Immunogenicity and Protection of the mXCL1-PyCSP Fusion Protein Prime-and-Trap DNA Vaccine in a Murine Malaria Immunization/Challenge Model

Kenneth Boey

A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2024

Committee:

Sean C. Murphy

Thea L. Brabb

Brian M. Iritani

Program Authorized to Offer Degree:

Comparative Medicine

©Copyright 2024

Kenneth Boey

University of Washington

Abstract

Immunogenicity and Protection of the mXCL1-PyCSP Fusion Protein Prime-and-Trap DNA Vaccine in a Murine Malaria Immunization/Challenge Model

Kenneth Boey

Chair of the Supervisory Committee:

Sean C. Murphy

Department of Laboratory Medicine and Pathology

Malaria is a life-threatening parasitic disease caused by *Plasmodium* spp. and is transmitted by female *Anopheles* spp. mosquitoes. Annually, there are nearly 250 million cases worldwide causing over 600,000 deaths, primarily in children under 5 years of age in sub-Saharan Africa. Currently, no highly efficacious (>85–90%) vaccine exists, hence the development of such a vaccine against human malaria infection is of paramount importance. The chemokine ligand XCL1, also known as lymphotactin, binds to its chemokine receptor XCR1. Recent studies have shown that XCL1-antigen fusion proteins efficiently induce $CD8^+$ T cell responses by preferentially delivering antigens to cross-presenting dendritic cells expressing XCR1. In this study, we evaluated the immunogenicity of a fusion protein of the murine XCL1 chemokine and the *Plasmodium yoelii* circumsporozoite protein (mXCL1-PyCSP) in our "Prime-and-Trap" vaccine in a murine model of malaria. We hypothesized that this fusion protein vaccine would increase immunogenicity and protection outcomes compared to the standard unfused PyCSP vaccine. In summary, we showed

that the fusion of PyCSP with the mXCL1 chemokine enhanced cell-mediated immune responses and significantly increased immunogenicity in male and female BALB/cJ mice, while not hampering protection outcomes in female mice. Fusion with mXCL1 may also reduce the need for cluster priming and improve vaccine scheduling. Overall, the data obtained in the present study could contribute to the overall goal of improving the efficacy of pre-erythrocytic malaria vaccines.

Table of Contents

List of Figures i

List of Abbreviations ii

Introduction 1

Materials and Methods 7

Results 10

Discussion 15

Figures 22

References 27

List of Figures

Figure 1. Schematic diagram of the mXCL1-PyCSP fusion protein P&T DNA vaccine construct.. 22

Figure 2. Vaccination with separate PyCSP and mXCL1 plasmids do not efficiently target DCs.. 23

Figure 3. The mXCL1-PyCSP vaccine significantly increases immunogenicity in male and female mice.. 24

Figure 4. The mXCL1-PyCSP vaccine decreases the need for cluster priming in female mice..... 25

Figure 5. The mXCL1-PyCSP vaccine similarly protects female mice during PySPZ challenge..... 26

List of Abbreviations

ANOVA – analysis of variance

APC – antigen presenting cell

BLASTP – protein Basic Local Alignment Search Tool

cDC1 – conventional type-1 dendritic cell

CGIDR – Center for Global Infectious Disease Research

CO_2 – carbon dioxide

COVID-19 – coronavirus disease 2019

CP – cluster prime

CSP – circumsporozoite protein

DC – dendritic cell

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

ELISPOT – enzyme-linked immunosorbent spot

ggDNA – transdermal DNA vaccination by gene gun

ID – intradermal

IFN-γ – interferon-γ

IgG – immunoglobulin G

LNP – lipid nanoparticle

IV – intravenous

LT – *Escherichia coli* heat-labile toxin

mRNA – messenger ribonucleic acid

mXCL1 – murine X-C motif chemokine ligand 1

mXCL1-PyCSP – fusion protein of murine X-C motif chemokine ligand 1 and *Plasmodium yoelii* circumsporozoite protein

mXCL1-V21C/A59C or mXCL1.var – stabilized variant of murine X-C motif chemokine ligand 1

mXCL1-WT – wild-type murine X-C motif chemokine ligand 1

NCBI – National Center for Biotechnology Information

NHP – nonhuman primate

P&T – Prime-and-Trap

PyCSP – *Plasmodium yoelii* circumsporozoite protein

pDC – plasmacytoid dendritic cell

PyRAS – *Plasmodium yoelii* radiation-attenuated sporozoites

PySPZ – *Plasmodium yoelii* live non-attenuated sporozoites

PyWT – wild-type *Plasmodium yoelii*

RAS – radiation-attenuated sporozoites

RNA – ribonucleic acid

SCRI – Seattle Children's Research Institute

SD – standard deviation

SFU – spot-forming units

SP – single prime

TD – transdermal

T_{RM} cells – tissue-resident memory T cells

WHO – World Health Organization

XCL1 – X-C motif chemokine ligand 1

XCL2 – X-C motif chemokine ligand 2

XCR1 – X-C motif chemokine receptor 1

Acknowledgements

This study was supported by NIH/NIAID grants 1R01AI141857 and U01AI155313. I thank Dr. Brad Stone for the early conception of this project, as well as Anya Kalata, Haley Masters, Dr. Melanie Shears, and personnel from the Seattle Children's Research Institute CGIDR Insectary for their technical support in this project.

I would also like to thank members of my Supervisory Committee Dr. Thea Brabb and Dr. Brian Iritani, as well as all the members of the Murphy Laboratory for their support and critical evaluation of my research work over the years.

Finally, I would like to express my gratitude to my mentor and Chair of my Supervisory Committee, Dr. Sean Murphy, for his continual guidance and without whom none of this research would have been possible.

Dedication

This work is dedicated to my parents, brother, and significant other who have all supported me relentlessly throughout my entire academic pursuit.

Introduction

Malaria is a potentially life-threatening tropical and subtropical disease caused by intracellular protozoan parasites from the genus *Plasmodium* [1, 2]. The disease is transmitted by female *Anopheles* spp. mosquitoes in warm and humid environments with freshwater bodies that facilitate propagation of the vector [1]. In 2022, an estimated 249 million human cases of malaria occurred worldwide in 85 malaria-endemic countries, causing around 608,000 deaths [3]. There are at least five known species of *Plasmodium* that can infect humans, including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* (subsp. *wallikeri* and *curtisi*), and *P. knowlesi* [1, 2]. Most human deaths are caused by *P. falciparum* in sub-Saharan Africa [1], with greater than 50% of all deaths occurring in merely four countries in 2022 – Nigeria (31%), the Democratic Republic of Congo (12%), Niger (6%), and Tanzania (4%) [3]. In Asia and South America, *P. vivax* causes the greatest human morbidity and mortality [1]. Malaria remains as a major public health and socioeconomic problem in many regions, with direct costs estimated to be at least US$12 billion per year.

The life cycle of the malaria parasite is extremely complex and involves many stages. It begins with a female *Anopheles* mosquito taking a blood meal and injecting *Plasmodium* sporozoites into the bloodstream, which are then transported to the liver to invade hepatocytes and undergo asexual replication [1, 2]. This refers to the pre-erythrocytic (hepatic) stage, which lasts approximately 5–6 days in humans. The infected hepatocytes then rupture and release thousands of merozoites, which egress into the bloodstream and invade erythrocytes, beginning another

asexual replication cycle [1, 2]. This stage refers to the erythrocytic (blood) stage, which is when clinical malaria presents itself with clinical signs and symptoms of malaria infection. Merozoites reproduce into trophozoites and then into schizonts, which erupt from the erythrocytes and release even more merozoites to reinvade other erythrocytes, continuing the asexual replication cycle [2]. This causes massive destruction of erythrocytes responsible for the clinical symptoms of malaria. In a portion of the trophozoites, the sexual reproduction cycle occurs when they mature to male and female gametocytes. During a blood meal by another female *Anopheles* mosquito, these gametocytes are taken up into the mosquito midgut, which eventually get converted into oocysts during the mosquito stage. Finally, the oocysts mature, releasing sporozoites, which migrate to the salivary gland of the mosquito [1, 2]. This completes the whole life cycle of the parasite when the sporozoites are transmitted into another human host.

