New approaches to pre-erythrocytic malaria vaccination

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New approaches to pre-erythrocytic malaria vaccination

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Abstract

Malaria is an infectious parasitic disease and a global threat responsible for an estimated 200 million cases and half a million deaths every year. Although progress has been made in recent years with a number of vaccines advancing into later stages of clinical trials, a vaccine with both high efficacy and durability still remains a target to achieve. This thesis presents the results of pre-clinical studies performed with the aim to obtain an improved version of the existing R21 vaccine capable of enhancing immunogenicity, protective efficacy and durability. With this aim, a set of novel R21 variants were designed exploring all immunogenic regions of the *Plasmodium falciparum* circumsporozoite protein in a VLP vaccine platform. Along with creating new variants, an alternative strategy of using pre-formed immune complexes consisting of R21 complexed with monoclonal antibody 2A10 to the central NANP repeats has been explored. With this strategy already in use for animal vaccines, promising published results obtained with Zika virus, and still unexplored for parasitic infections, I considered to apply this approach to malaria and have obtained promising results. Glycans linked to parasitic surface proteins remain relatively less explored in comparison to proteins and are capable of eliciting immune response that might hold protective potential. With this aim, the last results chapter of this thesis describes results obtained from using sporozoite surface specific glycan alpha- galactose as a vaccine candidate.

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List of abbreviations

ALP	Alkaline phosphatase
AMA1	Apical membrane antigen 1
AnAPN1	*Anopheles* alanyl aminopeptidase N
AOX1	Alcohol Oxidase 1
AOX2	Alcohol Oxidase 2
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BCR	B cell receptor
BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered methanol-complex Medium
BSA	Bovine serum albumin
CD	Cluster of differentiation
CE	Cleavage epitope
CHMI	Controlled human malaria infection
COVID	Coronavirus disease
CR1	Complement receptor 1
CR2	Complement receptor 2
CSP	Circumsporozoite protein
CTD	C- terminal domain
CTL	Cytotoxic T lymphocyte
CVac	Chemoprophylaxis Vaccine
DC	Dendritic cell

DNA	Deoxy-ribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTP	Diphtheria Tetanus Pertusis
DW	Distilled water
EBA	Erythrocyte binding antigen
EBL	Erythrocyte-binding-like
EDTA	Ethylenediaminetetraacetic acid
EEF	Exo erythrocytic forms
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
EMP1	Erythrocyte membrane protein 1
EPA	Pseudomonas aeruginosa ExoProtein A
FDC	Follicular dendritic cell
GC	Germinal Center
GPI	Glycosylphosphatidylinositol
GRAVY	Grand average of hydropathy
GSK	GlaxoSmithKline
GT	Galactosyltransferase
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HEV	Hepatitis E virus
HGF	Hepatocyte growth factor
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human Papilloma virus

HSA	Human serum albumin
HSPGs	Heparan sulfate proteoglycans
IBD	Infectious bursa disease
IC	Immune complex
IEDB	Immune epitope Database
IFN	Interferon
IL	Interleukin
IM	Intra- muscular
IP	Intra- peritoneal
IRS	Indoor residual spray
IV	Intra- venous
JE	Junctional epitope
KO	Knock- out
LB	Luria- Bertani
LLIN	Long lasting insecticidal nets
LN	Lymph node
LPS	Laemmli protein sample
Mab	Monoclonal antibody
MD	Minimal dextrose
MDA5	Melanoma differentiation-associated protein 5
ME- TRAP	Multi-epitope Thrombospondin-related anonymous protein
MHC	Major histocompatibility complex
MM	Matrix-M
MSP	Merozoite surface protein
MVA	Modified vaccinia Ankara
NBT	Nitro blue tetrazolium

NDV	Newcastle Disease Virus
NHP	Non-human primate
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NK	Natural killer
NLRs	NOD like receptors
NOD	Nucleotide- binding oligomerization domain
OD	Optical density
PADRE	pan HLA DR-binding epitope
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate- buffered saline
PBST	Phosphate- buffered saline tween
pNPP	p-Nitrophenyl Phosphate, Disodium Salt
PRRs	Pattern recognition receptors
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
RH	Reticulocyte-binding-like homolog
Rh	Rhesus
RIC	Recombinant immune complex
RIG-1	Retinoic acid- inducible gene 1
RLRs	RIG-1 like receptors
RNA	Ribonucleic acid
RPMI	Rosewell park memorial institute
RT	Room temperature
SC	Subcutaneous
SDS- PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SEC	Size exclusion chromatography
SIV	Simian immunodeficiency virus
SOC	Super Optimal broth with Catabolite repression
SPZ	Sporozoites
TAE	Tris-acetate-EDTA
TBS	Tris- buffered saline
TEM	Transmission electron microscopy
TLRs	Toll- Like receptors
TNF	Tumor necrosis factor
TRAP	Thrombospondin-related anonymous protein
TSR	Thrombospondin type-1 repeat
VLP	Virus-Like Particle
WB	Western blot
WHO	World Health Organization
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

Disclosure statement

The results for chapter 3 relating to expression and production and chapter 4 illustrating immunogenicity and efficacy of R21 variant vaccines was done by me and Ahmed Salman. Ahmed Salman designed, cloned the constructs and the performed the animal experiments, while I performed the expression analysis for clone selection, downstream processing and analysis of purified material along with ELISA for immunogenicity assessment. Growth and scale up of cultures were performed by both of us.

For the other two chapters i.e. Immune complex (chapter 5) and Alpha-gal (chapter6) vaccination, I conceptualized the experiments, planned and performed them. Challenge studies were undertaken with help from Ahmed Salman.

Potential publications from this thesis

1. R21 vaccine variants are more efficacious in pre-clinical mouse models and hold the potential for in-human clinical trials.
2. Immune complex vaccination with R21 VLP complexed with monoclonal antibody 2A10 is immunogenic and induces sterile protection.
3. Alpha-galactose administered with adjuvant Matrix- M1 via intra-muscular route is immunogenic and a potential malaria pre-erythrocytic stage vaccine candidate.

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Chapter 1

Introduction

1. Introduction

1.1 Malaria: Epidemiology

Malaria remains a global threat with 229 million cases recorded in 87 endemic countries, and causing 409,000 deaths in 2019 [1]. Malaria is transmitted in 97 countries in the world and factors like frequency of contact between infected mosquitos and humans depending upon vector density, their location and feeding habits play a determining role in the transmission [2]. Although a number of endemic countries in Asia, Americas and Africa have been declared malaria free, progress with improved malaria control has slowed down in high transmission areas [3]. Malaria is caused by parasites of the genus *Plasmodium* [4], and five species that cause malaria in humans are *P. falciparum*, *P. vivax*, *P. malarie*, *P. ovale*, *and P. knowlesi* [5]. *P. falciparum*, which is the most fatal and drug resistant of the five species that infect humans [6], accounted for 99.7% cases in Africa and 94% of the cases globally in 2019 [7, 8]. *P. vivax* malaria is more common in tropical, subtropical and temperate zones, especially in Asia and Latin America [9] with the highest burdens found in India, Pakistan, Ethiopia, and Sudan [10]. Africa is the most affected of all (figure 1a) due to a combination of factors like highly efficient *A. gambie* vector transmitting *P. falciparum*, weather conditions allowing year round transmission and scarcity of malaria control resources [11]. With about 125 million pregnancies are at risk of malaria every year, and 272,000 children aged less than five years dying due to malaria, pregnant women and children form the most vulnerable groups affected by malaria [12]. Among children over 5 years of age, over 200 million are at the risk of disease affecting not only their health and education, but also contributing to malaria transmission [13].

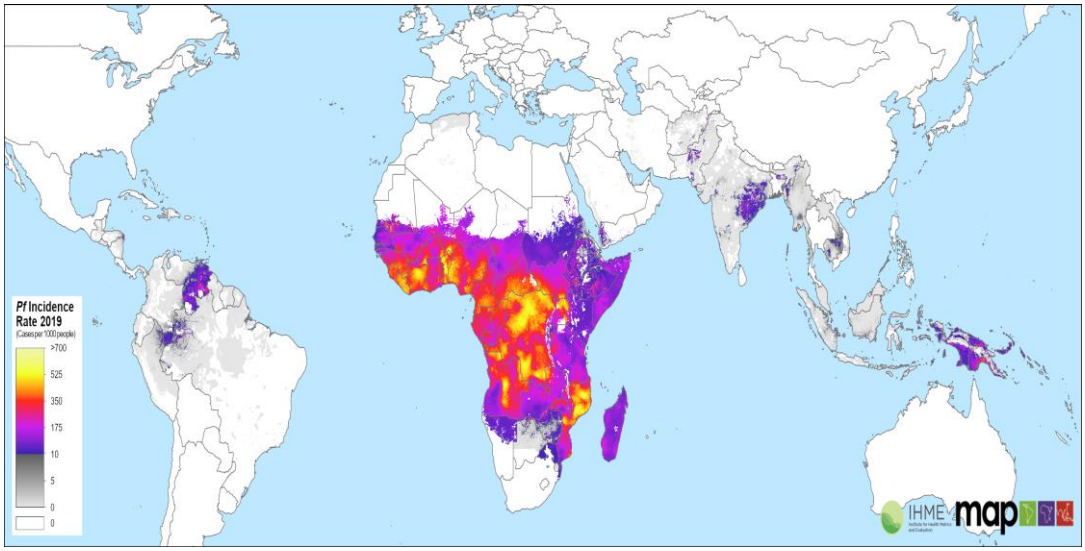


Figure 1a *P. falciparum* malaria global incidence rate for 2019 from the Malaria Atlas Project Directory [14].

1.2 Malaria: Eradication methods and challenges

Scale-up in malaria control efforts over the past 20 years have significantly reduced the disease burden [15]. Long- lasting insecticidal nets (LLIN) used as bed nets, treated with insecticides like pyrethroids, and indoor residual spray (IRS) have helped reduce malaria cases for over 15 years [16]. But over the years, use of the same pesticides in farming has led to resistance development in *Anopheles [17]*. Other factors rendering use of LLINs and IRS ineffective are changes in the biting behaviour of the vector, limited free distribution coverage for LLINs and higher demands than availability for IRS leading to poor usage adherence.[15]. Genetic mutations in the parasite leading to antibiotic resistance and those affecting diagnosis have an adverse effect on malaria control. A number of deletions for example in histidine-rich protein (pfhrp)2 and pfhrp3 genes have been identified in 11 countries rendering the parasite undetectable on rapid diagnostic testing, while mutations in *Pf*Kelch13 have been identified which confer partial resistance to artemisinin [8, 18]. Challenging efforts to identify novel targets that are safe, address the issue of drug- resistance and can cure malaria with single dose [12] have created promising new treatments like Artefenomel and Cipargamin which are parasite enzyme

and ion channel blocker respectively [19]. Climate change associated with global warming has also been highlighted as a concern as studies suggest that the resulting effect of this in the form of increased temperature, rainfall, and humidity might help the mosquito develop faster leading to increased transmission not only in malarious areas, but establish in non- malarious areas too [20]. Since 2000, approximately 10 clinical trials were registered for and 145 trials were completed targeting different stages of malaria life cycle, with most trials targeting the pre-erythrocytic stage. A number of vaccine trials have reached into advanced stages for e.g. pre-erythrocytic vaccines like RTSS/ AS01E (recommended for use by WHO), R21/MM (Phase III), whole sporozoite vaccine PfSPZ (Phase II). More than 20 blood stage vaccine clinical trials were registered between 2006-2010 with the number decreasing to 3 between 2016-2020 and have shown disappointing efficacy. The only promising candidate has been *Pf*RH5 (Phase II) which with the adjuvant AS01B has demonstrated antibody response longevity over 2 years and significant reduction in parasite growth rate in CHMI trial[21]. Since 2016, 2 trials related to placental malaria vaccines targeting chondroitin sulfate A -binding proteins on the parasites that uniquely sequester in the placenta, have been conducted and have been found to be safe, immunogenic and hence promising [22, 23]. Out of a total 11 clinical trials registered between 2000-2020, the candidate *Pfs*230 has entered Phase II[24]. More about development in the field of malaria vaccines is further described in 1.5 and current status of clinical trials is as in table 1a.

Vaccine	**Dosage**	**Phase**	**Population**	**Outcome; follow-up**
Vaccines targeting sporozoites				
PfSPZ	3 doses of 9×10^5	1	18–45 years, Tanzania	100% efficacy against CHMI at 23–79 days
	3 doses of 9×10^5	1	5–12 months, Kenya	Safe and immunogenic over 29 days

PfSPZ-CVac	2 doses of 2×10^5 with chloroquine	1	Malaria-naive adults	100% efficacy against CHMI at 3 months
GAP3KO	Bites from 200 infected mosquitoes	1	Malaria-naive adults	Vaccine efficacy pending (unpublished)
RTS,S	3 doses of 25 μg	3	5–17 months, multiple African sites	56% efficacy against first or only malaria episode over 12 months
	2 doses of 50 μg, 1 delayed dose of 10 μg	2	Malaria-naive adults	87% efficacy against CHMI at 3 weeks
	2 doses of 25 μg, 1 delayed dose of 5 μg	2	5–17 months; Ghana and Kenya	Vaccine efficacy pending (unpublished)
	RTS,S with seasonal malaria chemoprevention	3	5–17 months; Burkina Faso and Mali	Vaccine efficacy pending (unpublished)
R21	3 doses of 5 μg	2	5–17 months; Burkina Faso	71–76% efficacy against at least one malaria episode over 12 months (depending on adjuvant dosage)
	3 doses of 5 μg	3	5–36 months; multiple African sites	Vaccine efficacy pending (unpublished)
Vaccines targeting blood stages				
RH5.1	3 doses of 10 μg	2	Malaria-naive adults	1-day delay in parasitemia following CHMI at 14 days
	Dose escalation study	1	5–17 months, 18–45 years, Tanzania	Vaccine safety and immunogenicity pending (unpublished)
PAMVAC	3 doses of 50 μg	1	Malaria-naive adults	Safe and immunogenic over 6 months
PRIMVAC	3 doses of 100 μg	1	Burkina Faso	Safe and immunogenic over 35 days
Transmission-blocking vaccines				
Pfs230D1M	2 doses of 40 μg	1	Malaria-naive adults	Safe and immunogenic over 56 days
	3 doses of 40 μg	2	1 year and older; Mali	Vaccine efficacy pending (unpublished)

Table 1a: Current status of malaria vaccine trials. Modified from [24]

Of all, RTS,S is the only vaccine to have completed Phase III trial reducing clinical incidence by 39% and severe malaria by 31.5% among children aged 5–17 months [25]. RTS,S has been authorized for use as part of safety and efficacy assessments at some sites within 3 African countries Malawi, Kenya and Ghana. This implementation programme is aimed to address the safety concerns linked to increased number of deaths in girls and meningitis and cerebral malaria in vaccinated children [26], before approving it for likely wide-scale implementation, but only 10-15 million doses of RTS,S/AS01 are due to be manufactured per year in the 2020s [27]. In a Phase IIb trial, another vaccine R21 has shown excellent efficacy of 77% over 1 year in children, has entered Phase III trials and holds the potential to be the best vaccine developed so far [28]. The challenge in making a vaccine against malaria relates in part to the complex life cycle of the parasite. The malarial parasite life cycle includes (i) the pre-erythrocytic stage in humans, in which the parasite infects hepatocytes as sporozoites; followed by (ii) erythrocytic stage, in which the parasite reproduces asexually in RBCs in blood; and (iii) the sexual and sporogonic stage, in which the parasite reproduces sexually in Anopheline mosquitoes [29].

1.3 *P. falciparum* life cycle

P. falciparum has a complex life cycle alternating between *Anopheles* mosquitoes and vertebrate host that require formation of unique zoite forms to invade different cell types at specific stages [2].

1.3.1 Pre-erythrocytic stage

Sporozoites and skin

The dermis of the skin is the first site of sporozoite entry, since this is the depth to which the mosquito's proboscis reaches [30]. The sporozoites can stay active in the skin for several hours before initiating the liver stage infection [31]. Fewer than 50 sporozoites

per bite on average are likely inoculated during an infected mosquito bite in the wild [32]. Studies on *P. berghei* sporozoites injected in mice ear pinna indicate that ~ 25% of the sporozoites leave the skin by invading blood vessels and ~ 15% by lymphatic vessels, while ~60% remain in the skin [32]. The sporozoite motility of 1-2 μm/sec in dermis is maintained for initial 30 minutes and though sporozoites can move for more than 1 hour, their speed decreases with time [32]. Sporozoites glide in the dermis along the blood vessels wall are carried into the blood stream at 40-50 μm/sec similar to the speed of erythrocytes in the dermal capillary bed. This invasion concludes in less than 1 minute [33]. The dermal exit is aided by the motile nature of sporozoites along with their ability to traverse cells [30].

Sporozoites and Liver

The malarial parasite belongs to the phylum Apicomplexa, an intracellular parasite, growing and replicating within a nonphagosomal parasitophorous vacuole, a compartment bound to the membrane and segregated from most intracellular traffic routes [34]. Once the sporozoites have reached the blood, they are transported via the hepatic artery or the portal vein towards the hepatic sinusoids [34]. The liver architecture and blood flow are the key reasons for the parasite to infect the hepatocytes. Liver structure consists of fenestrated sinusoids [35], narrow capillaries with an average diameter of 10μm, in which the blood flow is slower than other capillaries [36]. Both the fenestrae and slowing of blood flow aids the malarial parasite in reaching hepatocytes. Another important feature is the presence and the high degree of sulfation of the Heparin sulfate proteoglycans (HSPGs) on liver cells which play a crucial role in this process through their interactions with the sporozoite surface protein called the circumsporozoite protein (CSP) [37]. To infect the hepatocytes, sporozoites have been shown to traverse through liver macrophages called Kupffer cells, which are located on the sinusoids; and

can provide sporozoites with direct access to space of Disse and hepatocytes [38]. The sporozoites also traverse through a number of hepatocytes, before initiating infection in one hepatocyte. Also, traversal of sporozoites make hepatocytes secrete Hepatocyte growth factor (HGF) which renders them susceptible to infection [39]. Once the sporozoite has established an infection in a hepatocyte, it transforms into an intracellular trophozoite within a parasitophorous vacuolar membrane (PVM) [40] and 10,000-30,000 of merozoites per invading sporozoite are released into the blood stream [41, 42]. The duration of this stage of the life cycle is ~2-3 days in rodent *Plasmodium* parasites and 7-14 days for humans depending on the parasite species, with 7 days typical of *P. falciparum* [43].

1.3.2 Blood stage

P. falciparum merozoites released in the blood initiate the asexual blood stage of the life cycle by invading the circulating RBCs, while *P. vivax* prefer and generally requires reticulocytes [44]. Merozoites attachment is likely to be mediated by proteins like the MSPs, EBLs and RHs, followed by attachment of *Pf*RH5 to the RBC receptor basigin [45]. After a series of successive events like initial low affinity interaction, followed by apical orientation of parasite, and formation of tight junctions mediated by these proteins, the invading merozoite enters the cell within ~ 60 seconds of initiation of invasion, forming a parasitophorous vacuole [44]. Here the merozoite undergoes ring, trophozoite and schizont stages multiplying to form 16 to 32 daughter merozoites [46]. During maturation to a trophozoite, the infected RBC is modified by PfEMP1 proteins which forms knobs on the surface, help the RBC to adhere to the microvasculature (sequestration) and prevent parasite clearance by the spleen[47]. The daughter merozoites are released into the blood stream after rupturing the RBC to invade other RBCs, thus continuing the asexual stage of the life cycle [46]. This leads to the

presentation of clinical symptoms and as the parasite biomass increases, can lead to severe anaemia, blood acidosis along with splenomegaly and hepatomegaly [42]. During this stage, some of these multi-nucleated forms within the RBCs commit to gametocytogenesis forming male and female gametocytes within 10-12 days. The transmission is ensured when these are taken up by the Anopheles mosquito where the sexual stage of life cycle ensues [48].

1.3.3 Sexual stage in mosquito

After ingestion by the mosquito during a blood meal, the gametocytes get activated when triggered by the midgut environment which involves a drop in temperature, rise in pH and calcium concentration and availability of the metabolite xanthurenic acid [49]. The activated male and female gametocytes fertilize to form a zygote which transforms into an ookinete. The ookinetes penetrate the midgut epithelium and transform into oocysts on the basal side of the midgut. Mature oocysts rupture within 12 to 14 days releasing thousands of sporozoites which invade the salivary glands [50]. These then reside in the salivary ducts and are delivered via the infected mosquito during the blood meal [51] as shown in figure 1b.

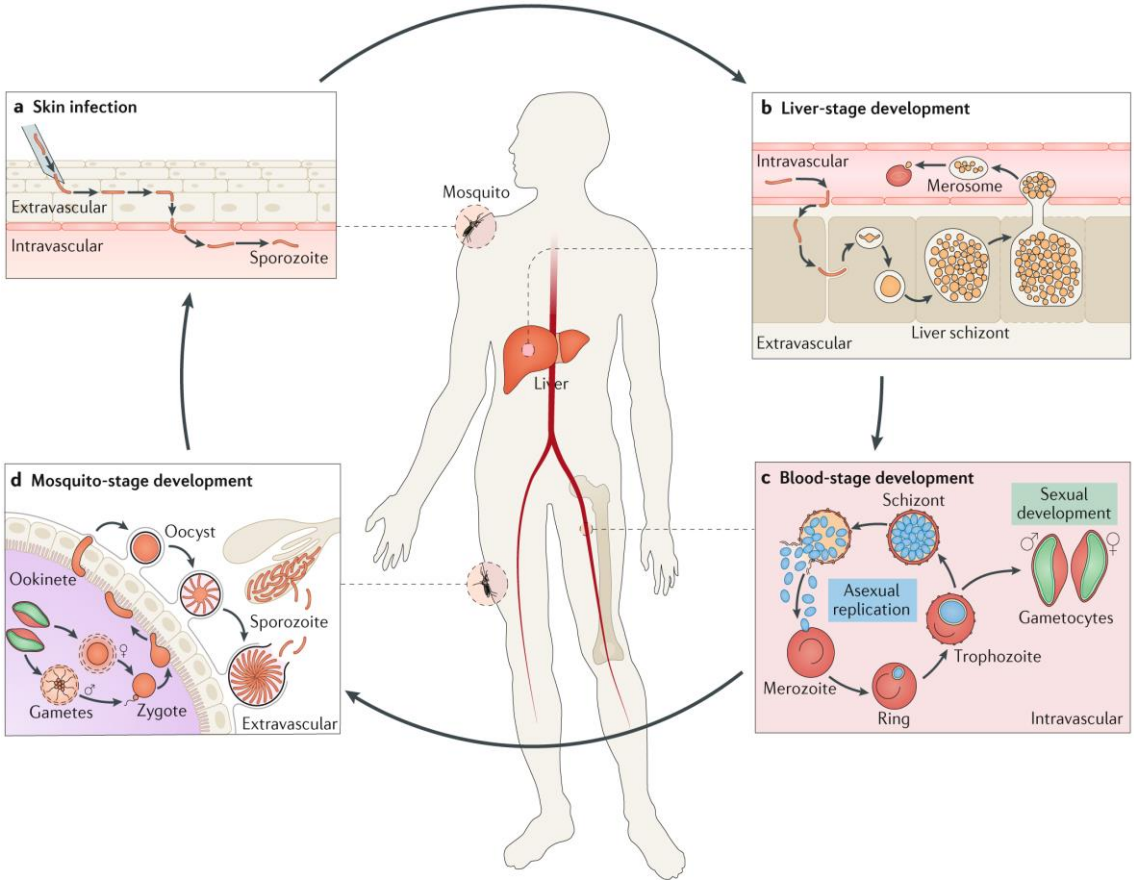


Figure 1b: Life cycle of *Plasmodium falciparum* in human and mosquito [52]. *P. falciparum* life cycle begins with the inoculation of sporozoites by *Anopheles* mosquito into humans during a blood meal. Sporozoites enter the blood stream and reach the liver to establish the clinically silent liver stage, part of the pre-erythrocytic stage of infection. Each sporozoite invading a hepatocyte produces thousands of daughter merozoites that are released in the blood to infect RBCs. Each infected RBC produces ~32 merozoites able to initiate a new round of RBC infection, some of which transform into gametocytes which are taken up by the mosquito during a blood meal. The sexual stage in mosquito forms sporozoites which travel to salivary glands ready to infect the human host during the next blood meal.

1.4 Malaria immunology

Both innate and adaptive immune responses play a critical role at different stages of malaria from prevention of establishment of hepatocyte infection in the pre-erythrocytic stage, to blocking merozoites invading RBCs and, at transmission stage, blocking gametocytes in humans and mosquito.

1.4.1 Immune response

1.4.1.1 Innate immune response

The innate immune response is the first line of defense against pathogens and is activated on recognition of unique conserved features in pathogens [53]. These are called the pathogen associated molecular patterns (PAMPs) which are recognized by pattern recognition receptors (PRRs), for example soluble receptors like the complement system components or membrane bound like the toll-like receptors [53, 54]. Components of pathogen like proteins, lipopolysaccharides, glycans, and nucleic acids can act as PAMPs [55]. The TLRs recognizing these are expressed by the cells of the innate immune system namely antigen presenting DCs, phagocytic macrophages and granulocytes, cytotoxic natural killer (NK) cells, and γδ T lymphocytes [56]. Macrophages become activated by phagocytosing pathogens and secrete cytokines and chemokines which helps to recruit neutrophils and monocytes[57]. Cytosolic PAMPs like pathogen DNA and RNA are also recognized by non-TLR receptors like the retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). This recognition activates downstream signaling pathways and stimulates a type I IFN response. This in turn promotes activation of a number of genes, the products of which have been known to enhance pathogen detection and restrict intracellular replication of pathogens [58].

1.4.1.2 Innate immune response to malaria

Neutrophils are the first to respond to the damage inflicted by the insect bite, and the numbers increase steadily in the skin 1-2 hours after the bite [59] Inflammatory monocytes numbers have been seen to increase both in skin and draining lymph nodes [60]. Once in hepatocytes, although the malarial sporozoites are largely shielded from recognition by cells of the immune system, the growing parasites are recognized by the cytosolic PRRs of hepatocytes, initiating antiparasitic type I IFN response. The parasite RNA is known to interact with the RIG-I family protein homolog called MDA5, also leading to type I IFN production [42]. Type I IFN has been shown to lead to activation and infiltration of leukocytes into the liver critical for parasite elimination [42, 61]. NK, NKT, and γδT cells inhibit the EEF growth by secretion of IFN gamma [62]. Malaria parasites are capable of inducing type I IFN responses independent of TLR3 and TLR4 receptors and their signaling proteins (MyD88 and TRIF) [63]. Thus, IFNs have a prominent role in innate immune response against the sporozoites. These molecules along with TNF-α and IL-12 have been shown to play an important role in the blood stage innate response [64]. DCs play a critical role in both innate and adaptive immune response by acting as APCs, which after uptake of antigen migrate to the lymphoid organs and initiate an immune response. It has been reported that 50% of the parasites entering the LNs were found closely associated with DCs, 4 hours post mosquito bite [33]. DCs and monocytes not only recognize and engulf merozoites, DNA, RNA and hemozoin released from the infected RBCs, but also Glycosylphosphatidylinositol (GPI)via the TLR1/2 and TLR4 toll-like receptors present on their surface [47].

1.4.1.3 Adaptive immune response

An adaptive immune response is initiated 4-7 days after initial innate responses [57] and is mediated by T and B lymphocytes [65]. The innate immune response plays an

important role in initiating the adaptive immune response. The professional APCs like the DCs and macrophages process the antigens and present them via the MHC molecules to CD4+ and CD8+ T cells [66]. CD4+ T cells also help CD8+ T cell proliferation by secreting IL-2 [67]. CD8+ T cells clear pathogens mainly by producing granzymes capable of inducing apoptosis and perforin capable of forming pores in the pathogen cell membrane [57]. B cell activation occurs when they recognize circulating antigen or PAMPs via BCR or PRRs respectively or MHC linked antigenic peptides via CD4+ T cells [66, 68]. Cytokines like IL4, IL21 and IFN-gamma secreted by CD4+ T cells stimulate B cells proliferation and differentiation into plasmablasts and memory B cells. B cells can also act as professional APCs and present antigen to both CD4+ and CD8+ T cells and contribute in formation of long- lasting memory T cells [69]. Secreted antibodies not only bind to the antigen on the pathogen surface and block binding to the host cellular receptor, but also mark the pathogen for destruction via phagocytosis by macrophages [53].

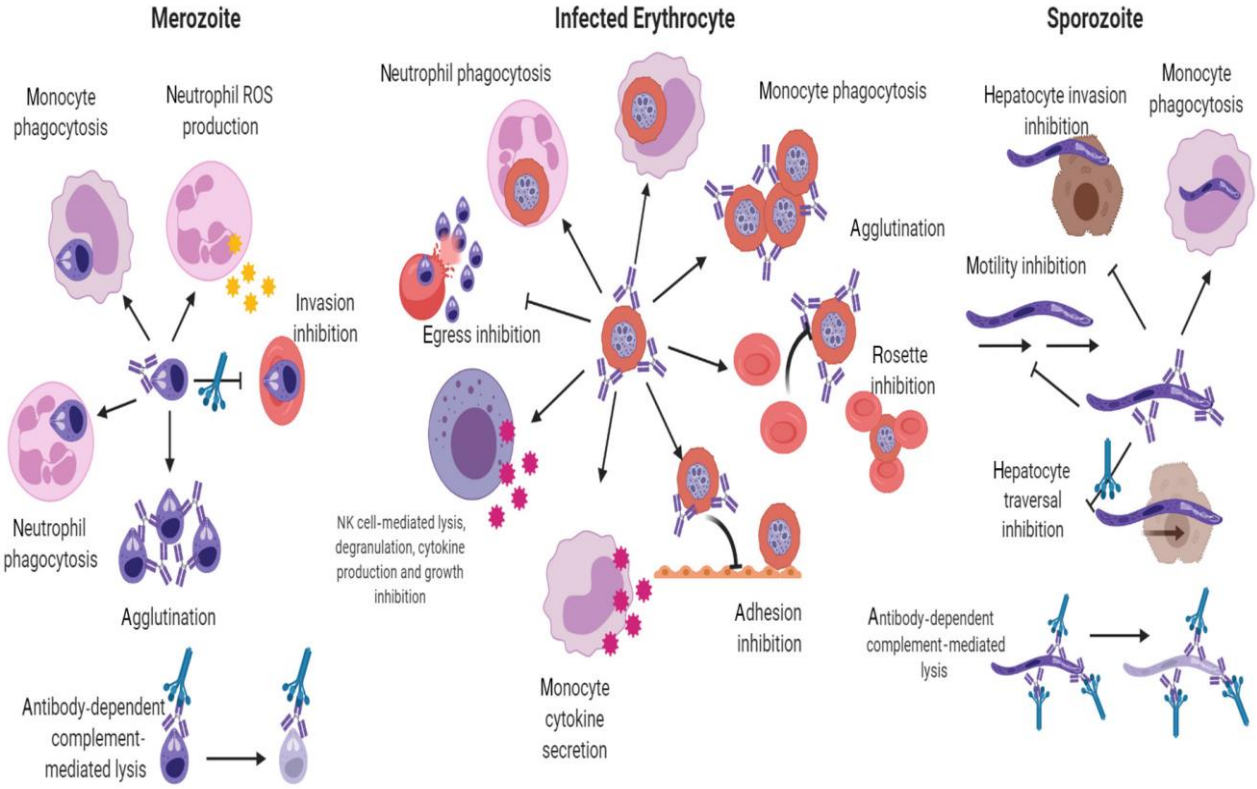
1.4.1.4 Adaptive immune response to malaria

Both cellular and humoral responses that form the adaptive immune response are important for defense against malaria. Various mouse and human studies involving viral vectors and attenuated sporozoites as vaccines, have highlighted the critical role of CD8+ T cells in conferring protection against the liver stage of malaria [70]. The first activation of CD8+ T cells has been observed in skin draining LNs ~48 hours after inoculation of sporozoites, from which they migrate to other lymph and non- lymphoid organs including the liver [71]. Antibodies also play a crucial role in limiting the number of sporozoites routing to establish an infection. Antibodies have been shown to affect sporozoite gliding and cell transversal causing over 50% of the sporozoites unable to leave the injection site[30]. Studies with irradiated sporozoites have highlighted the

important role of antibodies to major surface proteins like CSP in retarding sporozoite mobility in the skin[72]. In the liver, these cells recognize parasite epitopes associated with the MHC class I and kill the infected hepatocytes [73]. CD4+ T cells also play an important role by priming phagocytic cells to capture and kill malaria parasites by producing IFN-gamma (Th1 cells), and helping B cells produce antibodies against the parasite (particularly Tfh cells) [74]. However, this process is often less effective in natural infections than post vaccination owing to two of the important factors that help the parasite subvert a T cell encounter. These are: a) a short intra hepatic lifecycle (~2 days in mice and ~7 days in humans) and b) the small number of hepatocytes that get infected during natural infection, in humans often tens of cells in a liver that has billions of hepatocytes[36, 75]. Also, after completing a successful liver-stage infection, the merozoites are released from the hepatocyte protected by the host cell membrane escaping phagocytosis by macrophages and sinusoidal Kupfer cells [76].

Strong antibody responses have been observed to pre-erythrocytically-expressed antigens like *Pf*CSP, TRAP and *Pf*AMA1 after both naturally occurring and vaccine-induced immune response [77]. Antibodies to blood stage merozoite antigens like the MSP1, MSP2, EBA175 and Rh2A9 and VSAs found on the surface of infected RBCs, have been associated with naturally acquired protection in children [78]. Antibodies to VSAs lead to uptake of infected RBCs by monocytes and macrophages and clearance from circulation [79]. The establishment of blood stage infection has been shown to affect the CD8+ T cells induced during liver stage. The interaction of infected RBCs with the DCs interferes with the secretion of IL-12 responsible for initiation and maintenance of T cell responses and the decreased levels of which have been attributed to inhibit the activation of liver- stage specific CD8+ T cells, rendering the host susceptible to new infection [80, 81]. During the sexual stage, a number of antigens expressed by the gametes during the pre- (in humans) and post- (in mosquitoes) fertilization phase have been identified.

