Acyline was injected to select groups on days 4 and 5 (300 µg each dose) prior to vaccination or challenge. To confirm suppression, testosterone levels of the groups that received acyline were monitored through submental bleeds and subsequent ELISA before and after injections.

Figure 8: *Acyline P&T optimization experimental layout*. The presence of a square indicates whether or not an event took place, while the color of the square indicates if acyline was administered on days 4 and 5 prior to the event.

Group 1 was our positive control and received acyline prior to both vaccination and challenge, simulating a castrated male mouse. Groups 2 and 3 were our experimental groups that received acyline prior to either vaccination or challenge respectively. Group 4

was our vaccine control group that did not receive acyline, and Group 5 was our naive infectivity control group.

3.2.2 Acyline at challenge protects male mice from liver stage infection better than at vaccination

Two replicate experiments were performed. Due to unforeseen circumstances, replicate 1 received two additional doses (300 µg each) of acyline on days 11 and 12 prior to challenge. Serum ELISA confirmed suppressed testosterone levels during prime, trap, and challenge compared to baseline measurements in all groups tested **(Supplementary Figure 1)**.

In replicate 1, the positive control (Group 1) showed the highest level of protection amongst all groups with 100%. The naïve group (Group 5) was completely unprotected as expected, and the vaccine control group (Group 4) was partially protected at 40% (2 out of 5 mice). Amongst the experimental groups, the group that received acyline prior to challenge had improved protection outcomes (80%) compared to the group that received acyline prior to vaccination **(Figure 9A)**. This difference was not statistically significant (p= 0.2063), likely due to the low sample size. However, we did see a statistically significant difference between protection outcomes between Groups 1 and 2 (p= 0.0476) and Groups 3 and 5 (p= 0.0476).

In replicate 2, amongst the controls, the positive control (Group 1) was the most protected at 66.7%. No protection was observed amongst the vaccine control (Group 4) and naïve infectivity (Group 5) groups. Additionally, the naïve mice also showed the highest 18S burden as quantified via qRT-PCR, with a mean of 10. log 18S copies/mg of liver **(Figure 9D)**. The experimental groups showed potentially conflicting results in comparison with the previous replicate. Group 2 (acyline at vaccination) displayed 100% protection, while Group 3 (acyline at challenge) had 2/3 uninfected mice (67%) **(Figure 9C)**.

Despite the potentially conflicting results, after aggregating the data from both replicates, overall Group 3 that received acyline prior to challenge was better protected (75%) compared to Group 2 that received acyline prior to vaccination (50%) **(Figure 9E)**. While this difference was not statistically significant, both Groups 2 and 3 were significantly better protected than the naïve controls ($p= 0.0229$ and $p= 0.0015$ respectively). In conclusion, this experiment indicates that the immunosuppressive effects of testosterone reduces protection in male mice to a greater extent during challenge compared to during vaccination. However, more replicates are still needed to draw a statistically significant conclusion.