Currently, a highly effective or efficacious (>85–90%) malaria vaccine has not been developed, though it is encouraging that the World Health Organization (WHO) has recommended the very first two malaria vaccines for broad use in children – RTS,S/$AS01_E$ (Mosquirix™, GlaxoSmithKline) in October 2021 [4], and R21/Matrix-M™ (University of Oxford/Serum Institute of India) in October 2023 [5]. The vaccines, which are recombinant vaccines consisting of the circumsporozoite protein (CSP) antigen and targeting sporozoites, were added to the WHO's List of Prequalified Vaccines in July 2022 and December 2023, respectively [6, 7]. Although promising, these vaccines do have important limitations. They require a complicated age-based vaccination schedule of an initial three doses one month apart, followed by a booster 12–18 months after [5, 8]. There is a modest to good 12-month efficacy of ~55% (RTS,S/$AS01_E$) [9] to 68% (R21/Matrix-

M™; 75% in seasonal locations) [10], and this protective immunity rapidly wanes thereafter. The window of action is also very brief (minutes to hours) since the vaccines mainly target the sporozoites during the post-blood meal period (sporozoite stage) prior to entry into the hepatocytes. These vaccines thus currently do not replace, but rather complement all the other public health tools available in malaria-endemic countries.

There are several potential targets of malaria vaccines at different stages of the *Plasmodium* life cycle, including antibodies blocking hepatocyte invasion (sporozoite stage, such as in the WHO-recommended vaccines), $CD8^+$ T cell activation and antibody production (pre-erythrocytic stage), antibodies blocking invasion (erythrocytic stage), and antibodies blocking fertilization/transmission (gametocyte/mosquito stage) [11]. As targeting the pre-erythrocytic stage could prevent the erythrocytic stage and downstream life cycle from occurring, preventing clinical disease, it is considered an attractive or ideal target for malaria vaccine design. Furthermore, there are 5–6 days available during the pre-erythrocytic stage for all infected hepatocytes to be killed. However, it is important for 100% of these infected hepatocytes to be eradicated prior to the erythrocytic stage, as a single merozoite that gets released back into the bloodstream would allow the asexual replication cycle in erythrocytes to begin. Although high titers of CSP-binding antibodies alone can confer high levels of protection [12], induction of $CD8^+$ T cells, specifically liver tissue-resident memory $CD8^+$ T cells (T_{RM} cells), appears to be critical for reliable and durable sterile protection [13].

Our laboratory has previously developed a two-step heterologous vaccine strategy called "Prime-and-Trap" (P&T) in an immunization/challenge mouse model, which combines priming the periphery with a DNA-based vaccine to induce peripheral antigen-specific $CD8^+$ T cells, followed by expression of the antigen in the liver [14]. This vaccine strategy involves priming the skin using plasmid DNA encoding the rodent malaria parasite *Plasmodium yoelii* CSP antigen (PyCSP), followed by a single intravenous (IV) dose of radiation-attenuated sporozoites (RAS) to "trap" the activated $CD8^+$ T cells in the liver, generating $CD8^+$ T_{RM} cells. Our P&T strategy has been shown to be able to induce robust CSP-specific $CD8^+$ T_{RM} responses in the liver and confer durable sterile protection in female mice using this rodent malaria immunization/challenge model for at least four months [15].

The X-C motif chemokine ligand 1 (XCL1), also known as lymphotactin, is one of only two in the C class of chemokines. It differs in structure and function from all chemokines in the other three classes (CXC, CC, CX3C), as it has only two cysteines and one disulfide bond [16, 17]. As a result, it can undergo a reversible conformational change between two forms due to the lack of a second disulfide bond. Through this conformational change, XCL1 may be found in two states – Ltn10 and Ltn40, where the former is a monomer at 10°C the latter is a dimer at 40°C [17]. As such, murine XCL1 (mXCL1) is considered less stable or more metamorphic in its natural form, referred to as wild-type mXCL1 (mXCL1-WT). To effectively utilize mXCL1 in vaccine studies, a highly active and stabilized variant with two disulfide bonds, referred to as mXCL1-V21C/A59C, was previously generated [18]. The receptor associated with XCL1 is XCR1, and it needs the chemokine-like Ltn10 conformation for the structure to bind and activate XCR1 [19]. XCL1 is produced mainly by T, NK

and NKT cells during infectious and inflammatory responses, whereas XCR1 is exclusively expressed by conventional type 1 DCs (cDC1s) [16, 20, 21].

The XCL1-XCR1 axis plays an important role in both innate and adaptive (DC-mediated cytotoxic) immune response. XCL1 is capable of inducing strong chemotaxis of cDC1s, which exclusively express XCR1, and are antigen presenting cells (APCs) capable of cross-presentation [16, 20, 21]. In mice, these XCR1-expressing cDC1s have been identified as CD8α$^+$ residential DCs and CD103$^+$ interstitial DCs [22, 23]. This expression is conserved on homologous DC subsets in humans as CD141$^+$ DCs [24, 25], and in macaques as CADM1$^+$ DCs [26]. These DCs efficiently process cell-associated antigens and present them to CD8$^+$ T cells, which mediate cytotoxic T lymphocyte response to intracellular viral and tumor antigens [16, 18, 20, 27]. Recent studies have evaluated XCL1-antigen fusion DNA vaccines against various other diseases, including influenza [28, 29], COVID-19 [30], foot-and-mouth disease [31], porcine reproductive and respiratory syndrome [32], and even for tumor models and cancer immunotherapy [33-36]. Prior to this study, no other study has been known to evaluate the use of XCL1 in a vaccine against malaria infection.

In this study, we aimed to evaluate the immunogenicity and protection of the murine XCL1-*P. yoelii* circumsporozoite protein (mXCL1-PyCSP) fusion protein P&T vaccine in a murine malaria immunization/challenge model. Specifically, we investigated if we could improve the immunogenicity of a PyCSP-based murine malaria vaccine in our P&T vaccine strategy by fusion with mXCL1 and administering to male and female BALB/c mice. In addition, we evaluated if

fusion with mXCL1 would decrease or remove the need for cluster (multiple) priming in our P&T regimen, as single priming would be much more advantageous in a vaccine with decreased complexity to the vaccination schedule. We also investigated the protection induced by the mXCL1-PyCSP vaccine compared to the standard unfused PyCSP vaccine in our standard P&T regimen. This study is part of the ongoing efforts to improve the current tools available in developing an effective and efficacious vaccine against human malaria infection.

Materials and Methods

Animals

Male (*n* = 40) and female (*n* = 135) 4–6-week-old BALB/cJ mice (*Mus musculus*) were acquired from The Jackson Laboratory (Bar Harbor, ME) and housed under standard conditions at the University of Washington. All animals were acclimated for at least 2 weeks prior to the start of each experiment. All animal procedures were performed as part of an approved scientific protocol in accordance with the University of Washington Institutional Animal Care and Use Committee.

DNA vaccination via gene gun

The PyCSP DNA vaccine plasmids were constructed in the pUb.3 vector with the mXCL1-WT or mXCL1-V21C/A59C tag (collectively referred to as mXCL1 if not specified) as previously described [14, 37], with the exception of mXCL1 replacing ubiquitin (Figure 1), or with separate PyCSP and mXCL1 plasmids, and then purified as described [38]. The PyCSP-minigene encodes the codon-optimized SYVPSAEQI epitope and the PyCSP plasmid encodes the full-length codon-optimized CSP protein without the major repeat regions, GPGAPQ and PPQQ. All plasmids were Sanger sequenced (GENEWIZ, Azenta Life Sciences, Burlington, MA) before use. Gene gun DNA vaccine cartridges were constructed by loading purified DNA plasmids onto 0.8–1.5µm diameter gold powder (Technic Inc., Cranston, RI) and then coating the loaded gold onto tubing as previously described [37], without the incorporation of *Escherichia coli* heat-labile toxin (LT)-encoding plasmid adjuvant. Following shaving of the ventral abdominal fur, the mice were vaccinated

transdermally on the ventral abdomen using a PowderJect-style gene gun. The number of cartridges used and the frequency of vaccinations are described in the respective results sections.