Antibodies with transmission reducing properties to the major surface proteins like *Pfs*230 and *Pfs*48/45 have been identified in naturally infected individuals [82]. Immunity to two post- fertilization proteins associated with the ookinete, *Pfs*25 and *Pfs*28, have shown to block transmission [83]. The antibodies to these along with mosquito midgut protein Anopheline alanyl aminopeptidase N (AnAPN1) hold the potential to disrupt the sporogonic cycle [84] when taken up during the blood meal by the infecting mosquito. The overall protective effect of antibodies during malaria life cycle is summarized in the figure 1c.



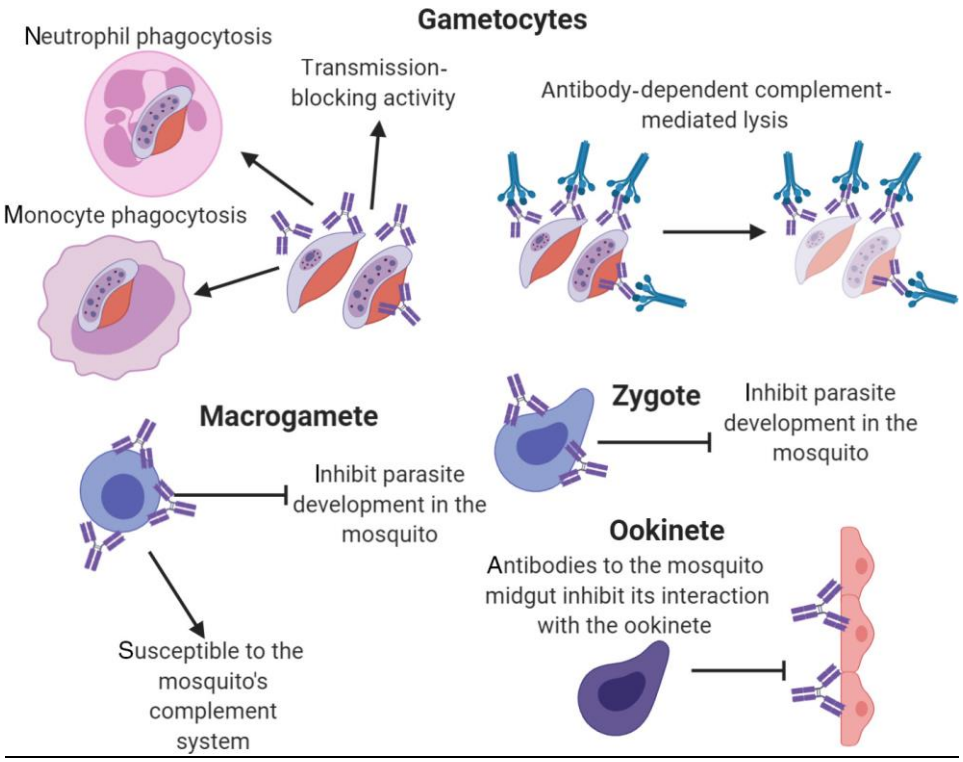


Figure 1c: Protective role of antibodies during pre-erythrocytic, blood stage and transmission stage. During the pre-erythrocytic stage, sporozoite protein specific antibodies coat the sporozoite surface retarding their motility and inhibiting hepatocyte traversal and invasion along with making them susceptible to phagocytosis and complement-mediated lysis. At blood stage, antibodies recognizing merozoites promote clearance by phagocytosis and complement mediated lysis and infected RBCs along with this inhibit adhesion and rosette formation. Aided by antibodies, the gametocytes are cleared by phagocytosis and complement mediated killing in humans while antibodies inhibit the development of the parasite in the mosquito[85].

1.5 Malaria vaccines: development so far

The malaria vaccines currently in clinical trials and pre-clinical development are whole sporozoites, recombinant protein or subunit, virus-like particles and viral vector [23], with some initial pre-clinical work on RNA vaccines. Whole sporozoite vaccines have

been developed as a pre-erythrocytic vaccine, while subunit and viral vector vaccines have been developed targeting all three stages of the parasite life cycle. Complete protection against malaria challenge was achieved by radiation-attenuated whole sporozoites more than 40 years ago [86] and over the years a number of field trials have demonstrated that the efficacy conferred by whole sporozoite vaccine is dose dependent and requires intravenous administration [23]. Viral vector vaccines, used in heterologous prime-boost regimens, with combinations of DNA vaccines and a heterologous vector, for example Modified Vaccinia Ankara (MVA), have been found to be safe and capable of eliciting high levels of CD8+ T cell mediated immunity, but failed to induce protection in most vaccinees in controlled human malaria infection (CHMI) trials [87-89]. Subunit vaccines comprising immuno-dominant B and T cell epitopes of the circumsporozoite protein have demonstrated encouraging efficacy results in pre-clinical and clinical trials. Subunit vaccines can be manufactured with low cost and high purity [90] and hence hold the potential of a more deployable vaccine format than the whole sporozoite vaccines. Developments in the field of bioinformatics have enabled availability of database for e.g. the IEDB (The Immune Epitope Database, NIAID) of over 500,000 B and T cell epitopes that include the *P. falciparum* and *P. vivax* malarial parasite proteins, have provided potential targets for subunit vaccines [90]. A subunit vaccine as a peptide conjugated to tetanus toxoid (TT) was the first malaria vaccine to reach clinical trials [91, 92] and peptides linked to VLPs based on fusion to the HBsAg and produced in yeast, or linked chemically as in Qβ VLP and bonded using the SpyTag-SpyCatcher platform, have been developed since then [93, 94]. As the major work described in this thesis is using R21 and R21 variants which are VLP vaccines, it is important to highlight it's advantages as a vaccine platform to support the research strategy adopted in this thesis.

1.5.1 VLPs as vaccines

VLPs are non-infectious nanoparticles which are multimeric in nature and do not contain any genetic material [95], expressing the desired antigen on the surface. Hence, VLPs morphologically in some ways resemble viruses [96]. As many as 110 viral proteins from over 30 viruses have been identified that can self-assemble into VLPs and resemble empty viruses, which can be explored as vaccines. A few examples of these are Hepatitis B virus, Papilloma virus and bacteriophage Qβ [97]. This has enabled modifying not only the external VLP surface and use as vaccines by expression of repeated array of antigenic peptides, but also internal surface modifications to encapsulate drugs and nucleic acids for targeted delivery [95]. As VLPs lack genetic material [98], have no replication capacity they are generally considered potentially safe, although this must be confirmed in clinical trials. Currently, there are approximately 12 VLP based licensed vaccines for use in humans including Recombivax HB and Engerix-B for hepatitis B virus (HBV), Gardasil, Cervarix, and Gardasil-9 for human papillomavirus (HPV), and Hecolin for hepatitis E virus (HEV) [95]. VLPs have been successfully combined as in Gardasil 9® [99], a nine-valent HPV vaccine, into a safe and efficacious vaccine, providing protection against multiple strains of closely related viruses in a single formulation [100].

The biggest advantage of VLP vaccine is the expression of desired immunodominant epitope on the surface eliciting high antibody [101], effective CD4+ proliferative responses and, at least pre-clinically, CD8+ cytotoxic T lymphocyte (CTL) responses [102]. VLPs commonly have a diameter of 20-200nm, which is suitable for recognition by dendritic cells and other antigen-presenting cells (APCs), and are readily transported to the lymph nodes [103], where they can induce a strong immune response [95]. The VLPs that reach the LNs are endocytosed by APCs, enter the T cell zone and are processed and efficiently presented by MHC molecules eliciting cell mediated immune

responses [104]. Unmodified VLPs entering the B cell zone of the LNs, are able to induce B cell receptor cross linking triggering endocytosis, B cell proliferation and activation. Following this activation, the B cells interact with Th cells leading to immunoglobulin class switching and generation of B cell memory [105].

1.5.2 Pre-erythrocyte stage vaccines

Vaccines targeting the pre-erythrocytic stage of malaria have received the most success so far. RTS,S (made by GSK and soon Bharat Biotech, from 2028) is nearing WHO pre-qualification which should lead to subsequent licensure in several African countries , followed by R21(made by The Jenner Institute, University of Oxford, now in partnership with the Serum Institute of India as a large scale manufacturer) in Phase III trials are both CSP subunit vaccines developed using HBsAg VLP platforms. While RTS,S vaccine efficacy was relatively modest, 39% in its phase III trial higher efficacy can be achieved with seasonal administration [106, 107], R21 has demonstrated an excellent efficacy of 77% in a highly seasonal malaria transmission setting in Phase IIb trials in comparison to RTS,S which was 56% over one year [28] in seven African countries [108]. For both the vaccines, antibody titres to the NANP repeats wane over time. Another approach that has demonstrated high efficacy is the use of whole sporozoites as radiation attenuated or genetically attenuated which are arrested in the liver and unable to establish blood stage infection, and wild type parasites administered under chemoprophylaxis (e.g. chloroquine) which eliminate parasite during initial blood stage infection [109]. These have been used as *Pf*SPZ vaccine by Sanaria Inc., has shown remarkable efficacy of up to 100% over 1 year in malaria naïve adults with homologous strain challenge, but as seen with RTS,S and R21, the antibodies responses wane over time [110] and have demonstrated reduced efficacy in malaria- exposed adults in Mali [111]. In a Phase I CHMI trial, another Sanaria vaccine, *Pf*SPZ-CVac vaccine which consists of live and

infectious sporozoites administered via DVI under chloroquine prophylaxis demonstrated an efficacy of 75% [112]. In a recent Kenyan trial in older infants irradiated sporozoites from Sanaria showed no efficacy, likely excluding their utility in a key target population[113]. Genetically attenuated parasites (GAP) developed in recent years have shown better immunity than irradiated parasites and have surpassed the possible issue of breakthrough infections [114]. One such vaccine, *Pf*SPZ-GA1 was able to induce sterile protection in mice and found safe and immunogenic in healthy adults, but the efficacy was suboptimal [115].

For whole parasite vaccines like *Pf*SPZ, manufacturing costs associated with extraction of sporozoites in high numbers for administration of 3-5 doses, storage and administration via IV route for a large population is very problematic in terms of large scale deployability[116], but might be feasible for travellers' vaccination. Some other candidates as recombinant proteins in Phase I stage clinical trial are LSA-3-rec, FMP012 (*Pf*CelTOS), and full length CSP [23, 117].

1.5.3 Blood stage vaccines

Most blood stage vaccines in development aim to elicit antibodies blocking the invasion of RBCs by merozoites, and RH5 has been found to be the only protein so far to demonstrate this for multiple parasite strains [118]. *Pf*RH5 as vaccine has been explored using heterologous viral vector platforms (ChAd63- MVA) with induction of high antibody titres in humans to the levels found similar to or higher than prolonged natural exposure with clinical trials ongoing for assessing vaccine efficacy [45, 119]. Recent results from recombinant *Pf*RH5/AS01B Phase I/IIa clinical trial show high antibody longevity (over 2 years) and reduction in parasite growth rate in CHMI [21]. Owing to their extensive polymorphism, other essential proteins involved in the RBC invasion such as MSP1 and MSP3, AMA1, and EBA-175 have been shown to induce apparently high

antibody titres when explored as vaccines but have either failed completely or led to just limited protection [120]. With promising results from whole sporozoite vaccine for the pre-erythrocytic stage, pre-clinical studies are being undertaken using lysate of parasitized RBC which can not only induce protection against blood stage, but also cross-protect against both the liver and sexual-stages [121]. Some examples of blood stage vaccines in various stages of development are AMA1-RON 2 (subunit vaccine, pre-clinical), MSP3 (synthetic peptide, Phase I), AdCh63-MSP1 (viral vector, Phase I/II) and MSP3-LSP (sub-unit, Phase II)[117].

1.5.4 Transmission blocking vaccines

Transmission blocking vaccines (TBV) hold the potential to reduce malaria transmission by inhibiting the development of *Plasmodium* parasites in the *Anopheles* vector by eliciting antibodies against the antigens related to asexual and sexual stages of the parasite [122]. A leading TBV candidate *Pfs25*, a protein expressed on the surface of zygotes and ookinetes, has been explored in a number of clinical trials with limited success as a recombinant protein, fusion protein and VLP after having shown promising level of antibody responses in pre-clinical studies [123, 124]. When fused with a molecular adjuvant IMX-313, *Pfs25* was more immunogenic both as a protein and in viral vectors, with significantly better transmission blocking activity than the monomeric form [124]. Combination of this vaccine with the pre-erythrocytic vaccine RTS,S as a part of multi-stage vaccination regime has been found to be safe with no negative effect on the functional antibody titres for both antigens in *in vitro* assays, holding the potential to be translated to human vaccination [123]. Some other transmission blocking subunit vaccines in development are *Pfs230* (Phase II) and *Pfs48/45* and AnAPN1 (pre-clinical) [23, 125].

1.6 Correlates of protection

Identification of immunological correlates of protection is very important in vaccine development. It has been defined by WHO as 'a type and amount of immunological response that correlates with vaccine-induced protection against a clinically apparent infectious disease and that is considered predictive of clinical efficacy' [126]. It can be a mechanistic correlate which is a specific immune function directly linked to protection, or non- mechanistic that is not directly linked to protection, and has a correlation with the immune response that is mechanistic [127]. Immune correlates of protection have been attributed to levels of antigen specific antibodies, CD4+ and CD8+ T cell levels, and with advances in analytical techniques, linked to antibody isotype, avidity and T cell subsets. Vaccine antibody levels that correlate to protection against disease have been well defined for a number of viral and bacterial pathogens using a range of analytical techniques [128]. Development of high throughput techniques like micro-chip arrays have provided genomic, proteomic and transcriptomic data have helped in identification of protective correlates in naturally exposed individuals, and both protected and unprotected individuals post vaccination [129]. Passive transfer of antibodies and T cell depletion studies in pre-clinical models have also provided a guideline to possible correlates of protection in human studies [130]. In malaria, identifying these in population with naturally acquired protection, though not sterilizing, have provided useful insights into vaccine development. This has been attributed to both antibody and T cell responses to blood stage antigens in both pre-clinical and clinical studies [131]. Most of the studies highlight the non- mechanistic contribution of one or more components of the immune response in conferring protection. For radiation attenuated sporozoite vaccines, anti-CSP antibody levels have been reported as a correlate of protection in humans as in many vaccine protected individuals CD8+ T cells were not consistently induced [131], while studies in mice and non-human primates (NHP) models

show that protection was achieved by CD8+ T cells independent of antibody responses [132, 133]. For the leading RTS,S vaccine, passive transfer of human anti-CSP MAbs at the same concentrations as in vaccine recipients, conferred protection in humanized mice on challenge provided supporting evidence for anti-CSP antibody levels as a correlate [134]. In RTS,S CHMI and field studies, along with anti-CSP antibody levels, CD4+ T cell, IFN-gamma, IL-2 and TNF-alpha levels have been correlated to protection [134]. Anti-CSP titres of ~200 EU/mL in infants and ~500 EU/mL were measured in older children at 4 weeks after 3rd dose of RTS,S and have been correlated to protection. The antibodies wane to ~10% of this peak titre within 1 year of vaccination bringing it below the estimated protective threshold of 121 EU/mL.[131]. Thus, maintenance of protective levels of the identified mechanistic correlate linked to protection by a vaccine is also crucial. In ChAd63- MVA ME-TRAP vaccine CHMI trial and field trials in Kenya, the protective efficacy corelated with levels of CD8+ vaccine-induced T cells induced by vaccination[135]. For promising blood stage vaccine candidates like *Pf*RH5, a study in NHP that received protein-in-adjuvant and/or a viral vectored vaccine targeting either *Pf*RH5 demonstrated that the concentration of anti-PfRH5 IgG required to achieve an *in vivo* growth inhibition of 50% (IVGI EC50) was estimated at 185 µg/ml[103]. In a human CHMI trial involving recombinant *Pf*RH5, similar results were reported suggesting comparability between two species [21]. With transmission blocking vaccines in early stages of development, a Phase I trial involving *Pfs*25-EPA/Alhydrogel® vaccine suggested a correlation between IgG antibody titres of 88 µg/mL two weeks after 4th vaccination in humans and transmission blocking in standard membrane feeding assays [136].

1.7 Circumsporozoite protein

The work covered in the first 3 results chapters of this thesis involves screening, production, immunogenicity and efficacy of variants of R21 vaccine and use a vaccination strategy using R21 that has not been explored at the Jenner Institute before. As *P. falciparum* CSP forms the base of the R21 and variant vaccines, it would be important to highlight its structure and role in the parasite life cycle.

During its 2-host life cycle, in human and mosquito, *P. falciparum* undergoes numerous different morphological changes in five different host tissues [29]. The proteins expressed during the mosquito cycle have been targeted for use as transmission blocking vaccines and those in humans as blood stage and liver stage vaccines. The malarial parasite genome encodes for ~ 5500 proteins [6] expressed at different stages of the parasite life cycle. One of the most abundant proteins is CSP. CSP is a 397 amino acid [137] major surface antigen on the sporozoite of the malaria parasite [138]. CSP is embedded in the plasma membrane via a GPI anchor at its C-terminus, exposing the protein to the extracellular space. The protein consists of an N-terminal domain, a region of tandem repeats, and a C-terminal domain that is homologous to the TSR superfamily (figure 1d). The N- terminus of CSP lacks predicted secondary structures, adopting a flexible configuration. The number of repeats varies from 37 to 44 in different strains of *P. falciparum* forming β-turn structures and exists in an equilibrium between folded and extended states [139]. Recent structural studies have revealed that region III and the TSR fold into a single "αTSR" domain (also referred to as the C-terminal domain, CTD) [140].

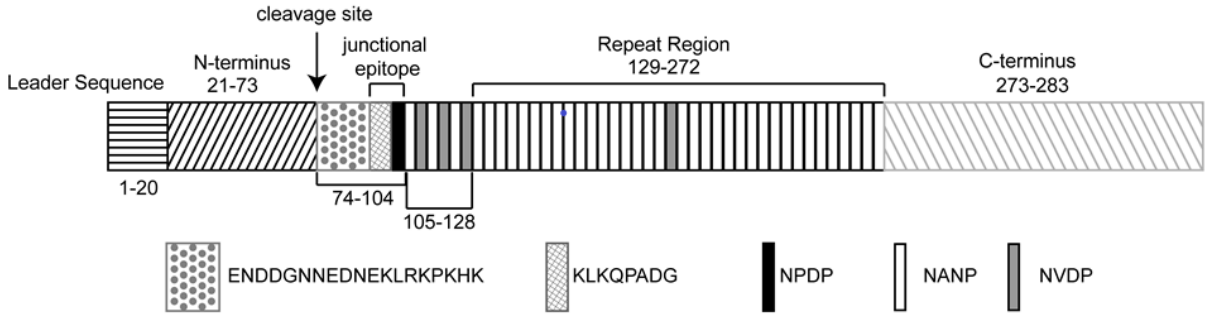


Figure 1d: Structure of *Pf*CSP consists of and N consisting of cleavage site and junctional epitope and C terminus region containing thrombospondin-like type I repeat (TSR) domain. The two regions are separated by NANP, NVDP and NPDP repeats. Figure made by A. Spencer.

CSP is crucial in the formation of sporozoites in the mosquito midgut [141]. CSP also has a role in binding of sporozoites to liver cells, and has been shown to inactivate the protein synthesis machinery of the host cell [142] by binding to RNA-associated sites on the ribosomes [143]. In *P. falciparum*, the central repeat of CSP protein mainly consists of many copies of the tetrapeptide Asn-Ala-Asn-Pro (NANP). This is conserved between parasite variants and is the target of protective antibodies in rodent malaria models [144]. CSP is one of the key proteins recognized by the human immune system, with a highly conserved NANP that is a major target of protective antibodies, and hence has been one of the most important anti-malarial vaccine candidates [142]. The sporozoites coming in contact with the HSPGs, triggers a signal to proteolytically cleave the N terminus sequence 68-87 'NSRSLGENDDGNNDENEKLR' [34] of the protein by parasite-derived cysteine protease and exposing the thrombospondin alpha – 1 repeat (TSR) domain [2]. This switches the sporozoites from migratory to invasive state [138] to initiate liver stage infection.

Antibodies are produced to all the regions of the CSP protein which include the N terminus containing the cleavage and junctional epitopes, the central NANP repeats and to a lesser extent to the C terminus[145-147]. Naturally acquired anti- CSP antibodies through repeated infections in humans and vaccinated animals in pre-clinical studies have pre-dominantly been IgG1, IgG3 and IgM. Anti- CSP IgG1 and IgG3 to the central NANP region have been shown to be potent complement pathway activators contributing to sporozoite cell traversal inhibition and death, and the possibility of the same with the other regions of CSP needs to be investigated[148]. Anti-CSP antibodies to the central repeat region have been associated with protection for RTSS vaccine[149] with subclasses IgG1 and IgG3 being considered as main corelates[150].

1.8 Thesis aims and outline

The malaria vaccine that has reached the furthest so far, RTSS shows modest efficacy with antibody levels waning over time. With the most promising Phase IIb results from R21 vaccine, WHO's target of 75% or more efficacy in children over a 12-month period appears to have been met [151]. Another vaccine, PfSPZ-CVac where chemically attenuated whole malarial sporozoites were administered with chloroquine chemoprophylaxis has shown 77% efficacy against heterologous CHMI challenge 12 weeks post immunization [152]. Despite very promising results, the antibodies wane over time and a vaccine with higher durability and better longevity is desirable. With this aim, this thesis presents the outcome of including previously unexplored regions the sequence of the sporozoite dominant protein CSP like the Junctional and Cleavage epitopes in R21 variant vaccines, alone or as multimers or in different possible combinations with other immunodominant sequences like the central repeats. Along with this, the effect of reducing NANP repeats, deleting the C- terminus region and addition

of CD4+ T cell helper sequences have also been considered for generation of new variants.

With the new R21 variants generated, the effect of mixing variants to study the potential synergistic or antagonist effect on efficacy and reducing the vaccine dose of promising variant VLPs on immunogenicity and efficacy has been explored thereafter.

With promising results from licensed poultry viral vaccines, immune complex immunization has been explored for human viruses and autoimmune diseases. As this approach was unexplored in field of malaria, I have used it as a mode of vaccination to compare the immune response and efficacy in comparison to the standard R21 vaccine. Previously unexplored at the Jenner Institute, a promising glycan called the Alpha-Gal present on the surface of many parasites including *Plasmodium* but absent in humans, holds the capacity to act as a potential vaccine candidate for the pre-erythrocytic stage parasite. The aim here was to study the immune response to Alpha-Gal when injected in transgenic mice that will provide a direction to planning further studies using this glycan.

These aims are addressed in the four results chapters and a final discussion chapter as below:

Chapter 3: Expression and production of R21 vaccine variants

Chapter 4: Assessment of immunogenicity and efficacy of R21 vaccine variants

Chapter 5: Immune complex generation as strategy for vaccination

Chapter 6: Alpha-Gal glycan as a potential pre-erythrocytic vaccine candidate

Chapter 7: Discussion and Future Directions

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Media

Table 2a: Media

Name	Components	Use
Bacterial growth media		
LB- Carb	LB broth (Sigma, L7275) / agar (Sigma, L7025) + 100µg/mL Carbenicillin (Sigma, C1613)	Growth of *E.coli* cultures containing an Ampicillin resistance gene
SOC	SOC Outgrowth medium (Invitrogen)	Recovery medium for transformed *E.coli.*
Pichia growth media		
YPD medium/ agar (Yeast Extract Peptone Dextrose)	1% yeast extract, 2% peptone, 2% dextrose (glucose)	Selection and growth of transformed colonies
BMGY (Buffered Glycerol-complex Medium)	1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% YNB, 0.0004% biotin, 1% glycerol	Growth of *P. pastoris* for protein expression.
BMMY (Buffered Methanol-complex Medium)	1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% YNB, 0.0004% biotin, 1% methanol	Initiate and maintain protein expression in *P. pastoris.*
MD (Minimal Dextrose) agar	1.34% YNB, 0.0004% biotin, 2% dextrose	Selection of transformed yeast colonies

2.1.2 Buffers

Table 2b: Buffers

Buffer	Components	Use
Pichia Resuspension buffer	20mM Tris pH 8, 5mM EDTA	Re-suspend frozen Pichia pellets prior to homogenization.
Pichia Liberation buffer	20mM Tris pH 8, 5mM EDTA, 0.5% Tween 20	Facilitate liberation of VLPs from Pichia membranes post homogenization.
Pichia wash buffer	20mM Tris pH 8	Pellet wash post homogenization and before liberation step.
TBS (Tris Buffer Saline)	150mM NaCl, 20mM Tris	C- tag and SEC column equilibration, column wash and SEC elution.
C- tag Elution buffer	2M $MgCl_2$	Elution of VLPs from C- tag column
C- tag cleaning buffer	0.2M Glycine, pH 2	Cleaning C- tag column post elution
Column storage buffer	20% Ethanol	Storing C- tag and SEC column resin post use.
PBS (Phosphate Buffer Saline)	Gibco DPBS powder dissolved in dH2O.	To make 2 and 1% BSA/ PBS Tween for ELISA blocking and sample dilution, PBS/Tween (0.05%) for washing plates.
Carbonate-bicarbonate coating buffer, (pH 9.5)	0.05M: 1 Capsule (Sigma C3041) dissolved in 100mL dH2O.	To dissolve peptide to coat ELISA plates.
	0.5M: 10 Capsules (Sigma C3041) dissolved in 100mL	To dissolve Alpha- Gal BSA to coat ELISA plates.

2.1.3 Antibodies

Table 2c: Antibodies

Antibody	Use
2A10 (anti NANP Mab)	WB (1:1000), ELISA (1:20), used in IC experiment for vaccination as a complex with R21 and un-complexed.
Mouse anti Hepatitis B Surface Antigen antibody (Genetex, GTX40707)	WB (1:1000)
CaptureSelect™ Biotin Anti-C-tag Conjugate (Thermo Scientific, 7103252100)	WB (1:1000)
Anti-Mouse IgG (whole molecule) –Alkaline Phosphatase antibody produced in goat (Sigma, A3562)	WB (1:1000), ELISA (1:3000)
Mouse anti IgG Streptavidin-ALP antibody (Mabtech, 3310-8)	WB (1:1000)
Biotin anti-mouse IgM antibody (BioLegend, 406503)	Secondary antibody for anti- Alpha- Gal IgM ELISA (1:1000)
Anti-alpha-Gal, Mouse IgG1, Kappa (Absolute antibody, Ab01163-1.1)	Positive control for anti- Alpha- Gal IgG Standard ELISA
Anti-alpha-Gal, Mouse IgM, Kappa, (Absolute antibody, Ab01163-21.0)	Positive control for anti- Alpha- Gal IgM Standard ELISA

2.1.4 Plasmids

Table 2d: Plasmids

Name	Expression system	Promoter	Use
pPIC3.5K	*Pichia pastoris* (GS115)	AOX1	Used for expression of all R21 variant constructs

2.1.5 Expression system

Pichia pastoris is a methylotropic eukaryotic organism and can use methanol as carbon and energy source. Invitrogen™ GS115, *Pichia pastoris* Yeast Strain (Product Code.10707484) has been used throughout for the production of all the VLPs described in this thesis. It has two encoding genes (AOX1 and AOX2) of alcohol oxidase enzyme (AOX). Methanol induces the transcription of the enzyme and also the gene of interest downstream [153]. pPIC3.5K is a ~ 9000 bp vector which consists of the marker gene His4 and phenotype mut +.

2.1.6 Adjuvants

Matrix-M1 adjuvant, lot M1-118 (0.75 mL, 0.375 mg/mL) has been provided by Novavax to be used in the vaccination studies. Matrix M is a mixture of Matrix-A and Matrix-C at a ratio of 85:15. Both these components have been purified from *Quillaja saponaria* Molina tree, formulated with cholesterol and phospholipid into matrix particles of ~ 40 nm in size [154]. The adjuvant has shown to induce high and long lasting levels of broadly reacting antibodies, a T cell response and promotes cellular drainage to local lymph nodes [155]. The formulation with Matrix-M might contain a total of 100 to 200

μg of total material including antigen, while conventional adjuvant formulations may contain > 10 mg of these materials [156].

2.1.7 Chemicals

Table 2e: Chemicals

Chemical	Use
4b-Galactotriose (Carbosynth Ltd., OG00715 3a) and 1-3-1-4-galactotriose-BSA (Dextra Laboratories Ltd., NGP0434)	Immunization
Galα1-3Gal-BSA, 3 atom spacer (Dextra Laboratories Ltd., NGP0203)	Coating ELISA plates@ 10μg/mL)
Di-Potassium hydrogen Phosphate (Merck, 105104)	Phosphate buffer
Potassium phosphate monobasic (Sigma, P5655)	Phosphate buffer
Glycerol (Sigma, G5516)	Constituent of BMGY media and to make glycerol stocks of clones.
Sterile DPBS (Sigma, D8537)	For dilution of peptides and vaccine preparation for immunization.
DPBS powder (Gibco, 21600)	To make PBS and PBS/ Tween for ELISA and western blotting wash steps.
Magnesium Chloride (Sigma, M8266)	2M solution used for elution of VLPs from C-tag column.
Difco Select Phytone (BD Biosciences, 210391)	For making BMGY and BMMY media.
Bacto™ Yeast extract (BD Biosciences, 288620)	For making BMGY and BMMY media.
Tween 20 (Sigma, P7949)	ELISA wash buffer component
1M Tris solution	Purification buffer component

Methanol (Fisher Scientific, M/3950/17)	To maintain methanol levels in culture for ongoing expression.
Glycine (Sigma, G7126)	0.2M solution for C-tag resin regeneration.
Ethanol (Fisher Scientific, E/600DF/17)	20% in distilled water for column storage.
NaOH (SLS, CHE3370)	0.5M solution for cell disrupter and SEC column sanitization.
EDTA (VWR, 20309.296)	Component of Pichia resuspension and VLP liberation buffer.
NaCl (Merck, 1.06400.5000)	Component of TBS
Dextrose (VWR, 0188)	Component of YPD media/agar
Biotin (Sigma, B4639-5G)	To supplement BMGY and BMMY
Yeast nitrogen base (Sigma, Y0626)	To supplement BMGY and BMMY
1M Sorbitol (G Biosciences, R038)	Revival of cells post electroporation

Peptides used in R21v immunogenicity assessment (@2 μg/mL) working concentration		
Peptide sequence	Supplier	Reference no.
NANP6c	Mimitopes	3772801
KLKQPADGNPDPNANPNVDP	Think peptides	CPD65915
NVDP6c	Mimitopes	2995902
ENDDGNNEDNEKLRKPKH	Think peptides	CPD56887

2.1.8 Chimeric Malaria Parasites used for the challenge model

The chimeric parasite used in the challenge studies express *P. falciparum* CSP wild-type gene coding sequence (CDS) instead of the *P. berghei CSP*. *P. berghei CSP* has been knocked out using the standard two-step GIMO-transfection procedure whereby there is first replacement of PbCSP CDS with a positive-negative selectable marker, prior to insertion of the *P. falciparum* CSP wild-type CDS. The chimeric parasite line *P. bergei* ANKA (*PbANKA-PfCSP(r)*$_{PbCSP}$ (2257 cl2)) expressing wild-type coding sequence for PfCSP under control of the endogenous PbCSP promoter was used for the all challenge experiments. Generation and analysis of the chimeric parasite line 2257 cl2 is described details is published [157, 158].

2.2 Methods

2.2.1 Gene synthesis

The genetic sequence was ordered as a GeneArt DNA plasmid from ThermoFisher Scientific with a final concentration of 5µg. Modifications requested were the addition of the BamHI restriction enzyme site and the Kozak sequence for Pichia pastoris yeast -respectively- upstream to the PfCSP based vaccine sequence optimized for *P. pastoris* yeast. The antigen sequence was preceded by the Kozak sequence to aid translation in yeast cells. C-tag sequence, a STOP codon and NotI restriction enzyme site added respectively at the 3' DNA appendix and codon usage was optimized for yeast expression.

2.2.2 Digestion step

The plasmid was resuspended in 100µL of autoclaved DNAse/RNAse free water and the final DNA concentration was confirmed using Nanodrop. The plasmid was digested to obtained insert in linear form. The restriction enzymes-based digestion reaction was set

up in a DNAse/ RNAse free tube in a total volume of 50 µL with the following restriction enzymes:

DNA (~2 µg)	40 µL
Not I	2.5 µL
BamH I	2.5 µL
Eco R1	2.5 µL
CutSmart buffer (10X)	5 µL

The tubes were incubated overnight for digestion at 37^{0}c in incubator. A 5 µL of sample was removed to confirm the correct size of the insert on an agarose gel. The remaining digest was incubated at 65^{0}c heating block for 20 minutes to inactivate the restriction enzymes.

2.2.3 Agarose gel electrophoresis

Digestion step DNA products were separated by 1% agarose gel (with SYBR safe for visualization of bands) electrophoresis (95V, 50-70min) in 1x Tris-acetate-EDTA [15] buffer. Visualization of the DNA was accomplished by the use of transilluminator. The size of bands was estimated by comparison to the DNA marker smart ladder.

2.2.4 Ligation

The remaining digest (~ 45 µL) was cleaned up using Qiagen QIAmp kit. 275 µL (5x) of PB buffer was added to the remaining digest and loaded onto the spin column. The column was spun at 4000 rpm for 1 minute, and flow through was discarded. The column was washed by adding 0.5 mL of PE buffer and spun at 13000 rpm for 1 minute. Then column was then dry spun at 13000 rpm for 1 minute to remove any remaining buffer. 50 µL of autoclaved DNase/ RNase free water was added to the column and the tube was incubated at 80^{0}c for 1 minute. The DNA was eluted by spinning the column at 13000

rpm for 1 minute. The cleaned digest (~ 100ng) was used for ligation into pPIC3-5IC (8979 bp) vector.

dH20	12 µL
10 X buffer	2 µL
vector	2 µL
insert	2 µL
ATP	1 µL
ligase	1 µL

Total 20uL

The reaction was incubated at 25°C or RT for 3 to 4 hours, if O/N then in temperature block at 16C.

2.2.5 Transformation into *E.coli*

Chemically competent sub-cloning maximum efficiency DH5-alpha *E. coli* competent cells (Invitrogen, Cat. no. # 12297-016) were transformed with the ligation product. 50 µL of *E.coli* competent cells was kept on ice and all of the ligation product (20 µL) was added to it. The mix was incubated at 42°C for 40 seconds and then on ice for 2 minutes. 200 µL of SOC medium was added to the mix and incubated at 37°C for 30 minutes. The culture was streaked out onto LB agar plates containing the relevant antibiotic for the resistance gene in the transformed plasmid and the plates were left in a 37°C incubator overnight to grow. 10 colonies were picked up and grown in 5mL of LB media with ampicillin. After overnight growth, the cultures were spun down at 4000 rpm for 10 minutes. The supernatant was discarded and the pellets for processed for plasmid extraction using Qiagen Miniprep kit and protocol. The concentration of plasmid DNA was checked using Nanodrop. The plasmid DNA was digested with NotI+BamHI and the correct band size was confirmed on agarose gel electrophoresis.