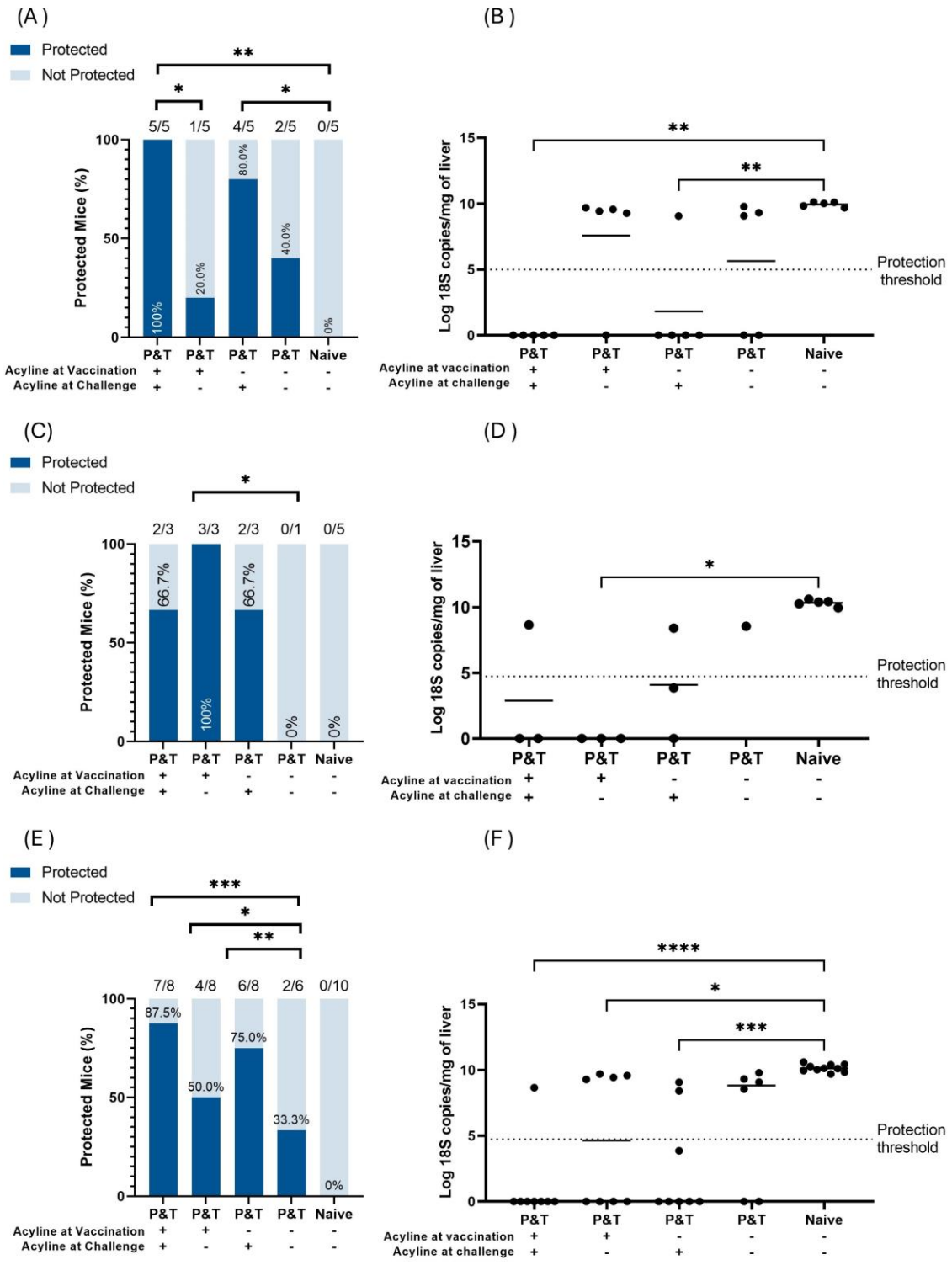

Figure 9: *Acyline P&T optimization results*. Percent protection bar graphs and log 18S copies/mg of liver scatter plots are shown for replicate 1 (A and B respectively), 2 (C and D respectively), and aggregate of both replicates (E and F respectively). The horizontal bars on the scatter plots denote group means.

3.3 Testosterone addback

The first two replicates of the acyline P&T optimization experiments indicated that the hormone environment at challenge may be of importance. Since testosterone suppression at this timepoint improved protection outcomes compared to no suppression, we next asked if adding exogenous testosterone back could reverse the protective effects of acyline. This would further test that testosterone, and not another unintended effect of acyline administration, was the driving factor behind our observed differences in protection outcome in acyline treated mice.

3.3.1 Dose determining pilot

To determine an appropriate testosterone dose for an addback, we performed a pilot experiment with two different doses of testosterone: 10 uμ/g and 100 μg/g body weight of the mouse. These doses were selected after consulting literature about testosterone addback experiments to castrated male mice[83]. Male BALB/cJ mice (4-6 weeks old) were divided into 2 groups, with 5 per group. Acyline was administered as two injections (300 ug each) on consecutive days. At 24 hours after the last acyline injection, mice were subcutaneously injected with a single dose of testosterone dissolved in sesame oil. Serum was collected via submental bleeds at specific timepoints to establish testosterone levels at baseline, 24 hours post injection, and 48 hours post injection.

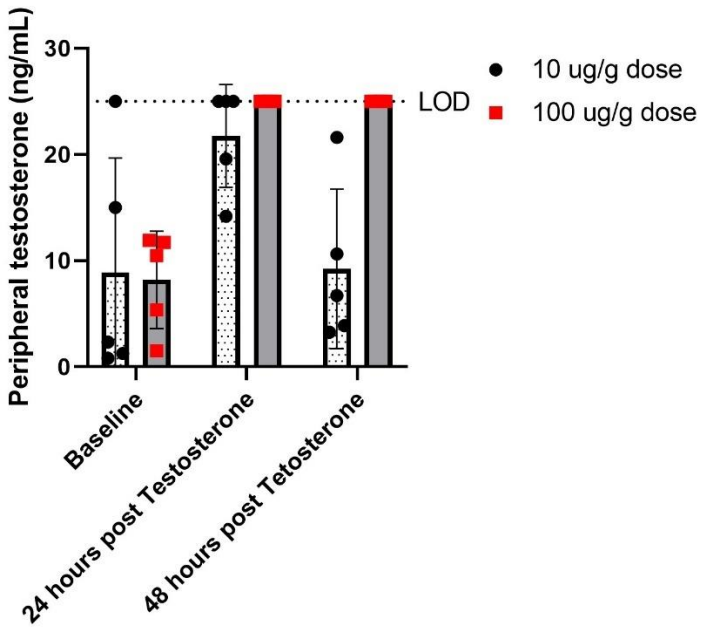

Figure 10: *Testosterone addback pilot.* The upper limit of detection is at 25 ng/mL.

Both doses of exogenous testosterone increased peripheral levels of the hormone compared to baseline at 24 hours post injection **(Figure 10)**. At the 48 hour timepoint, the peripheral hormone levels of the 10 ug/g body weight group (mean = 9.2 ng/mL) had dropped close to their baseline levels of measurement (mean = 8.9 ng/mL). However, for the 100 µg/g body weight group, the testosterone levels of all the mice continued to exceed the upper LOD of 25 ng/mL.

We reasoned that the higher testosterone dose would ensure any observable differences, if any are present, between groups. Additionally, the high dose would reduce the number of times mice would need to be injected to sustain high levels of testosterone in future experiments. Over a time period of 6 days from the last acyline injection to the liver harvest,

we would need to inject the mice with testosterone daily at the lower dose (6 injections), whereas the higher dose would require injections every other day (3 injections). This would reduce the stress of injections experienced by the animals. Thus, we decided to proceed with the 100 µg/g body weight dose of testosterone for subsequent experiments.

3.3.2 Testosterone addback experimental layout

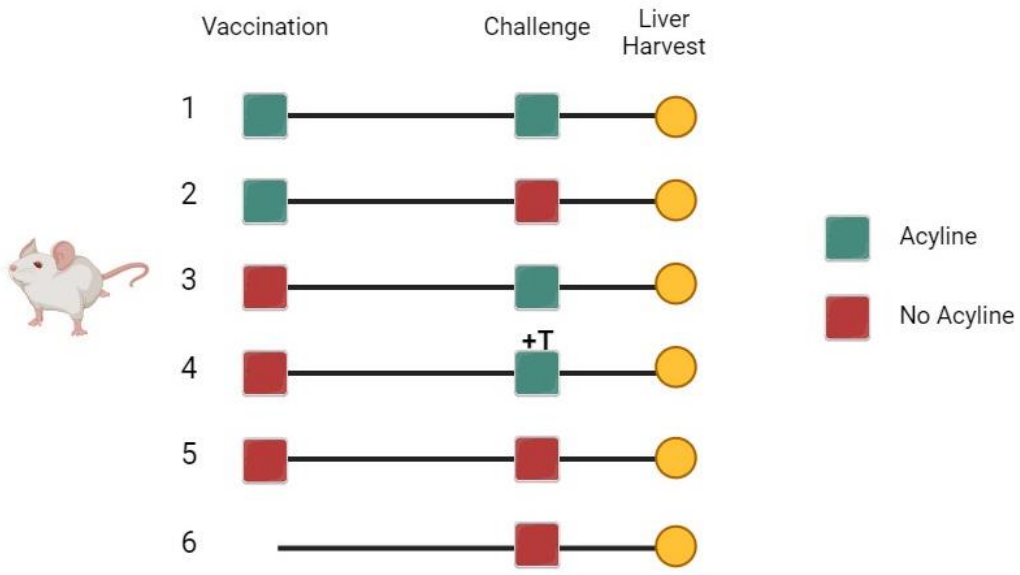

Figure 11: *Testosterone addback layout*. The presence of a square indicates whether or not an event took place, while the color of the square indicates if acyline was administered on days 4 and 5 prior to the event. Group 4 also received 3 injections of exogenous testosterone prior to and after challenge.