Vaccination and challenge using *P. yoelii* sporozoites

Female *Anopheles stephensi* mosquitoes infected with wild-type *P. yoelii* 17XNL (PyWT) were reared at the Seattle Children's Research Institute (SCRI) Center for Global Infectious Disease Research (CGIDR) Insectary (Seattle, WA). Fresh sporozoites were obtained by salivary gland dissection 14–18 days post-infection, followed by density gradient purification using Accudenz (Accurate Chemical & Scientific Corporation, Carle Place, NY) as described [39]. For the trapping (vaccination) procedure in the P&T regimen, freshly-dissected and purified sporozoites were then irradiated using a dose of 10,000 rads of X-ray exposure (Rad Source, Buford, GA) to generate *P. yoelii* radiation-attenuated sporozoites (PyRAS). For the challenge procedure in the P&T regimen, freshly-dissected and purified live non-attenuated *P. yoelii* sporozoites (PySPZ) were used. All PyRAS and PySPZ were administered to mice retro-orbitally (intravenously) diluted in 100 µL of Schneider's insect media (Gibco, Thermo Fisher Scientific, Waltham, MA). A dose of 2×10^4 PyRAS was used for trapping procedures, and a dose of 1×10^3 PySPZ was used for challenge procedures. Erythrocytic stage protection after PySPZ challenge was assessed by Giemsa-stained thin blood smear microscopy 3–14 days post-challenge (Days 59–70). Blood was obtained by tail prick using a 30G hypodermic needle either daily or once every other day. Mice were considered protected from challenge if blood smears remained negative for parasites up to Day 14 post-challenge.

Ex vivo IFN-γ ELISPOT assay

PyCSP peptide (SYVPSAEQI) was synthesized (Genemed Synthesis Inc., San Antonio, TX) and reconstituted in dimethyl sulfoxide (DMSO). Mouse interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assays (eBioscience, San Diego, CA) were conducted by stimulating cells with PyCSP peptide (or DMSO vehicle control) at 1 μg/ml for 18 h at 37°C and developed following manufacturer guidelines as described previously [14, 40]. The number of spot-forming units (SFU) in each well was calculated using an ImmunoSpot 5.1 Analyzer (Cellular Technology Ltd, Shaker Heights, OH). SFU were normalized to DMSO control wells and SFU per 10^6 splenocytes were reported.

Statistics

Comparisons of ELISPOT groups were done using ordinary one-way analysis of variance (ANOVA) with Šídák's multiple comparisons test. Protection data was evaluated using Fisher's exact test. Error bars in figures are reported as standard deviation (SD) with individual mouse samples shown if applicable. All p-values and individual experiment statistics are listed in the corresponding figure legends. Statistical significance was defined as $p < 0.05$. GraphPad Prism 10.0.2 Software (San Diego, CA) was used for all calculations.

Results

Vaccination with separate PyCSP and mXCL1 plasmids do not efficiently target DCs

To assess the feasibility of incorporating mXCL1 into our P&T vaccine regimen in order to harness its strong chemotactic effects on XCR1-expressing DCs, we conducted a pilot immunogenicity experiment using separate mXCL1 and PyCSP plasmids in our P&T DNA vaccine. Female BALB/cJ mice (*n* = 20) were utilized in this pilot immunogenicity experiment, divided equally into 4 groups (*n* = 5 per group) – Ubiquitin Parent (negative control), mXCL1 + PyCSP (separate mXCL1-WT and PyCSP plasmids), PyCSP (SP) (PyCSP group with single priming), and PyCSP (CP) (PyCSP group with cluster priming; positive control as previously optimized [14]). This experiment involved transdermal (TD) DNA vaccination by gene gun (ggDNA) using two vaccine cartridges on Days 0 +/- 2 (single prime only on Day 0; cluster prime on Days 0 and 2), followed by a ggDNA boost on Day 28, also using two cartridges. On Day 56, mice were humanely euthanized using carbon dioxide (CO_2) asphyxiation and their spleens harvested for an IFN-γ ELISPOT assay endpoint to assess levels of PyCSP-specific IFN-γ^+ T cells (Figure 2A). One of the initial five mice in the PyCSP (CP) group sustained an unrelated paw injury on Day 1 and was thus euthanized and excluded from the rest of the experiment (*n* = 19 at the end of the experiment).

While there generally seemed to be an increased mean of SFU per 10^6 splenocytes of PyCSP-specific IFN-γ^+ T cells in the mXCL1 + PyCSP group compared to the PyCSP (SP) and PyCSP (CP) groups, none of the comparisons among these three groups were statistically different (Figure 2B). Based on the results, this means that using a P&T vaccine with separate mXCL1 and PyCSP

plasmids did not significantly increase immunogenicity in female mice compared to the standard unfused PyCSP vaccine, regardless of the priming schedule (single or cluster prime). Given the inconclusive results, we looked to develop a DNA vaccine incorporating the fusion mXCL1-PyCSP protein for our P&T regimen to utilize a vaccine that could enable mXCL1 and PyCSP to be transcribed and translated together. In addition, we also looked to utilize the stabilized and more active variant of the mXCL1 with two disulfide bonds, referred to as mXCL1-V21C/A59C [18] or mXCL1.var from this point forward.

The mXCL1-PyCSP vaccine significantly increases immunogenicity in male and female mice

To evaluate the immunogenicity of the mXCL1-PyCSP fusion protein P&T vaccine compared to the standard unfused PyCSP vaccine, we conducted four immunogenicity experiments, two using male mice and two using female mice (n = 80 total). In each experiment, male or female BALB/cJ mice (n = 20) were divided equally into 4 groups (n = 5 per group) – mXCL1.var Parent (negative control), mXCL1-PyCSP (mXCL1-WT and PyCSP fusion protein), mXCL1.var-PyCSP (mXCL1.var and PyCSP fusion protein), and PyCSP (positive control as previously optimized [14]). Mice were ggDNA vaccinated using two vaccine cartridges on Day 0 (single prime only on Day 0). On Day 28, mice were humanely euthanized using CO_2 asphyxiation and their spleens harvested for an IFN-γ ELISPOT assay endpoint to assess levels of PyCSP-specific IFN-γ^+ T cells (Figure 3A).

The mXCL1-PyCSP and mXCL1.var-PyCSP groups had significantly higher mean SFU per 10^6 splenocytes of PyCSP-specific IFN-γ^+ T cells (p<0.01) compared to the standard unfused PyCSP

group in both female (Figure 3B) and male mice (Figure 3C), with the mXCL1.var-PyCSP group in the male experiments being the most statistically different ($p<0.001$) compared to the PyCSP group amongst all experiments (Figure 3C). This suggested that fusion with mXCL1 significantly increased the immunogenicity of the PyCSP P&T vaccine in both male and female mice, which could potentially provide an impetus to investigate its effectiveness in challenge and protection experiments. However, there were no statistically significant differences observed between the mXCL1-PyCSP and mXCL1.var-PyCSP groups in both male and female mice (Figures 3B and 3C), suggesting that utilization of either the mXCL1 or mXCL1.var tags were similar in effectively increasing the immunogenicity of the PyCSP vaccine using this immunization model.

The mXCL1-PyCSP vaccine decreases the need for cluster priming in female mice

To investigate the effects on immunogenicity using single priming compared to cluster priming with the mXCL1-PyCSP and standard unfused PyCSP vaccines, we conducted two immunogenicity experiments in female mice (*n* = 50 total). In each experiment, female BALB/cJ mice (*n* = 25) were divided equally into 5 groups (*n* = 5 per group) – mXCL1.var Parent (negative control), mXCL1.var-PyCSP (SP) (mXCL1.var and PyCSP fusion protein with single priming), mXCL1.var-PyCSP (CP) (mXCL1.var and PyCSP fusion protein with cluster priming), PyCSP (SP) (unfused PyCSP with single priming), and PyCSP (CP) (unfused PyCSP with cluster priming; positive control as previously optimized [14]). Mice were ggDNA vaccinated using two vaccine cartridges on Days 0 +/- 2 (single prime only on Day 0; cluster prime on Days 0 and 2). On Day 28, mice were humanely euthanized using CO_2 asphyxiation and their spleens harvested for an IFN-γ ELISPOT assay endpoint to assess levels of PyCSP-specific IFN-γ^+ T cells (Figure 4A).