Confirmed clones were regrown as 3mL LB (+ ampicillin) cultures overnight at 37^{0}C. 1mL of culture was mixed with 1mL of LB Ampicillin media containing 40% glycerol for glycerol stock. One confirmed clone was grown in 150mL media and processed using QIAgen Midiprep kit and protocol to obtain higher concentration of DNA to be used for electroporation. The DNA was eluted in 300 µL of autoclaved DNase/ RNase free water and the concentration was measured using Nanodrop. The DNA was reconfirmed by restriction enzymes digestion analysis.

2.2.6 Linearization

The plasmid was linearized using the restriction enzymes SalI and digested overnight at 37^{0}c. The digest was incubated at 65^{0}c heating block for 20 minutes to inactivate the restriction enzyme. The plasmid DNA was cleaned up as mentioned in the ligation step.

2.2.7 Electroporation

2.2.7.1 Competent Yeast Cells

For preparation of GS115 yeast competent cells for transfection, one isolated colony from the reference Invitrogen™ GS115, *Pichia pastoris* Yeast Strain (Product Code.10707484) was picked from the MD plate and inoculated into 25mL of YPD media. The flask is incubated at 28^{0}c at 250rpm till the OD600 reaches 0.1-0.2. 10mL of this culture is inoculated in 200mL of YPD media and harvested when the OD600 reaches 0.6- 0.8. GS115 *P. pastoris* yeast pellet was processed using Invitrogen™ Pichia EasyComp™ Transformation Kit (Ref. # 45-0373) as per manufacturer's protocol to generate competent yeast cells. Competent yeast cells were then aliquoted and stored in -80 freezer as single use aliquots for future use.

2.2.7.2 Electroporation procedure

80 µL (~ 2 x 10^6 cells) of cells were mixed with 20 µg of linearized DNA in a 0.2 µm cuvette on ice and incubated for 5 minutes. The cells were pulsed at using Bio-Rad Gene Pulser Xcell Electroporation unit using a pre-set program (2000 volts, 25 µF, 200 Ω, cuvette (mm) 2). 1mL of ice- cold 1M Sorbitol was added to the cuvette. The contents of the cuvette were transferred to a microcentrifuge tube and incubated at 28°C for 1 hour. Each construct was spread onto 3 MD plates with 200 µL – 600 µL of culture and incubated at 28°C until colonies appeared (3 to 5 days).

2.2.8 Colony culture

Five to ten isolated colonies were picked up from each plate and inoculated into 5mL of BMGY in a tube and grown at 28°C in a shaking incubator at 250 rpm. After 48 hours, the cultures were spun down at 2000 rpm for 5 min and pellets were re-suspended in 2mL of BMMY. The cultures were then grown for 48 hours with methanol feed every 12 hours with a gradually increasing methanol concentration starting by 0.5% up to a final concentration of 2% (40µL).

2.2.9 Expression analysis

After 48 hours, the expression of the protein was confirmed by western blot analysis using anti NANP, anti HepB and anti C- tag antibodies. 78µL of sample was mixed with 12µL of NUPAG reducing buffer and 30 µL of 4 x LDS sample buffer and incubated at 100°C for 15 minutes. The samples and R21 positive control were run on Invitrogen NuPage 4-12% Bis- tris Midi gel alongside pre-stained colour marker 245 kDa (NEB). Western blotting was performed using Transblot Turbo on 0.2µm nitrocellulose membrane and blocking the membrane is 2% BSA/PBS for 1 hour. The membrane was incubated in primary antibody anti- NANP, anti C- tag and anti HepB for 1 hour. After

washing the membrane 3 x with PBS/T and 1 x with PBS, the membranes were incubated in anti- mouse IgG with alkaline phosphatase, and then washed again for developing. One BCIP/ NBT tablet (Sigma) was dissolved in 10mL DW for developing the membranes. The membranes were washed in DW after the development of bands.

2.2.10 Selection of clone, expansion and induction

The clone confirmed positive on all three blots was chosen for expansion. The culture was initiated by picking a portion of the colony from the plate and inoculating into 50mL of BMGY in a 250mL shaker flask. The flask was incubated at 28°C, 250rpm for 48 hours. 10mL of culture was transferred to a 2L flask with 1L BMGY. The culture was induced when the OD600 reached 10- 15 absorbance units. The culture was spun down at 1500g for 15 min. Supernatant was discarded and pellet was re-suspended in 500mL of BMMY. The cultures were fed with 1% (5mL) methanol 12 hours, and 2% (10mL) every 12 hours thereafter. The culture was harvested 12 hours after the 4th feed and pellets were frozen at -80°C.

2.2.11 VLP Purification

2.2.11.1 Cell lysis:

The pellets were thawed at RT in 5 times their weight with lysis buffer and lysed at 1000 bar in a high-pressure homogenizer. The lysed material was clarified in 20mM Tris to remove debris and homogenized again in liberation buffer to release VLPs. The clarified and 0.22 µm filtered material was loaded onto affinity C- tag column.

2.2.11.2 C- tag affinity purification:

The VLPs were bound to the C–tag resin containing the low molecular weight camelid antibodies to the EPEA tag on the VLPs. The C-tag resin was bought (Thermo Scientific,

Cat. # 1943072250) and ~ 20 mL of resin was packed in XK 16/20 column (GE Healthcare Life Sciences, Code # 28-9889-37). TBS was used as the equilibration buffer and the VLPs were eluted using 2M MgCl2 as fractions.

2.2.11.3 Size exclusion chromatography:

The eluted C- tag affinity column peak was pooled, filtered through 0.22µm filter and loaded (~ 10mL) onto HiLoad® 16/600 Superdex® 200 pg SEC column (GE Healthcare Life Sciences, Cytiva # 28-9893-35) for buffer exchange into TBS and collected as fractions. The fractions were pooled after western blot analysis to confirm the presence of the protein.

2.2.12 Formation and confirmation of ICs

Different concentrations of anti-NANP IgG monoclonal antibody 2A10, were mixed with a fixed concentration of R21 in separate tubes to achieve the target Ab:Ag ratio, and incubated at 37°C for1hour, 50 rpm. Half of the tube contents were taken into a separate tube processed with reducing agent as in section 2.2.9 by incubation at 100°C for15 minutes and half left un-processed. The R21 control was processed the same way. The Ab: Ag ratios are as in table 2.6.

Table 2f: Antibody- Antigen ratios for IC formation

Ab: Ag ratio	2A10 concentration	R21 concentration
1:1	0.2µg	0.2µg
1:2	0.1µg	0.2µg
2:1	0.4µg	0.2µg
5:1	1µg	0.2µg

2.3 Analysis

2.3.1 Confirmation of particle

The presence of particles was confirmed by Transmission electron microscopy (TEM) and was performed by Ahmed Salman or Errin Johnson at the Dunn school of Pathology facilities.

2.3.2 Protein quantification

Protein quantification was performed using Pierce BCA kit (Thermo Scientific, catalogue no. 23227) as per kit instructions.

2.3.3 Confirmation of presence of protein

To confirm the presence of vital protein sequences for the purpose of pooling fractions post purification, samples were processed and run on a denaturing gel as described in section 2.2.9. One of the gels was stained with Coomassie stain to detect bands of correct size and other gels were processed for Western blotting and detection of bands with antibodies as described in section 2.2.9.

2.3.4 ELISA for quantification of immune response

Nunc Maxisorp plates were coated with 100µl/well of 2µg/ml peptide in coating buffer, sealed with clingfilm and stored at + 4^0C overnight. Next day, the plates were washed 6x with PBST (0.05%). The plates were then blocked 200µl/well with 2% BSA/PBS and incubated for 1 hour at RT. Serum samples were thawed and diluted in 1% BSA-Tween. Anti NANP 2A10 Mab, anti- Alpha gal IgG1 and IgM were used as a positive control depending on the analysis and naïve samples were used as negative control. The blocking solution was discarded the pre- diluted serum samples and control were added and plates were incubated for 1 to 2 hours at RT. Plates were then washed 6x with PBST and goat

anti-mouse alkaline phosphatase (1 in 3000) diluted in PBS was added to each well and incubated for 1 hour at RT. The plates were washed 6x with PBST and 1x with PBS. pNPP substrate was added to each well and plates were read using Gen 5 software at 405nm.

2.3.4.1 Standard ELISA

For standard ELISA, plates were coated and blocked as mentioned above. Diluted serum samples were added in triplicates. A standard curve was generated by including dilutions of positive control Mabs in duplicates. The standard corresponding to value of 1 was also used as an internal control and added to the plate in six wells. Plates were washed and read as mentioned above.

2.3.4.2 Endpoint ELISA

For endpoint ELISA, the diluted serum samples and 2A10 Mab positive control were added in duplicates on each plate and serially diluted row down with the last row on the plate left as blank. The endpoint titre was defined as the dilution at which the OD of the sample reaches the cut-off. The cut-off is defined as the average absorbance in the no serum control wells plus 3x stdev of this average.

2.3.5 Statistical Analysis and Software

Statistical analysis was performed using GraphPad Prism version 9 (Graphpad, USA) unless indicated otherwise. Prior to statistical analysis to compare two or more populations, the Kolmogorov-Smirnov test for normality was used to determine whether the values followed a Gaussian distribution. An unpaired t-test was employed to compare two normally distributed groups, whereas Mann-Whitney rank test was used for comparing two non-parametric groups. Survival and protective efficacy to in challenge

experiments was presented using Kaplan-Meier curves and significance tested using the Log-Rank (Mantel-Cox) Test. The value of $p<0.05$ was considered statistically significant (and further levels of significance denoted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$).

2.4 Murine experiments

2.4.1 Animals

All animal work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Oxford Animal Care and Ethical Review Committee for use under Project License P9804B4Fl [107 I51D6A718, and PIL # I8CAB5A2A]. Animals were group housed in individually ventilated cages under specific pathogen free conditions, with constant temperature, humidity and with a 12:12 light-dark cycle (8am to 8 pm). For induction of short-term anesthesia, animals were either injected IM with xylazine and ketamine or anaesthetized using vaporized IsoFlo®. All animals were humanely sacrificed at the end of each experiment by an approved Schedule 1 method. All efforts were made to minimize suffering. Following table shows the type of mice used for assessment of immune response and efficacy of vaccine variants or novel vaccination approach.

Table 2g: Mouse strains used for experiments

Mouse strain	Study
BALB/c mice	Comparison of R21 variants to R21 vaccine
	Immune complex experiments
C57/BL6 Alpha gal Transferase knock out mice (AgalT KO)	Alpha- Gal experiments

2.4.2 Vaccination plan and blood sampling

For all the vaccinations, 5 µg of adjuvant Matrix-M (MM) was used as recommended by Novavax Inc. The volumes were made up with sterile PBS when required. Vaccination was performed within 1 hour of formulation preparation which was kept on ice till injected. All groups had 6 mice, unless mentioned otherwise. Mice were vaccinated (prime) and boosted 3 weeks post prime (Figure 2a). A second boost was administered in some experiments and has been described separately when done. Blood sampling was done 3 weeks post prime and 3 weeks post boost. In case of a 2nd boost, blood was sampled 3 weeks post 2nd boost. Blood samples were collected via tail vein bleed in a microcuvette tube or by cardiac puncture with a 26gauge needle to withdraw blood from the heart under anaesthetic (3.5% isoflurane, 2L/minute oxygen). Blood was allowed to clot by storing it at 4°C overnight before centrifuging it at 13000 rpm for 10 minutes to separate the sera. Sera were carefully removed in individual tubes and stored at -20°C until use.

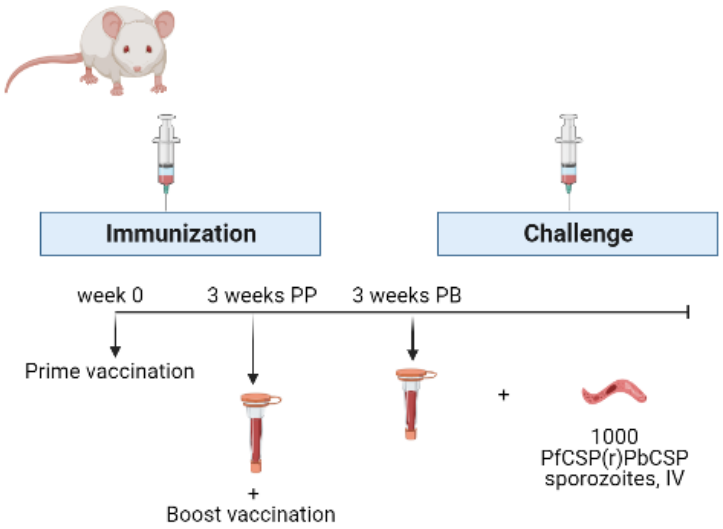


Figure 2a: Prime-Boost immunization plan. PP= Post Prime, PB= Post Boost. The mice were immunized and a boost vaccination was done 3 weeks PP with

blood sampling 3 weeks PP and PB. The mice were challenged with 1000 *Pf*CSP®*Pb*CSP sporozoites 3 weeks PB.

2.4.2.1 Vaccination plan for R21v experiments

For R21v and R21v PADRE experiments, all mice were injected with 1µg of R21v and MM through IM route for both prime and boost vaccination. In reduced dose experiments, R21v was administered at 0.1 µg with 5 µg of MM. In case of mixed vaccination, two different VLPs were mixed in the same tube with MM adjuvant before injection.

2.4.2.2 Vaccination plan for IC experiments

Mice were immunized with IC as in table 4 and 5 in two separate experiments 1 and 2. ICs were first formed by mixing 1µg each of R21 and 2A10 (1:1 in a 2 mL tube. The tubes were incubated at 37°C, 1h at 50 rpm. MM and PBS were added thereafter, and tubes were kept on ice till use for injections. For experiment 2, additional 1 µg of R21 was added to the IC mix before immunizing some groups. For experiment 1, IP route of immunization was also performed along with IM. This plan was followed for all 3 immunizations, prime, 1st boost and 2nd boost. The vaccination plan followed is as in figure 2b. For experiment 2, IM and SC routes were chosen and 2 immunizations were performed instead of 3 (table 4).

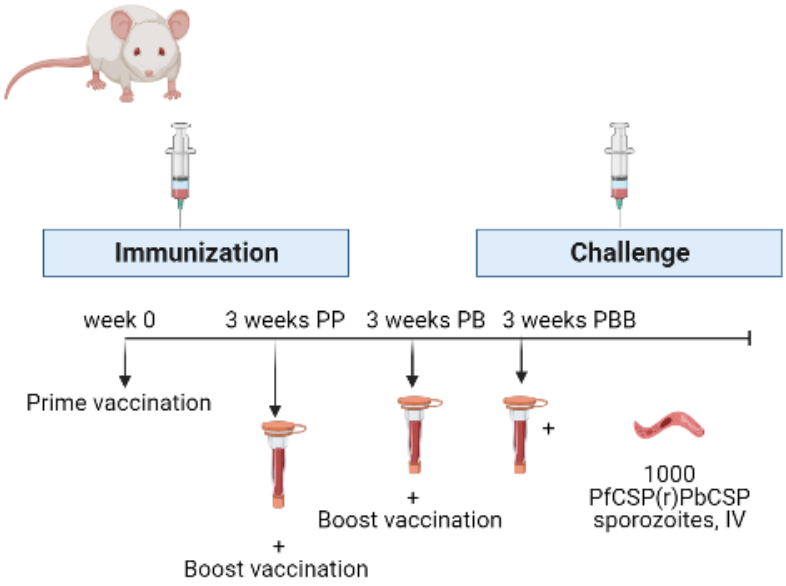


Figure 2b: Prime-Boost immunization plan. PP= Post Prime, PB= Post Boost. The mice were immunized and a boost vaccination was done 3 weeks PP with blood sampling 3 weeks PP and PB. For experiment 2, only 2 immunizations were performed. The mice were challenged with 1000 *Pf*CSP®*Pb*CSP sporozoites 3 weeks post final boost vaccination.

Table 2h: Vaccination groups for IC experiments

Experiment 1		
No.	Group	Route (volume injected)
1	R21+2A10 IC, No MM	IM (50µL)
2	R21+2A10 IC + MM	IM (50µL)
3	R21+2A10 IC + MM	IP (200µL)
4	2A10, No MM	IM (50µL)
5	2A10, No MM	IP (200µL)
6	R21 + MM (control group)	IM (50µL)
7	Naïve	

Experiment 2		
1	R21 + 2A10 IC +R21 +MM	IM (50µL)
2	R21 + 2A10 IC +R21 +MM	SC (50µL)
3	R21 + 2A10 IC +MM	IM (50µL)
4	R21 + 2A10 IC + MM	SC (50µL)
5	R21 + MM	IM (50µL)
6	R21 + MM	SC (50µL)
7	Naïve	

2.4.2.3 Vaccination plan for Alpha-Gal experiments

For the 1st experiment, AgalT KO mice were injected SC (200µL) with 75µg of Alpha Gal trisaccharide along with MM and R21 depending on the group as described in table 2.8 below. Prime and boost vaccination plan (Figure 2a) was followed for this experiment. The mice were challenged with sporozoites 3 weeks post boost as in section 2.4.5.

Table 2i: Vaccination groups for Alpha-gal experiment 1

Group	Vaccination, SC
1	alpha gal + MM
2	R21 +alpha gal + MM
3	R21 + MM
4	Naïve

For experiment 2, AgalT KO mice were injected with 75µg of Alpha Gal trisaccharide-BSA along with MM and R21 depending on the group as in the table 2.9 below with

different routes of immunizations. As the aim of this experiment was to know the immune response to BSA conjugated Agal trisaccharide with and without the adjuvant MM and the best route of immunisation and not efficacy, no challenge with sporozoites was performed. Each group was immunized 3 times as prime, boost and 2nd boost (Figure 2b).

Table 2j: Vaccination groups of Alpha-gal experiment 2

No.	Group	Route (volume injected)
1	Agal-BSA	IM (50µL)
2	Agal-BSA + MM	IM (50µL)
3	Agal-BSA	SC (200µL)
4	Agal-BSA + MM	SC (200µL)
5	Agal-BSA	IP (200µL)
6	Agal-BSA + MM	IP (200µL)
9	Naïve	

2.4.3 Sporozoite production and challenge

Frozen *P. berghei* pRBC of the chimeric parasite line *PbANKA-PfCSP(r)*$_{PbCSP}$ (2257 cl2) were thawed and 100-300 µl injected intra-peritoneal (i.p.) into a naive BALB/c mouse. Five to six days later the parasitaemia and gametocytaemia were determined by analysing Geimsa stained thin blood films and addition of a drop of blood to exflagellation media enabled the analysis of exflagellation. Anaesthetized mice were placed onto pots of starved 4-7 days old female *Anopheles stephensi* mosquitoes for approximately 10-15 minutes. Mosquitoes infected with the chimeric *P. berghei* parasite were maintained at 19-23°C in a humidified incubator and fed on fructose solution. At approximately 21-23 days

post-feed mosquito salivary glands were dissected to obtain infectious sporozoites in the appropriate cell culture media and homogenized on ice. Sporozoite numbers were counted using a haemocytometer under phase contrast.

To test the efficacy of the vaccines, vaccinated and naïve control mice groups were injected with 1000 sporozoites by intravenous (IV) injection into the lateral tail vein approximately 3 weeks post the final boost vaccination. Mice were monitored from day five post-injection via thin blood films. Once parasite positive blood films had been confirmed on three consecutive days, mice were sacrificed. The parasitemia level from three blood smears allowed calculation of time to 1% parasitemia via linear regression. If thin blood films were negative fourteen days post-infection mice were classes as "protected" and were sacrificed.

2.4.5 Preparing Thin-blood Smears

Thin-blood smears were prepared on glass slides from a drop of blood obtained from the tail snip. The blood smear was allowed to air-dry, then fixed with methanol, and stained for 0.5-1 h with 5% Giemsa diluted in Milli-QH2O. Slides were then removed and allowed to air-dry at room temperature. The slide was viewed on a light microscope at 100x under oil immersion.

2.4.6 Statistical model for parasitaemia prediction

To predict the time to 1% blood-stage infection a linear regression modelling was used as described previously [159]. The logarithm to base 10 of the calculated percentage of parasitaemia was plotted against the time post-challenge and GraphPad Prism 5 statistical analysis software used for generating linear regression model on the linear part of the blood-stage growth curve. This approach has been previously used; because time

to blood-stage parasitaemia reflects number of parasites erupting from the liver, provided there is no blood stage immunity [160].

Chapter 3

Expression and production of R21 vaccine variants

3. Expression and production of R21 vaccine variants

3.1 Introduction

VLPs have been proved to be generally safe and a number of vaccines have been licensed for use in humans using this platform, such as hepatitis and papilloma virus vaccines. Expression of the desired antigen with a protein sequence capable of self- assembling into particles have made development of VLP vaccines possible using mammalian cells, yeast and bacteria. VLPs have been known to induce robust antibody and also a cell mediated immune response making them an ideal vaccine platform. The two malaria vaccines in the most advanced stage of development currently RTS,S and R21 have been developed using the hepatitis B surface antigen VLP platform. Both the vaccines elicit protective antibody response to the central NANP repeats in the CS protein. R21 displays the immunogenic sequence (18 x NANP + C terminus) fused to every HBsAg in contrast to RTS,S, in which one fusion protein is found for every four native unfused HBsAg molecules. Thus R21 has the advantage of increased antigenic display on the surface of the VLP and almost all the immune response is limited to immunogenic sequence rather than HBsAg as seen in RTS,S. Collins et al showed that there was no staining of R21 with a Mab to HBsAg presumably because the VLP surface is coated with CSP sequence[161]. In pre-clinical studies, when challenged with transgenic sporozoites, R21 elicited sterile protection in BALB/c mice after 3 vaccination doses [162]. R21 vaccine has completed a number of clinical trials assessing safety of the vaccine with the adjuvant Matrix M1 (NCT02925403) and with/without MM (NCT02572388), safety and tolerability of the vaccine with the adjuvant AS01B (NCT02600975), a safety and efficacy trial with ChAd63/ MVA ME-TRAP (NCT02905019), while Phase I/II/III field trials involving both children and adults are at different stages of progression (NCT03580824, NCT03896724, NCT03947190; NCT04704830). Another interesting ongoing trial

(NCT03970993) is looking into different doses of R21/MM and assessment of efficacy by CHMI[163]. The most recent Phase IIb trial in 5-17 month old children living in a malaria endemic high transmission region of Burkina Faso demonstrated an excellent efficacy of 77% 1year post vaccination[28], reaching above the 75% efficacy goal set by WHO in African children over 1 year. With a follow up Phase II trial, R21 Phase III trial is underway in five African sites [151] and NCT04704830. For both RTS,S and R21 the protective antibody levels and efficacy wane over time [28] and a malaria vaccine with higher durability still remains a target to achieve. As the R21 vaccine was developed at the Jenner Institute, excellent data obtained from pre-clinical and clinical trials have provided a base for the development of improved malaria vaccines. Recent studies have highlighted the protective potential of antibodies generated to the cleavage site and junctional region [164] of CSP, which do not form part of either the RTS,S or R21 vaccine. These two regions lie in the N- terminal half of the CSP near to the junction of the central NANP major repeat region. The junctional epitope (KQPADGNPDP) is focused on a unique NPDP four amino acid at amino acids 101- 104 [165, 166], to which high avidity monoclonal antibodies (CIS43 and MGG4) have been identified, which cross react with NANP repeats. The former has recently shown high efficacy in a human CHMI trial[167]. The sequence of interest in the cleavage site is amino acids 81- 91: EDNEKLRKPKH, the antibodies to this linear epitope (MAb5D5) when combined with those to anti NANP repeat sequence (MAb 2A10) provide greater efficacy [168]. To explore the full potential of the protective antibody generation by including these sequences, new R21 variants were designed to compare with the existing R21 vaccine. It has also been proposed that repetitive antigens especially on parasite surface proteins can be important vaccine components, but can also interfere with the affinity maturation of antibodies against protective antigens [139]. In *P. falciparum*, it was observed that a synthetic peptide that contained three copies of NANP bound monoclonal antibodies as

effectively as did intact sporozoites [169]. The presence of immune dominant repeats and presence of high levels of antibodies to the repeats in individuals still susceptible to infection, show that the repeats have been evolutionary selected to evade host immune response by hyperstimulation of irrelevant B cell responses and diverting away from more critical epitopes [170]. Hence, reducing the number of NANP repeats has also been considered while designing the R21v constructs. In addition to this, constructs that demonstrated high efficacy were further modified to include a universal CD4+ T cell helper non-natural sequence called the PADRE (pan HLA DR- binding Epitope) sequence. Based on the findings of Jardetzky et al., PADRE sequence was engineered by introduction of poly- D- alanine at N and C termini and stabilizing derivatives of alanine in influenza hemagglutinin epitope 307-319 [171]. PADRE binds to 15 to 16 most common human HLA- DR types with high affinity and activates CD4+ T cells [172]. CD4+ T cells play an important role in humoral immunity by providing B cell help, enable antibody class switching and affinity maturation [173], which can further enhance the antibody response. The sequence has been constructed to be immunogenic in humans and was found to be 100 fold more potent on a molar basis than a control tetanus toxoid-derived universal epitope, when evaluated in a human T cell proliferation assay [174]. The sequence being inserted in the new constructs is AKFVAAWTLKAAA (Ala-Lys-Phe-Val-Ala-Ala-Trp-Thr-Leu-Lys-Ala-Ala-Ala) [175].

Both RTS,S and R21 vaccines have been produced using different strains of yeast. RTS,S has been produced using *Saccharomyces cerevisiae*[176], while *Pichia pastoris* was used for the production of R21 [161]. *P. pastoris* holds a number of advantages over *S. cerevisiae* such as production of high cell biomass and 10-100-fold higher protein yield driven by strong promoters like AOX1. Also, in comparison to *S. cerevisiae*, the length of glycans added during post translational modifications is smaller and glycosylations like the terminal α1,3 glycan linkages that can lead to adverse immunogenic reactions are absent

in *Pichia* [177]. GS115 is the most commonly used *P.pastoris* strain in biopharmaceutical industry due to its ability to grow high cell densities in large scale fermenters [178]. Pre-clinical batch of R21 was produced using the *P. pastoris* strain PichiaPink and owing to aggregation issues and low yield, switched to GS115 strain.

3.2 Results

3.2.1 Vaccine construct design

The R21 variants were made keeping the established R21 construct backbone. This was made at the Jenner Institute by Katherine Collins [161]. R21 sequence is a fusion of 18 NANP repeats and C terminus of the *Pf*CSP from the NF54 strain and HBsAg. This construct was modified to add a C-tag (amino acids EPEA) to the C terminus of R21 (R21c) to allow affinity-based purification of highly pure particles using a column with anti-EPEA antibodies linked matrix. This was developed by Florian Brod at the Jenner Institute and led to the generation of the first R21c batch produced for Phase I clinical trials. Addition of the tag did not interfere with particle formation and yielded 10 fold more vaccine product than the previous un- tagged version [179]. All the R21 variants sequences were designed at the Jenner Institute by Ahmed M. Salman, and include the C-tag sequence. These were all generated across different time periods so that results from the initial constructs helped inform the design and generation of later candidates. The amino acid sequences used to make R21 variants are as described in table 3a. The combination in which these sequences were included to form the 25 R21 variants is as in table 3b.

Start of the sequence: Part of *Pf*CSP sequence present depending on variant	
N terminus of *Pf*CSP (aa 21-73)	QEYQCYGSSSNTRVLNELNYDNAGTNLYNELEM NYYGKQENWYSLKKNSRSLG
Cleavage epitope (aa 81-91)	ENDDGNNEDNEKLRKPKH
Junctional epitope (aa 95- 104)	KLKQPADGNPDP
Repeat region (between aa 105 to 272)	NANP and NVDP
C- terminus (aa 273-377)	NKNNQGNGQGHNMPNDPNRNVDENANANSAV KNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVT CGNGIQVRIKPGSANKPKDELDYANDIEKKICKM EKCSSV
Sequence not a part of *Pf*CSP, present depending on variant construct design	
PADRE sequence	AKFVAAWTLKAAA
Followed by: Non *Pf*CSP sequence present in all constructs	
4 amino acids from the pre-S2 region of the hepatitis B virus	PVTN
226 amino acids of the HBsAg	MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSW WTSLNFLGGSPVCLGQNSQSPTSNHSPTSCPPICP GYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLP VCPLIPGSTTTNTGPCKTCTTPAQGNSMFPSCCC TKPTDGNCTCIPIPSSWAFAKYLWEWASVRFSW LSLLVPFVQWFVGLSPTVWLSAIWMMWYWGPS LYSIVSPFIPLLPIFFCLWVYI

4 amino acids of C-tag	EPEA

Table 3a: Amino acid sequence of *Pf*CSP and other sequences present in R21 variants. The sequence of *Pf*CSP broken down as sequences that form the R21 variants depending on construct design.

Construct	Presence of sequence							
	N ter	CE	JE	NANP	NVDP	C ter	NPDP	PADRE
R21				+(18)		+		
G1		+	+	+(3)	+(3)			
G2		+	+	+(9)	+(3)			
G3		+	+	+(21)	+(3)	+		
G4	+			+(18)		+		
G5*	+			+(38)		+		
G6			+(5)	+(5)	+(5)			
G7		+(5)	+(5)	+(5)	+(5)			
G8				+(18)				
G9				+(18) @ c ter				
G9'R				+(18) rev				
G10				+(36)				
G11							+(18)	
G12		+(18)						
G13					+(18)			
G14		+	+	+(6)				
G15*		+	+	+(6)				+
G16			+	+(6)				+
G17				+(18)		+		+
G18				+(18)				+
G19*		+	+	+(3)	+(3)			+

G20		+	+	+(3) + PNAN				
G21			+	+(18)		+		
G22			+	+(18)		+		+
G23				+(6)	+(6)		+(6)	
G24				+(12)	+(12)		+(12)	

Table 3b: R21 variants design. R21 variant constructs designed to include specific region of the CSP protein, including 6 containing the T cell helper PADRE sequence. The order of sequences in the table indicate the order in which these are present in the construct followed by amino acids from Pre S2 region of HBsAg, HBsAg and C-tag as indicated in table 3a. (N ter: N terminus, CE: Cleavage epitope, JE: Junctional epitope, C ter: C terminus).

3.2.2 Expression, production and purification of R21v VLPs

The steps involved in production of VLPs from cloning to final purified VLPs is described in the process flow as below in figure 3a.

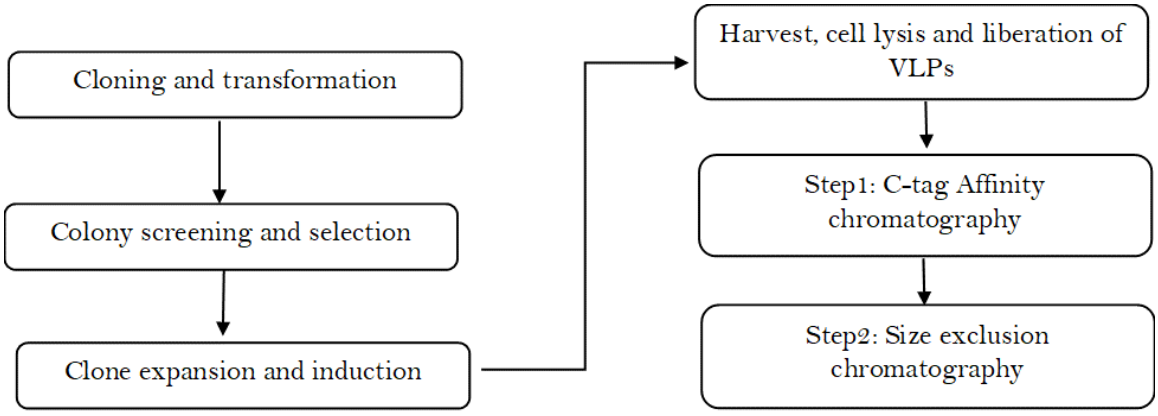


Figure 3a: Overview of process for production of R21v VLPs. After cloning of target gene sequence into pPIC3.5k plasmid and transforming into GS115, *Pichia pastoris* competent yeast cells made by Ahmed M. Salman using Invitrogen™ Pichia EasyComp™ Transformation Kit. Following the growth of the

transformed competent yeast cells, isolated colony were grown, induced and screened for presence of target sequence. Once confirmed clone was expanded and induced to produce the protein intracellularly. The cells were harvested and subjected to lysis followed by 2 purification steps, affinity based on C-tag followed by size exclusion chromatography to obtain the purified product.

3.2.2.1 Cloning and transformation

The codon optimized sequence obtained as a GeneArt plasmid was digested using restriction enzymes as BamHI and NotI with additional EcoRI digestion to break the plasmid backbone and prevent the plasmid re-ligation. The digested product was ligated into linearized pPIC3.5K vector (9004 bp) with the same restriction enzymes BamHI and NotI. pPIC3.5K is a plasmid designed to allow identification of transformants with multiple integrations of the desired gene in the *Pichia* genome. It is ideal of intracellular protein expression, hence suited to the VLP platform where protein particles retained intracellularly assemble to form particles on lysis. Post transformation into *E.coli* and amplification, the plasmid was extracted by midi-prep and linearized by SalI restriction enzyme. SalI linearizes the plasmid at His4 site and facilitate insertion at His4 site in the *P. pastoris* (GS115) genome. As GS115 is a histidine requiring auxotroph, only transformed clones grow in histidine deficient media. Competent GS115 cells transformed using electroporation were plated on Minimal Dextrose (MD) agar and kept at 28°c incubator. Positive colonies were chosen on after 3-5 days for small scale culture growth for expression analysis.

3.2.2.2 Screening of clones and selection for expansion

Isolated colonies were picked up from each plate and inoculated into BMGY medium and protein production was induced under AOX1 promoter by transferring cultures from

glycerol containing BMGY medium to methanol containing BMMY medium, 48 hours after growth initiation. The cultures were then induced for 48 hours with methanol feed every 12 hours. After 48 hours, the expression of the protein was confirmed by Western Blot analysis using anti NANP, anti HBsAg and anti C-tag primary antibodies. The clones confirmed positive on all three blots were chosen for expansion. A representative example of western blot analysis of R21 variant G4 clones using anti-HBsAg (Figure 3b (a)), anti- NANP (Figure 3b (b)) and anti- C-tag (Figure 3b (c)) antibodies is as described in Figure 3b.