The layout for experiment was similar to that of the acyline P&T dose optimization experiment, with the addition of another group (Group 4), the testosterone addback group **(Figure 11)**. Mice in this group received acyline at challenge as previously described (days -

1 and 0), and then 3 doses of subcutaneous testosterone at 100 µg/g body weight of the mice on days 1, 3, and 5.

Peripheral levels of testosterone were monitored through submental bleeds before and after an event and quantified via serum ELISA. As before, all mice were challenged with WT *P. yoelii* spz and humanely euthanized 44 hours post infection for 18S liver burden quantification. Due to the similarity of layouts with replicates 1 and 2 of the acyline P&T optimization experiment, the two replicates with the testosterone addback groups will be here on referred to as replicates 3 and 4.

3.3.3 Exogenous testosterone prior to challenge reverses protective effect of acyline

Both the combined protection data from replicates 3 and 4 **(Figure 12A)** and the aggregate protection data from all 4 replicates **(Figure 12C)** saw similar trends in results. The naïve group was completely unprotected, while the vaccine control group showed partial protection. Amongst the groups that received acyline, the positive controls (Group 1) had relatively high protection levels, and mice that had their testosterone suppressed during challenge (Group 3) were better protected than those with hormone suppression during vaccination (Group 2). Finally, exogenous testosterone administration after acyline completely abolished protection in Group 4 **(Figure 12)**. qRT-PCR data also showed a higher mean liver log 18S burden in group 4 (7.1 log 18S copies/mg of liver) compared to all the other vaccinated groups **(Figure 12B and 12D)**. The decrease in protection due to

testosterone addback was significant when compared to Group 3 ($p= 0.018$ for replicates 3 and 4, and $p= 0.0009$ for aggregate data). Suppressed (Groups 1-3) or elevated (Group 4) levels of testosterone after acyline or exogenous testosterone administration respectively were confirmed with serum ELISA **(Supplementary figure 2).** In conclusion, these 4 replicates suggest that testosterone associated immunosuppression during malaria challenge decreases protection in male mice to a greater degree than during vaccination. This trend is not statistically significant, likely due to the small number of mice, and additional replicates may help to reach a more conclusive answer. Finally, the addback experiment lends credence to the fact that the immunosuppressive effects are testosterone specific, as exogenous addition of the hormone after depletion abolished the protection observed with testosterone suppression.