The mXCL1.var-PyCSP (CP) group had the overall highest mean SFU per 10^6 splenocytes of PyCSP-specific IFN-γ^+ T cells among the experimental groups, which was significantly higher compared to the PyCSP (CP) group ($p<0.01$), PyCSP (SP) group ($p<0.0001$), and the mXCL1.var-PyCSP (SP) group ($p<0.05$) (Figure 4B). The mXCL1.var-PyCSP (SP) group, on the other hand, had no statistically significant differences compared to the PyCSP (SP) and PyCSP (CP) groups (Figure 4B). This shows that while cluster priming still elicits greater immunogenic responses, single priming may potentially be sufficient using this P&T vaccine regimen. The immunogenicity elicited by single priming with the mXCL1-PyCSP fusion protein vaccine is comparable to cluster priming with the standard unfused PyCSP vaccine, suggesting the potential for a refinement in the regular cluster priming regimen to a single priming regimen, improving the overall vaccine scheduling efficiency. In addition, the individual data points of the mXCL1.var-PyCSP (SP) and mXCL1.var-PyCSP (CP) groups showed less animal-to-animal variation compared to the PyCSP (SP) and PyCSP (CP) groups (Figure 4B), further enhancing the vaccine by eliciting more consistent immunogenic responses.

The mXCL1-PyCSP vaccine similarly protects female mice during PySPZ challenge

To evaluate the protection induced by the mXCL1-PyCSP fusion protein P&T vaccine compared to the standard unfused PyCSP vaccine, we conducted a P&T experiment using the immunization/challenge model. Female BALB/cJ mice (*n* = 25) were divided equally into 5 groups (*n* = 5 per group) – mXCL1 Parent (negative control), mXCL1.var Parent (negative control), mXCL1-

PyCSP (mXCL1-WT and PyCSP fusion protein), mXCL1.var-PyCSP (mXCL1.var and PyCSP fusion protein), and PyCSP (positive control as previously optimized [14]). Mice were ggDNA vaccinated using two vaccine cartridges on Day 0 (single prime only on Day 0), and then vaccinated (trapped) with 2 x 10^4 freshly-dissected PyRAS administered retro-orbitally on Day 28. On Day 56, a challenge dose of 1 x 10^3 freshly-dissected PySPZ was administered retro-orbitally. Subsequently, 3–14 days post-challenge (Days 59–70), the mice were bled by tail prick and thin blood smear microscopy performed to ascertain if they were protected from challenge (Figure 5A). The mice were then humanely euthanized using CO_2 asphyxiation either following a positive blood smear result or at the end of the experiment on Day 70.

The mXCL1-PyCSP and mXCL1.var-PyCSP groups had 100% protection rates post-challenge, which was the same as the standard PyCSP group (Figure 5B). This suggested that fusion with mXCL1 did not reduce the efficacy of the PyCSP P&T vaccine. However, without additional studies to dose de-escalate the standard unfused PyCSP vaccine to achieve sub-therapeutic outcomes, we cannot determine if fusion with mXCL1 has a favorable effect on protection because the standard unfused PyCSP vaccine already achieved complete sterile protection in our female mice using our standard P&T immunization/challenge model [14].

Discussion

The estimated number of global malaria cases in 2022 exceeded pre-COVID-19 pandemic levels in 2019 by 7%, and the number in 2022 was 55% higher than it should have been if the 2025 Global Technical Strategy for Malaria targets are to be met [3]. This highlights the importance of a more effective and efficacious vaccine that can prevent clinical disease and stop further transmission of malaria. Fusing antigens to chemokines to target antigen presenting cells is a promising method for enhancing immunogenicity of DNA vaccines [41]. XCL1 fusion protein vaccines selectively target XCR1-expressing DCs, which excel at cross-presenting antigens to naïve $CD8^+$ T cells and enhancing proliferation of $CD4^+$ T cells [16, 20, 21]. In this study, we assessed immunogenicity and protection outcomes using the mXCL1-PyCSP fusion protein P&T vaccine through a series of immunogenicity and P&T immunization/challenge experiments in male and female BALB/cJ mice.

Recent studies assessing the use of XCL1-antigen fusion DNA vaccines in other infectious diseases have shown to enhance immune responses and induces protection against infection. For example, a SARS-CoV-2 immunogenicity study of the mXCL1-spike fusion DNA vaccine published in 2022 revealed that vaccination of 8-week-old C57BL/6 mice using the fusion vaccine significantly increased the number of IFN-γ^+ T cells in the spleens by ELISPOT assay [30]. In an influenza study published in 2015, vaccination of 6–10-week-old BALB/c mice with the mXCL1-hemagglutinin fusion vaccine also saw significant increases in the numbers of IFN-γ^+ T cells in the spleens by ELISPOT assay, while vaccination mediated full protection against lethal challenge with

influenza A virus [29]. Similarly in the present study, vaccination with the mXCL1-PyCSP fusion vaccine in both male and female BALB/cJ mice significantly increased the numbers of IFN-γ^+ T cells in the spleens by ELISPOT assay ($p < 0.0001$ and $p < 0.01$, respectively) compared to the standard unfused PyCSP vaccine. These studies highlight the fusion of XCL1 to antigens' capability of enhancing the immunogenicity of DNA vaccines, and the XCR1 receptor as a very promising target for vaccine delivery. While other receptors on murine CD8α^+ DCs, such as CD205 (DEC-205) and Clec9A , have also previously shown good induction of CD8$^+$ T cell responses [42-44], they lack the specificity for a functional DC subset to elicit highly specific immune responses as compared to XCL1. CD205 is expressed in a variety of cells, including B cells as well as thymic and intestinal epithelia [45], while Clec9A is also expressed on plasmacytoid DCs (pDCs) and a subset of CD24$^+$ blood DCs [43, 44]. By being expressed only on the subset of DCs (cDC1s) preferentially interacting with components of cytotoxic immunity (NK cells and CD8$^+$ T cells), XCL1 might be able to direct an entry port for vaccines against intracellular pathogens such as *Plasmodium* or *Mycobacterium* [20].

Interestingly, there were no statistical differences between the groups that received vaccines fused with the less stable mXCL1-WT and the highly active mXCL1-V21C/A59C across all experiments that compared them in the present study. This is in contrast to the original study assessing the newly generated mXCL1-V21C/A59C variant, where intradermal (ID) administration of mXCL1-V21C/A59C, but not that of mXCL1-WT, significantly increased the accumulation of XCR1-expressing DCs at the injection site [18]. Perhaps, the disparity could have been attributed to the differences in routes of administration (TD injection-less gene gun vs ID injection). Indeed,

further studies could be conducted to assess the differences associated with the routes of administering mXCL1 in how they affect recruitment and stimulation of XCR1-expressing DCs as well as the associated immune response activation. More likely, the mere fusion of chemokine to antigen might be the impetus in increasing immunogenicity and act as a molecular adjuvant of sorts, regardless of the degree of stability or functional activity of the chemokine in its unfused state. This is consistent with our experiments in the present study utilizing separate mXCL1 and PyCSP plasmids, where vaccinating with separate plasmids did not show any statistically significant differences compared to the other groups that were vaccinated with the PyCSP vaccine. The structural stability of mXCL1-WT or mXCL1-V21C/A59C after fusion with an antigen has also not been studied before, and elucidation of this could be helpful in understanding the structural conformation of XCL1 when part of fusion protein DNA vaccines.

Further insight into assessing the immunogenicity of the mXCL1-PyCSP vaccine as well as the expression of vaccine constructs could be performed to better characterize the cell-mediated and humoral immune responses generated by this vaccine. For example, investigating the PyCSP-specific humoral immune responses using antigen binding ELISAs for IgG antibody levels would be important in evaluating the humoral effects of the mXCL1-PyCSP vaccine. Bone marrow could also be harvested post-vaccination to evaluate whether PyCSP fusion with mXCL1 could increase long-lived antibody-secreting plasma cells in the bone marrow. In order to evaluate the difference in the frequency of IFN-γ^+ $CD4^+$ and $CD8^+$ T cells post-vaccination, a flow cytometry endpoint should also be considered. In addition, western blot assays could also be performed on muscle

tissues and draining lymph nodes in order to analyze the expression and function of the mXCL1-PyCSP fusion protein and DNA vaccine constructs after transfection.