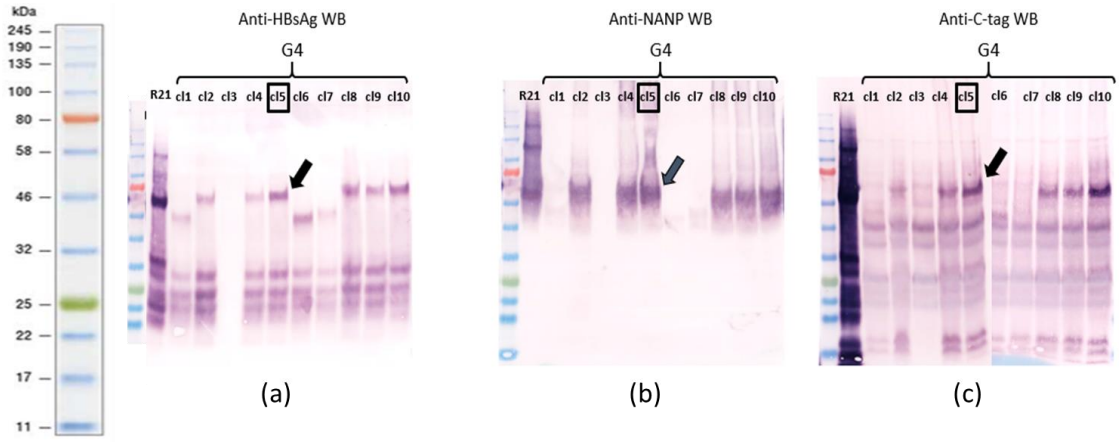


Figure 3b: Western blot analysis of R21 variant G4 (~56kD) using ant- HBsAg (a), anti- NANP (b) and anti-C-tag (c) antibodies. Clone was chosen for expansion after confirmation of band of correct size on all three blots.

For expansion, the culture was initiated by picking a portion of the confirmed clone colony from the plate and inoculating into BMGY in a 250 mL shaker flask. After 48 hours, 10 mL of culture was transferred to a 2 L flask with 1 L BMGY and grown for 2-3 days. The culture was induced when the OD600 reached 10- 15 absorbance units. For induction, the culture was spun down, and the pellet was re-suspended in BMMY. The cultures were fed with methanol as mentioned in 2.2.10, harvested 3 days post induction and the pellets were stored at -80°c until purification.

3.2.2.3 Purification of VLPs

The pellets were thawed at RT, resuspended in lysis buffer and lysed in a high-pressure homogenizer (~1000 bar). The lysed material was clarified to remove debris and homogenized again in liberation buffer to release VLPs. The clarified and 0.22 µm filtered material was loaded onto affinity C-tag column. The VLPs were eluted as fractions (Figure 3c (a)) and pooled for final purification step using size exclusion chromatography to get the product as uniform particles buffer exchanged in TBS. The purified material from SEC was collected in form of fractions (Figure 3c (b)). The SEC fractions were analysed using SDS-PAGE and Western Blot analysis as described in section 2.2.9. Figure 3d shows the electrophoresis and blot profile of all purified constructs indicating the purity of the material.

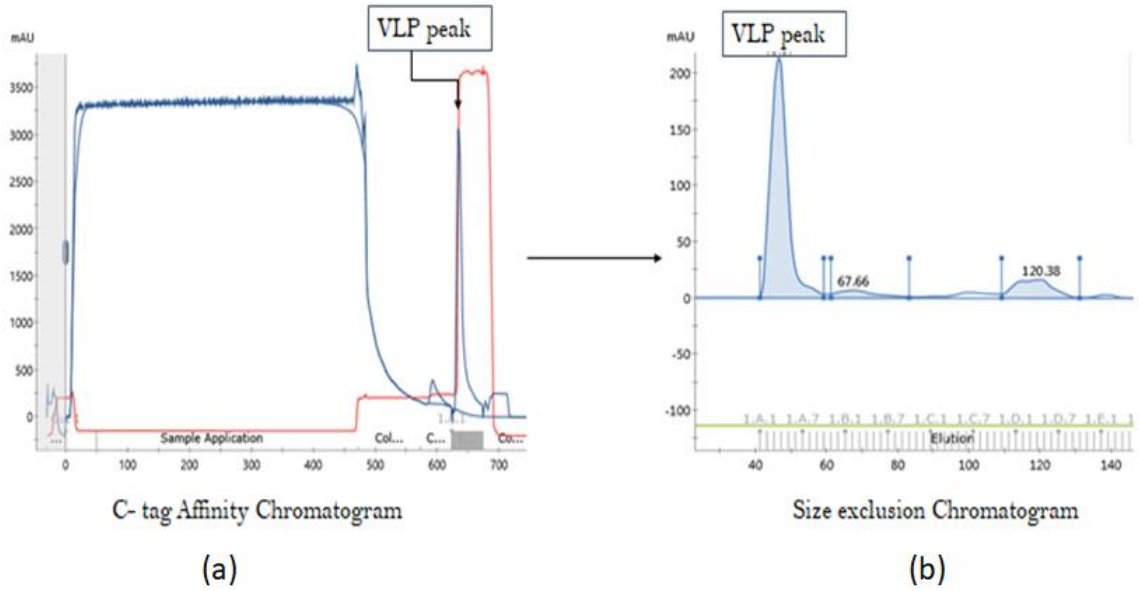


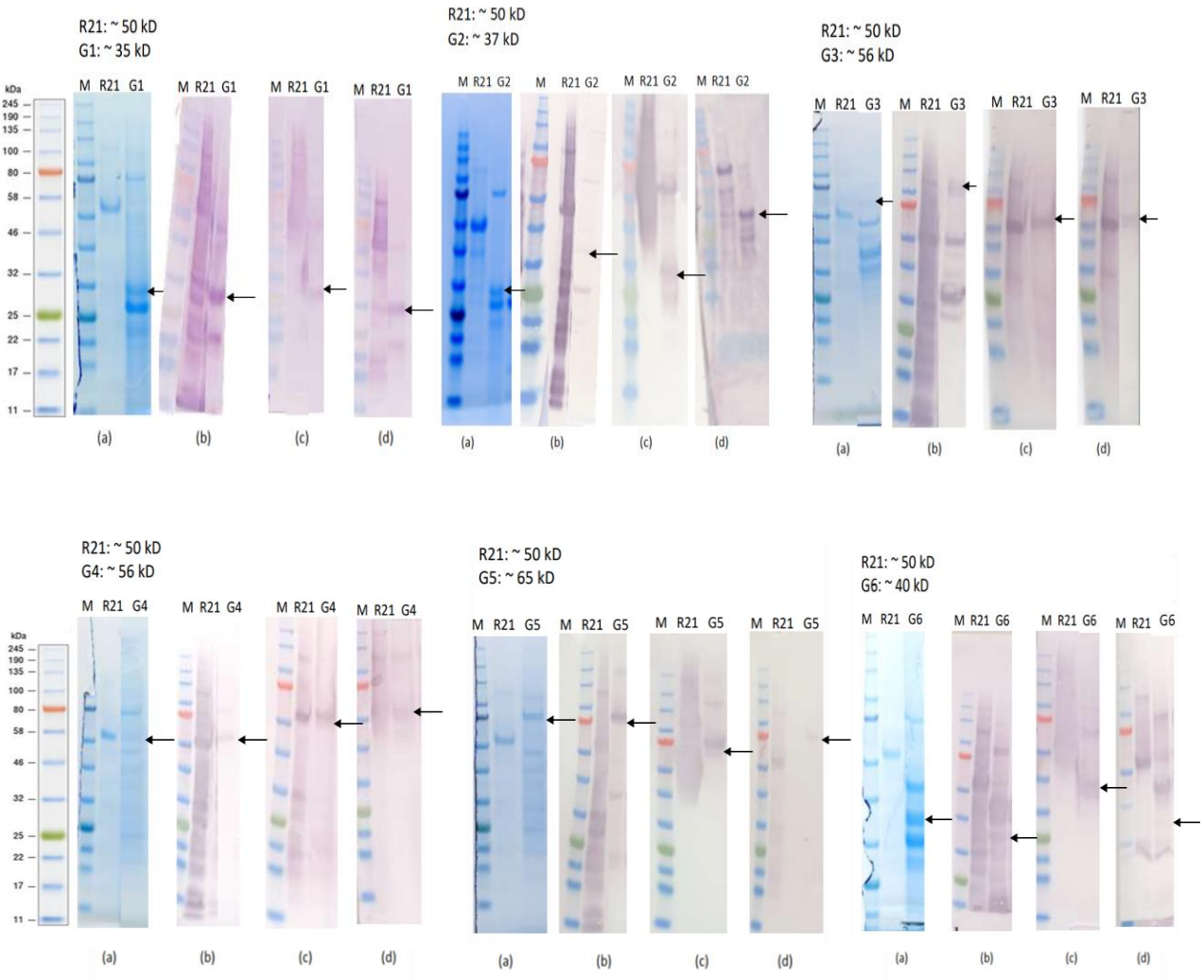
Figure 3c: C-tag affinity and Size exclusion chromatograms from a representative R21v G4. The eluted peak of VLPs was collected from C-tag column as fractions. The fractions from 50mAu to peak to 50mAu were pooled and loaded onto SEC

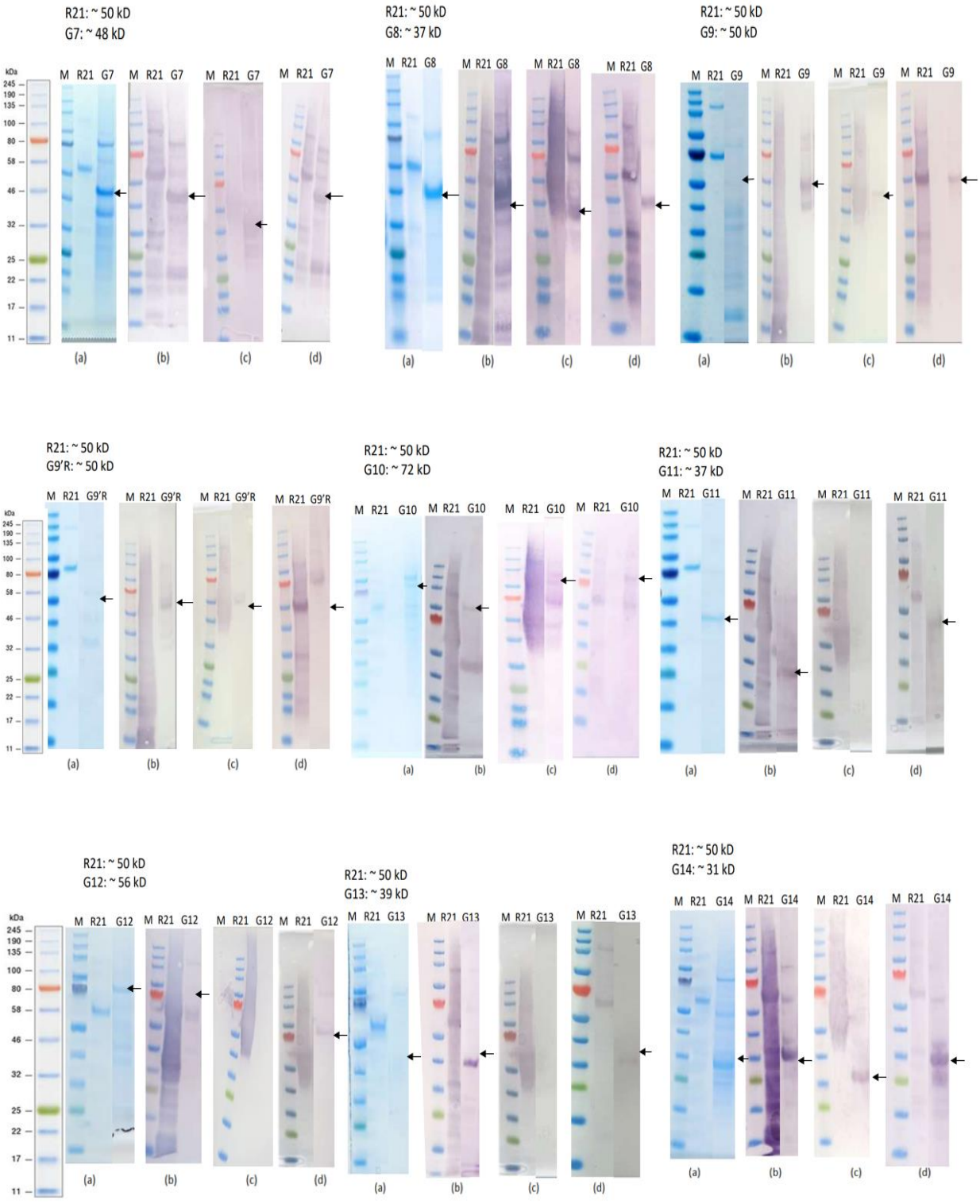
column. Post SEC, the buffer exchanged VLP fractions were analysed for confirmation presence of amino acid sequence before pooling them.

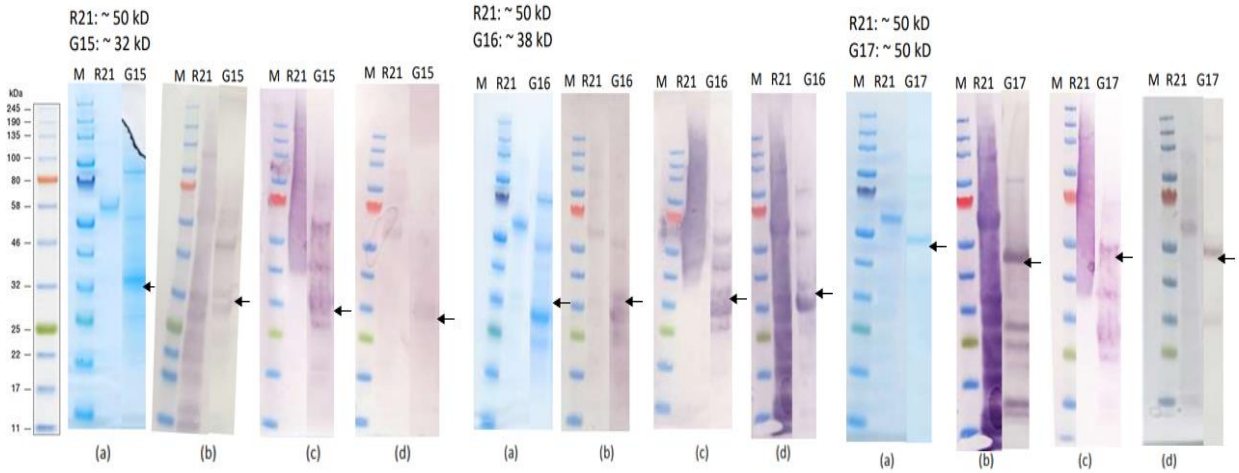
3.2.3 Analysis of purified VLPs

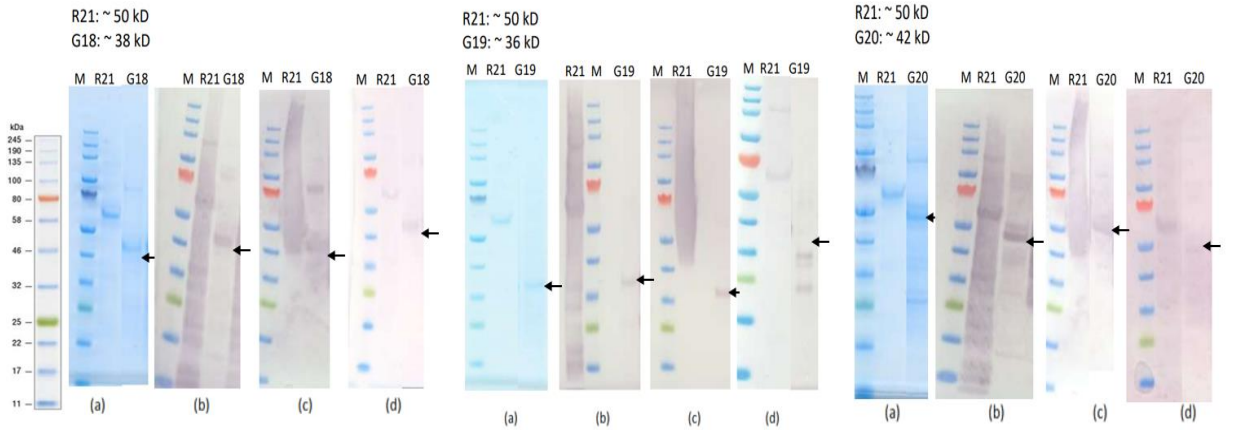
3.2.3.1 Confirmation of protein expression

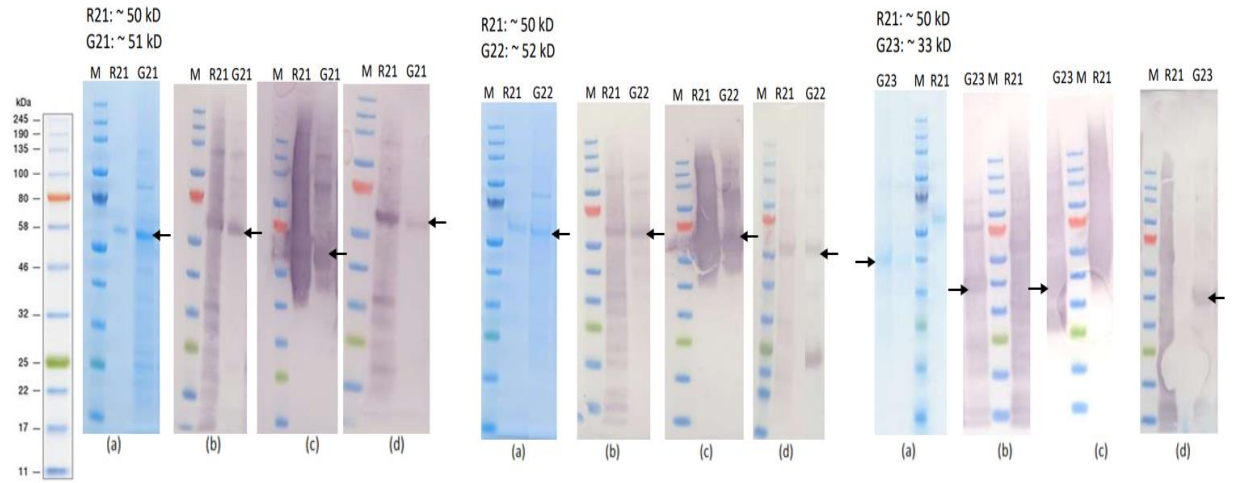
The expression of the protein was confirmed via Coomassie gel staining (a) and western blotting with anti C- tag (b), anti- NANP (c) and anti- HBsAg (d) antibodies. The R21v was considered for immunogenicity assessment only after confirming the presence of correct size molecular weight band on the gel and the blot. Figure 3d below shows expression analysis results for the R21v constructs used in vaccination.




R21: ~ 50 kD
G7: ~ 48 kD
R21: ~ 50 kD
G8: ~ 37 kD
R21: ~ 50 kD
G9: ~ 50 kD
R21: ~ 50 kD
G9'R: ~ 50 kD
R21: ~ 50 kD
G10: ~ 72 kD
R21: ~ 50 kD
G11: ~ 37 kD
R21: ~ 50 kD
G12: ~ 56 kD
R21: ~ 50 kD
G13: ~ 39 kD
R21: ~ 50 kD
G14: ~ 31 kD
kDa
245
190
135
100
80
58
46
32
25
22
17
11
M R21 G7
M R21 G8
M R21 G9
M R21 G9'R
M R21 G10
M R21 G11
M R21 G12
M R21 G13
M R21 G14
(a)
(b)
(c)
(d)


R21: ~ 50 kD
G15: ~ 32 kD
R21: ~ 50 kD
G16: ~ 38 kD
R21: ~ 50 kD
G17: ~ 50 kD
M R21 G15
M R21 G16
M R21 G17
(a)
(b)
(c)
(d)


R21: ~ 50 kD
G18: ~ 38 kD
R21: ~ 50 kD
G19: ~ 36 kD
R21: ~ 50 kD
G20: ~ 42 kD
M R21 G18
M R21 G19
M R21 G20
(a)
(b)
(c)
(d)


R21: ~ 50 kD
G21: ~ 51 kD
R21: ~ 50 kD
G22: ~ 52 kD
R21: ~ 50 kD
G23: ~ 33 kD
M R21 G21
M R21 G22
G23 M R21
(a)
(b)
(c)
(d)

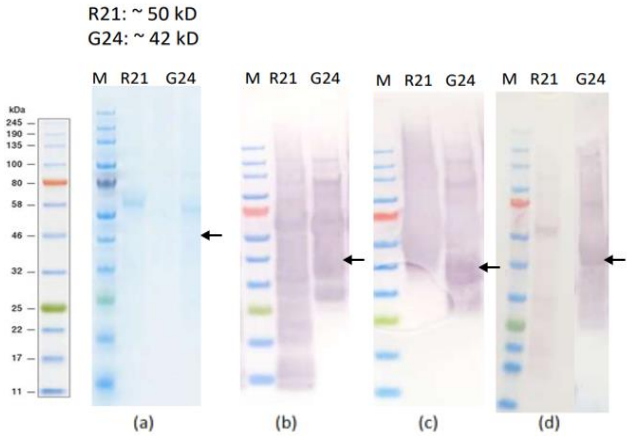
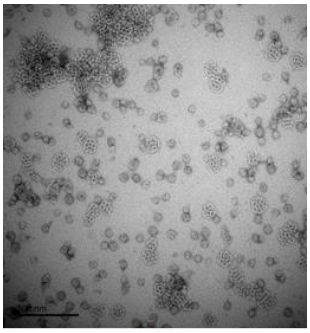


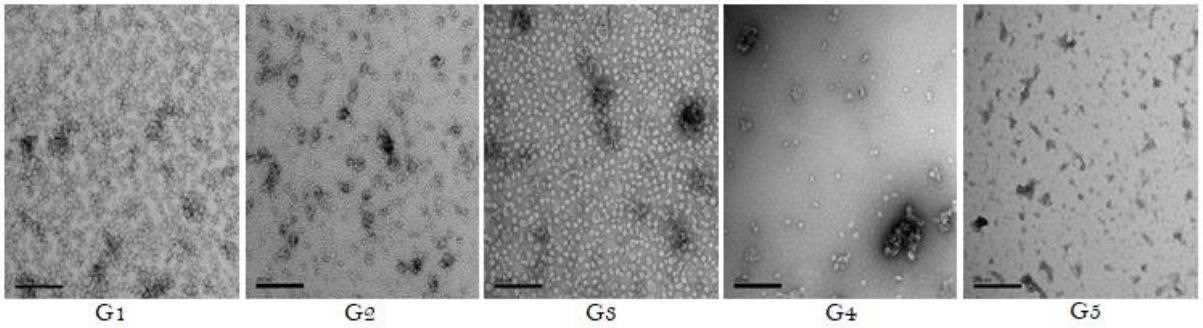
Figure 3d: Confirmation of protein expression by Western blot using Coomassie stain (a), anti C- tag (b), anti- NANP (c) and anti- HBsAg (d) antibodies. All the purified constructs were analysed for the presence of approximately correct size of band in comparison to the positive control R21. Presence of more than one band indicates possible dimer formation or presence of oligomers with varied levels of glycosylation.

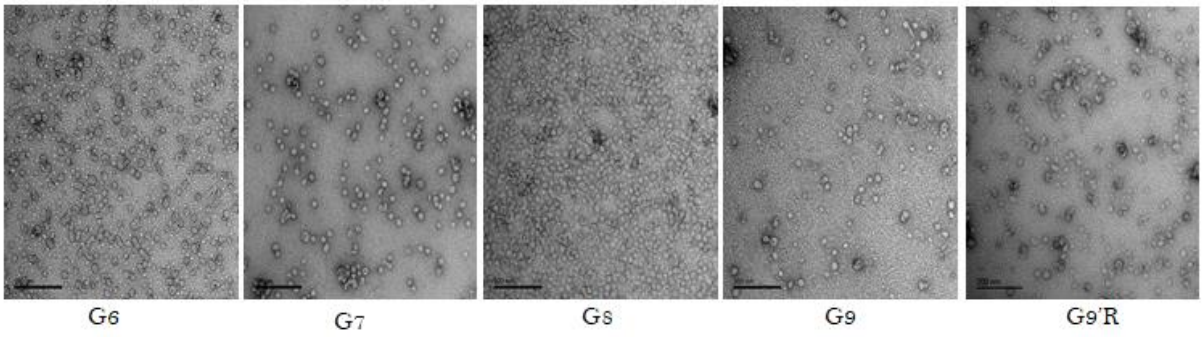
3.2.3.2 Confirmation of VLP formation

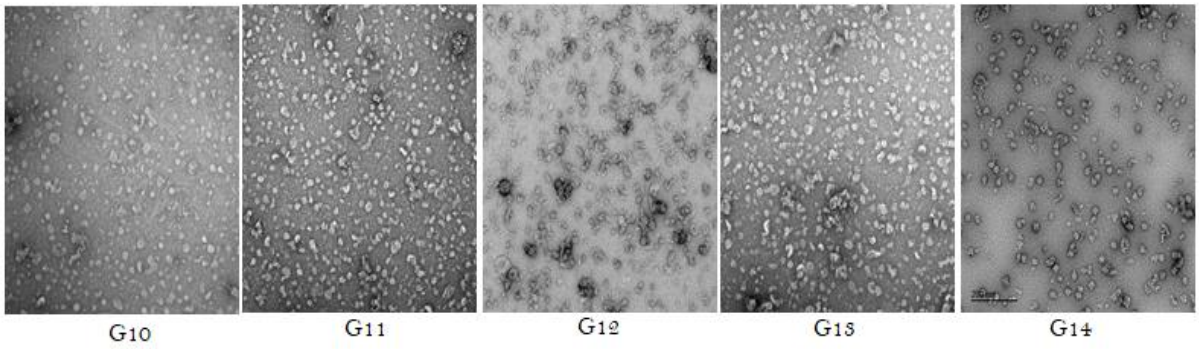
All the R21 variant VLPs were imaged and analyzed by Ahmed M. Salman using TEM at the Sir William Dunn School of Pathology EM Core Facility, to confirm the presence of particles (Figure 3e).

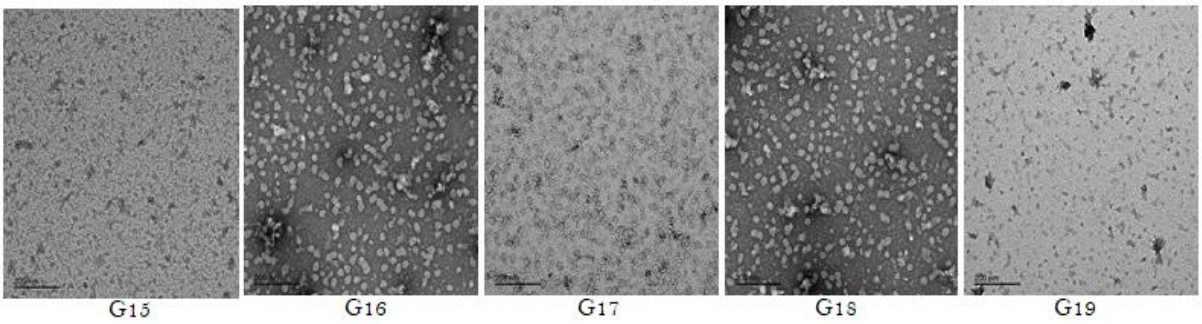


R21


G1
G2
G3
G4
G5


G6
G7
G8
G9
G9'R


G10
G11
G12
G13
G14


G15
G16
G17
G18
G19

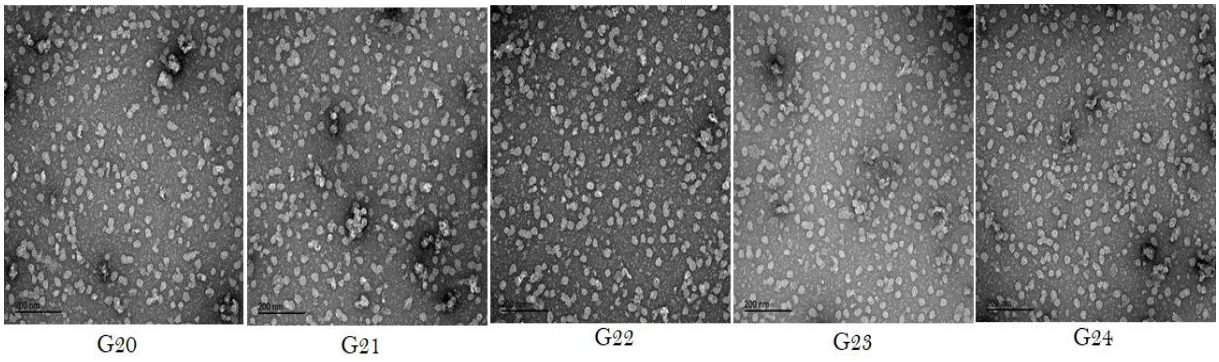


Figure 3e: TEM images of purified R21v VLPs with positive control R21 shows presence of particles ~20nm in size. G5, G15 and G19 failed to form VLPs as evident from the images. Scale 200nm.

3.3 Discussion

This chapter describes the production and quality control steps for R21 variants that has been used in pre-clinical studies in this thesis to assess the immunogenicity and protective in comparison to the current gold standard malaria VLP vaccine R21. As highlighted before, even though R21 has demonstrated excellent efficacy of 77% over 1 year in children, levels of protective antibodies and efficacy wane over time, identifying a persistent need for even better vaccine with longer durability that can help reduce repeated immunizations to maintain the efficacy. With new immunogenicity data on previously unexplored regions of the CSP available along with their synergistic effect with already known and well-studied regions like the NANP repeats, this opened an avenue to include them in R21 variants. Including these sequences as repeats to understand the immune response elicited and in combination with other sequences to understand potential synergistic or antagonist effect was kept in mind while designing the variants. This led to the generation of 25 new R21 variants. All the purified VLPs were subjected to post purification analysis using SDS PAGE, Western Blotting and TEM. Other methods routinely used for VLP characterization are analytical size

exclusion chromatography and Dynamic light scattering to determine particle size and level of aggregation. For Western blot and SDS PAGE profiles that show bands but slightly higher than the expected molecular weight due to varied levels of glycosylation, Mass spectrometry can used to confirm the sequence. These were not feasible to perform due to unavailability of or access to the equipment due to COVID pandemic restrictions. All the purified VLPs show varied degrees of purity and degradation. From previous experiments, it has been shown that R21 administered at an ultra- low dose of 0.05µg with the adjuvant AS01, elicited anti-NANP antibodies and sterile efficacy (at least 80%) of the same magnitude as higher doses [179]. Hence, looking into process development steps to improve purity was not explored and current level of purity was considered sufficient to include the VLP candidates in a pre-clinical study. Three of the constructs G5 (N terminus+ 38 NANP + C terminus), G15 (JE + CE + 6 NANP+ PADRE) and G19(JE + CE+ 3x NANP-NVDP + PADRE) did not form a VLP as evident from the EM images. Considering that a number of clones were tried from each these constructs and all did not form VLPs, shows that this issue is not clone and process dependent. In-process EM analysis to ascertain the possible cause is not possible due to presence of detergents during lysis steps and high salt during affinity purification step. Analysis with these samples would require buffer exchange ideal for EM, and as this happens during the final purification step, EM was only performed after SEC. There is also a possibility that the unique arrangement of sequences in these constructs might be interfering with a stable VLP formation. One of my hypotheses that can be linked to this is possible hydrophobic or hydrophilic changes arising due to this unique arrangement. If the R21 variant sequence attached to the hydrophobic HBsAg sequence is itself hydrophobic in nature, it might be incorporated into the lipid membrane and thereby interfere with the stability of the VLP structure. To assess this, using the Expasy ProtParam online tool, GRAVY (Grand Average of Hydropathy) number was determined which indicates

protein hydrophobicity as negative value and hydrophilicity as a positive value. It is calculated as the sum of hydropathy values of all amino acids in a protein or peptide sequence divided by the number of amino acid residues in it[180]. It helps to determine the possibility of a sequences to be transmembrane or buried in the protein scaffold due to high hydrophobicity value or freely available which might be for e.g. able to bind to antibodies. Unfortunately, the GRAVY number determined for each construct as a fusion protein including HBsAg and EPEA sequence, was inconclusive. Post purification strategies for stable VLP formation such as use of reducing agents with optimal low ionic strength and high pH conditions [181] and use of KSCN-heat induced particle maturation [182] could be tried as a possible way to form VLPs from purified material for these constructs. All the 25 new variants of R21 were included in a prime-boost vaccination regimen for the pre-clinical studies including R21 as a positive control to identify the differences in immunogenicity and protective efficacy between these vaccine candidates. The results of this work are described in the next chapter.

Chapter 4

Assessment of immunogenicity and efficacy of R21 vaccine variants

4. Assessment of immunogenicity and efficacy of R21 vaccine variants

4.1 Introduction

With the aim to generate a VLP vaccine with better efficacy and protective antibody durability than R21, 25 new R21 variant vaccines were generated, as described in the previous chapter . This chapter describes the immunogenicity and efficacy results obtained from the pre-clinical studies on BALB/c mice. For the first stage candidate screening, all the variants along with the positive control R21 vaccine, were assessed in a prime-boost immunization regime at a dose of 1µg with 5µg of adjuvant Matrix-M1. Matrix-M1 was the obvious choice for an adjuvant considering its safety and excellent protective immune response enhancing properties as demonstrated in a number of pre-clinical studies in mice and clinical trial studies in humans [151, 161]. This adjuvant was thus kept as a constant not only for initial screening, but also for further assessment of better performing candidates in different formats and combinations, which is described in length further in this chapter.