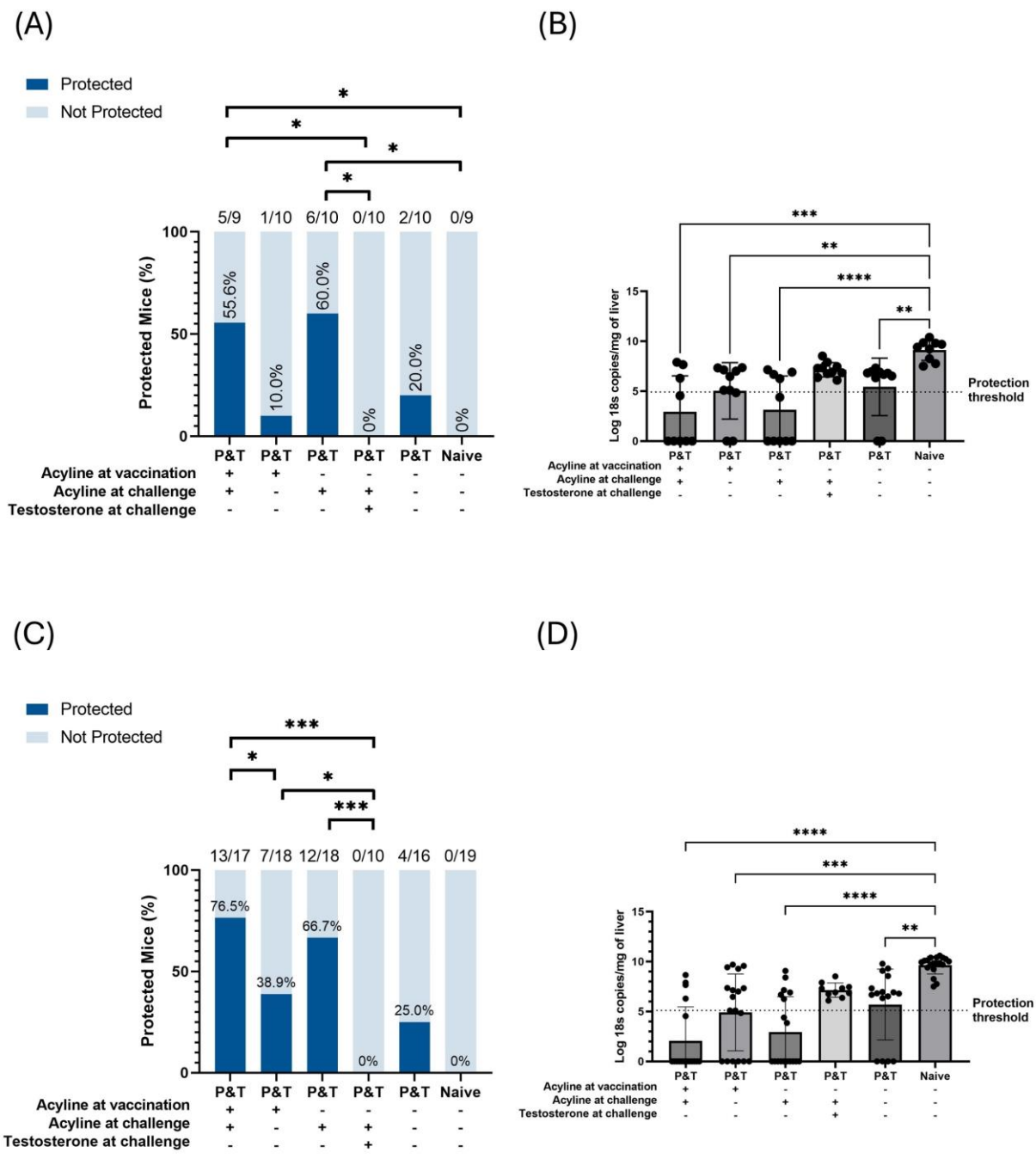

Figure 12: *Testosterone addback results*. Percent protection bar graphs and log 18S copies/mg of liver scatter plots are shown for replicate 3 and 4 (A and B respectively) and aggregate of all 4 replicates (C and D respectively). The horizontal bars on the scatter plots denote group means, while error bars show +/- standard error. In 5C, statistical significance of comparisons with the naïve group are not shown.

3.4 Androgen receptor expression quantification

In the above experiments, we had been using serum ELISAs to measure peripheral testosterone levels. However, we were concerned that the peripheral hormone levels may not be an accurate reflection of the liver hormone environment. Since the P&T vaccine targets the liver stage of malaria infection by generating protective liver-resident memory T-cells[34], we rationalized it would also be crucial to ensure that our hormone manipulation methods are affecting testosterone levels within the liver and not just circulating levels of peripheral hormone.

Although the gold-standard of quantifying steroid hormone levels is with liquid chromatography tandem mass spectrometry (LC-MS/MS), one of the major limitations of this approach is the high cost[84]. Therefore, we instead decided to approach this question by analyzing the expression of a gene that is known to be modulated by androgens, the androgen receptor (AR)[85]. In support of this approach, prior experiments carried out by Takeda *et al*[86] showed decreased AR levels via *in situ* hybridization in rat and mouse prostrates 3 days after castration, which were restored after treatment with dihydrotestosterone (DHT), a testosterone metabolite[87].

Based on these data, we hypothesized that if acyline and exogenous testosterone were affecting the hormone environment in the liver, peripheral testosterone levels would be correlated with AR expression in the liver. Thus, acyline administered male mice would

show decreased AR expression compared to control males, while testosterone treated male mice would show increased expression.

3.4.1 Experimental layout

Male and female BALB/cJ mice (4-6 weeks old) were divided into 4 groups, 3 per group per replicate (**Figure 13**).

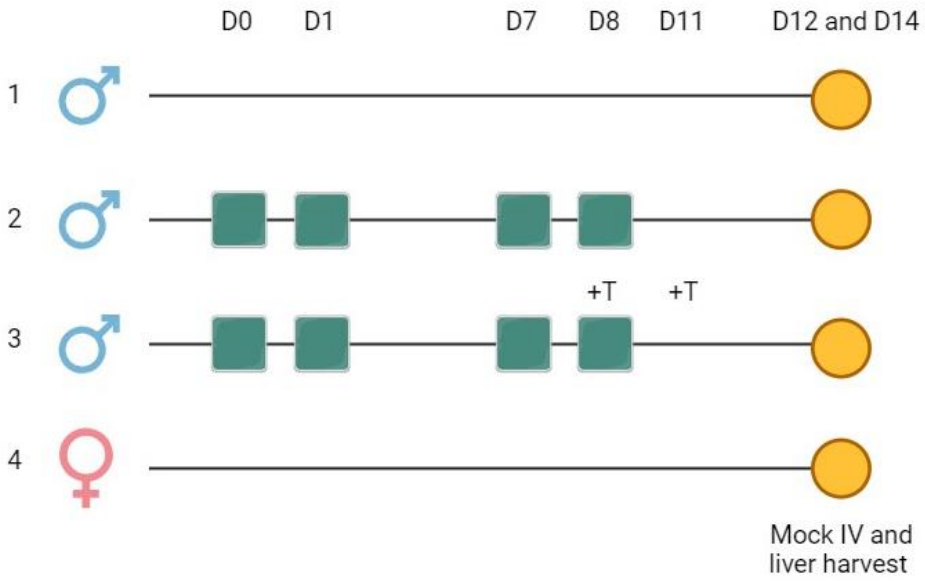

Figure 13: *Androgen receptor expression quantification experimental layout*. The green squares represent acyline injections.

Groups 2 and 3 received 4 doses of acyline on days 0, 1, 7, and 8 of 300 µg each. Group 3 was injected with 2 doses of exogenous testosterone dissolved in sesame oil on days 8 and 11. All other groups received only sesame oil on days 8 and 11 as a vehicle control. Groups 1 and 4 were our intact male and female controls respectively with no hormone

manipulation. Finally, on day 12, all the mice were mock challenged with IV injections of Schneider's media and their livers were harvested for AR expression qRT-PCR quantification 44 hours post injections. The mock injections were to simulate a challenge while minimizing the risk of an infection impacting hormone levels. Peripheral testosterone levels on the day of harvest were measured through submental bleeds and serum ELISA.

3.4.2 Acyline administration decreases AR expression in the liver

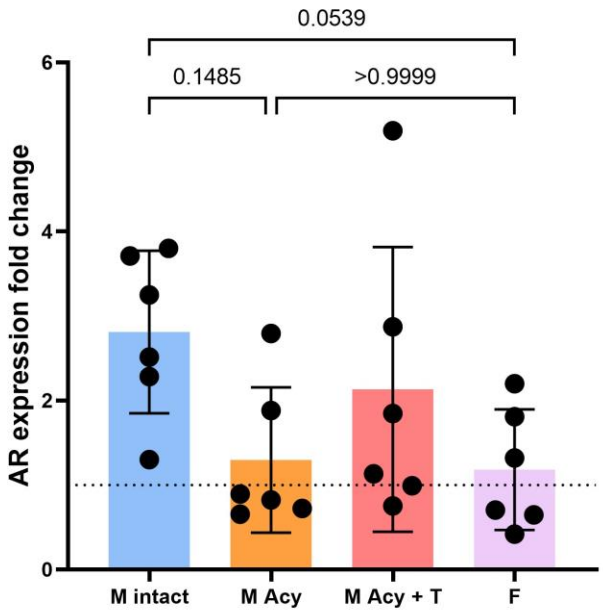

Figure 14: *Androgen receptor expression fold change compared to female control group.* Results from two replicates (n=6). The dotted line represents a fold change of 1. The height of the bars represents the group means. Error bars represent +/- standard error.