An important thing to bear in mind is that increased immunogenicity may not necessarily correlate to increased protection in malaria vaccinology. In this study, we have shown that vaccination with the mXCL1-PyCSP vaccine maintains the 100% protection rate in female BALB/cJ mice as with the standard unfused PyCSP vaccine which has previously been optimized [14]. While this suggests that fusion with mXCL1 is unlikely to adversely impact protection outcomes, the current data is unable to show that it improves protection outcomes when all mice under this P&T regimen are already protected 100% of the time. Instead, further studies using suboptimal priming and/or trapping doses with significantly increased challenge doses should be performed in order to investigate any differences in protection outcomes. There is also a lack of male animal usage in the present study, mainly due to the known low P&T protection rates and sex-specific differences seen in male mice [46, 47]. We have recently identified that the presence of androgens at the time of challenge inhibits protective cellular immune responses, despite forming functional memory responses following P&T vaccination [47]. It will also be pertinent to conduct standard P&T experiments with the mXCL1-PyCSP vaccine in male mice to assess if fusion with mXCL1 significantly increases protection outcomes.

While the XCL1 chemokine can be found in many different animal species, including non-mammalian species such as birds and reptiles, its expression is conserved among mammalian

species [16, 48]. This could be very useful in the translational aspects of XCL1 studies, where investigations into the expression and function of the chemokine in mice may translate feasibly well into large animal studies and human clinical trials. However, murine XCL1 is still fairly different compared to human XCL1, where they only share 61.4% amino acid identity and are 84% similar (NCBI BLASTP) [16, 20]. Expression patterns of surface receptors also often vary between species, making it difficult to relate observations in mice to other animal species, including humans. In addition, X-C motif chemokine ligand 2 (XCL2), the only other chemokine in the C class of chemokines, is only found in humans and several large animal species including sheep, pigs, macaques, and other nonhuman primates (NHPs), while not being present in mice. Based on amino acid sequencing, it also appears that the XCL1 and XCL2 sequences in humans, macaques, and other NHPs are more closely related compared to that of sheep and pigs [48]. The presence of XCL2 in humans and NHPs may present significant differences in the interactions within the XCL1-XCR1 axis compared to that of species that do not have XCL2, including mice. Thus, the XCL1-CSP fusion protein vaccine will eventually need to be evaluated in NHPs preclinically if we hope to one day bring this to human clinical trials. To date, there are currently no known studies evaluating XCL1-antigen fusion vaccines in NHPs at all. With the commercial availability of macacine XCL1 for research use, albeit the less stable wild-type variant, further studies evaluating the use of XCL1-antigen fusion vaccines in NHPs would be warranted.

Due to major advancements in vaccine technology, nucleic acid-based vaccines have been evaluated for use in a number of clinical applications, including cancer, allergy, and infectious diseases in the past few decades [49]. Another nucleic acid vaccine platform that achieved global

awareness due to its first commercialization and emergency use following the COVID-19 pandemic is the messenger RNA (mRNA) vaccine, and its application to a wide variety of other diseases is now being investigated. The mRNA molecule, unlike DNA, has no risk of unintentional insertion into genomic DNA [50]. mRNA vaccines also have the potential for rapid, cost-effective, and scalable manufacturing mainly due to the high yields of in vitro transcription reactions [51]. In addition, mRNA does not require nuclear delivery and can efficiently express the encoded genes following proper introduction into the cytoplasm. However, this can be difficult to achieve due to the properties of mRNA, including instability in an in vivo environment and cell membrane impermeability that prevent it from reaching the cytoplasm efficiently, where it functions [49]. Thus, a variety of carriers have been developed for in vivo degradation protection and efficient cytoplasmic delivery, of which lipid nanoparticles (LNPs) are currently favored [49, 51, 52]. However, the major drawback is that the mRNA vaccine in itself is not cell-specific and can be taken up by almost any cell type, lacking specificity for DCs, which are the most important and effective cells for antigen presentation. Recent studies have proposed several methods for mRNA vaccines to target DCs more efficiently and overcome this barrier, including regulating the size of mRNA-loaded LNPs to between 200–500 nm which showed to target splenic DCs [49], as well as designing a DC-targeting virus-like particle with an engineered Sindbis-virus glycoprotein that recognizes a surface protein on DCs [52]. Since XCL1 specifically targets cDC1s which exclusively express XCR1, we could leverage the interactions within the XCL1-XCR1 axis and investigate the possibility of designing an XCL1-CSP (or other malarial antigen) mRNA fusion vaccine. To date, there are currently no known studies evaluating XCL1-antigen mRNA fusion vaccines or the usage of XCL1 in mRNA-based vaccines.

In summary, we have shown that the fusion of PyCSP with the mXCL1 chemokine enhances cell-mediated immune responses and significantly increased immunogenicity in male and female BALB/cJ mice, while not hampering protection outcomes in female mice. Fusion with mXCL1 may also reduce the need for cluster priming and improve vaccine scheduling. Due to the strong cellular immune responses generated, fusion with mXCL1 could be considered for usage as a standard molecular adjuvant in all future studies. Further studies are needed to assess the immunogenicity and protection induced by the mXCL1-PyCSP fusion protein P&T DNA vaccine using other experimental setups and endpoints as discussed. Overall, the data obtained in the present study could contribute to the overall goal of improving the efficacy of pre-erythrocytic malaria vaccines.

Figures

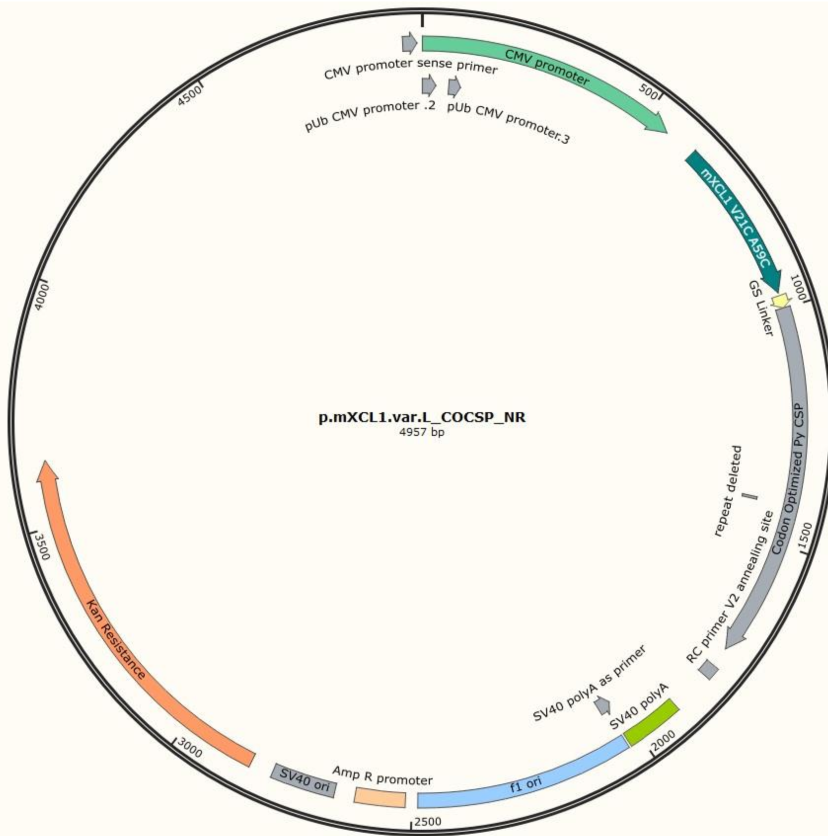


Figure 1. Schematic diagram of the mXCL1-PyCSP fusion protein P&T DNA vaccine construct.

The mXCL1 sequence begins with a Kozak consensus sequence (with the start codon but without the stop codon), followed by a glycine-serine (GS) linker that connects mXCL1 to PyCSP (full-length, without repeat regions GPGAPQ and PPQQ), and ends with a stop codon.