It is crucial that for a protective vaccine that the longevity and durability of immune correlate(s) is maintained to avoid repeated immunizations. The longevity of antibodies has been found to be varied depending on type of antigen and individual responses [183]. Long term natural immunity developed in absence of re-exposure to pathogen has been observed after measles, yellow fever and polio infection and has been seen to last for more than 40 years. In a study in Sweden, differences in persistent antibody titers have been seen post vaccination in inactivated polio Salk vaccine (> 10 years) in different age groups, while those for diphtheria and tetanus in DTP vaccination were seen to wane earlier, indicating difference in immune memory generation [184, 185]. Figure 4a gives an idea

about the varied antibody durability associated with vaccination as analyzed from outbreaks post vaccination from US, Europe, Canada and Asia [186].

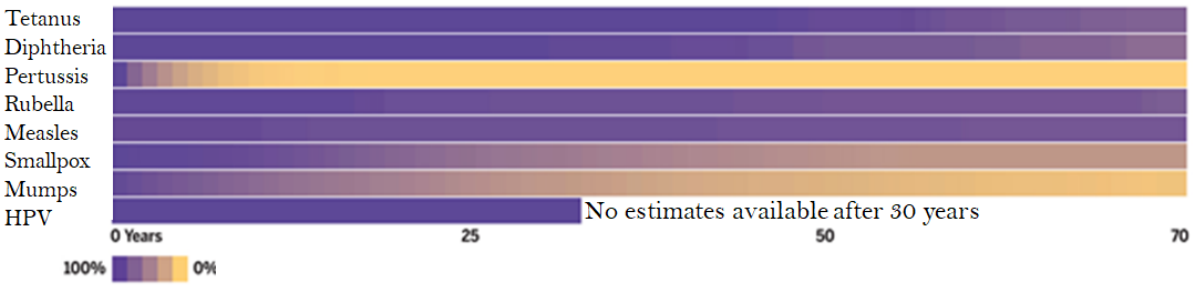


Figure 4a: Variations in vaccine durability in terms antibody levels as measured in four regions of the world.

In parasitic infections like malaria, the factors like complex life-cycle associated number of proteins and antigenic variation [187], contribute to the challenges in making an effective vaccine. As mentioned earlier, the three malaria vaccines PfSPZ, RTS,S and R21 have shown promising efficacy so far, but the anti-CSP antibody levels linked to protection wane over time and re-boosting becomes necessary to bring the antibody titre to protective levels. Hence, a vaccine with not only high efficacy but one with long-lasting high efficacy is still desirable. During the initial screening of first 14 vaccines (R21v G1 to G13), it was evident that R21v G1 (CE + JE + 3 x NANP-NVDP) performed the best in terms of efficacy and was used in an antibody longevity and durability study for head to head comparison with R21, the results for which are described in section 3.2.2.

Dose fractionation is a useful strategy in vaccination for dose sparing and cost reduction. This has been studied in many vaccines for diseases like influenza, polio and yellow fever, with resulting immunogenicity equivalent or higher than a full standard dose [188]. A phase I trial on R21 vaccine by the Jenner Institute in UK and Burkinable volunteers showed that 3 doses of 10 µg elicited significantly higher antibody responses at 6 months

than full dose of 50 μg [189]. Earlier, in RTS, S vaccine trial with different adjuvant formulations, reduced dose approach ($1/5^{th}$) was adopted in response to adverse reactions after standard 2nd dose of 50 μg for 2 out of 3 formulations; and found to be more immunogenic and efficacious than the full dose [190]. Based on this, in a Phase IIa RTS,S CHMI trial, higher efficacy of 86.7% was observed when three doses (0,1 and 7 months) were administered when third dose was a delayed fractional dose ($1/5^{th}$) of the 50 μg dose, in comparison to 3 full doses a month apart (0,1, and 2 months, 62.5%)[191]. This strategy has shown to increase efficacy against homologous CHMI, antibody somatic hypermutation and avidity in malaria-naïve adults [192]. The protective effect of delayed fractional dose has been attributed to possible reasons like the full third dose with one-month spacing regime might hinder the selection of high-affinity B and T cell clones, either through overstimulation and anergy, or through weak selection pressure resulting from high antigen availability[193]. The promising pre-clinical stage results from the R21 variant G1 and G18 at 1μg dose and reduced dose results from the human trial, provided a base to assess immunogenicity of these vaccine variants at reduced dose in a pre- clinical experiment. In the following experiment, this was tested comparing G1 and G18 with R21 at 1 μg and one- tenth i.e. 0.1 μg and reducing it further to one-hundredth (0.01 μg), with the standard Matrix- M dose of 5 μg.

Co-administration of more than one vaccine delivered by mixing two or more vaccines targeting different diseases has proven safe and potent as in diphtheria, tetanus, and pertussis vaccine which has been used in pediatric populations over the past 50 years[194]. These mixtures are often expanded to include more antigens like in the hexavalent Infanrix hexa® vaccine (GSK), which is a multicomponent inactivated vaccine targeting hepatitis B and two serotypes of poliomylitisin addition to the above three[195]. This vaccine consists of vaccine components as a combination of toxoid, recombinant protein, inactivated virus and polysaccharide adsorbed on toxoid, providing

an excellent example of component compatibility and safety when mixed together. With the aim to assess if the two best performing candidates can give a synergistic or antagonist effect when combined together, a mixed vaccination strategy was employed. One of the groups was injected with a mixture of G1 and R21 (1 µg each) with Matrix M (5 µg) and another group with G1 and G18 (1 µg each) with Matrix M (5 µg). The R21v candidate G11 (18 x NPDP) was also included in the study to co-administer with R21, even though G11 did not produce any efficacy in prime- boost vaccination challenge experiment. This has been further described in the section 3.2.3.2.

4.2 Results

4.2.1 New R21 variant vaccines are immunogenic with non- correlative efficacy

Twenty- five R21 variant vaccines were designed to study the inclusion of N- terminus region, Cleavage epitope, Junctional epitope, NVDP repeats and PADRE sequence, increasing and decreasing the number of NANP repeats and sometimes removing the C-terminal region of CSP. Table 4a describes these constructs according to the presence of a sequence indicated by +, while (n) indicates number of repeats of that sequence; and efficacy obtained in a pre-clinical study with 1000 sporozoite IV challenge post prime-boost vaccination. Delay to development of 1% parasitemia in comparison to naïve was observed in all constructs except G2, G9, G11, G12, G13. The constructs have been further categorized and grouped first on the basis of efficacy, then on uniqueness of the sequence and compared to R21 along with post prime and boost immunogenicity data. Statistical analysis was performed using Mann- Whitney test and a value of $p>0.05$ and less was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$). The constructs that did not form VLPs (G5, G15 and G19) have been excluded from further analysis as comparing them with R21 VLP would not be a suitable

comparison. For immunogenicity analysis, the antibody responses to the central NANP repeat region and junctional epitope KLKQPADGNPDP with additional NANP and NVDP were assessed. The rationale for including the additional repeats in the JE sequence was the in-conclusive results and cross-reactive antibody responses obtained from NVDP and NPDP to NANP. Also, after analysis using an extended peptide that included the cleavage epitope ENDDGNNEDNEKLRKPKH, and concluding that although the sequence is immunogenic (Figure 4f), it does not contribute to efficacy (table 4a), it was not included in further analysis for any constructs. As all the R21 variant constructs with CE included JE epitope (except G12), analysis was focused on antibodies to JE as mentioned above. Grouping of constructs has provided useful insights into possible antagonist and synergistic effects of antibodies to different sequences present in a construct with each other and a possible efficacy-enhancing role of T cell help. This has been discussed with each group below.

Along with this, immunogenicity and efficacy data for each construct as a part of individual experiment instead of sequence similarities have been detailed in the appendix. This shows that R21 efficacy ranges from 16.7% to 67% while for G1 it ranges from 50 % to 100% post 2 immunizations.

Construct	Presence of 1. Cleavage epitope: EDNEKLRKPKH 2. Junctional epitope: KLKQPADGNPDP 3. PADRE sequence: AKFVAAWTLKAAA 4. N terminus: QEYQCYGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWYSLKKNSRSLG 5. C terminus: NKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKCSSV								
	N ter	CE	JE	NANP	NVDP	C ter	NPDP	PADRE	Efficacy (%)
R21				+(18)		+			44.4^
G18				+(18)				+	100
G22			+	+(18)		+		+	83
G21			+	+(18)		+			80
G16			+	+(6)				+	67
G17				+(18)		+		+	67
G14		+	+	+(6)					67
G1		+	+	+(3)	+(3)				66.7^
G24				+(12)	+(12)		+(12)		33
G8				+(18)					16.7
G10				+(36)					16.7
G15*		+	+	+(6)				+	16.7
G2		+	+	+(9)	+(3)				0
G3		+	+	+(21)	+(3	+			0
G4	+			+(18)		+			0
G5*	+			+(38)		+			0
G6			+(5)						0
G7		+(5)	+(5)						0
G9				+(18) @ c ter					0
G9'R				+(18) rev					0
G11							+(18)		0
G12		+(18)							0
G13					+(18)				0
G19*		+	+	+(3)	+(3)			+	0
G23				+(6)	+(6)		+(6)		0

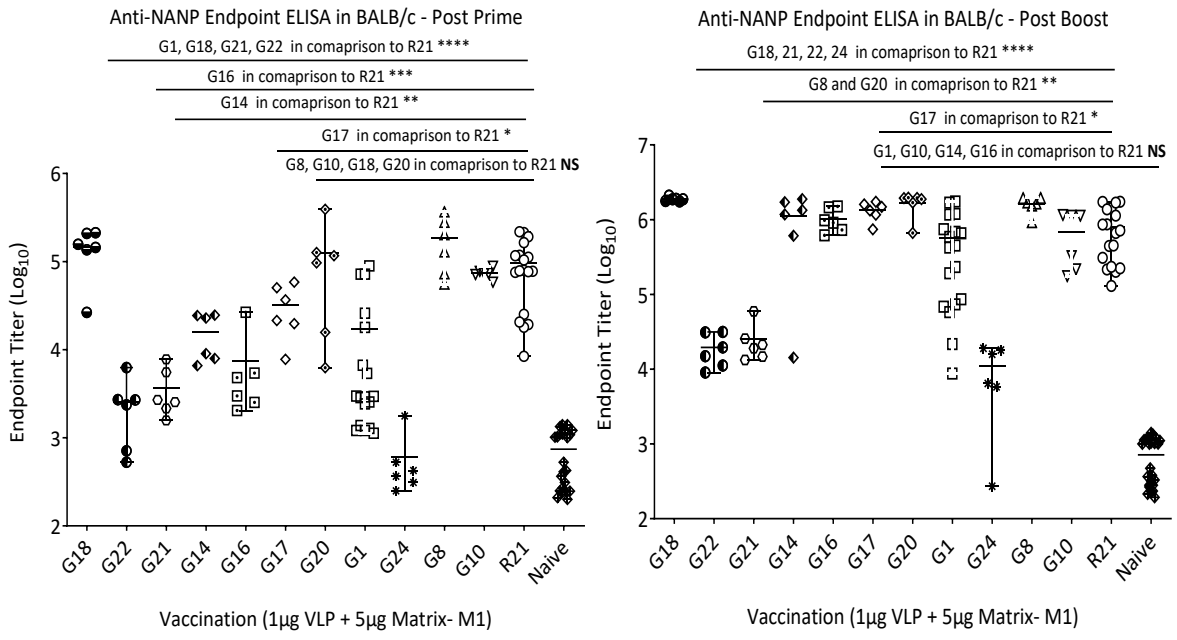
Table 4a: Sequence and related efficacy in BALB/c mice of 25 R21 variant constructs.

*Constructs that did not form VLP.

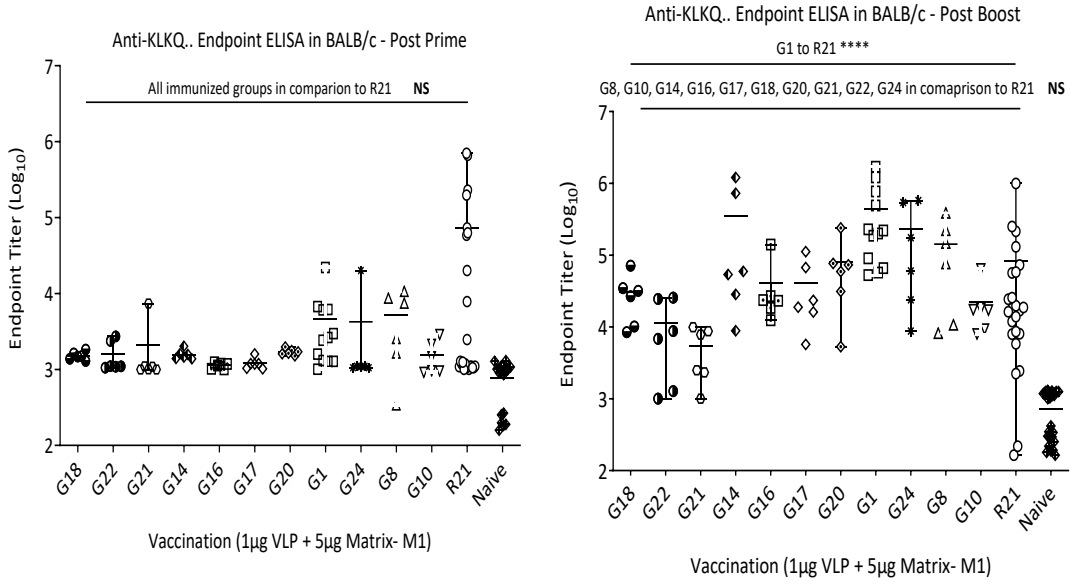
^ Average of 7 experiments

Construct	N ter	CE	JE	NANP	NVDP	C ter	NPDP	PADRE	Efficacy (%)
R21				+(18)		+			44.4
G18				+(18)				+	100
G22			+	+(18)		+		+	83
G21			+	+(18)		+			80
G14		+	+	+(6)					67
G16			+	+(6)				+	67
G17				+(18)		+		+	67
G20		+	+	+(3) + PNAN (18)					67
G1		+	+	+(3)	+(3)				66.7
G24				+(12)	(12)		(12)		33
G8				+(18)					16.7
G10				+(36)					16.7

Table 4b: Group 1 R21 variant constructs in order of positive efficacy in decreasing order in comparison to R21



(a) (b)



(c) (d)

Figure 4b: Immunogenicity data for group 1 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP response (G18, G21, G22 and G24****, G8, G20**, G17*, rest ns) and for anti- KLKQP…response (G1****, G21*, rest ns).

Looking into the efficacy obtained from the constructs above and relating this with the immunogenicity data, below are some possible observations and inferences:

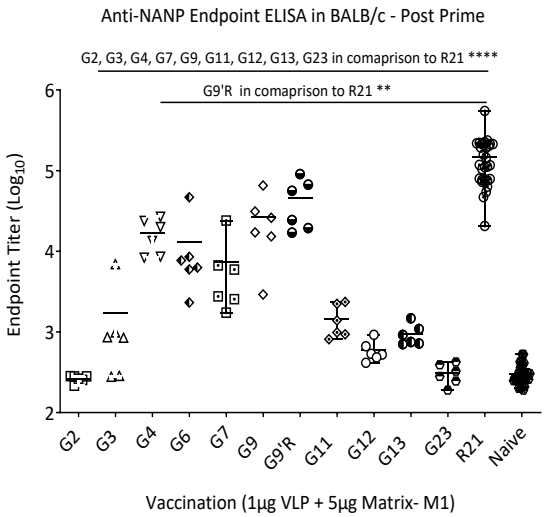
1. Number of NANP repeats critical for efficacy: Reduction in number of NANP repeats as seen in G14, G16 and G1 produces higher than or equivalent response to NANP repeats in comparison to constructs with greater than 6 repeats, but show less efficacy than constructs with 18 repeats. On the other hand, construct with more than 18 repeats as in G10 (36 repeats) show even reduced efficacy than ones with 18 repeats or less. This indicates 18 repeats are critical for higher efficacy with possible association to high affinity of anti- NANP antibodies than the levels.

2. Reversing sequence does not affect efficacy: Reversing the NANP sequence to PNAN (G20) does not affect the anti-NANP antibody levels and efficacy post boost.
3. Junctional epitope antibodies enhance efficacy in some sequence combinations: Higher efficacy of G22 in comparison to G17, which only differ by presence of JE, indicate that antibodies to JE do contribute to efficacy. On the other hand, comparing all constructs with same number of JE copies, high JE responses were seen for G1, G14 and G16 (3 or 6 NANPs) but with less efficacy than other constructs with 18 x NANP (G22, G21, G20, G16). This suggests that the possible enhancement of efficacy by JE is only when NANP are present as 18 repeats.

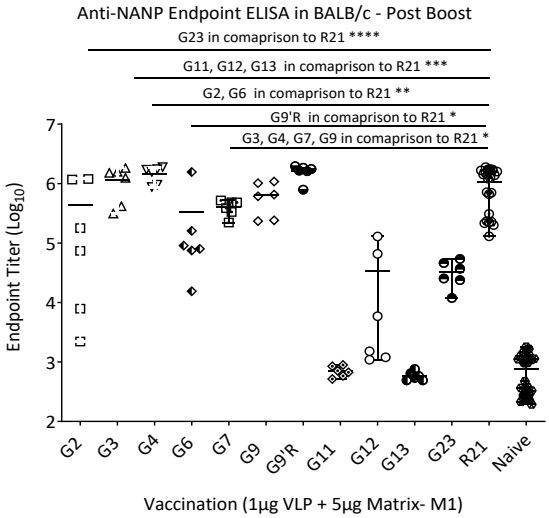
CD4+ T cell help is critical for better efficacy: Comparing efficacy of the three constructs with 18 NANP repeats, R21 (44.4%, + C terminus), G18 (100%, +PADRE) and G8 (16.7%, No C terminus or PADRE), which differ by the presence or absence of PADRE or C terminus, indicate T cell help may be useful for production of efficacy enhancing protective antibodies and PADRE may be a strong inducer of CD4+ T cell help than the C terminus epitopes. To add, anti-NANP immune responses remain unchanged between G18 and R21 indicating anti- PADRE antibodies do not exhibit any antagonistic effect and remains to be assessed in future. Also, with G10 which is different from G8 only in terms of double the number of NANP repeats gave the same level of efficacy, strongly suggesting that 18 or higher number of repeats of NANP without T cell help is not sufficient to produce good efficacy.

Construct	N ter	CE	JE	NANP	NVDP	C ter	NPDP	Efficacy (%)
R21				+(18)		+		44.4
G2		+	+	+(9)	+(3)			0
G3		+	+	+(21)	+(3)	+		0
G4	+			+(18)		+		0
G6			+(5)					0
G7		+(5)	+(5)					0
G9				+(18) @ c ter				0
G9'R				+(18) rev				0
G11							+(18)	0
G12		+(18)						0
G13					+(18)			0
G23				+(6)	+(6)		+(6)	0

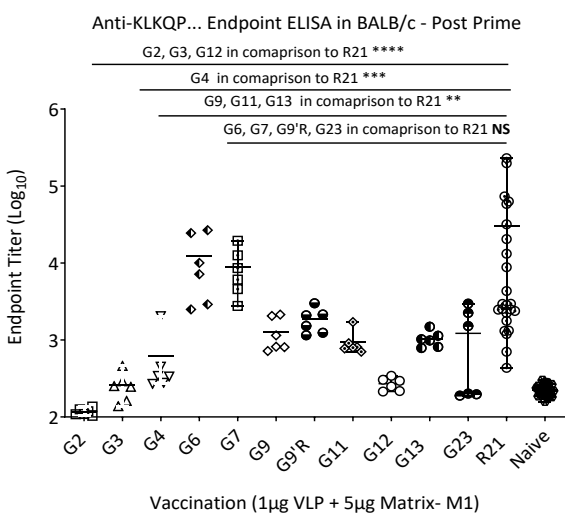
Table 4c: Group 2 R21 variant constructs with zero efficacy



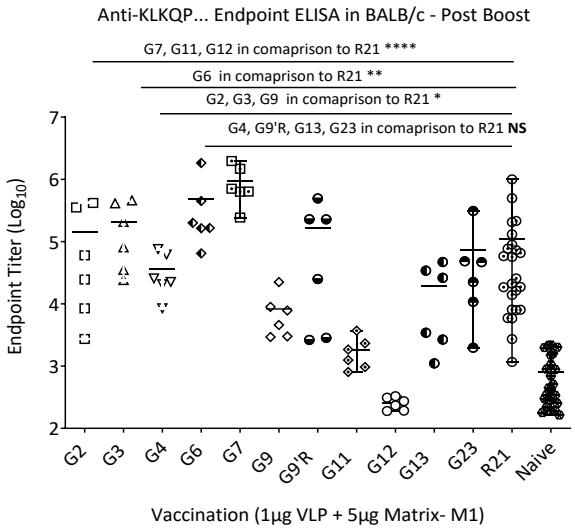
(a)



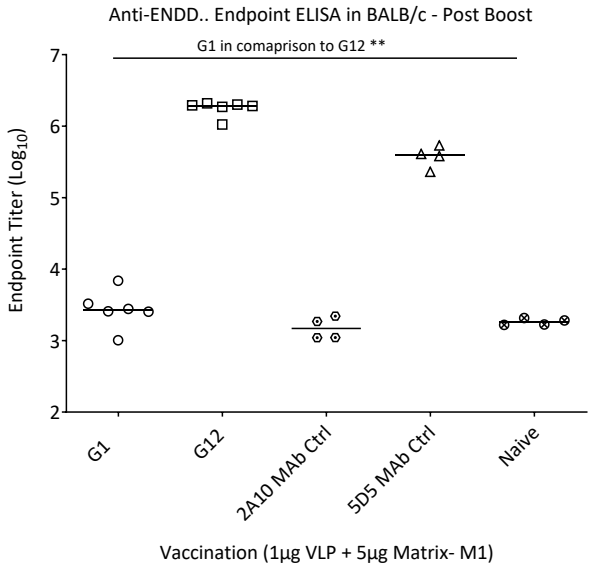
(b)



(c)



(d)



(e)

Figure 4c: Immunogenicity data for group 2 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels, (c) and (d) anti- KLKQP.. (JE) IgG levels and (e) show anti- ENDD…(CE) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP response (G23****, G11, G12, G13***, G2 and G6**, G3, G4, G7, G9 ns, G9'R *) and for anti- KLKQP…response (G7, G11 and G12****, G6** and G9*, rest ns). For CE ELISA, significantly higher immune response obtained than G1 (**).

A number of constructs failed to confer any protection on sporozoite challenge. Looking at the presence and number of repeats of sequences possible conclusions that can be drawn are as below:

1. NANP repeats not sufficient for efficacy: Both the constructs G9 and G9'R that consist of 18 NANP repeats but either placed differently as in G9 at the C terminus and in G9'R as a reversed sequence (PNAN) failed to confer any protection, indicates that the placement of or the arrangement of amino acids in NANP is not critical, but the repeats only on their own are not sufficient to confer any protection indicating additional help is likely required from other sequence region of CSP and/ or CD4+ T cell help, as mentioned above. This can be seen with G20 which also has 18 repeats of PNAN, as described in previous group, conferred high efficacy of 67% when in combination with JE and CE.
2. Antibodies to N terminus are non-efficacious: As seen with the construct G4, which differs from R21 with additional N terminus sequence, the measured efficacy indicates a possible antagonistic effect from the antibodies to the NANP antibodies. Also, for the construct G5, which did not form VLPs and hence is not included in the analysis, is similar to G4 differing by having 38 copies of NANP repeats instead of 18. This indicates that presence of N terminus might have possible effect on VLP formation with higher NANP repeat copy numbers, as G10 with just 36 NANP repeats was able to form a VLP.
3. NVDP and NPDP antibodies do not confer protection: Both the constructs G11 and G13 containing 18 repeats of NPDP and NVDP respectively failed to provide any protection on sporozoite challenge. The immune response to both constructs could only be measured on anti-NANP ELISA as antibodies as a cross-reactive response and in the anti- KLKQP….ELISA, which had both NVDP and NPDP sequences present along with NANP and JE. For G11, there was no significant increase in immune

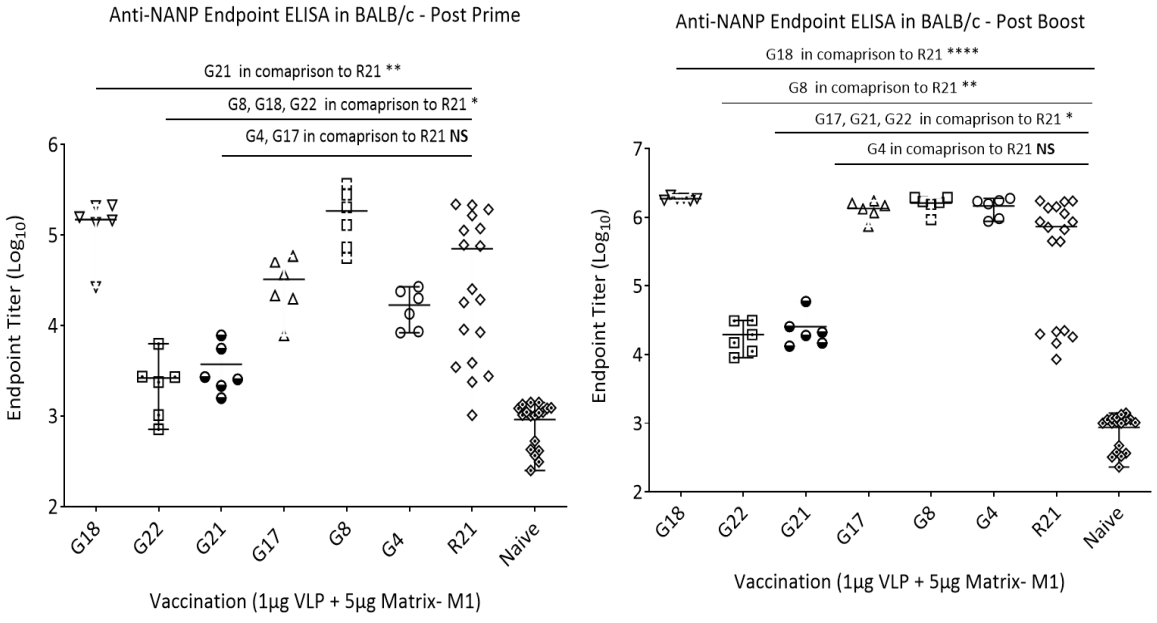
response for G11 after boost, while there was a moderate increase for G13. It can be concluded that inclusion of NVDP or NPDP in a construct containing immunodominant NANP sequence is hence not required.

4. Number of NANP repeats appears critical for JE antibodies: As described in the previous section, higher efficacy was associated with the JE present in a construct with 18 NANP repeats. As seen here, with constructs G2 and G3, which along with JE contain 9 and 21 NANP repeats respectively both failed to confer any protection. This indicates that lower and higher than 18 NANP repeats affect the activity of JE antibodies, despite similar levels of endpoint titters post boost on anti-NANP and anti-KLKQP.. ELISAs. JE antibodies do not affect the anti-NANP tires induced, but combination of higher or lower number of NANP repeats with JE has a surprising marked effect on efficacy. This is intriguing and can only be possibly associated with fine balance of protective antibodies levels obtained when 18 NANP repeats are present, which is somehow not achieved with different number of repeats.
5. JE and CE are not sufficient to produce efficacy: Three of the constructs that include repeats of just the JE (G6) or CE (G12) or both (G7) failed to confer any protection, indicating antibodies to these sequences are not sufficient in BALB/c mice. Similar immune responses were obtained in anti- NANP ELISA for G6 and G7 post prime and boost, while some non-specific response was observed for G12. In anti- KLKQP…. ELISA, G6 and G7 induced similar immune response post prime and boost, while G12 was close to naïve values. In a CE ELISA using the extended peptide as mentioned earlier, and Mab5D5 against the CE as a positive control, higher immune response was obtained for G12 with 18 repeats of CE in comparison to G1 with one repeat. It can be concluded that CE is capable of generating high antibody response, which is non- protective with a high possibility that its presence in a construct in combination with other sequences does not contribute to efficacy.

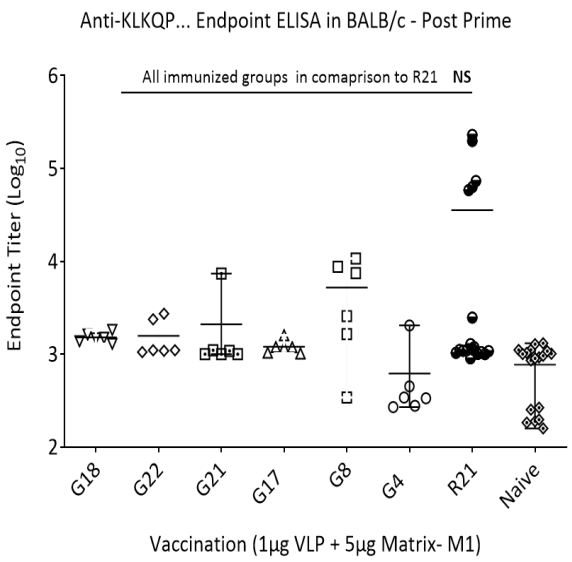
6. Presence of different numbers of cross-reactive sequence repeats affects immune response and efficacy: as mentioned earlier, lower of number of NANP repeats generate equivalent or higher anti-NANP immune response than constructs with 18 repeats, but are not protective. When cross-reactive sequences NANP, NVDP and NPDP were included in a construct as 6 equal number repeats (G23) this generated statistically higher (*p<0.05) anti-NANP responses in comparison to the construct with same sequences but with 12 repeats (G24). Despite this response, G23 failed to confer any protection, while G24 showed moderate protection (33%).

Construct	N ter	NANP	C ter	PADRE	JE	Efficacy (%)
R21		+(18)	+			44.4
G18		+(18)		+		100
G22		+(18)	+	+	+	83
G21		+(18)	+		+	80
G17		+(18)	+	+		67
G8		+(18)				16.7
G4	+	+(18)	+			0

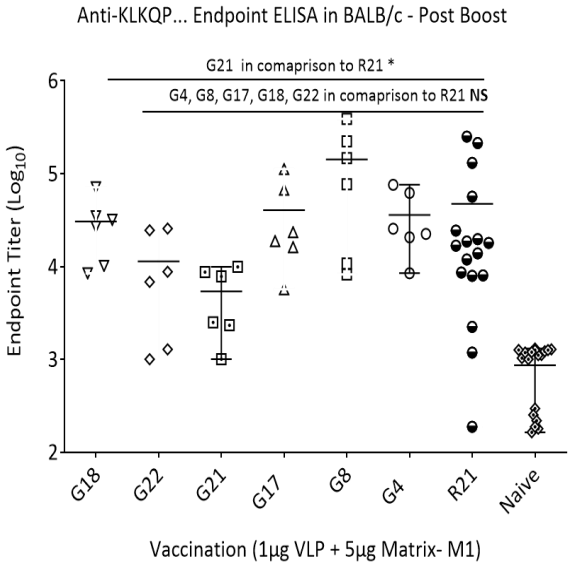
Table 4d: Group 3 Efficacy of R21 variant constructs with modifications to R21



(a) (b)



(c)



(d)

Figure 4d: Immunogenicity data for group 3 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP response (G18****, G8**, G22, G21 and G17*, rest ns) and for anti- KLKQP…response (G21*, rest ns).

All the constructs containing 18 NANP repeats show varied positive efficacy depending on which other sequence is present in the construct, except G4. As explained earlier, the possible antagonist nature of N- terminus antibodies to the anti- NANP repeats could be a reason for losing efficacy when compared to R21. The only other sequences, the present or absence of which is explored here are the N terminus, C terminus, PADRE and the JE. The possible observations from this group are:

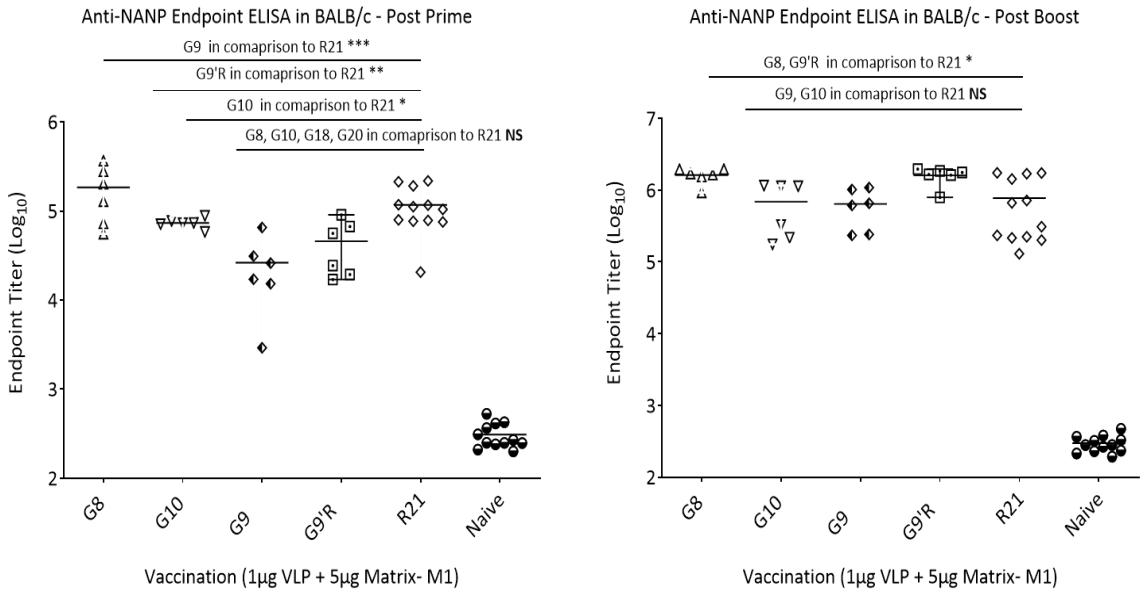
1. C- terminus enhances the efficacy from 18 NANP repeats: It can be clearly seen comparing R21 with G8 that C- terminus T cell help contributes to the efficacy when combined with 18 NANP repeats, as it reduces markedly when absent as seen in G8.

Also, as mentioned earlier, NANP repeats alone are not capable of producing high levels of protective antibodies that can translate into efficacy.