We hypothesized that the female mice would have the lowest levels of AR expression, thus decided to use this group as a comparator to see how much greater of a fold change (FC)

we would observe amongst the other groups. FC data of AR expression levels compared to the mean expression data of intact female mice (Group 4) were plotted on bar graphs **(Figure 14)**. Intact males without hormone manipulation (Group 1) had the highest FC amongst the male groups, with a mean FC of 2.8. Group 2 that received exogenous testosterone after acyline suppression recorded the next highest AR expression levels with a mean FC of 2.1. Interestingly, while Group 2 recorded much higher levels of testosterone compared to the intact males, their AR expression levels were lower. Finally, mice that received acyline had the lowest levels of AR expression amongst the male groups, averaging at a FC of 1.3 compared to the female control group. None of the differences were statistically significant, likely due to the low sample size. Testosterone suppression via acyline (Group 2) and excess peripheral testosterone levels after exogenous hormone injections (Group 3) were confirmed on ELISA **(Supplementary Figure 3)**. In conclusion, based on this experiment, while peripheral testosterone levels correlated with lower AR expression levels in intact females and acyline administered male mice, the same trend did not hold true when comparing intact males and testosterone administered males (Group 2). Intact males had lower levels of peripheral testosterone compared to Group 2, but higher AR expression FC, although this difference was not statistically significant.

3.5 Validation in C57BL/6 and *P. berghei* model

Finally, we aimed to test acyline mediated suppression of testosterone during challenge in a different mouse and parasite model. For this experiment, we used C57BL/6 mice and the

P. berghei mouse malaria model. *P. berghei* causes symptoms of cerebral malaria and early lethality in C57BL/6 mice[88–90]. Notably, C57/B6 mice develop more severe symptoms from *P. berghei* infections compared to BALBc/J mice, which are generally more resistant to infection[91]. RPL6 (ribosomal protein) and TRAP (spz adhesin) antigens were used for priming, both of which have been documented in literature to be effecting in vaccinating against *P. berghei*[92,93]. This experiment would test whether the effects of increased protection due to suppressed testosterone during challenge is generalizable to other mouse parasite models despite diverse host and parasite genetics.

3.5.1 Experimental layout

Figure 15: *Experimental layout for C57BL/6 and P. berghei model validation*. The presence of a square indicates whether or not an event took place, while the color of the square indicates if acyline was administered on days 4 and 5 prior to the event.

Male and female C57BL/6 mice (4-6 weeks old) were divided into 5 groups, 5 mice per group **(Figure 15)**. Mice were gene-gunned with RPL6 and TRAP antigens, trapped with irradiated *P. berghei* spz and challenged with WT *P. berghei*. Group 1 and 2 consisted of males and females respectively that were vaccinated and challenged but did not receive acyline. Group 3 was vaccinated and received acyline prior to challenge. Finally, groups 4 and 5 were our female and male naïve infectivity control groups respectively, that were challenged but neither vaccinated nor administered acyline. At 44 hours post infection, mice were humanely euthanized and their livers collected for 18S quantification by qRT-PCR.

3.5.2 Acyline improves protection in male vaccinated C57/B6 mice infected with *P. berghei*

Neither of the naïve control groups (Groups 4 and 5) showed any protection. The female vaccinated mice (Group 2) were the most protected group with 50% protection, followed by the male P&T acyline group which had 1 uninfected mouse out of 4 **(Figure 16A).** The male P&T no acyline group (Group 1) demonstrated no protection. Male vaccinated mice that received acyline also had a lower 18S burden in the liver (6.3 log 18S copies/mg of liver) compared to vaccinated males without testosterone suppression (7.4 log 18S copies/mg of liver). None of the differences were statistically significant, likely due to the low number of mice in the experiment. While the trend in data indicates generalizability of the protective

effects of testosterone suppression, since the results were not statistically significant, more research is warranted.

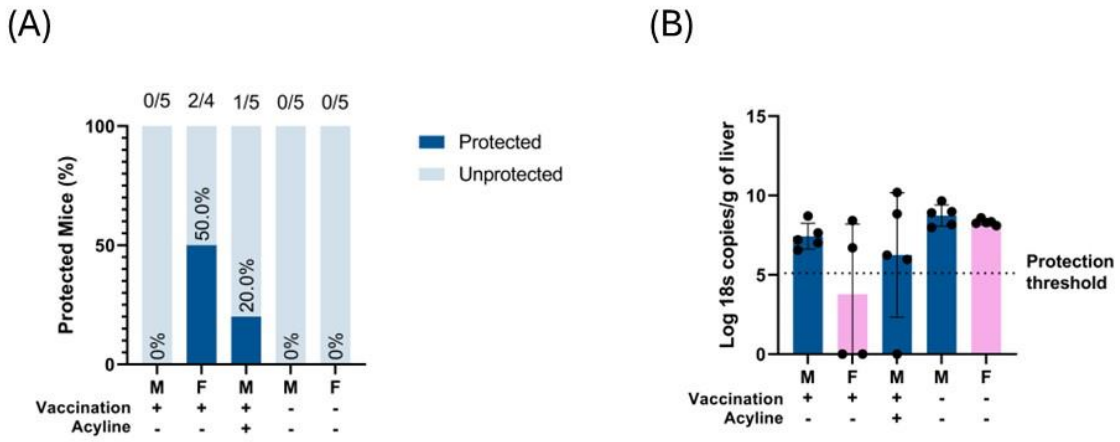

Figure 16: *Acyline validation in C57BL/6 and P. berghei model results*. Results from one experiment. Percent protection bar graphs (A) and log 18S copies/mg of liver scatter plots (B) are shown. Error bars in 11B show +/- standard error.