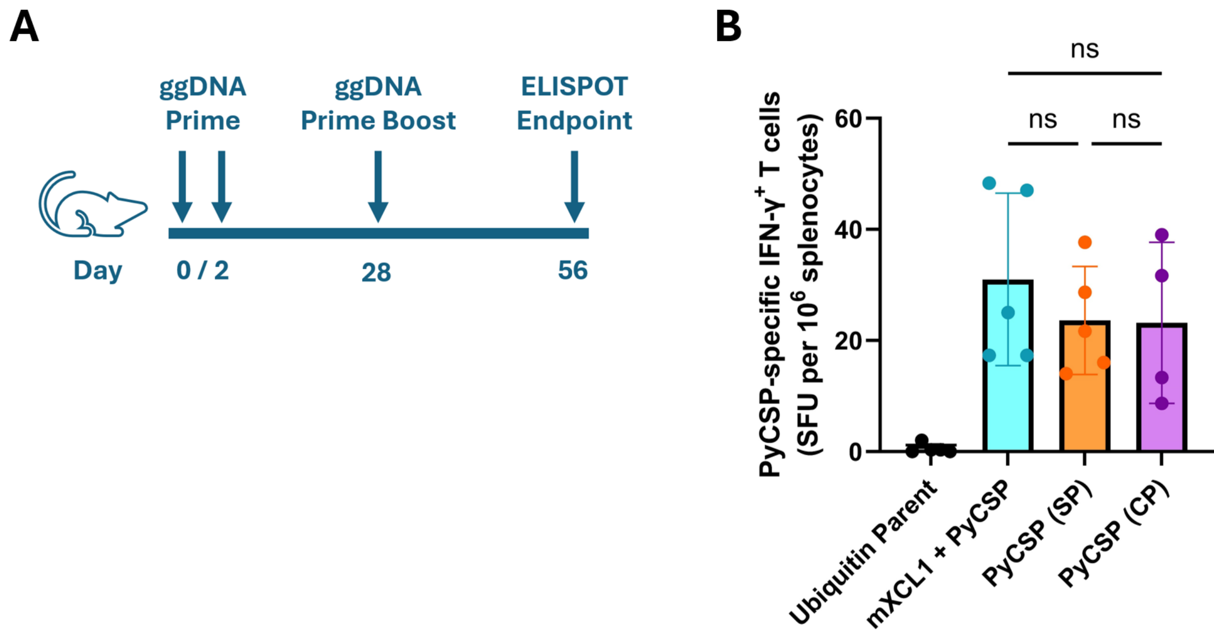


Figure 2. Vaccination with separate PyCSP and mXCL1 plasmids do not efficiently target DCs. (A) Experimental design of pilot immunogenicity experiment using separate PyCSP and mXCL1 plasmids. **(B)** PyCSP-specific IFN-γ^+ T cells in the spleen by ELISPOT assay from pilot immunogenicity experiment using female BALB/cJ mice stimulated with PyCSP peptide (SYVPSAEQI) or DMSO vehicle control. Error bars represent mean ± SD from n = 4–5 mice per group. Data was analyzed using ordinary one-way ANOVA with Šídák's multiple comparisons test; ns $p > 0.05$.

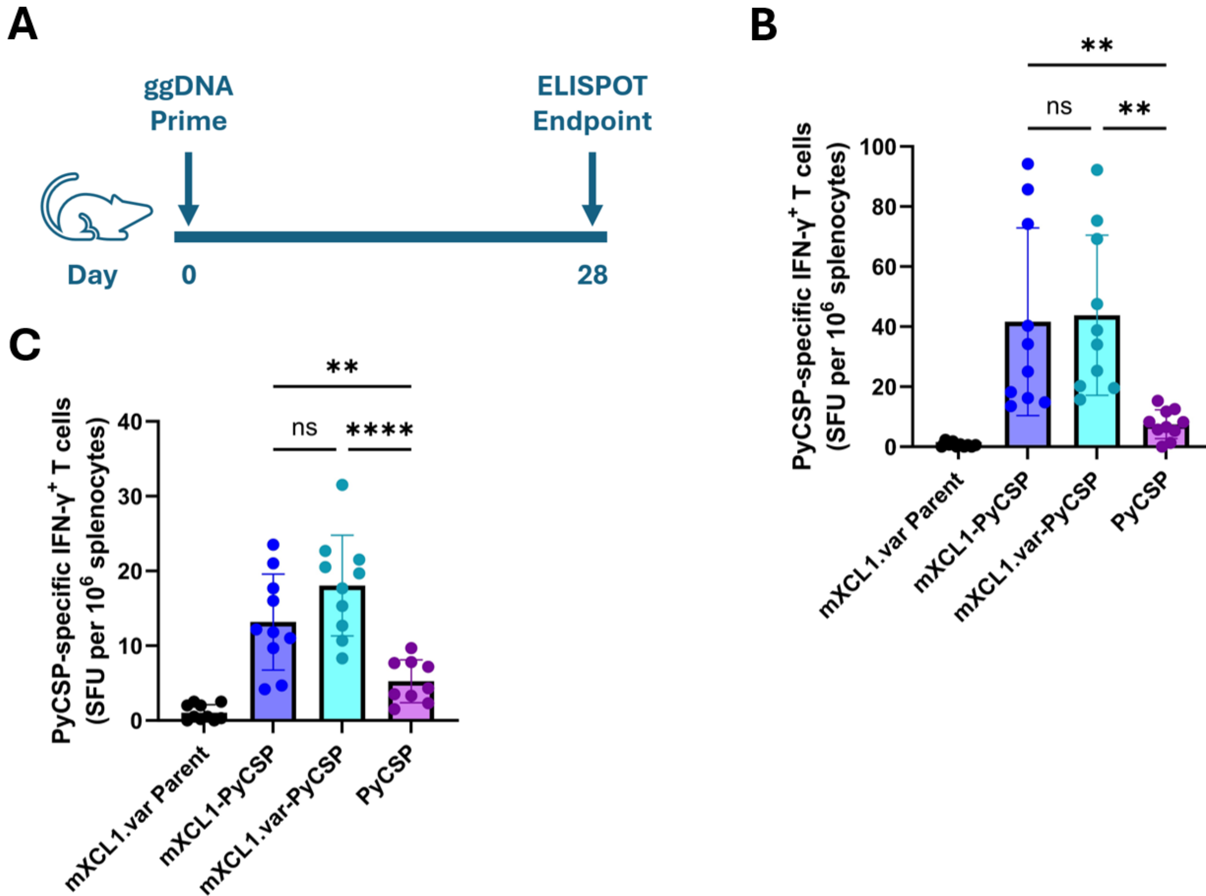


Figure 3. The mXCL1-PyCSP vaccine significantly increases immunogenicity in male and female mice. (A) Experimental design of immunogenicity experiments in male and female BALB/cJ mice using the mXCL1-PyCSP fusion protein vaccine. **(B)** PyCSP-specific IFN-γ$^+$ T cells in the spleen by ELISPOT assay from immunogenicity experiments using female BALB/cJ mice stimulated with PyCSP peptide (SYVPSAEQI) or DMSO vehicle control. Error bars represent mean ± SD from n = 10 mice per group across two experiments. **(C)** PyCSP-specific IFN-γ$^+$ T cells in the spleen by ELISPOT assay from immunogenicity experiments using male BALB/cJ mice stimulated with PyCSP peptide (SYVPSAEQI) or DMSO vehicle control. Error bars represent mean ± SD from n = 9–10 mice per group across two experiments. Data was analyzed using ordinary one-way ANOVA with Šídák's multiple comparisons test; ****p < 0.0001, **p < 0.01, ns p > 0.05.