2. PADRE provides better CD4+ T cell help than C- terminus: Comparing the constructs that contain only PADRE (G18) and only C- terminus (R21) along with 18 NANP repeats, it is evident that PADRE sequence is much more potent in providing CD4+ T cell help than the C- terminus in mice. It is also possible that antibodies to PADRE sequence might also have an added synergistic effect to the anti-NANP antibodies and this needs to be investigated. Two other constructs G22 and G17 contain both C- terminus and PADRE, with higher efficacy observed in G22 than G17. G22 consists of an additional JE region and there might be a possible synergistic effect from the antibodies to this region with the anti- NANP antibodies.
3. Antibodies to JE affect anti-NANP levels: As observed with G22 and G17, higher efficacy was obtained with the presence of JE. When the anti-NANP responses were compared G22 produced statistically significant less antibodies than G17 post boost. Also, there was no significant increase in anti- NANP antibody levels for G22. This indicates that presence of JE inhibits production of high titres of anti- NANP antibodies, but antibodies to this region appear to synergistically improve efficacy.

Construct	NANP	C ter	Efficacy (%)
R21	+(18)	+	44.4
G8	+(18)		16.7
G10	+(36)		16.7
G9	+(18) @ C ter		0
G9'R	+(18) as PNAN		0

Table 4e: Group 4 Efficacy of R21 variant constructs with NANP repeats only



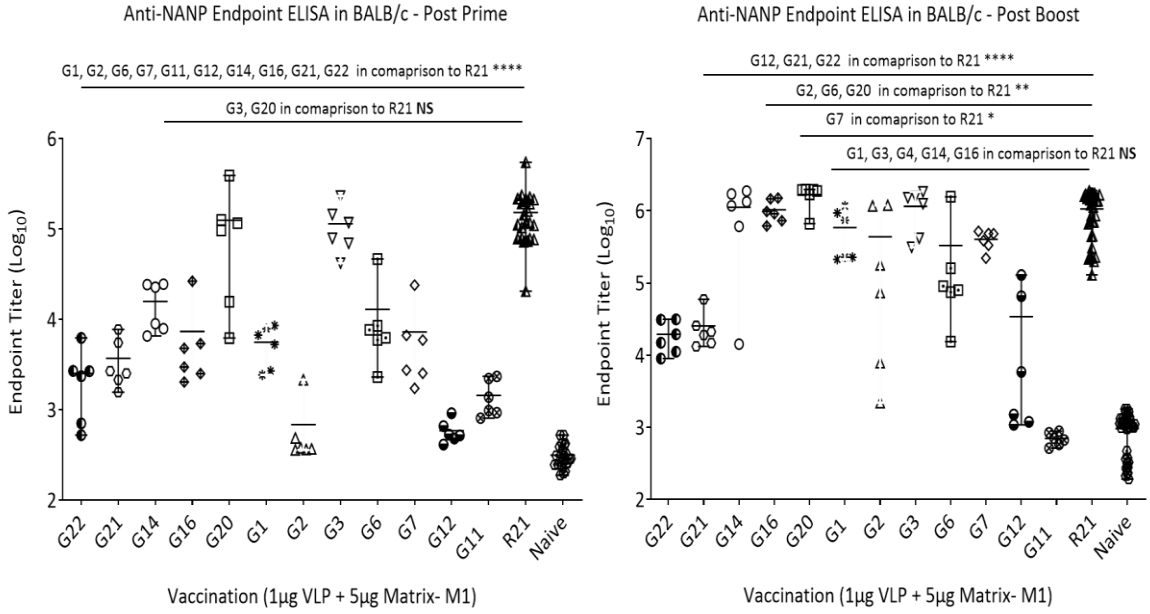
(a) (b)

Figure 4e: Immunogenicity data for group 4 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP response (G8 and G9'R*, G9 and G10 ns) and for anti- KLKQP…response (G9**, G8, G10 and G9'R ns).

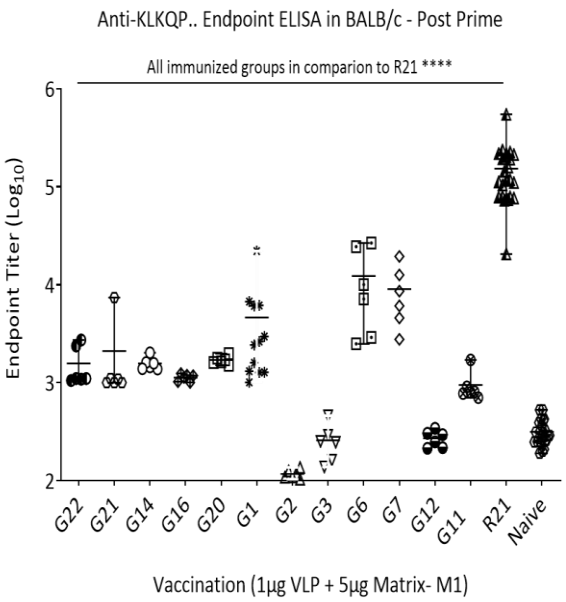
While comparing the previous groups, the possible conclusion that number of NANP repeats of 18 is critical and any number of repeats without presence of other sequence like C- terminus, PADRE, and cross- reacting repeats produces lower or no efficacy, is further supported by grouping constructs with NANP repeats only. In terms of anti- NANP reponses no significant difference was seen with any of the constructs despite differences in number of NANP repeats, change in postion of repeats at the C- terminus instead of N- terminus and presence in a reverse order.

Construct	NANP	CE	JE	NVDP	NPDP	C ter	PADRE	Efficacy (%)
R21	+(18)					+		44.4
G22	+(18)		+			+	+	83
G21	+(18)		+			+		80
G14	+[53]	+	+					67
G16	+[53]		+				+	67
G20	+(3) + PNAN (18)	+	+					67
G1	+(3)	+	+	+(3)				66.7
G2	+(9)	+	+	+(3)				0
G3	+[53]	+	+	+(3)		+		0
G6			+(5)					0
G7		+(5)	+(5)					0
G12		+(18)						0
G11					+(18)			0

Table 4f: Group 5 Efficacy of R21 variant constructs with cleavage and junctional epitopes



(a) (b)



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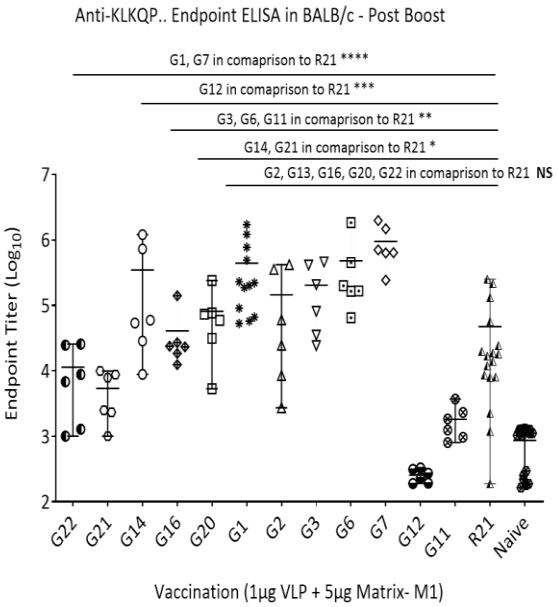

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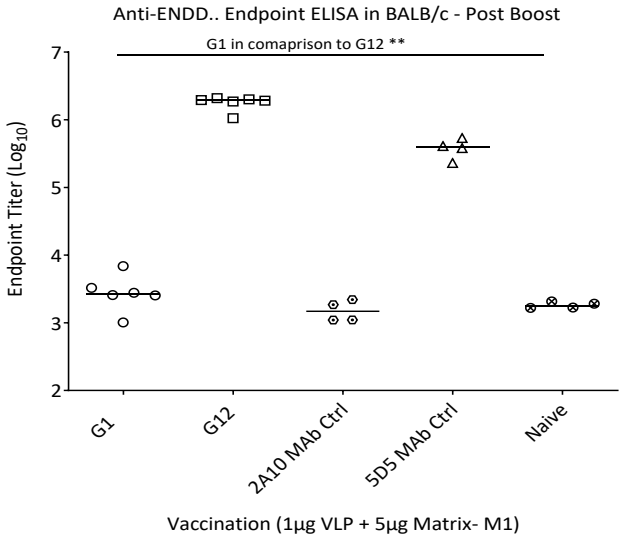


(e)

Figure 4f: Immunogenicity data for group 5 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels, (c) and (d) anti- KLKQP.. (JE) IgG levels and (e) show anti-ENDD…(CE) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP

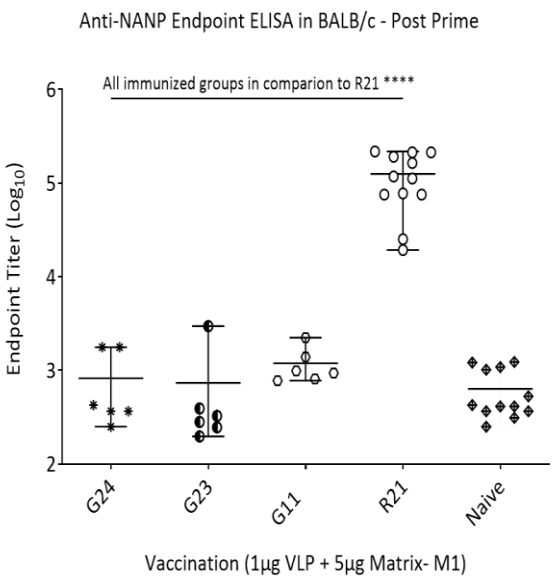
response (G12, G13, G21 and G22****, G2, G6 and G20**, G7*, G1, G3, G4, G14 and G16 ns) for anti- KLKQP…response (G1 and G7****, G12***, G3 and G6**, G14 and G21*, rest ns). For CE ELISA, significantly higher immune response obtained than G1 (**).

As the R21 variants were designed with new promising published data on JE and possibly the CE of CSP, capable of eliciting production of protective antibodies, grouping constructs with either JE or CE or both would provide useful information on this when present in the construct alone or in combination with NANP repeats, NANP cross reacting repeats and T cell epitopes. Also described earlier, some observations can be summarized as follows:

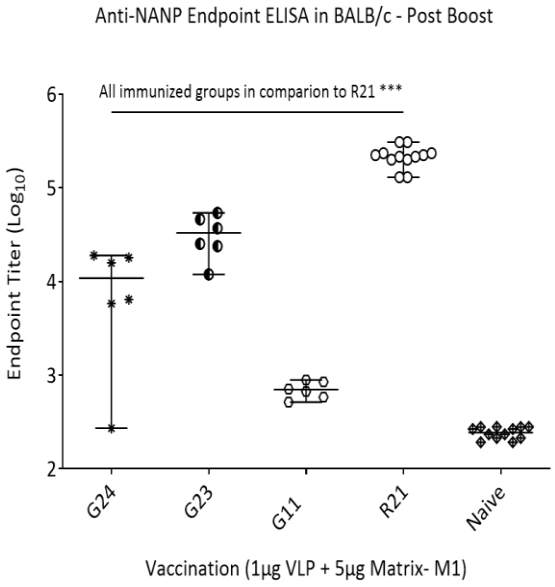
1. Immune response to the CE increases with number of repeats present, but antibodies are often non- protective. Higher efficacy associated with G22 and G21 can be associated with T cell help, as this is despite G22 lacking CE.
2. JE and CE are capable of producing an immune response that can be correlated with number of repeats, but both regions on their own are not sufficient to confer protection.
3. Comparing constructs G22 (18 NANP) and G16 (6 NANP), which both have JE and T cell helper sequences, JE seems to supresses the immune response to NANP when the latter is present as 18 repeats, but produces higher efficacy. On the other hand, it does not affect the immune response when NANP are present as 6 repeats, but shows reduction in efficacy. This indicates JE antibodies might have a role in affinity maturation of protective antibodies.

Table 4g: Group 6 Efficacy of R21 variant constructs with NPDP repeats

Construct	NANP	NVDP	NPDP	Efficacy (%)
R21	+(18)			44.4
G24	+(12)	+(12)	+(12)	33
G23	+(6)	+(6)	+(6)	0
G11			+(18)	0



(a)



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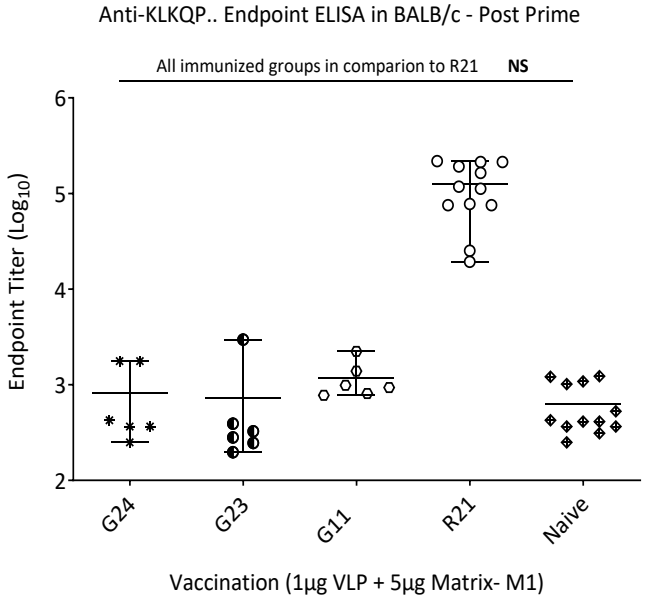

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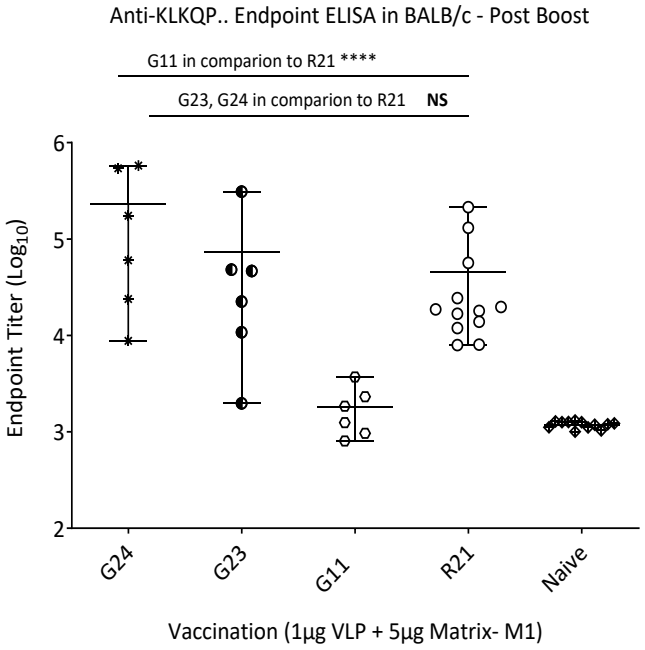


(d)

Figure 4g: Immunogenicity data for group 6 with constructs arranged from left to right with decreasing efficacy. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance to R21 as analysed by Mann- Whitney test post boost for anti-NANP response (All***) for anti- KLKQP…response (G11****, rest ns).

Focusing just on the NPDP sequence of the JE, 3 constructs can be compared for immune response and efficacy. When exclusively present as 18 repeats NPDP fails to enhance any immune response post boost when analyzed in an ELISA when used with an extended KLKQP.. sequence. This indicates that the moderate efficacy of G22 is mainly focused on the immunodominant NANP repeats and halving the number of repeats as in G23 does not bring a statistically significant change to the level of anti-NANP antibodies, but diminishes the protective potential.

4.2.2 Comparison of antibody longevity and durability of R21 variant G1 with R21

R21 has demonstrated an excellent efficacy of 77% over a year after 3 vaccinations in children 5-17 months of age in Africa. The protective anti- NANP antibody titers wane after 1 year and an additional dose is required to bring the titers back to peak levels [151]. It was crucial that exploring other potential immunogenic sequences of CSP that might along with anti-NANP antibodies confer long lasting protection and help reduce additional booster doses, was required. This became possible with availability of data on promising immunogenicity and potential protective role of JE and CE of the CSP [164], which helped design a number of R21 variant vaccines. During the initial screening of first 13 R21 variant constructs G1 to G13, G1 demonstrated higher efficacy than R21 after two immunizations. This was promising and a study was initiated to compare

longevity and durability of antibodies to NANP repeats as well JE and CE regions. The immunization regime was kept the same as original experiment and both the vaccines were administered at 1µg dose with 5 µg of adjuvant Matrix-M1 during prime and boost immunizations with a 3-week interval between the two immunizations. The longevity was assessed using ELISA by analyzing antibody titers to these sequences 3 weeks post prime vaccination and every month after the boost vaccination till 13 months. The durability of these antibodies was checked by challenging the mice intravenously with 1000 *P. berghei* transgenic sporozoites containing *Pf*CSP sequence. As evident from figure 4h, anti-NANP antibodies follow the same profile for both G1 and R21 over a 13-month period, with antibody titers remaining high for 8 months and reducing steadily but still remaining higher than titers measured 3 weeks post prime. For KLKQP… ELISA using the extended peptide containing JE sequence along with 1 repeat each of NANP, NVDP and NPDP, though involving cross- reactive responses, remain approximately a log higher for G1 in comparison to R21 and are maintained at same level as seen for 2 months post boost vaccination (Figure 4h).

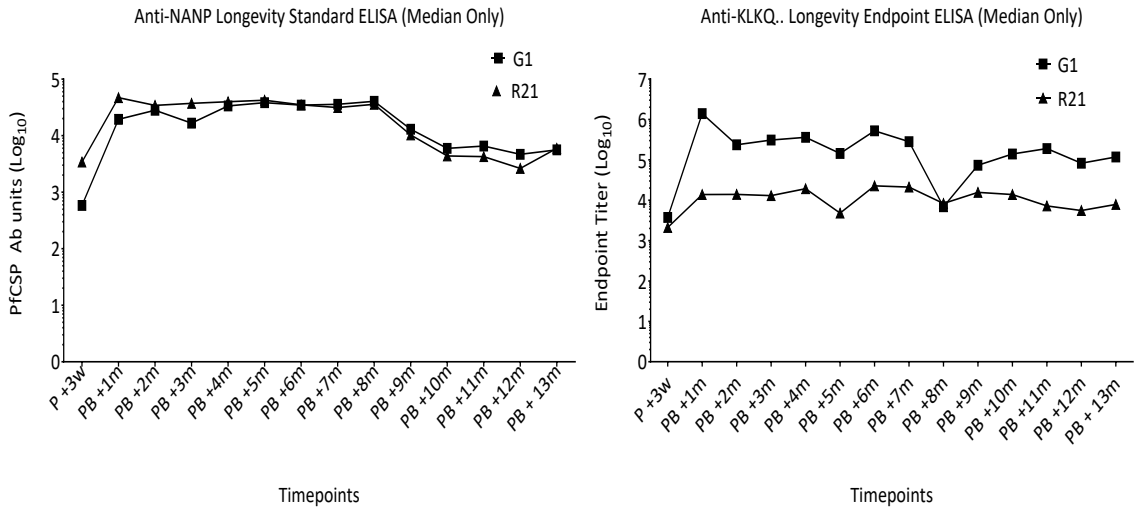
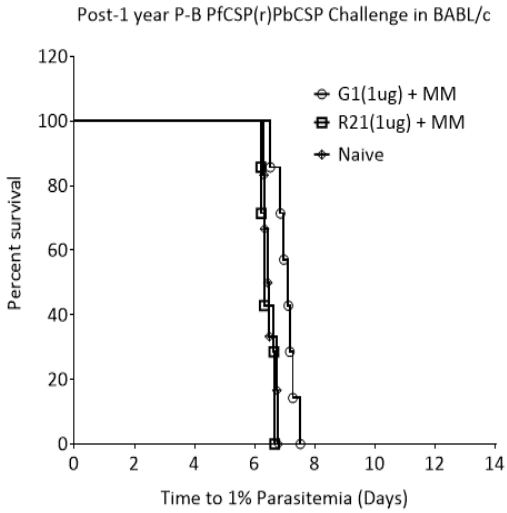


Figure 4h: Endpoint titers from KLKQP..(JE) ELISA and antibody units from anti-NANP (central repeats) standard ELISA analyzed for G1 and R21 over a 13-

month period. Over 13 months, for both constructs, the anti- NANP titers are maintained till 8 months to post boost levels and steadily reduce to plateauing levels towards 13 months post boost vaccination. Anti-KLKQP….response was higher for G1 considering the presence of JE in G1 while for R21 with no JE, the lower responses measured are from the anti-NANP IgG binding to extended KLKQP… sequence containing NANP and cross reacting sequence NVDP.

With this promising longevity of antibodies, the mice were challenged to see if the efficacy still remains higher for G1 when challenged 3 weeks post boost as seen in previous experiments. Figure 4i shows the efficacy data for G1 and R21 13 months post boost. All the challenged mice succumbed to infection and there no protection observed for both R21 and G1 after 13 months. There was significant delay in parasitemia (**p<0.01) observed with G1 in comparison to both naïve and R21, while for R21 in comparison to naïve it remained non- significant.



Groups	Efficacy	Delay to 1% vs Naive	P value Log-rank (Mantel-Cox) Test
G1 (1ug) + MM	0/7 (0%)	Yes	0.0012 (**)
R21 (1ug) + MM	0/7 (0%)	NS	0.3099
Naïve	0/7 (0%)	NA	NA
		G1 vs R21 Delay to 1%	
		Yes	0.0018 (**)

Figure 4i: Efficacy results from G1 and R21 challenged with 1000 sporozoites 13 months post boost.

With the promising data for JE and CE, it was useful finding how the immune response elicited by these sequences in a VLP format translate to efficacy. Despite the maintenance

of immune response as seen for JE region throughout the period and to NANP repeats for 8 months, these responses fail to lead to protection against sporozoite challenge. With efficacy data available for more promising candidates like G18, G22 and G21, assessing them in the same experimental format would provide data on whether presence of C-terminus and PADRE sequence contribute to antibody longevity and durability.

4.2.3 Co-delivery of VLPs is safe, and immunogenic with varied translation to efficacy

With promising efficacy obtained with G1 and G18, a co-delivery experiment was conducted to assess whether presence of protective antibodies from one vaccine can synergistically or antagonistically affect the immune response and efficacy of the other vaccine. Here, two different VLP vaccines were mixed in equal 1µg concentration with 5 µg Matrix-M1 and administered in a prime-boost vaccination regimen. The two VLPs were mixed with Matrix-M1 prior to vaccination in a tube and co-delivered using a syringe. All the co-delivery groups were found to be safe with no adverse effects noticed in BALB/c mice. The efficacy obtained after challenging the mice with 1000 sporozoites 3 weeks post boost vaccination for R21 and its variants G1, G11 and G18 administrated alone and mixing 2 VLPs together are listed in table 4h.

Co-delivery of VLPs								
	CE	JE	NANP	NVDP	C ter	NPDP	PADRE	Efficacy (%)
R21			+(18)		+			72.3+
G18			+(18)				+	91.5^
G1	+	+	+(3)	+(3)				77.6+
G11						+(18)		0
G1 + R21	+	+	+[53]	+(3)	+			83.5^
G1 + G18	+	+	+[53]	+(3)	+		+	50
G11 + R21			+(18)		+	+(18)		16.7
^Average of 2 experiments, + Average of 3 experiments								

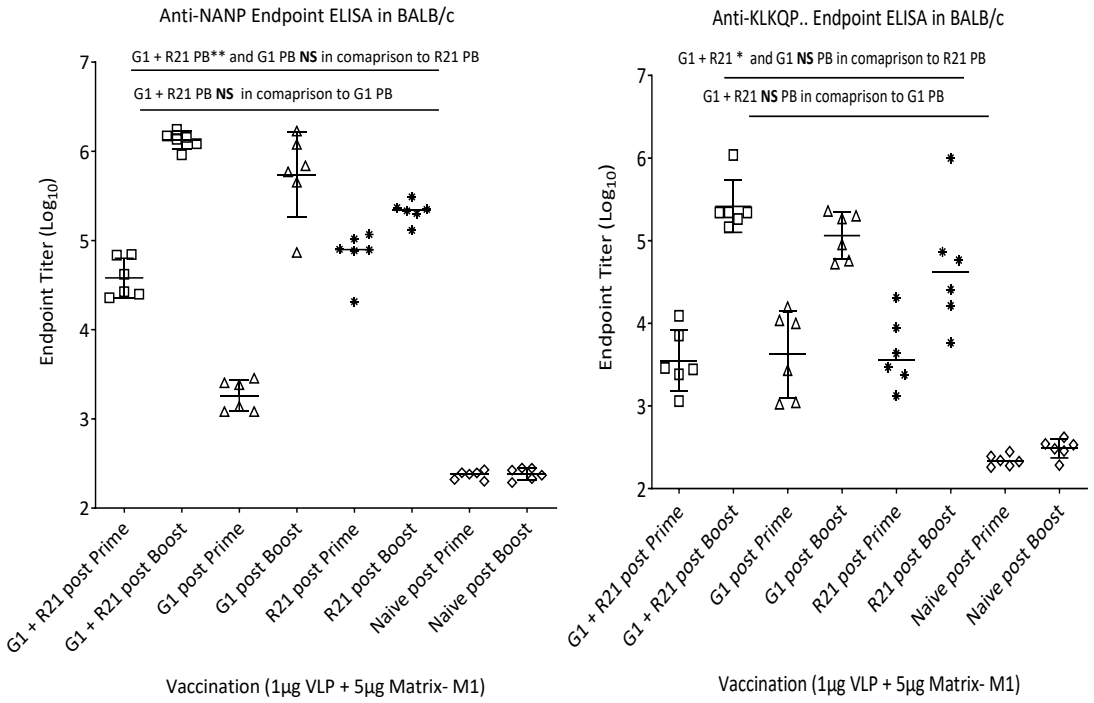
Table 4h: Efficacy obtained on co-delivery of VLP vaccines in comparison to the same vaccines delivered alone

4.2.3.1 Co-delivery of G1 with R21

In one of the studies, G1 (JE+ CE+ 3 x NANP-NVDP) was co-delivered with R21 (18x NANP+ C-terminus). The average efficacy of G1 from 7 experiments is 66.7%, but for better comparison only the average from experiments that involved co-delivery groups was considered for analysis. Averaging this way gave an efficacy of 72.3% for R21 and 77.6% for G1. The hypothesis here was that the antibodies from JE of G1 and T cell help from R21 might enhance the immune response and efficacy. There was a significant increase post boost in anti-NANP antibodies for G1 + R21 when compared to R21 (**) and but not for G1 (ns), while for JE antibodies, there was non- significant change when compared with both R21 and G1 (Figure 4j). This indicates that the enhancement of efficacy seen (83.5%) is a likely contribution from the elevated anti-NANP antibody levels.

Comparing the sequences present when G1 and R21 are mixed there are 1 copy each of JE, CE, and C-terminus, 21 repeats of NANP (3 from G1 and 18 from R21) and 3 repeats of NVDP. Interestingly, together these are present as a fusion sequence in the R21 variant G3 displayed together on the VLP surface. Despite this, there is a remarkable difference in efficacy between them with G3 failing to demonstrate any efficacy compared to G1 + R21 with 83.5% efficacy. Comparing the immunogenicity of G3 and G1+ R21, there was non-significant difference for anti JE and anti-NANP antibodies between the groups post boost. This shows that when present as fusion sequence on the VLP surface none of the immunogenic sites are hidden or unable to produce an immune response. With a lack of published data on the efficacy of co-delivery of VLP vaccines compared to a VLP displaying the same sequences differently, assessing antibody subclass, affinity and avidity of antibodies elicited by the two groups might help explain the observed difference

in efficacy. This can also be assessed for unique sequence combination for R21 and G1 individually differing from G3.



(a) (b)

Figure 4j: Anti-NANP and anti- KLKQP…immune responses post prime and boost for G1 and R21 co-delivered in comparison to G1 and R21 administered alone. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance was analysed by Mann- Whitney test post boost for anti-NANP response (R21 to G1: ns, R21 to G1 + R21**, G1 to G1 + R21: ns) and (R21 to G1: ns, R21 to G1 + R21*, G1 to G1 + R21: ns) for anti-KLKQP…response.

4.2.3.2 Co-delivery of G11 with R21

It has been demonstrated by Tan et al., that antibodies that target epitopes in both the central repeat region and N- terminal region of CSP simultaneously are more potent than

antibodies that exclusively recognize each individual site. They showed that the Mabs that recognized both NANP and NPDP were able to reduce parasite liver burden by up to 98.3% [166]. With this rationale, even though antibodies produced to R21 variant vaccine G11 (18 x NPDP) failed to confer any protection, was included in this co-delivery experiment with R21 to see if the anti-NPDP antibodies which also bind to NANP repeats were able to improve efficacy of R21 devoid of this sequence. Unfortunately, it did not demonstrate any enhancement and the efficacy reduced to 16.7%. This shows the difference between results obtained from an experiment where promising results were obtained on sporozoite challenge of mice 16-24 hours post injection of 150 μg of Mabs[166] to a sporozoite challenge 3 weeks post boost of vaccination with 2 VLPs displaying cross- reacting sequences. Figure 4k shows the immune response elicited by the G11 + R21 mixed vaccine group in comparison to G11 and R21. Though anti-NANP responses cannot be truly compared due to cross-reactivity, responses were significantly higher (**) for the mixed group when compared to G11 and non-significantly different to R21 post boost. In anti-KLKQP.. ELISA, which consists of NPDP as a part of the peptide used for coating plates, this profile remained the same, indicating no contribution from anti-NPDP antibodies to efficacy. On the other hand, reduction in efficacy indicates antagonist nature of these antibodies that might have a possible effect on formation of memory B cell to NANP repeats.

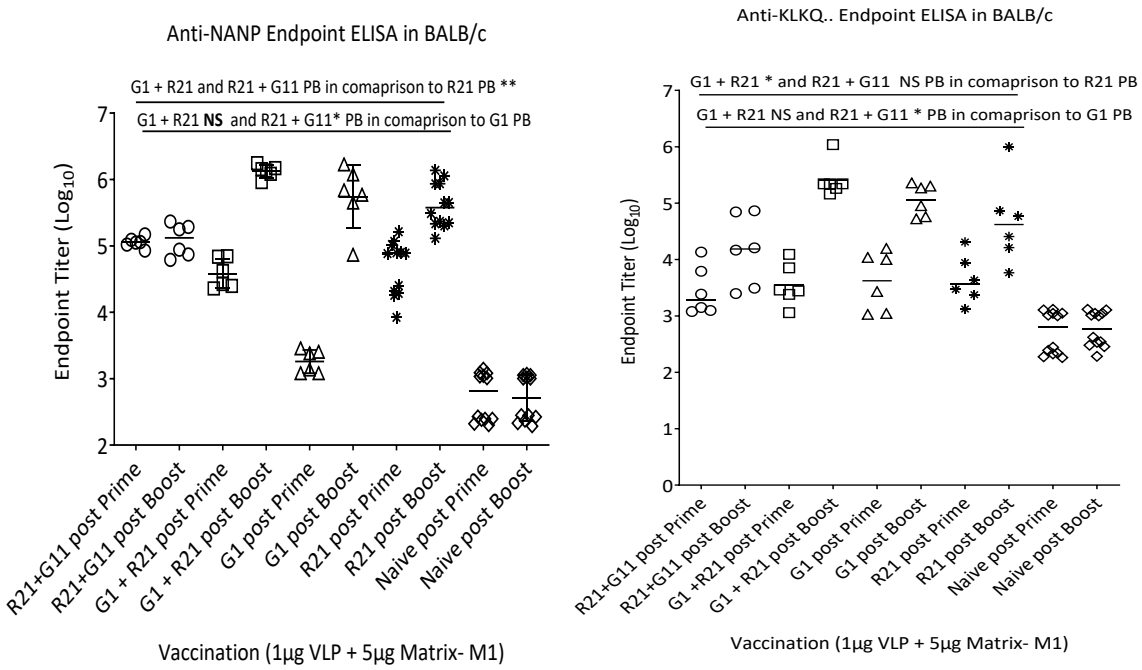
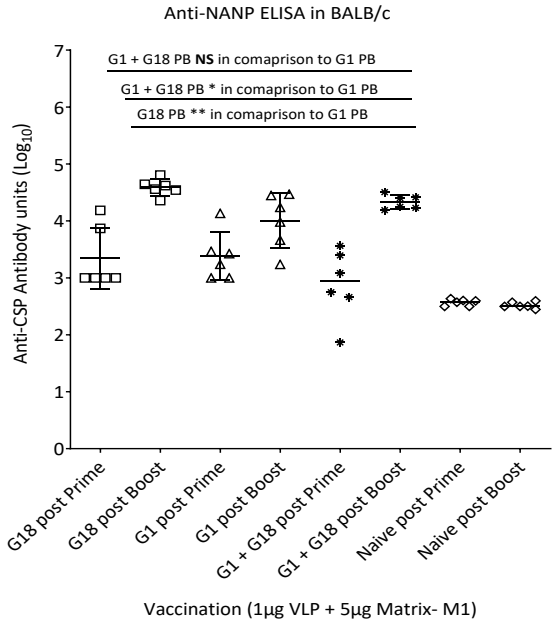


Figure 4k: Anti-NANP and anti- KLKQP…immune responses post prime and boost for G11 and R21 co-delivered in comparison to G11 and R21 administered alone. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance was analysed by Mann- Whitney test post boost for anti-NANP response (R21 to G11**, R21 to R21+G11 ns and G11 to R21+G11**) and (R21 to G11**, R21 to R+G11 ns and G11 to R21+G11**) for anti-KLKQP…response.