4. Discussion

This thesis aimed to investigate the role of testosterone in influencing vaccine outcomes in mouse malaria models. Specifically, we asked if testosterone suppressed the immune system to a greater extent during vaccination or the subsequent challenge. To achieve this, our goal was two-fold: to validate a chemical model of reversible testosterone suppression in mice, and then to use it to suppress testosterone at either vaccination or at challenge and compare protection outcomes after infectious spz challenge.

To reversibly suppress peripheral testosterone, we used a GnRH antagonist acyline. We first performed a dose optimization experiment to both validate the acute and reversible effects of acyline in a mouse model, and to determine an appropriate dose for subsequent experiments. Based on our pilot experiment, we decided to proceed with a dose of 2 injections of 300 µg each on successive days that suppressed peripheral testosterone levels for approximately 7 days. However, we confirmed that testosterone suppression occurred in a reversible and dose-dependent manner at all tested dosages.

Once we had a tested and validated dose of acyline, we next used it to suppress peripheral levels of testosterone at either vaccination or challenge in a series of P&T experiments. Our key findings were that male vaccinated mice that were administered acyline prior to challenge showed a trend for better protection than mice that received acyline prior to vaccination, although this difference was not statistically significant. Administration of

exogenous testosterone to acyline suppressed mice prior to challenge reversed the protective effect of acyline, further confirming the effect was testosterone specific. This indicated that testosterone is suppressing the immune response the most at challenge. However, a key limitation to our assays was that we were unable to directly measure the hormone environment in the liver. In order to address this limitation, we decided to correlate peripheral testosterone levels with liver AR expression, a surrogate measure for liver testosterone.

We quantified AR expression in the liver through qRT-PCR and found that acyline treated male mice with near undetectable levels of peripheral testosterone had low levels of AR expression compared to untreated males. While AR expression broadly correlated with peripheral testosterone, it is important to recognize this is still only a surrogate measure that does not give us conclusive answers about testosterone levels. Interestingly, testosterone administration after acyline suppression increased AR expression, but not as high as was observed in untreated males. While this difference is not statistically significant, it is unexpected since in our P&T experiments, vaccinated mice without hormone treatment were better protected than vaccinated mice that received acyline and testosterone. Androgens have been implicated in affecting the immune system independent of functional ARs[94]. Such non-canonical immunosuppressive pathways could explain the decreased protection in testosterone administered mice compared to untreated males despite the reduced level of AR expression.

Finally, we tested the effects of acyline on a different mouse and parasite strain model, C57BL/6 with *P. berghei*, referred to in literature as the experimental cerebral malaria model. Male vaccinated mice that received acyline were better protected than untreated vaccinated or naïve males. These results suggested that the above findings from the BALB/cJ and *P. yoelii* model about the suppressive effects of testosterone on the immune responses to P&T vaccination and recall of these responses at challenge may be generalizable to multiple mouse strains. However, due to the low sample size, we are unable to draw any statistically significant conclusions.

Considering the broader implications of this work for studying the role of sex hormones in various biological processes, there are potential advantages of using acyline over other methods for sex hormone depletion. While castration is the traditional method for hormone depletion in rodents[66–68,70], the irreversible nature of this approach made it inappropriate to use for our experiments. We showed that like castration[59], acyline reduce testosterone levels to near undetectable quantities, but in an acute and reversible manner. However, as castration and acyline have different mechanisms of action, it is formally possible they may have differential effects on the immune system. This can be explored in future studies.

We also observed that testosterone suppression after acyline administration was followed by a transient spike in testosterone levels beyond baseline measurements, a phenomenon that has previously been documented in human males[72]. This temporary overshoot is hypothesized to be due to disruption of the negative feedback loop between testosterone and GnRH release. In extensive research performed on rams and rhesus monkeys, it has been observed that testosterone and its metabolites act directly on the hypothalamus to inhibit GnRH production. In rams, castration increased the frequency of GnRH pulses[95]. It is likely that once acyline suppression wanes, the excess GnRH causes a spike in downstream testosterone levels that eventually equilibrates over time. This phenomenon is not observed with castration, where removal of the gonads leads to irreversible suppression of testosterone[96]. Interestingly, during replicates 3 and 4, ELISA data captured this overshoot prior to challenge in Group 2 (acyline before vaccination). This raises the question of whether the lower levels of protection seen in Group 2 were correlated with the absence of testosterone suppression during challenge or the high levels of testosterone at that timepoint. This may be investigated in the future by lengthening the time between a RAS trap and challenge, allowing for testosterone levels to equilibrate after acyline.

The increased immunosuppression of testosterone during challenge holds great implications for how we approach prevention and treatment methods for infectious diseases, especially when considering the effect of sex on disease susceptibility. Different cellular processes take place during vaccination and challenge. Gene gun delivery of antigen encoding DNA induces the priming and expansion of naïve CD8+ T cells into

effector CD8+ T cells, and trapping positions these effector cells in the liver as Trms[34]. During challenge, there is an increase in inflammatory pathways and innate immune responses and Trms are activated in the recall phase. From our experiments, the hormone environment at challenge appears to be especially important in modulating protection outcomes during malaria infection. While we have not explored the mechanisms by which testosterone may be impacting the immune environment during challenge, there are two possible hypotheses: (1) testosterone is indirectly suppressing the immune response by changing the tissue microenvironment and downregulating inflammatory pathways, making it harder for the immune system to detect the pathogen, or (2) testosterone is directly affecting the effector functions of the CD8+ T cells responding to an infection. In support of these two hypotheses, the literature suggests that androgens such as testosterone can modulate tissue microenvironment and gene expression[66], and also suppress inflammation[37,94]. Androgens have also been documented to directly impact lymphocyte frequencies and proliferative capacity. While human males have higher frequencies of CD8+ T cells compared to females, females show greater T cell proliferation in the peripheral blood and cytotoxicity[37]. Similarly, castrated C57BL/6 mice have higher numbers of CD8+ T cells in the peripheral lymphoid tissues than intact males. While we do not yet know the mechanism by which testosterone suppresses the immune system during challenge, there are many plausible hypotheses that can be investigated in future works.