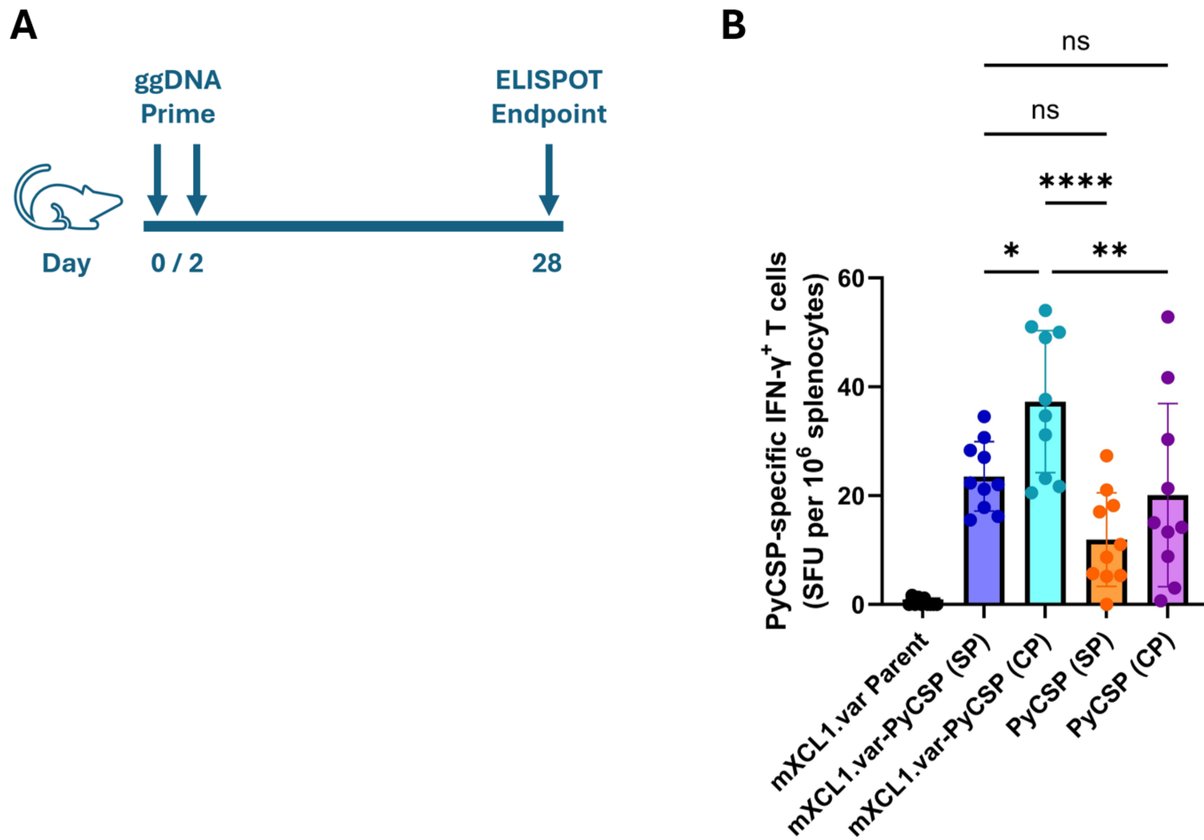
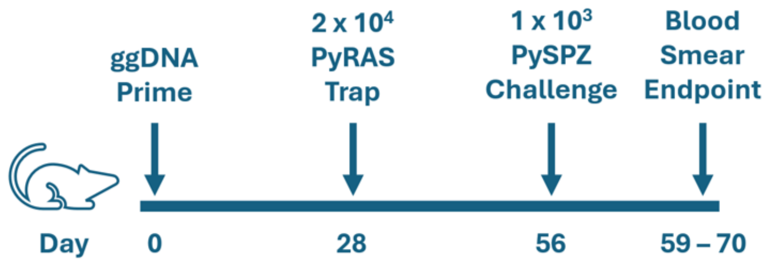


Figure 4. The mXCL1-PyCSP vaccine decreases the need for cluster priming in female mice. (A) Experimental design of single vs cluster prime immunogenicity experiments in female BALB/cJ mice using the mXCL1-PyCSP fusion protein vaccine. **(B)** PyCSP-specific IFN-γ^+ T cells in the spleen by ELISPOT assay from single vs cluster prime immunogenicity experiments using female BALB/cJ mice stimulated with PyCSP peptide (SYVPSAEQI) or DMSO vehicle control. Error bars represent mean ± SD from $n = 10$ mice per group across two experiments. Data was analyzed using ordinary one-way ANOVA with Šídák's multiple comparisons test; ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$, ns $p > 0.05$.



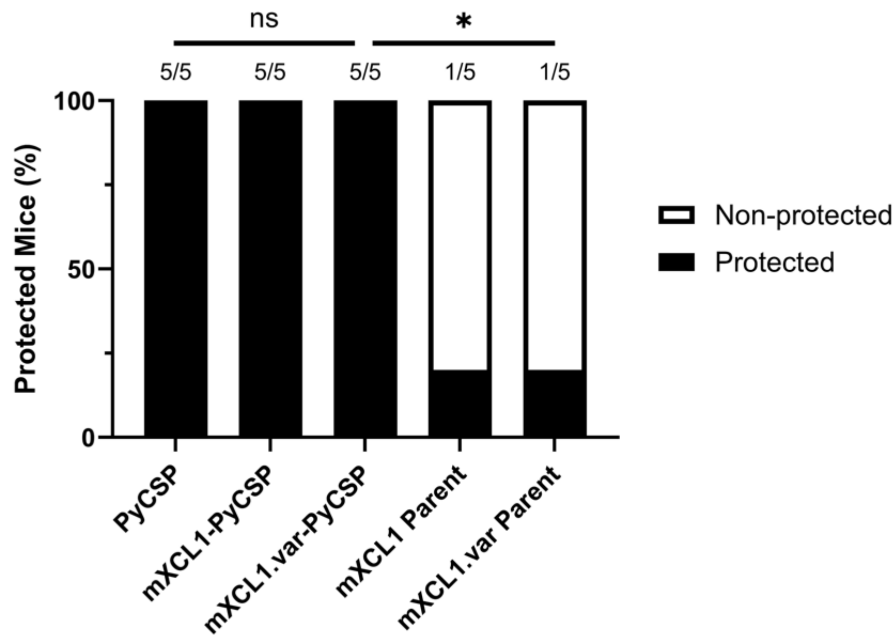


Figure 5. The mXCL1-PyCSP vaccine similarly protects female mice during PySPZ challenge. (A) Experimental design of P&T vaccine experiment in female BALB/cJ mice using the mXCL1-PyCSP fusion protein vaccine. **(B)** Percentages of mice protected against live challenge with 1x10^3 *P. yoelii* sporozoites (PySPZ) by blood smears through 14 days post-challenge. Data was analyzed using Fisher's exact test; *$p < 0.05$, ns $p > 0.05$.

References

1. Poespoprodjo, J.R., et al., *Malaria.* Lancet, 2023. **402**(10419): p. 2328-2345.

2. Fikadu, M. and E. Ashenafi, *Malaria: An Overview.* Infect Drug Resist, 2023. **16**: p. 3339-3347.

3. WHO, *World Malaria Report 2023*. 2023, World Health Organization: Geneva.

4. WHO. *WHO recommends groundbreaking malaria vaccine for children at risk*. 2021; Available from: https://www.who.int/news/item/06-10-2021-who-recommends-groundbreaking-malaria-vaccine-for-children-at-risk.

5. WHO. *WHO recommends R21/Matrix-M vaccine for malaria prevention in updated advice on immunization*. 2023; Available from: https://www.who.int/news/item/02-10-2023-who-recommends-r21-matrix-m-vaccine-for-malaria-prevention-in-updated-advice-on-immunization.

6. WHO. *Malaria vaccines (RTS,S and R21)*. 2024; Available from: https://www.who.int/news-room/questions-and-answers/item/q-a-on-rts-s-malaria-vaccine.

7. WHO. *WHO prequalifies a second malaria vaccine, a significant milestone in prevention of the disease*. 2023; Available from: https://www.who.int/news/item/21-12-2023-who-prequalifies-a-second-malaria-vaccine-a-significant-milestone-in-prevention-of-the-disease.

8. Beeson, J.G., et al., *The RTS,S malaria vaccine: Current impact and foundation for the future.* Sci Transl Med, 2022. **14**(671): p. eabo6646.

9. Samuels, A.M., et al., *Efficacy of RTS,S/AS01(E) malaria vaccine administered according to different full, fractional, and delayed third or early fourth dose regimens in children aged 5-17 months in Ghana and Kenya: an open-label, phase 2b, randomised controlled trial.* Lancet Infect Dis, 2022. **22**(9): p. 1329-1342.

10. Datoo, M., et al., *Safety and efficacy of malaria vaccine candidate R21/Matrix-M in African children: a multicentre, double-blind, randomised, phase 3 trial.* Lancet (London, England), 2024. **403**.