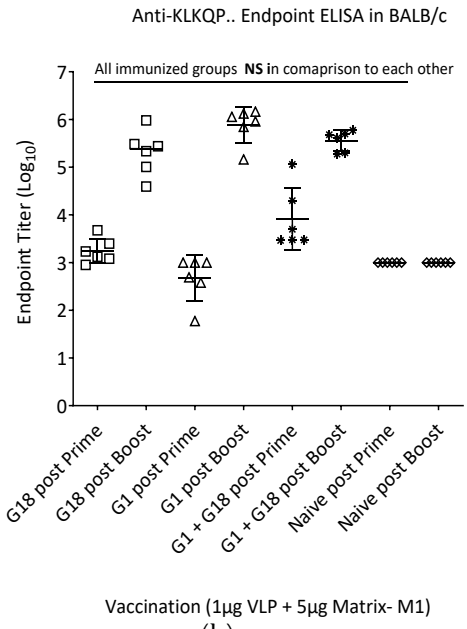
4.2.3.3 Co-delivery of G18 with G1

In a final study, G1 was mixed with G18 following the same vaccination regimen. The aim here was that considering the average efficacy for G1 from 7 experiments (66.7%), whether adding G18 (initial efficacy 100%) which only differs from R21 in terms of C-terminus replaced by PADRE sequence, can enhance its efficacy. Combining the two also formed the same sequence mix as G1 + R21 with additional contribution from PADRE sequence. Assessing the immune responses obtained through ELISA post boost, G18 was

statistically slightly better than G1+ G18 (*) but there was a non- significant difference between G1 and G1 + G18 for anti- NANP responses. There was a significant difference found post boost for anti- KLKQP... responses comparing G1 and G18 with the mix, post boost. This indicates, that in this format, the PADRE sequence does not provide any enhancement of immunogenicity or efficacy. The closest construct with sequences from G1 and G18 present in fused format is G22, which does not include the CE. With clear evidence from efficacy of G12 (18 x CE) that antibodies to CE do not contribute to efficacy, G22 can be compared to G1+ G18 mix. Surprisingly, G22 gives a high efficacy of 83% in comparison to G1 + G18 which is 50%. This is contrary to the observation from the previous G1 + R21 mix study. Comparing the immune responses post boost between G22 and G1+ G18, there is was a non- significant difference in anti-NANP antibodies, G1+ G18 mix elicited significantly higher (**) antibodies to JE region in comparison to G22 (Figure 4l). It is hard to address whether in a co-delivery format, antibodies to PADRE sequence or in combination with C- terminus have an overall antagonistic effect, but the combined T cell help from both sequences does not seem to be sufficient.



(a)



(b)

Figure 4l: Anti-NANP and anti- KLKQP…immune responses post prime and boost for G1 and G18 co-delivered in comparison to G1 and G18 administered alone. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance was analysed by Mann- Whitney test post boost for anti-NANP response (G1 to G18**, G18 to G1+ G18 mix*, rest ns) and (G1 to G18*, rest ns) for anti- KLKQP…response.

4.2.4 Immunogenicity and efficacy of R21 and its variants is not dose dependent

From initial promising efficacy from G1 and G18, both the constructs were assessed for immunogenicity and efficacy at low dose $1/10^{th}$ and $1/100^{th}$ of the standard 1µg dose. It has been demonstrated that R21 at an ultra-low dose of 0.05 µg when administered with adjuvant AS01 elicits the same levels of anti-NANP antibodies and efficacy (>80%) as with 10 times more dose of 0.5 µg with three immunizations in BALB/c mice [179]. Previously, higher than 80% efficacy was obtained with 0.5 µg R21 with 12 µg Matrix-M1, and evaluating at a reduced dose for R21 and Matrix- M1, has provided promising results, as highlighted in table 4i. In a single experiment, R21, G1 and G18 were administered at two reduced doses keeping the adjuvant Matrix-M1 amount constant at 5 µg. Comparing with specific immune responses within a group (Figure 4m), there was no difference in anti-NANP and anti-KLKQP… responses post boost, despite the efficacy ranging from 67 to 100% for all groups. Comparing similar doses within different groups, there was significant different between anti-NANP responses for G1 and R21 for all three doses, but anti-KLKQP…responses were significantly higher for G1 than R21 showing obvious co-relation with the presence of JE in G1. Putting this together with the efficacy obtained, G1 as at 1 µg dose is more efficacious (100%) than R21 (83%) at the same dose, while R21 gave a higher efficacy (100%) in comparison to G1 (67%) at 0.1 µg dose.

Further reducing the dose to 0.01 μg, an ~20% decrease in efficacy can be seen from 1 μg dose. Comparing R21 to G18, the anti-NANP responses were significantly higher for G18 than R21 at all three doses, while for anti-KLKQP.. response, it was higher for G18 at 1 μg dose and not significantly different at the two lower doses. Though not relevant for these two groups, anti-KLKQP.. response was measured to compare it with other groups as a part of the complete experimental analysis. Despite, higher anti-NANP responses of G18 in comparison to R21, both demonstrated the same efficacy (83%) at 1 μg dose. Reducing the dose to 0.1 μg, increased the efficacy of R21 (100%), but a reduction was seen for G18 (67%). Reducing the dose further to 0.01 μg, the reverse was observed with G18 conferring higher efficacy (83%) than R21 (67%) at the same dose. Lastly, comparing G1 to G18, it is clear from the efficacy table 4i that G1 gives higher efficacy (100%) than G18 (83%) at 1 μg dose, but reducing the dose to $1/10^{th}$ and $1/100^{th}$ does not affect the efficacy for both the variants. The anti-NANP response for G18 were significantly higher than G1 at all three doses, while only significantly higher at 0.1 μg dose for anti-KLKQP..antibodies, which is not reflected in efficacy.

Construct	Dose	CE	JE	NANP	NVDP	C ter	PADRE	Efficacy (%)
R21	1μg			+(18)		+		83
	0.1μg			+(18)		+		100
	0.01μg			+(18)		+		67
G18	1μg			+(18)			+	83
	0.1μg			+(18)			+	67
	0.01μg			+(18)			+	83
G1	1μg	+	+	+(3)	+(3)			100
	0.1μg	+	+	+(3)	+(3)			67
	0.01μg	+	+	+(3)	+(3)			83

Table 4i: Efficacy of R21 and two R21 variants at low dose in comparison to the 1μg dose.

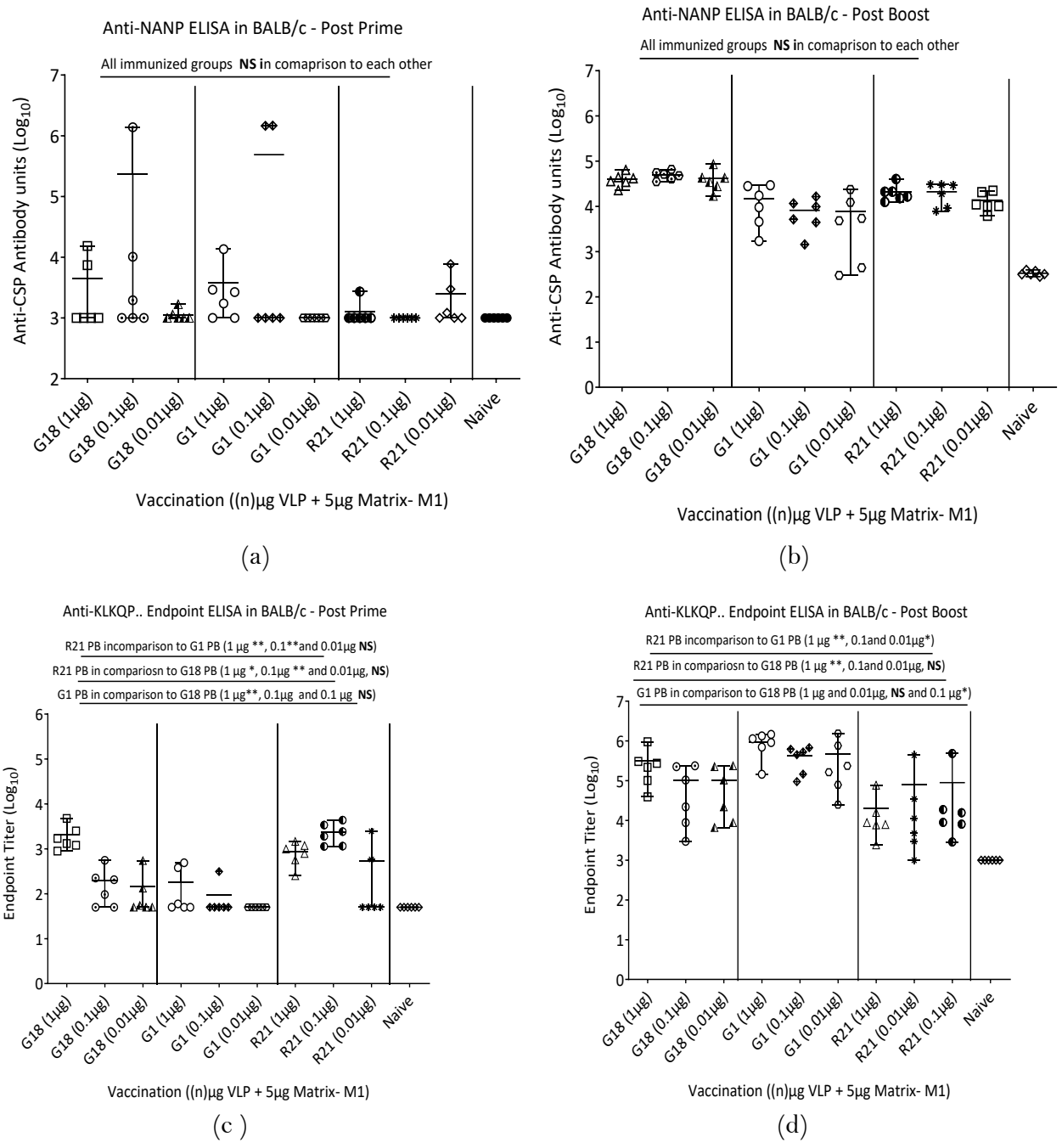


Figure 4m: Anti-NANP and anti- KLKQP…immune responses post prime and boost for R21, G1 and G18 administered at 1 µg, 0.1µg and 0.01µg with 5 µg Matrix-M1. Graph (a) and (b) show the anti-NANP (central repeats) IgG levels and (c) and (d) anti- KLKQP.. (JE) IgG levels post- prime and boost vaccination. Statistical significance was analysed by Mann- Whitney test post boost for anti-NANP response and anti- KLKQP…response within the dose group (ns). Comparison as made within different groups with same dose for anti-NANP ((R21 to G1, ns; R21 to G18 (1 µg *,

0.1and 0.01µg**); G1 to G18 (all doses**) and for anti-KLKQP…((R21 to G1(1 µg **, 0.1and 0.01µg*); R21 to G18 (1 µg **, 0.1and 0.01µg, ns); G1 to G18 (1 µg and 0.01µg, ns and 0.1 µg*))

4.3 Discussion

Analyzing the immune response and efficacy data from the 22 R21 variants that produced suitable VLPs it can be concluded that the efficacy might not correlate only to the induced antibodies titer generated against a specific epitope sequence and it might be dependent more on the quality of the induced antibodies in term of their avidity or affinity to bind to a specific epitope (which will be further investigated) which can be enhanced by the unique combination in which the sequence from different regions (epitopes) of CSP are fused and displayed on the VLP surface in a certain number and pattern.

One of the important observations is the criticality of number of NANP repeats to 18 when present in association with other sequences. Low number of repeats ((G1 (3 NANP), G14 and 16 (6 NANP), G2 (9 NANP) gave similar anti-NANP response to constructs with 18 NANP repeats, while higher number of repeats ((G3 (21 NANP) and G10 (36 NANP)) failed to demonstrate any additive effect to it. In both cases, no or less efficacy was obtained in comparison to constructs with 18 NANP repeats. The contribution of CSP C- terminus antibodies to protection of the RTS,S and other CSP-based vaccines has been controversial. Data from PfSPZ-CVac trial, the low titres and inability of these antibodies to confer protection in mice do not support a role for CS-mediated humoral protection with whole sporozoite vaccines [196], while association of IgG1 and IgG3 subclass of antibodies to this region to efficacy in RTS,S Phase III clinical trial in children highlights its importance [150]. It was well demonstrated here that removing the C-terminus from R21 (G8) markedly reduces the efficacy from 44.4% to 16.7%, highlighting critical role of C- terminus. Replacing C- terminus with universal CD4 + T cell helper

sequence PADRE (G18) demonstrated excellent efficacy of 100% indicating T cell help is more critical for efficacy than the potential synergistic role of antibodies elicited against C- terminus or PADRE.

With the aim to enhance the longevity and durability of R21, it was interesting to include the JE and CE sequences in R21 variants. Though both the sequences produced immune response, none of them when present exclusively in a construct was able to confer any protection. Only JE demonstrated contribution to efficacy enhancement with higher efficacy seen when in combination with 18 NANP repeats and T cell epitopes (G22 and G21), and reduced efficacy seen when number of NANP repeats were reduced (G14 and G16) or T cell epitopes were removed (G20 and G1). Interesting to note here was that JE antibodies had an apparent antagonist effect on levels of anti- NANP antibodies when NANP was present as 18 repeats (G22 and G21), but either the JE antibodies compensated for or helped in selection of high avidity anti- NANP antibodies giving 80% or higher efficacy for these constructs. Despite this, looking at the efficacy from G18 which does not contain JE or CE, indicates that though they are important contributors to efficacy, 18 NANP repeats with a strong CD4+ T cell helper PADRE sequence is capable to inducing higher levels of protective antibodies. More studies using these three constructs in bigger groups of mice as well as in other mice strains like the CD1 and C57BL/6 need to be conducted to provide a better insight into construct performance.

Longevity and durability experiment on G1 and R21 provided a useful insight that, despite initial promising efficacy than the existing vaccine, long lasting efficacy is still questionable and only such long term pre-clinical studies can help decide whether the candidate can be taken further to in-human clinical trials. As R21 has already demonstrated an efficacy of 77% over 1-year period, G1 might only be considered for in human trials if the durability associated with antibody longevity was significantly higher

than R21. As G1 was more efficacious than R21 3 weeks post boost, looking into change in isotype, affinity and avidity of antibody through the entire period might provide an evidence for diminishing efficacy.

Experimenting with co-delivery approach by mixing two VLPs before administration leads to a conclusion that a particular immunogenic sequence presented to the immune system in two different ways might not change the immune response to these sequences, but can have a remarkable effect on production of protective antibodies. This was seen with G1 +R21 and comparing it to G3. Mixing G1 with R21 also confirmed the importance of T cell help in augmenting the efficacy obtained from G1. Despite promising published results with NPDP sequence, co-delivery of G11 and R21 did not lead to any efficacy enhancement for R21 and contrary reduction in efficacy indicates the antagonist nature of the non-protective antibodies from this sequence in a mixed- vaccination VLP format.

Co-delivery of G1 and G18 provided an unwelcome example of how the efficacy of two high efficacy vaccines can reduce when mixed together. The efficacy reduced to 50% on co-delivery from 91.5% for G18 and 77.6% for G1 when administered alone. None of the co-delivered vaccines show any reduced antibody response arguing against the possibility that the VLPs were aggregating leading to creation of buried antigenic sites. To further understand the positive and negative effects on efficacy when VLPs were co-delivered, possible changes in IgG isotype from the total IgG response can be assessed in future.

Demonstration of the same efficacy from a vaccine at reduced dose is potentially a major attribute that could allow important dose-sparing making it possible to vaccinate a relatively larger population with reduced cost. The immunogenicity and efficacy data from three constructs at different doses demonstrate that they are not clearly correlated.

The improvement of efficacy at low dose as seen for R21 and maintenance of efficacy at low dose as seen for G18 (1 μg and 0.01 μg dose) is promising. Further analysis into antibodies to PADRE sequence and changes in antibody isotype, affinity and avidity might provide a better understanding of the underlying mechanism that might be possibly associated with different efficacy outcomes. One thing to take into consideration is that promising reduced dose regimens also need to be investigated for longevity and durability of protective antibodies, as if this is found to lead to a greater number of doses required than the standard dose, the initial dose sparing and cost reduction will still remain an unachieved goal.

With the promising data obtained R21 variants G1, G14, G16, G17, G18, G21 and G22 that have shown higher efficacy than R21 hold the potential to be explored further.

Chapter 5

Immune complex generation as strategy for vaccination

5. Immune complex generation as a strategy for vaccination

5.1 Introduction

Immune Complex vaccination involves immunization with a pre-formed antigen-antibody complex at known concentration. This concept is over a 100 years old and was initially explored using antisera complexed to bacterial toxoids to decrease side effects in human vaccination [197]. Immune complexes (IC) are formed naturally when the antigen forms a complex with circulating antibodies in the body, leading to elimination of the pathogen by phagocytosis or complement activation [198]. ICs attach to the long membrane processes of follicular dendritic cells (FDCs) [197] and are taken up by the FDCs, stromal cells that reside in primary follicles and germinal centers of the secondary and tertiary lymphoid organs [199]. The ICs interact with the FDCs through the Fc domain of the Ig component with the Fc receptors on the FDCs. The antigen is then processed and presented via MHC molecules providing T cell help to activate B cells and production of antibodies as described in figure 5a below [200]. Complement C3 complex has been shown assemble with the IC aided by complement receptors CR1 and CR2, and play a crucial role in generating B cell memory [199, 201]. Thus, ICs engage both the innate and adaptive arms of the immune system. FDCs are the only known cell types that can trap ICs, retain them for long periods of time, and thereby preventing degradation [202]. This has been explained by another mechanism whereby the ICs trapped on FDCs form iccosomes or immune- complex coated bodies [203]. These beaded string-like structures release from the FDCs ~ 3 days post immunization into the germinal center (GC) where they

are taken up by B cells and processed to present to the T cells leading to induction of antibody- forming cells and B cell memory through production of lymphokines [204].

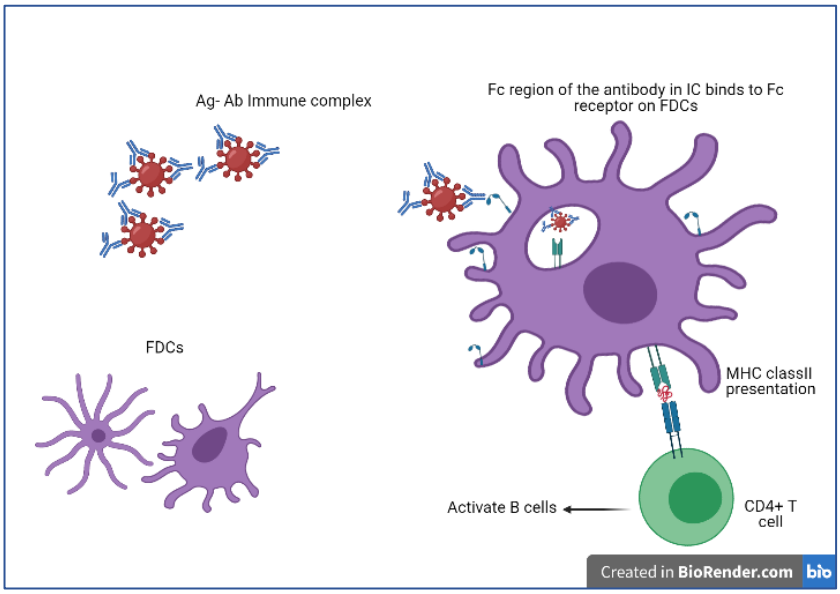


Figure 5a: Mechanism of immune response by ICs via FDCs. Attachment of IC Fc region to the FC receptor on FDC leads to internalization, processing and presentation via MHC class II to the CD4+ T cells. CD4+ T helper cells secrete cytokines and activate B cells.

IC vaccination has been successfully used in prevention of infectious bursal disease (Cevac Transmune IBD® and Bursaplex®) and this approach is being developed for protection against Newcastle Disease Virus (NDV) in poultry. For human diseases, this mode of vaccination has been explored in HIV, SIV, Chronic HBV, influenza, Ebola and cancer with promising outcomes at pre-clinical stages [205]. This mode of vaccination has also been tested at the pre-clinical level for Zika and HPV where codelivery of VLPs and RIC (Recombinant immune complex) resulted in a highly immunogenic, synergistic vaccine delivery system, with a 2-5-fold increase in antibody titers that corelated with virus neutralization in vitro [206, 207]. This

approach has also been used for adenovirus-antibody ICs and has shown to stimulate strong immune responses by an increase in the duration of antigen availability [208]. In a human study using HBsAg-HBIg IC on healthy adult volunteers, the complex was found to be safe and induced potent anti- HBs response [209]. With the aim of exploring the possibility of enhancement in the immunogenicity and efficacy of R21 vaccine, this chapter details the results obtained from two experiments,

1. Experiment 1: When IC formed combining R21 with 2A10 antibodies administered via IP and IM routes and;
2. Experiment 2: When R21:2A10 IC was co-administered with R21 administered via SC and IM routes.

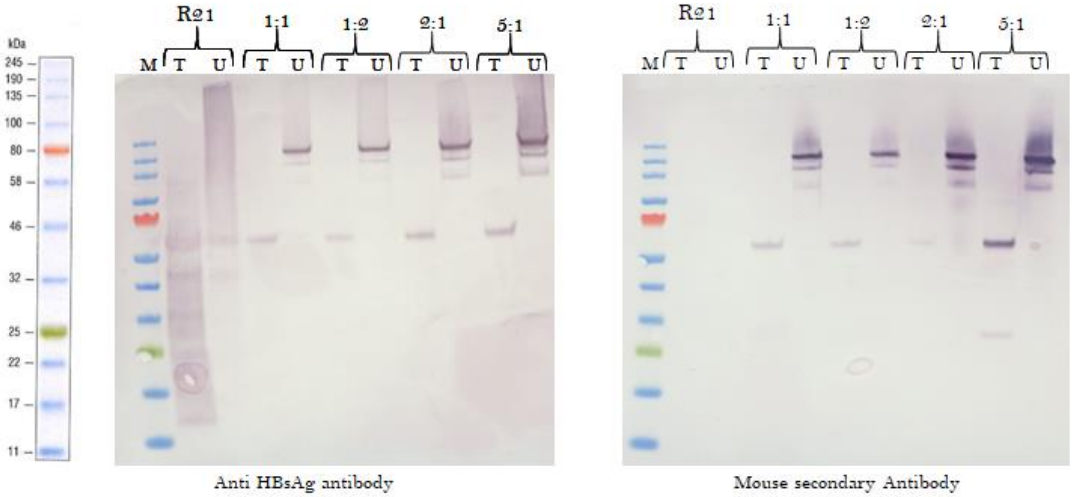
5.2 Results

Different amounts (µg) of MAb (2A10): Ag (R21) were combined and analyzed to select the best ratio that can be used for vaccination as described in 2.2.12 and table 2.6.

5.2.1 Western Blot analysis

The immune complexes were formed by incubating different ratios of R21 VLP and Mab 2A10 as described in section 2.2.1.2 and western blot analysis was performed as described in section 2.2.9. All the ratios show formation of complexes as seen in figure 5b. One of the two blots was stained with anti-HBsAg antibody (Figure 5b (a)) to confirm presence of R21 and the other with mouse secondary antibody (Figure 5b (b)) to detect 2A10 antibody. For the samples treated with reducing agent, the band of correct size (~50kDa) was detected which is the size of a R21 monomer. The presence of a high molecular weight band at ~190 kD in both the blots for untreated samples

confirmed immune complex formation. Other bands seen on both the blots at the same position indicate smaller complexes.



(a) (b)

Figure 5b: Western blot analysis of ICs. M= Marker, T= Sample treated with reducing agent, U= untreated sample. One of the blots was stained with anti-HBsAg antibody (a) to capture HBsAg sequence fused to the CS sequence followed by mouse secondary antibody (b) to develop bands. To confirm the formation of IC, a second blot was stained directly with mouse secondary antibody to bind to Mab 2A10.

5.2.2 Immune gold TEM

A sample of the IC from all ratios was sent to the Dunn School of Pathology Electron Microscopy Facility and processed for negative staining after tagging the IC with anti-mouse antibody conjugated to gold particles, which bound to the Fc region of the antibody forming a part of the IC. R21 VLP was used as a negative control to which no gold conjugated antibody would bind. As seen in figure 5c, the immune gold

labelling was clearly positive in IC ratios 1:1, 1:2, and 2:1, while in 5:1 it was not very clear and showed some aggregation. R21 appeared negative, confirming the IC formation only when R21 was mixed and incubated with Mab 2A10. Considering the availability of material (both R21 and 2A10), 1:1 ratio (1µg of each) was chosen and used in both the experiments.

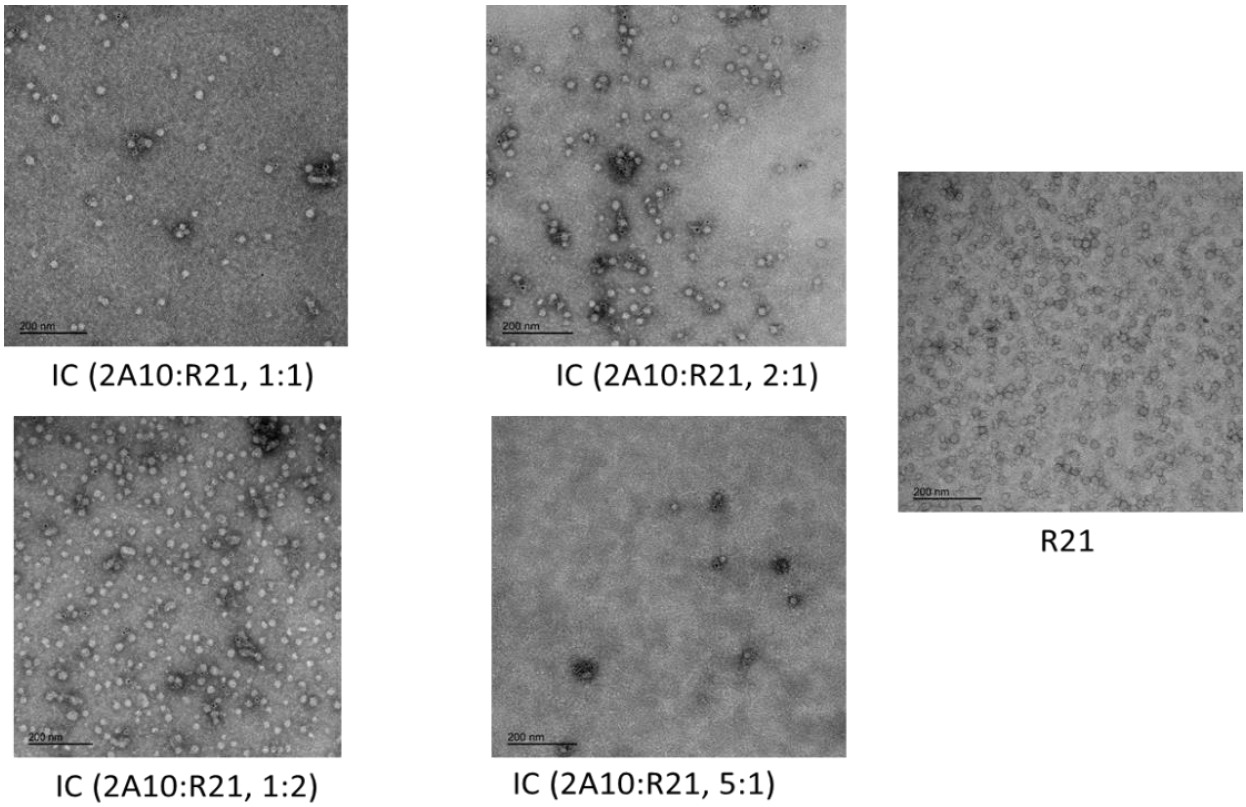


Figure 5c: Immune gold labelling of IC in different ratios and R21 VLP. Three ratios 1:1, 1:2 and 2:1 confirmed positive IC formation, while it was not clear in the ratio 5:1, with signs of aggregation. R21 VLP did not bind to the gold conjugated antibody and appears negative.

Based on the confirming results from Western blot and TEM, the ratio of 1:1 (R21:2A10, 1µg each) was chosen to be used for immunization.

5.3 Results from experiment 1: R21 VLP: 2A10 Mab Immune complex is immunogenic and protective against sporozoite challenge

Two routes of immunization were used in this experiment IM and IP. The rationale for choosing the IP route of immunization in this experiment was based on the preferred route chosen by Barabas et al. for treatment of an experimental autoimmune kidney disease called Heymann nephritis, and IM route to compare to as a standard route for R21 vaccine used in pre-clinical and clinical studies. The choice of IP route for administration of antigen for development of experimental disease and treatment might have been due to proximity to the kidneys targeting rapid absorption of antigen and treatment which can enter the systemic circulation within 10 seconds of administration [210]. Though this approach was used for the treatment of a disease rather than as a vaccine, my rationale to study the immune complex approach in malaria model stemmed from the published positive outcome of ability to induce the production of same antibody and specificity as in the IC. The aim here was to assess this approach to possibly induce higher levels of antibodies to the immunodominant NANP repeats and compare its translation to efficacy with R21.

5.3.1 Immunogenicity assessment

BALB/c mice were vaccinated as described in section 2.4.3 with an IC formed of 1µg each of Mab 2A10 and R21. Anti- NANP antibody ELISA and statistical analysis were performed as mentioned earlier. Figure 5d shows the endpoint titers, 3 weeks post prime (a), post 1st boost (b) and post 2nd boost (c). The IC group without the adjuvant MM produced slightly lower immune response, while IC + MM (IM and IP) groups produced similar response to NANP as the positive control R21 + MM group at all

three time points. The antibody responses after 1st and 2nd boost fall in the same log scale and 2nd boost does not seem to enhance the antibody response. The two 2A10 antibody groups fail to produce any response post prime vaccination. In the 2A10 IM group, post 1st boost, one mouse and post 2nd boost 2 mice show an anti-NANP response which is also visible in the 2A10 IP group in 1 mouse post 2nd boost. Owing to the serum half-life of 6-8 days[211] in adult mice for 2A10 which is IgG2a isotype [212], the response observed on ELISA may have likely come from circulating 2A10 that was used for vaccination, but after being recycled by the neonatal IgG Fc receptor (FcRn). IgG in circulation when taken up by the endothelial cells and monocytes bind to FcRn in an acidic endosome which helps IgG to escape degradation and is released back into the blood stream, prolonging its half-life [213]. Also, as 2A10 is produced from mouse hybridoma cells line the levels of sialylation may have also contributed in prolonging the half-life [214].

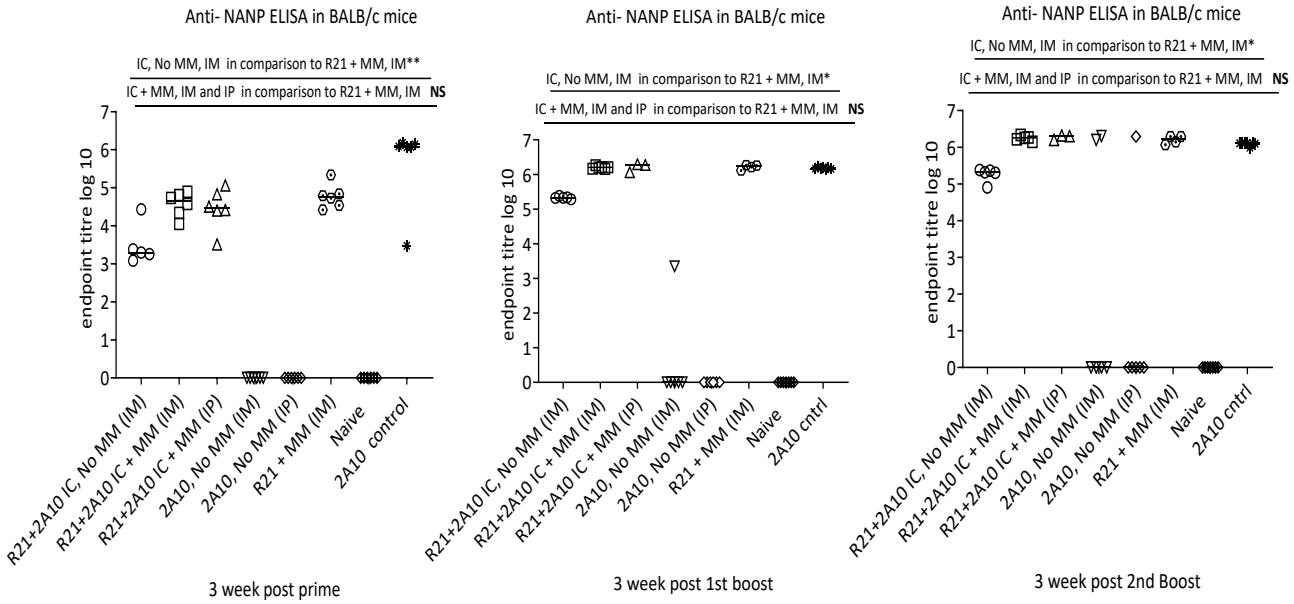
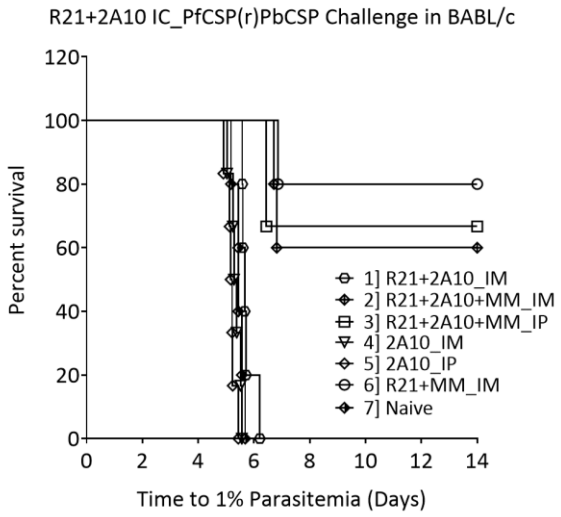


Figure 5d: Immune response to anti NANP antibodies after prime, boost and 2nd boost vaccination. The IC group without MM produced a moderate antibody response which increased significantly after the 1st boost and plateaued after the 2nd boost. The groups containing IC + MM (IM and IP) elicited higher antibody responses than the IC group without MM, and of similar magnitude when analyzed three weeks post prime. The antibody responses were enhanced after 1st boost but plateaued after the 2nd boost with no further enhancement. 2A10 groups failed to mount any response post prime while anti-NANP response was observed post 1st boost in IM group (1/6 mice) and post 2nd boost in IM (2/6 mice) and IP (1/6 mice) group. Groups were compared by Mann-Whitney test. * = $p < 0.05$.

5.3.2 Efficacy Results

Despite showing high immunogenicity in anti-NANP IgG ELISA after 3 immunizations, the efficacy of the two IC groups (IM and IP) was less (60 and 67% respectively) than, and significantly different to, the positive control group (R21 + MM, 80%). The antibody response difference between the two IC groups with and without MM injected IM was within a log, and yet no protection was observed in groups without MM. The IC groups 2A10 antibodies did not show any protection (figure 5e). This shows the important role of MM adjuvant here in elevating the antibody levels to the optimum level that can confer protection. MM has been shown to induce higher CSP specific CD4+ secreting high levels of IFNγ, TNF and IL2 contributing to higher avidity and optimum levels of protective IgG isotype [161].