Looking at these results in the context of infectious diseases in models more broadly, it should be noted that while there is significant precedence in literature of the immunosuppressive effects of testosterone for in many disease models, this phenomenon

does not always equate to increased disease severity. For example, administration of exogenous testosterone to castrated male mice infected with influenza A decreased lung inflammation, CD8+ T cell quantity and activity[94], leading to improved disease outcomes[97]. Whether testosterone plays a protective or detrimental role thus appears to be dependent on whether or not the disease pathology is primarily driven by damage caused by the pathogen or is immune mediated.

Finally, it is important to mention the clinical and scientific importance of this work. While sex-based differences in malaria vaccine efficacy have yet to be documented in humans, differential immunogenicity based on biological sex has been observed. For example, post pubertal females vaccinated with PfSPZ had higher levels of anti PfCSP antibodies compared to males[49]. Additionally, malaria incidence, severity, and clearance rate appear to be influenced by biological sex[46], as is the case with many other infectious and non-infectious diseases[37,39]. Thus, it is clear that a greater understanding of the impact of sex and sex hormones on the immune system is essential to engineer better treatment and prevention methods. This thesis aims to lay the foundation for future work on this topic.

Limitations of this study include the small number of animals used in some of our experiments, such as the AR expression quantification (n= 6 per group), and the C57BL/6 study (n= 4 or 5 per group). The low sample size made it difficult to draw statistically significant conclusions about the trends we observed. Another limitation is our lack of a direct method of assaying liver levels of testosterone. While the AR qRT-PCR data provided some validation about whether or not acyline and exogenous testosterone was likely affecting the liver hormone environment, our results lack statistical significance and do not

provide a conclusive answer about organ levels of testosterone. A superior approach, if feasible, would be to use LC/MS to directly measure testosterone quantity in the liver.

5. Conclusion:

This thesis aimed to investigate if testosterone suppressed the immune system to a greater extent during vaccination or infection in the context of a malaria mouse model of vaccination. Using an acute and reversible GnRH antagonist, we found that suppressing testosterone prior to challenge afforded better protection to male mice than hormone depletion prior to vaccination. Our goal was to set up the foundation for greater insight into the mechanisms by which testosterone impacts the immune system. In a broader context, we hope that this work will pave the way for a better understanding of the role of sex steroids in susceptibility to infectious diseases and to help engineer better vaccines and treatment methods.