11. Mariano, R., et al., *A Review of Major Patents on Potential Malaria Vaccine Targets.* Pathogens, 2023. **12**(2).

12. Foquet, L., et al., *Vaccine-induced monoclonal antibodies targeting circumsporozoite protein prevent Plasmodium falciparum infection.* J Clin Invest, 2014. **124**(1): p. 140-4.

13. Fernandez-Ruiz, D., et al., *Liver-Resident Memory CD8+ T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection.* Immunity, 2016. **45**(4): p. 889-902.

14. Olsen, T.M., et al., *Prime-and-Trap Malaria Vaccination To Generate Protective CD8(+) Liver-Resident Memory T Cells.* J Immunol, 2018. **201**(7): p. 1984-1993.

15. Watson, F.N., et al., *Cryopreserved Sporozoites with and without the Glycolipid Adjuvant 7DW8-5 Protect in Prime-and-Trap Malaria Vaccination.* Am J Trop Med Hyg, 2022. **106**(4): p. 1227-1236.

16. Lei, Y. and Y. Takahama, *XCL1 and XCR1 in the immune system.* Microbes Infect, 2012. **14**(3): p. 262-267.

17. Tyler, R.C., et al., *Native-state interconversion of a metamorphic protein requires global unfolding.* Biochemistry, 2011. **50**(33): p. 7077-9.

18. Matsuo, K., et al., *A Highly Active Form of XCL1/Lymphotactin Functions as an Effective Adjuvant to Recruit Cross-Presenting Dendritic Cells for Induction of Effector and Memory CD8+ T Cells.* Front Immunol, 2018. **9**.

19. Volkman, B.F., T.Y. Liu, and F.C. Peterson, *Chapter 3. Lymphotactin structural dynamics.* Methods Enzymol, 2009. **461**: p. 51-70.

20. Kroczek, R.A. and V. Henn, *The Role of XCR1 and its Ligand XCL1 in Antigen Cross-Presentation by Murine and Human Dendritic Cells.* Front Immunol, 2012. **3**.

21. de Souza-Silva, G.A., F.B. Sulczewski, and S.B. Boscardin, *Recombinant antigen delivery to dendritic cells as a way to improve vaccine design.* Exp Biol Med (Maywood), 2023. **248**(19): p. 1616-1623.

22. Crozat, K., et al., *Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8α+ type.* J Immunol, 2011. **187**(9): p. 4411-5.

23. Dorner, B.G., et al., *Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells.* Immunity, 2009. **31**(5): p. 823-33.

24. Crozat, K., et al., *The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells.* J Exp Med, 2010. **207**(6): p. 1283-92.

25. Bachem, A., et al., *Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells.* J Exp Med, 2010. **207**(6): p. 1273-81.

26. Dutertre, C.A., et al., *TLR3-responsive, XCR1+, CD141(BDCA-3)+/CD8α+-equivalent dendritic cells uncovered in healthy and simian immunodeficiency virus-infected rhesus macaques.* J Immunol, 2014. **192**(10): p. 4697-708.

27. Kamei, M., et al., *Transcutaneous immunization with a highly active form of XCL1 as a vaccine adjuvant using a hydrophilic gel patch elicits long-term CD8(+) T cell responses.* J Pharmacol Sci, 2020. **143**(3): p. 182-187.

28. Lysén, A., et al., *Intranasal delivery of a cDC1 targeted influenza vaccine with poly(I:C) enhances T cell responses and protects against influenza infection.* Scand J Immunol, 2022. **95**(3): p. e13128.

29. Fossum, E., et al., *Vaccine molecules targeting Xcr1 on cross-presenting DCs induce protective CD8+ T-cell responses against influenza virus.* Eur J Immunol, 2015. **45**(2): p. 624-35.

30. Qi, H., et al., *Immunogenicity of the Xcl1-SARS-CoV-2 Spike Fusion DNA Vaccine for COVID-19.* Vaccines (Basel), 2022. **10**(3).

31. Li, K., et al., *Molecular vaccine prepared by fusion of XCL1 to the multi-epitope protein of foot-and-mouth disease virus enhances the specific humoural immune response in cattle.* Appl Microbiol Biotechnol, 2017. **101**(21): p. 7889-7900.

32. Bernelin-Cottet, C., et al., *A DNA Prime Immuno-Potentiates a Modified Live Vaccine against the Porcine Reproductive and Respiratory Syndrome Virus but Does Not Improve Heterologous Protection.* Viruses, 2019. **11**(6).

33. Botelho, N.K., et al., *Combination of Synthetic Long Peptides and XCL1 Fusion Proteins Results in Superior Tumor Control.* Front Immunol, 2019. **10**: p. 294.

34. Chen, K., et al., *XCL1/Glypican-3 Fusion Gene Immunization Generates Potent Antitumor Cellular Immunity and Enhances Anti-PD-1 Efficacy.* Cancer Immunol Res, 2020. **8**(1): p. 81-93.

35. Mizumoto, Y., et al., *Anticancer effects of chemokine-directed antigen delivery to a cross-presenting dendritic cell subset with immune checkpoint blockade.* Br J Cancer, 2020. **122**(8): p. 1185-1193.

36. Zhang, K., et al., *The XCL1-Mediated DNA Vaccine Targeting Type 1 Conventional Dendritic Cells Combined with Gemcitabine and Anti-PD1 Antibody Induces Potent Antitumor Immunity in a Mouse Lung Cancer Model.* Int J Mol Sci, 2024. **25**(3): p. 1880.

37. Stone, B.C., et al., *Complex Minigene Library Vaccination for Discovery of Pre-Erythrocytic Plasmodium T Cell Antigens.* PLoS One, 2016. **11**(4): p. e0153449.

38. Imai, J., et al., *Purification of the subcellular compartment in which exogenous antigens undergo endoplasmic reticulum-associated degradation from dendritic cells.* Heliyon, 2016. **2**(9): p. e00151.

39. Kennedy, M., et al., *A rapid and scalable density gradient purification method for Plasmodium sporozoites.* Malar J, 2012. **11**: p. 421.

40. Murphy, S.C., et al., *A T-cell response to a liver-stage Plasmodium antigen is not boosted by repeated sporozoite immunizations.* Proc Natl Acad Sci U S A, 2013. **110**(15): p. 6055-60.

41. Lysén, A., et al., *Dendritic cell targeted Ccl3- and Xcl1-fusion DNA vaccines differ in induced immune responses and optimal delivery site.* Sci Rep, 2019. **9**(1): p. 1820.

42. Bonifaz, L., et al., *Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance.* J Exp Med, 2002. **196**(12): p. 1627-38.

43. Caminschi, I., et al., *The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement.* Blood, 2008. **112**(8): p. 3264-73.

44. Sancho, D., et al., *Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin.* J Clin Invest, 2008. **118**(6): p. 2098-110.

45. Witmer-Pack, M.D., et al., *Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues.* Cell Immunol, 1995. **163**(1): p. 157-62.

46. Watson, F.N., et al., *Sex-Specific Differences in Cytokine Induction by the Glycolipid Adjuvant 7DW8-5 in Mice.* Biomolecules, 2022. **13**(1).

47. Duncombe, C.J., et al., *Androgens inhibit protective CD8+ T cell responses against pre-erythrocytic malaria parasites*. 2024: Nat Commun, under review.

48. Fox, J.C., et al., *Structural and agonist properties of XCL2, the other member of the C-chemokine subfamily.* Cytokine, 2015. **71**(2): p. 302-11.

49. Sasaki, K., et al., *mRNA-Loaded Lipid Nanoparticles Targeting Dendritic Cells for Cancer Immunotherapy.* Pharmaceutics, 2022. **14**(8).

50. Pardi, N., et al., *mRNA vaccines - a new era in vaccinology.* Nat Rev Drug Discov, 2018. **17**(4): p. 261-279.

51. Clemente, B., et al., *Straight to the point: targeted mRNA-delivery to immune cells for improved vaccine design.* Front Immunol, 2023. **14**.

52. Yin, D., et al., *Dendritic-cell-targeting virus-like particles as potent mRNA vaccine carriers.* Nat Biomed Eng, 2024.