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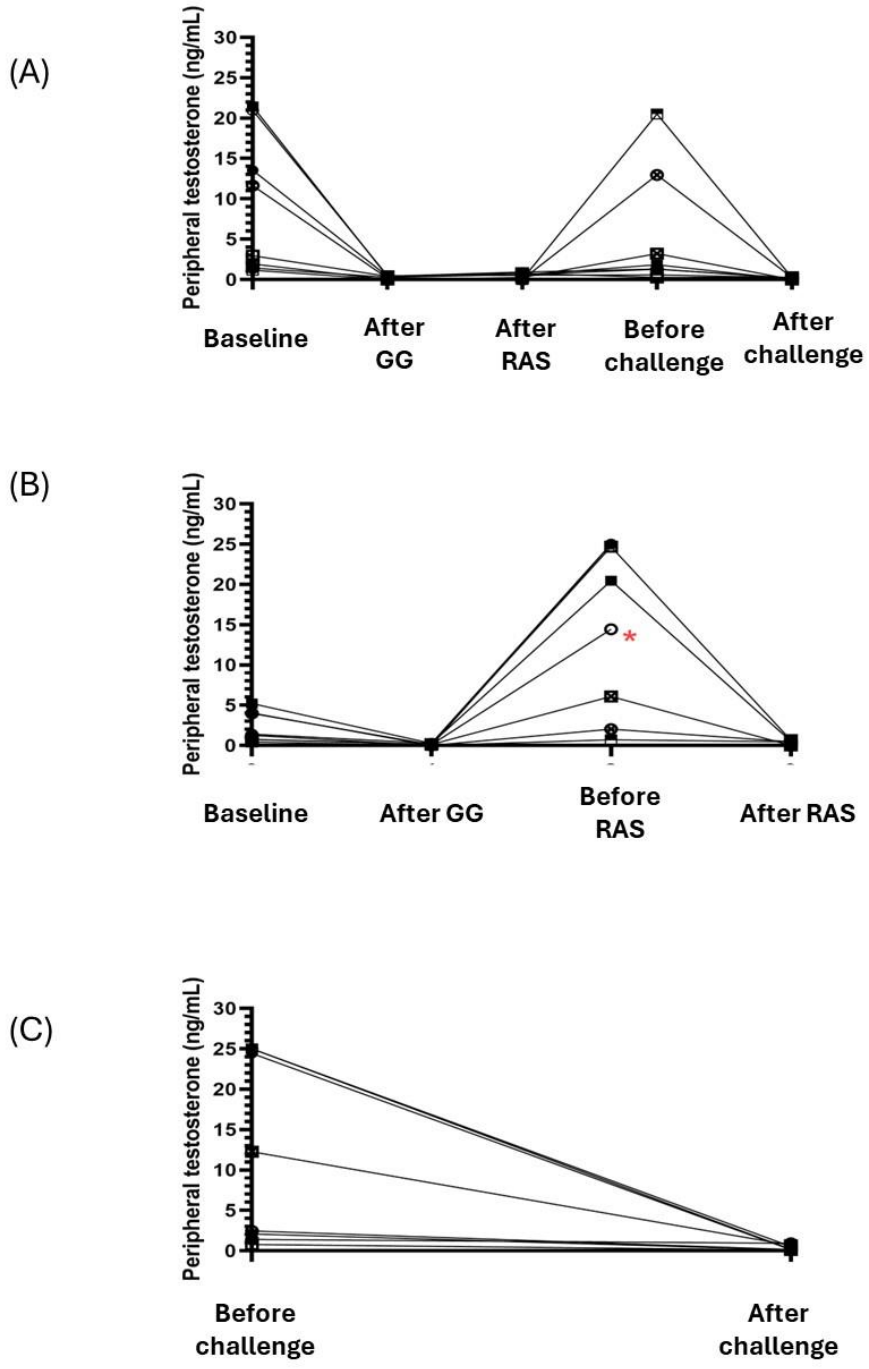
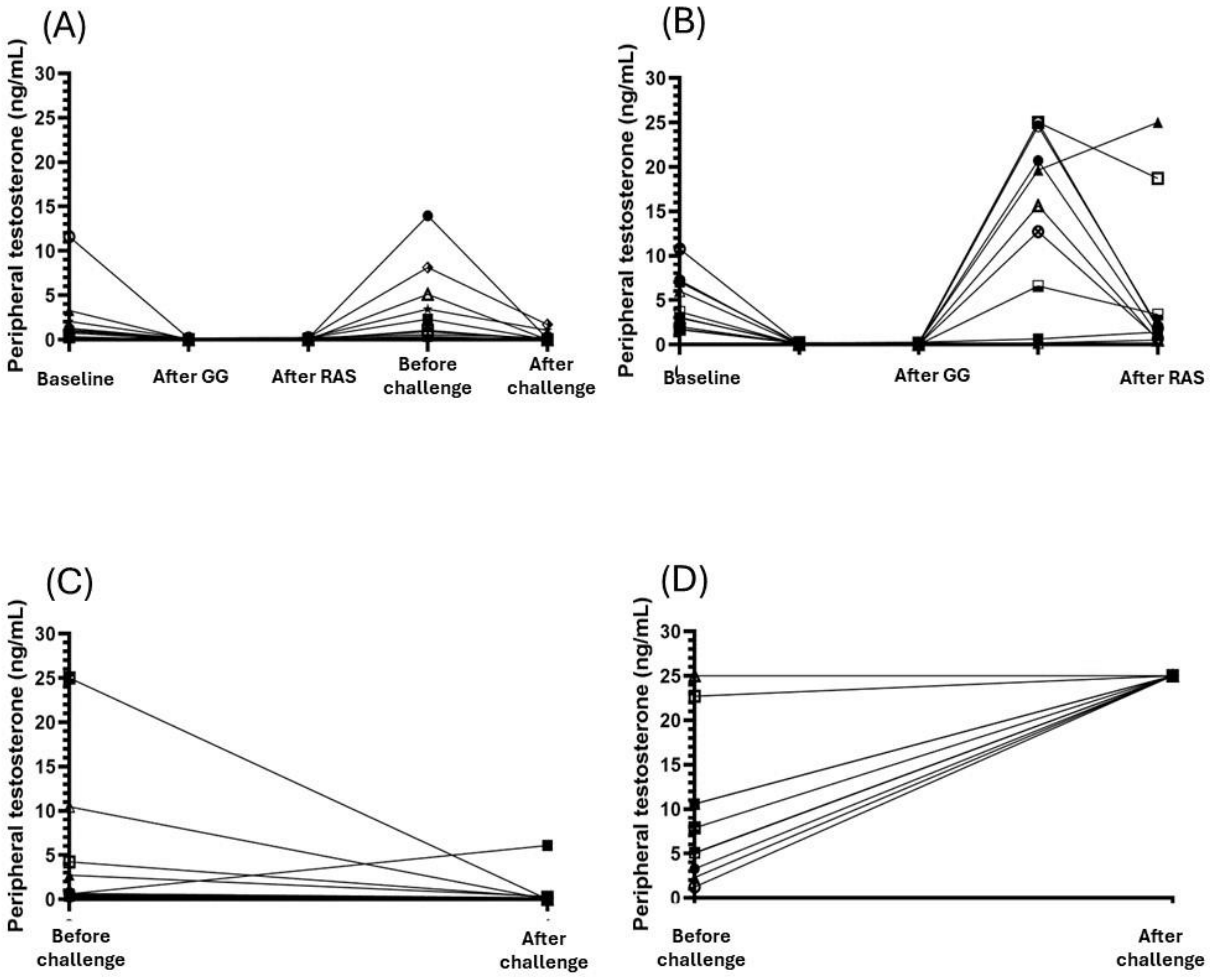
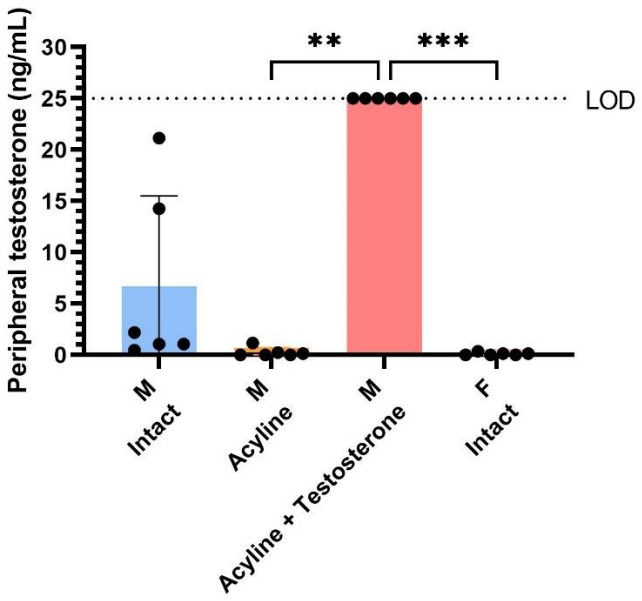
Supplementary figures:



Supplementary figure 1: *Testosterone suppression in acyline P&T optimization replicates 1 and 2.* Testosterone levels (y-axis) measured before and after acyline injections in group 1 (A), group 2 (B), and group 3 (C). X-axis indicates serum collection timepoints. The red asterisk in (B) represents a lack of post-RAS follow-up for that mouse due to the sample being lost after collection.



Supplementary figure 2: Testosterone suppression in acyline P&T replicates 3 and 4 with testosterone addback. Testosterone levels (y-axis) measured before and after acyline injections in group 1 (Sup A), group 2 (Sup B), group 3 (Sup C), and group 4 (Sup D). X-axis indicates serum collection timepoints.



Supplementary figure 3: *Peripheral testosterone levels at time of harvest of mice in AR expression quantification experiment*. The dotted line indicates the upper limit of detection of the ELISA kit used. Error bars indicate +/- standard deviation.

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