Group	Route of immunization	Efficacy	Delay to 1% v/s naïve	P- value Long-rank (Mantel- Cox) Test
R21 (1ug) + 2A10 mAb (1ug), No MM	IM	0/5 (0%)	NS	0.0565
R21 (1ug) + 2A10 mAb (1ug) + MM (5ug)	IM	3/5 (60%)	Yes	0.0018 (**)
R21 (1ug) + 2A10 mAb (1ug) + MM (5ug)	IP	2/3 (67%)	Yes	0.0136 (*)
2A10 mAb (1ug), No MM	IM	0/6 (0%)	NS	0.1967
2A10 mAb (1ug), No MM.	IP	0/6 (0%)	NS	0.0682
R21 (1ug) + MM (5ug)	IM	4/5 (80%)	Yes	
Naive		0/6 (0%)	NA	

Figure 5e and table 5a: Efficacy results after three immunizations with IC administered via IM and IP route. No protection was observed in IC group without MM, while 60% protection was observed in IC group with MM when administered IM. IC group with MM (IP) conferred 67% protection with R21 +MM (IM) giving 80% protection. All the groups showing protection also had significant delay to 1% parasitemia when compared to the naïve. Groups were compared by Mantel-Cox test. * = $p < 0.05$.

5.4 Conclusion and rationale for experiment 2

Despite induction of high levels of anti NANP antibodies, the control group R21 + MM (IM) showed highest efficacy (80%) after three vaccinations, followed by IC + MM (IP, 67%) and then IC + MM (IM, 60%). The likely explanation for this could be that IC immunization may not be able to improve the immunogenicity (and efficacy) of what is already a very potent immunization approach of use of a VLP with the potent adjuvant MM, as suggested by the immunogenicity data of figure 5d. After concluding this experiment, new published data on co-delivery of VLP with IC with Zika as a disease model was available[207] and has been described below. In my experiment, as the vaccine being used in IC formation is a VLP, I was keen to apply the concept used in Zika model to malaria and formed the basis of the next experiment. Considering the evident role of MM adjuvant in protection, it was included in all groups for the next experiment.

5.5 Results from experiment 2: Co-administration of R21 and IC (R21:2A10) is equally immunogenic but less protective than IC only with two doses via IM and SC route and R21 administered via SC route

In the previous experiment, R21 + MM gave 80% protection after three immunizations. In this experiment, two immunizations were planned to explore possible differences between the vaccinated groups using IM and SC route to assess co-administration of VLP with IC (VLP + Mab) and to assess any enhancement in efficacy compared to the standard two immunizations with R21 + MM via the same routes. Published data suggests that when this platform was used for Zika, 5-fold higher antibody titers were induced and correlated with in vitro virus neutralization while for HPV similar antibody titers were achieved in comparison to only IC groups,

but gave 5 fold higher virus neutralization *in vitro* when VLP and IC were co-administered [206, 207]. The high titers could be possibly due the combination of VLP with IC consisting of VLP and Mab. The VLP might be eliciting both a B cell response and the IC via internalization, processing and presentation on MHC II, and engagement of CD4+ T helper cells contributing to the B cell response.

5.5.1 Immunogenicity assessment

BALB/c mice were vaccinated as described in section 2.4.2.2. NANP ELISA and statistical analysis were performed as mentioned earlier. Figure 5f shows the endpoint titers, 3 weeks post prime, and 3 weeks post boost. The two groups immunized with VLP + IC show similar antibody responses 3 weeks post prime and boost when compared to the IC and R21 groups.

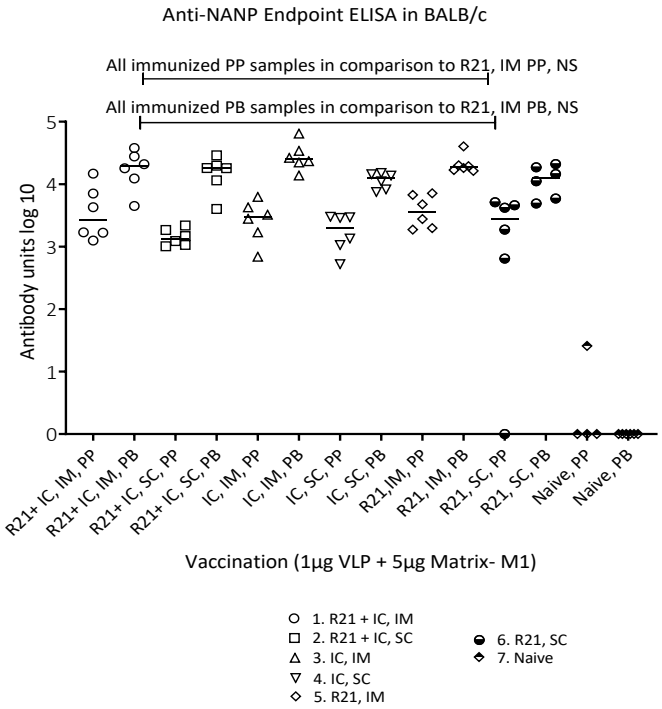
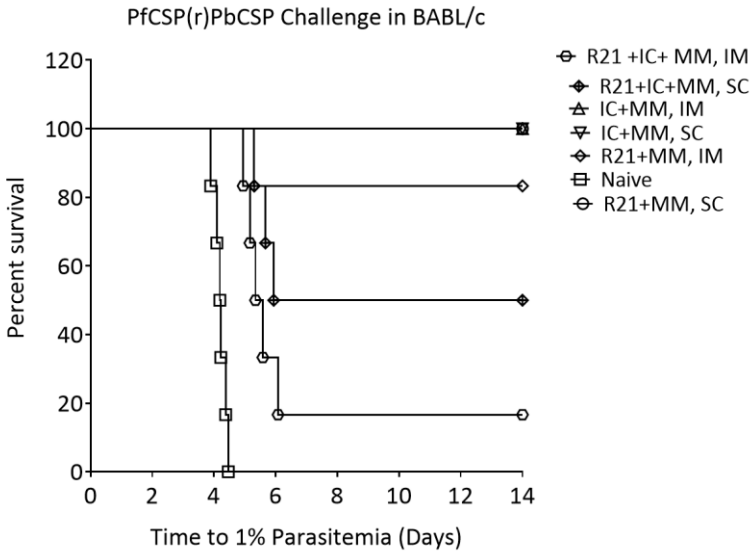


Figure 5f: Immune response 3 weeks after prime and boost vaccination: PP= Post prime and PB= Post boost vaccination. All the vaccinated groups elicited

anti- NANP response post prime and boost immunizations and were found to be statistically non-significant when compared to the standard R21+ MM (IM) group with respective groups. Groups were compared by Mann-Whitney test. * = $p < 0.05$.

5.5.2 Efficacy Results

The mice were challenged with 1000 sporozoites 3 weeks post the boost vaccination. Despite very similar immunogenicity responses to anti- NANP antibodies, the groups with co-administration of R21 VLP with IC, showed the lowest protective efficacy of 16.7% for IM group and 50% for SC group. Hence, the level of antibodies in these groups did not translate to efficacy. Three groups; IC via IM and SC routes and R21 via SC route showed 100% efficacy with all mice protected post challenge with 1000 sporozoites via IV route (Figure 5g). The control group R21 +MM via IM route gave better efficacy of 80% than the co- administration groups, which is also same as obtained after three immunizations in experiment 1.This demonstrates that the level of antibodies does not co-relate with protection and it seems that a fine balance of optimum antibody avidity and isotype might have been in the case of three groups giving 100% protection is not achieved to the same extent when VLP is co-administered with IC. Also, as the adjuvant MM was used in immunization of all the groups, the difference can be possible attributed to the VLP + IC co-administration platform, which might be skewing antibody ratio to less protective isotype. The published results so far for Zika and HPV has been from *in vitro* studies and would be interesting to know how the results translate to in *in vivo* model. The results of this experiment can also be attributed to the fact that the target organism here is a parasite and not a virus.



Groups	Route of immunization	Efficacy	Delay to 1% vs Naive	P value Log-rank (Mantel-Cox) Test
R21+IC (R21:2A10) +MM	IM	1/6 (16.7%)	Yes	0.0005 (***)
R21+IC (R21:2A10) +MM	SC	3/6 (50%)	Yes	0.0005 (***)
IC (R21:2A10) + MM	IM	6/6 (100%)	Yes	0.0001 (****)
IC (R21:2A10) + MM	SC	6/6 (100%)	Yes	0.0001 (****)
R21 + MM	IM	5/6 (83.3%)	Yes	0.0005 (***)
R21 + MM	SC	6/6 (100%)	Yes	0.0001 (****)
Naive		0/6 (0%)	NA	

Figure 5g and table 5b: Efficacy results from co-administration of VLP with IC in comparison to IC and VLP alone immunized through IM and SC routes. The VLP + IC groups gave low protection (IM 16.7% and SC 50%) and three groups IC (IM and SC) and R21 (SC) gave 100% protection in comparison to the control group R21 (80%) after two immunizations. All the groups showing protection also had significant delay to 1% parasitemia when compared to the naïve. Groups were compared by Mantel-Cox test. * = $p < 0.05$.

5.6 Discussion

The immune complex platform has been successfully employed for poultry vaccines and promising results have been published for human diseases. The aim of this chapter was to generate an Antigen: Antibody immune complex with R21 VLP vaccine and 2A10 Mab which is directed against the immunodominant NANP repeats and compare the immune response generated by immunization via different routes and compare it with the control vaccine R21 (IM route) for which well published results on immunogenicity and efficacy from pre-clinical and clinical studies are available. The first step was to analyze and select the best R21: 2A10 ratio that can be used in all immunization experiments. The western blot analysis and immune gold TEM confirmed IC formation. As 1µg of R21 has been a standard dose used in pre-clinical studies for comparing with new vaccines and platforms, a ratio of 1:1 (1 µg of R21 and 2A10 each) was chosen to compare with 1µg of R21. This ratio has been kept constant in both the experiments conducted for this chapter. In both the experiments the immunogenicity data in terms of anti NANP antibody response was inconclusive but differing efficacies were observed. In the first experiment the R21 + MM, IM group out performed IC groups after three immunizations. Within the IC groups (IM route), the contributing role of MM was very well evident in increasing efficacy as IC group without MM failed to provide any protection despite an immune response that was close to the IC group with MM. This was also seen in both the 2A10 immunization groups. Matrix M has been shown to induce strong immediate and long-term humoral response with increased frequencies of antigen specific B cells with the bone marrow along with CD4+and CD8+ T cells. [215, 216]. It was hence included in immunization of all groups in experiment 2.

The main aim of experiment 2 was to study possible antibody level and efficacy enhancement using a VLP + IC co-administration platform. Despite the promising in-vitro results obtained from Zika and HPV studies, it failed to provide any enhancement in terms of immunogenicity and efficacy when applied to malaria. On the other hand, IC only groups via IM and SC route and VLP only via SC route gave 100% protection. The common group between both experiments IC + MM (IM), gave an efficacy of 60% after three immunizations and 100% after two immunizations respectively. This outcome suggests that VLP + IC co-administration, although giving high immunogenicity, does not provide the fine balance of avidity and protective antibody isotype levels that were met when only IC was used and more conclusive results may be obtained by performing related assays. This was the first time R21 was administered via subcutaneous route in a challenge experiment in comparison to the standard IM route. The promising protective efficacy has opened an avenue to understand the underlying mechanism and compare the durability of antibodies over a longer period of time if any better.

With positive results obtained from IC experiments, more stable ways of generating ICs can be adopted for e.g. making it as a fusion protein where the antibody is linked to the VLP rather than making a complex by incubating two separate moieties together to form an IC. This has been successfully produced in plant cells [207] and would also be possible with the existing VLP platform using *P. pastoris* as described in previous chapter.

Chapter 6

Alpha-gal glycan as potential pre-erythrocytic vaccine candidate

6. Alpha-gal glycan as a potential pre-erythrocytic vaccine candidate

6.1 Introduction

Humans have high levels of circulating antibodies against xeno-glycans, sugars, and polysaccharides expressed on the surface of pathogens. This diversity and ability of glycans and sugars coating the surface of pathogens to elicit a protective antibody response in humans was demonstrated by Heidelberger and Avery in 1923 [217], where high antibody levels specific to pneumococcal polysaccharide were detected even 8 years after immunization [218]. As identified by use of microarray technology, human serum contains anti-carbohydrate antibodies capable of binding to >100 different carbohydrate antigens mostly associated with over 400 different strains of intestinal bacteria [219]. The immune response to glycans is T-cell-independent and mostly IgM in type which is short lived. Also, as glycosylation in not genetically driven, there is a lot of heterogeneity found leading to diverse glycoforms which can produce a weak antigenic response [217]. This is particularly important as no memory B cells are formed and poor immunogenicity in observed in human infants under the age of 18 months, in the elderly and in the immunocompromised [66, 220]. However, glycans or polysaccharides conjugated to a protein are capable of eliciting long lasting T cell dependent antibody responses. This can be attributed to the difference in the processing of unconjugated polysaccharide vaccines, which is different from protein conjugated polysaccharide vaccines when presented to the immune system. The unconjugated polysaccharide binds to the BCR on a B cell and the activated B cell differentiates into plasma cell that is T cell independent, leading to short lived immune response and no memory cell production. On the other hand,

the protein conjugated polysaccharides bind to the BCR on the polysaccharide-specific B cells while the linked protein is internalized, processed and presented via the MHC II molecules to carrier peptide specific T cells, which in turn help the polysaccharide specific B cells in affinity maturation, production of plasma and memory cells [66, 221]. This was very well demonstrated when capsular polysaccharide of *Haemophilus influenzae* type b was covalently linked to tetanus toxoid and then conferred 80% protection in 7- 14 months old infants [222]. So far, a number of multi-valent and conjugate polysaccharide vaccines have been licensed for human use for bacterial diseases like *H. influenza*e, *N. meningitidis*, typhoid and pneumonia [220], but none for viruses [223] or parasites [224].

Galalpha1-3Galbeta1-(3)4GlcNAc-R (α-gal) is one such glycan, and naturally found in the cells of marsupial and placental mammals like kangaroo, mouse, rat, rabbit, pig, cow, horse, cat, dog, and dolphin, on cells of prosimians (e.g., lemurs), and of New World monkeys (monkeys of South America) at a density of 1×10^6 - 30×10^6 epitopes/cell [225]. The expression of α-gal was suppressed in humans, apes and old world monkeys due to mutational inactivation of the gene by natural selection of the enzyme alpha1,3galactosyltransferase (alpha1,3GT) that synthesizes α-gal [226]. α-gal has been found in a range of pathogens to which no effective vaccine is yet available (Table 6a). Anti- α-gal antibodies constitute 1- 5% of circulating IgG and IgM in healthy adults [227] and are first generated when immune cells come in contact with the α-gal on the surface of bacteria from the gastrointestinal microbiota [228] such as some strains of Gram-negative *E. coli*, *Serratia* and *Klebsiella* and Gram-positive *Enterococcus* and *Streptococcus* [229]. Though anti- gal IgE has been associated with tick bites and meat allergy, called 'α-gal

syndrome', anti- α-gal IgG and IgM hold protective potential against many infectious diseases [230]. High antibody (~100-fold increase), CD4+ T cell response (> 100 fold higher) and CD8+T cell response (~6-fold increase) was seen when mice were immunized with inactivated influenza virus presenting α-gal epitopes in comparison to inactivated influenza virus lacking α-gal epitopes [231]. It has also been used in a number of other pre-clinical studies for cancer [232, 233] and parasitic diseases like Leishmania using a VLP platform [234], and Chaga's disease using human serum albumin [61]-conjugated α-gal [235] with promising results. Recently, it has been highlighted that there is a reduction in the α-gal antibody titers, which correlated with COVID- 19 disease severity, when assessed in patients at different disease stages in comparison with healthy controls [236].

Pathogen	Disease	Vaccine availability	Presence of α-Gal
Plasmodium	Malaria	No	Yes
Leishmania infantum	Visceral	No	Yes
Trypanosoma brucei	Sleeping sickness	No	Yes
Trypanosoma cruzi	Chagas disease	No	Yes
Borrelia burgdorferi	Lyme disease	No	Yes

Table 6a: Presence of α-Gal in Vector-Borne Pathogens and Vaccine Availability [237]

Alpha-gal has been found associated with GPI anchored proteins in malaria. In α1,3GT knock out mice, once bound to the surface of *Plasmodium* sporozoites, anti-α-gal IgM, IgG2b, and IgG3 MAbs have been shown to activate the classical pathway of complement, as assessed by C3 deposition and confer sterile protection [226]. Both

anti- α-gal IgG and IgM antibodies have been detected at a statistically higher level in healthy African populations than in Europeans [237] (Figure 6a). It was observed that individuals from Mali and Senegal who had higher level of anti- α-gal IgG and IgM antibodies were apparently less likely to be infected by *P. falciparum* [226, 237]. With this encouraging published evidence, alpha gal holds potential as a vaccine candidate for malaria that may help boost the antibodies to a level required for protection.

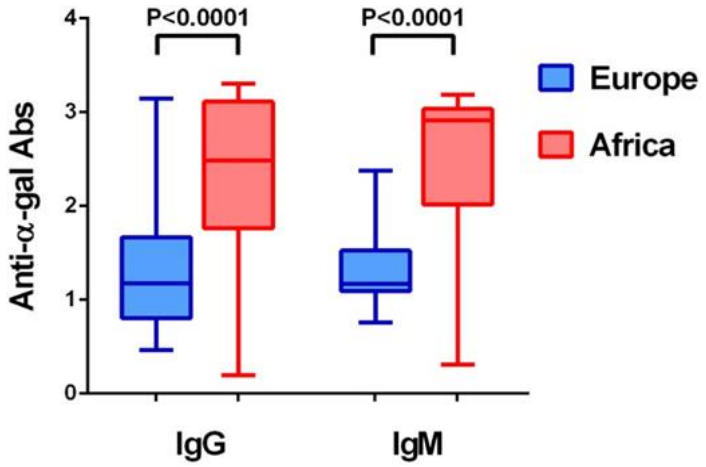


Figure 6a: Levels of anti-α-Gal IgM and IgG antibodies in healthy individuals from Africa and Europe [237].

6.2 Results

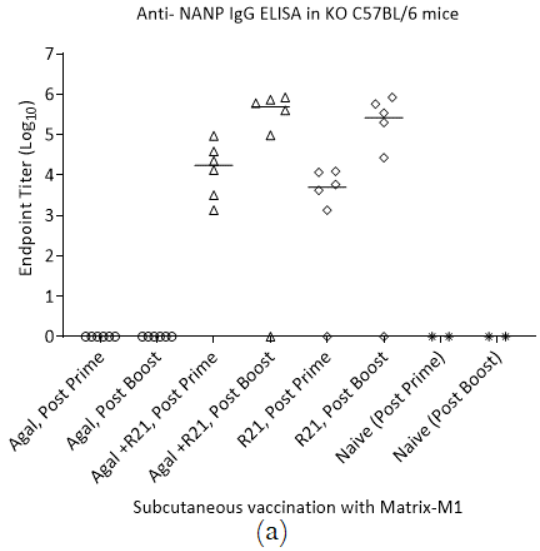
6.2.1 Unconjugated α-gal produces suboptimal immune response and is not protective

Glycans in free or un-conjugated form can only induce suboptimal immune response [66], but with no data on immunogenicity of free α-gal available, except higher affinity of natural anti- α-gal antibodies to free α-gal in trisaccharide form, it was important to investigate this. As this was assessed comparing it with R21, which

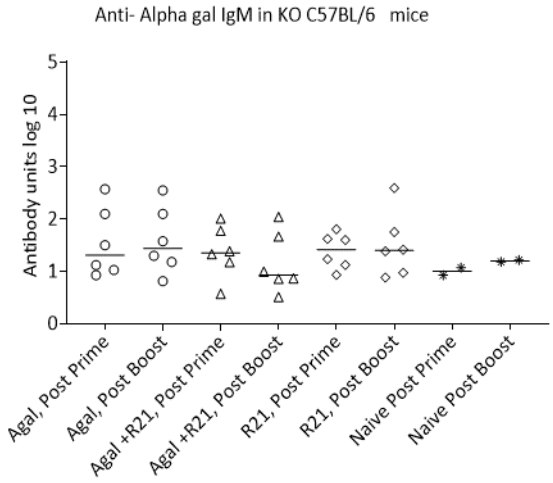
requires the Matrix- M1 adjuvant for higher immunogenicity and efficacy, it was included in all groups for this experiment, although immune responses to R21 remained unknown at this stage in these KO mice. The aim to co-administer R21 and α-gal was to assess whether the antibodies to both together can lead to possible enhancement in R21 efficacy or has any antagonistic effect. With the initial limited number of α-galT KO mice obtained (kindly provided by Miguel Soares, The Gulbenkian Institute, Portugal), inclusion of extra groups without Matrix- M1 was not feasible at that stage. Also, only SC route of immunization was tried owing to the same reason. KO mice were immunized as in section 2.4.4., with 75 μg of α-gal and 1 μg of R21 depending on the group. Matrix-M1 was administered at 5 μg in all groups. Figure 6b shows the immune responses to NANP (a) and α-gal ((b) and (c)). As seen from the figure, in the mice administered un-conjugated α-gal trisaccharide with and without R21 weak anti- IgM immune responses to α-gal were observed post prime and these remained unchanged post boost. Sub-optimal and non-significantly different immune response was observed with the R21 only group. R21 is produced using *P. pastoris* which is not capable of adding α-gal moieties as a post translational modification due to lack to transferase enzyme required for this. The levels observed were of the same order as the naïve mice indicating that this may not have been a response to the vaccine and that there may have been a contribution from the circulating anti- α-gal antibodies derived from the intestinal microbiome as seen in humans.

As expected, there was no NANP response in α-gal only group, while there was no significant difference between α-gal + R21 and R21 only group, post prime and boost. The anti-NANP IgG response for R21 observed with these KO mice is of the same

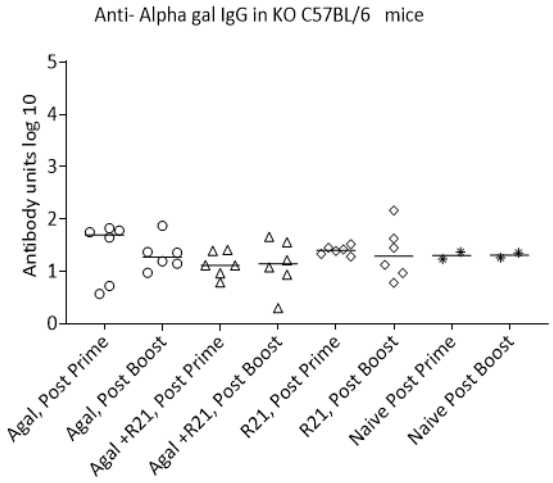
level as seen in the BALB/c mice. There was no significant difference seen in antibody levels between groups, while for the R21 + MM group significant increase (*p<0.05) was seen between prime and boost vaccination. This was non- significant in the α-gal + R21 + MM group, indicating a possible antagonistic effect on anti- NANP IgG induction when mixed together. Analyzing the anti- α-gal IgG response, no significant difference was observed between prime and boost vaccination within the same groups



(a)

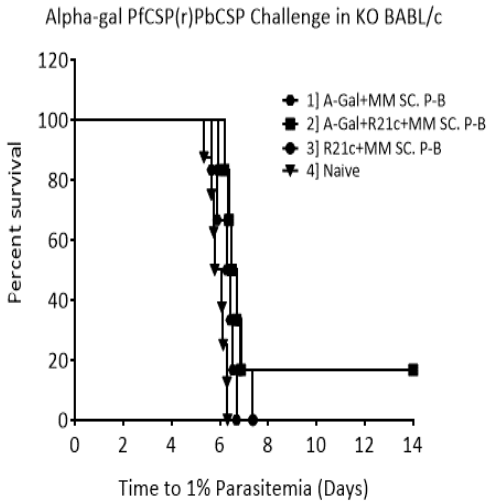


(b)



(c)

Figure 6b: Anti- NANP IgG (a) and Anti- α-gal IgM (b) and IgG (c) ELISA results for experiment 1: No anti-NANP IgG response was seen in α-gal only groups, while within the groups post prime and post boost the difference was non-significant for α-gal + R21+ MM group and significantly higher (*p<0.05) in R21 + MM group. Comparing post prime and boost anti-NANP responses there was a non- significant difference between the two groups. For anti- α-gal IgG and IgM, no significant differences were found between prime and boost.



Groups	Efficacy	Delay to 1% vs Naive	P value Log-rank (Mantel-Cox) Test
1. A-Gal (75ug) + 5 ug MM.	0/6 (0%)	NS	0.0668
2. A-Gal (75ug) + R21c (1ug) + 5 ug MM.	1/6 (16.7%)	Yes	0.0014 (**)
3. R21c+5 ug MM.	0/6 (0%)	Yes	0.0029 (**)
4. Naïve	0/8 (0%)	NA	NA

Figure 6c and table 6b: Efficacy results for Alpha-gal immunization experiment 1. Unconjugated Alpha-gal failed to confer any efficacy, while in combination with R21 was 16.7%. Significant (**) delay to 1% parasitemia was only observed in groups containing R21.

The overall suboptimal immune response to unconjugated α-gal was well reflected with the efficacy result (Figure 6c and table 6b) when the mice were challenged with 1000 sporozoites 3 weeks post boost, only α-gal + R21 group was able to show any efficacy (16.7%) of all groups. This might be possibly attributed to combined effect of

anti- α-gal and anti- NANP antibodies, but larger group sizes will be required to assess this. Despite induction of similar levels of anti- NANP IgG for R21 groups and presence of MM, the immunogenicity does not seem to translate to efficacy indicating mice strain differences with these being C57BL/6 KO mice. But this is likely to relate to the well known greater difficulty of protecting C57/BL6 mice with NANP-based vaccines that BALB/c mice. But, in conclusion, the required immunogenicity and efficacy has not been achieved here with unconjugated α-gal and R21 via SC route in this mouse model. With this conclusion, the next section describes the results obtained with protein- conjugated α-gal for immunization.

6.2.2 BSA- conjugated α-gal is capable of inducing higher immune response with Matrix- M1.

With clear conclusion from the previous experiment that unconjugated α-gal conferred no protection in my study, BSA-conjugated α-gal trisaccharide was used here to assess the immunogenicity via three different immunization routes IM, SC and IP. Although IM is the preferred choice of immunization route for vaccination, data on other immunization routes would provide an insight into the ability of this glycan to induce immune responses via these routes with and without the adjuvant Matrix-M1. Three immunizations were performed 3 weeks apart and the analysis of IgG and IgM anti-α-gal antibodies responses were performed using standard ELISA, results as in figure 6d ((a) and (b)) and 4e ((a) and (b)).

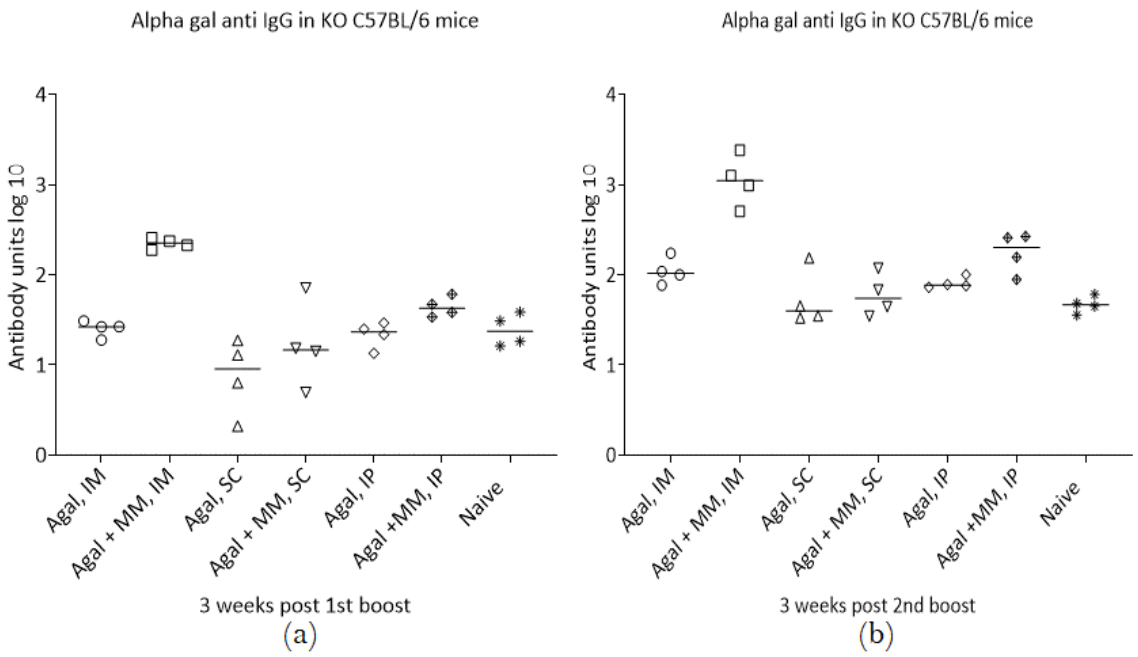


Figure 6d: Anti- α-gal IgG ELISA results for experiment 2 post boost vaccination 1 (a) and 2 (b). Comparing the immune response post 1st and 2nd boost vaccination, both IM and IP route with MM showed a significant increase in anti- α-gal antibodies (*$p<0.05$), while no significant difference was observed for SC route. Between the groups with and without the adjuvant, only IM route induced significantly higher (*$p<0.05$) antibodies post prime and boost.

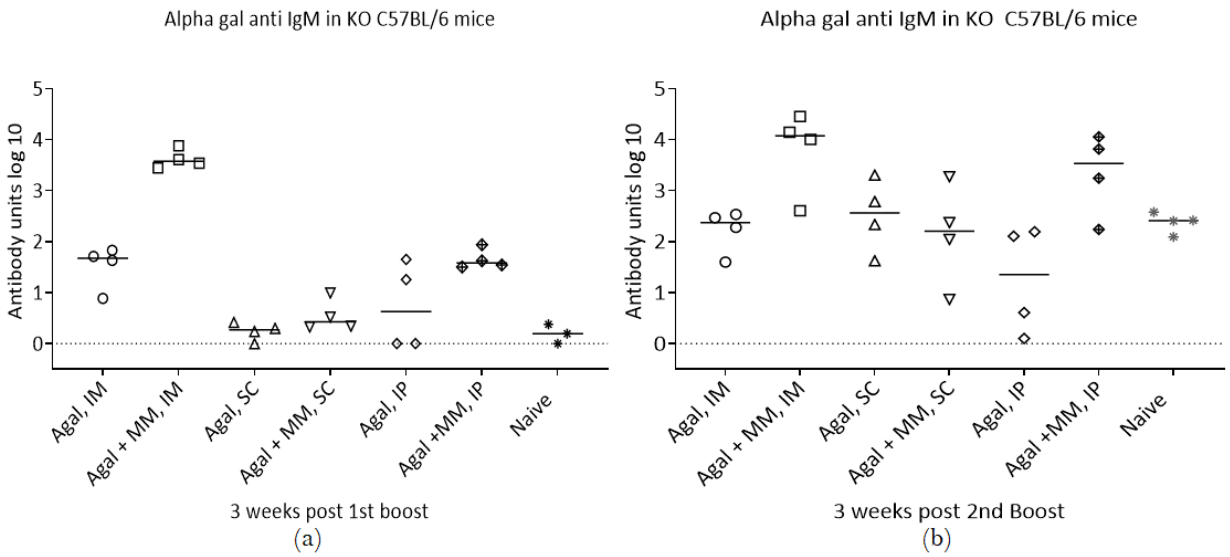


Figure 6e: Anti- α-gal IgM ELISA results for experiment 2 post boost vaccination 1 (a) and 2 (b). Comparing the immune response post 1st and 2nd boost vaccination, both IM and IP route with MM showed a significant increase in anti- α-gal antibodies (*p<0.05), while no significant difference was observed for SC route. Between the groups with and without the adjuvant, IM and IP route induced significantly higher (*p<0.05) antibodies post 1st and second boost.

For IgG, the group with IM route vaccination adjuvanted with MM provided the best immune response post 1st and 2nd boost in comparison to the other groups. Also, the IM group without MM induces antibodies, which are a log less than the group with MM. For IgM, group with MM via IM and IP route induced significantly higher titer than the non-adjuvanted group. Also, with the two groups, the titers were significantly higher than post 1st boost respectively. Considering both the IgG and IgM antibody responses, the IM route with MM seems to be the preferred choice to plan further experiments.

6.3 Discussion

Alpha-gal has been described both as a friend and a foe, with the capability to induce and enhance protective immune response as seen with pre-clinical studies for viruses, parasites and cancer, so a potential friend; However, it can also elicit an allergic immune response associated with tick bites and red meat [238]. Two important published papers fail to observe the production of anti- α-gal IgE, one by Yilmaz et al [226] demonstrating no circulating IgE on α-gal vaccination and by Roman-Carrasco et al [239] that α-gal linked delayed hypersensitivity is associated with lipid and not protein, increasing confidence in the use of α-gal as potential vaccine candidate.

As presented in the results section, it was clear from the two experiments conducted with unconjugated and conjugated α-gal that protein-conjugated α-gal with MM was able to induce t a higher level immune response to α-gal. Vaccination via different routes of immunization also has helped to identify the IM route as likely the best route of immunization, which can be kept constant for future experiments modifying other study parameters like α-gal quantity. With these encouraging initial results obtained with conjugated α-gal immunization, experiments are in-line to explore the level of efficacy that it might be able to confer alone and in combination with R21. This will be compared with the efficacy of the standard dose of 1 μg of R21. There are possible ways that the protein or VLP can be produced with α-gal post-translationally added, or use simple steps that can add α-gal moieties can be attached on bench via enzymatic reaction or addition of linker.

Chapter 7

Discussion and future directions

7. Discussion and future directions

Designing a highly efficacious durable vaccine against malaria has been a constant challenge for decades. A number of promising vaccines like RTS,S and *Pf*SPZ have been developed and have reached Phase II and III of clinical trials, created optimism for a licensed vaccine in coming years. With suboptimal efficacy of RTS,S vaccine in the field and the *Pf*SPZ vaccine showing minimal efficacy in infants in the field in Kenya [113]with also logistical issues of storage and intravenous deployment linked with it, the success of R21 meeting the WHO's malaria vaccine efficacy target has identified it as a leading vaccine candidate . Despite this, the common issue linked with all these vaccines is the waning efficacy over time needing the requirement of repeat vaccination to bring protective antibody levels to the efficacious levels. This makes it important not only to look into previously unexplored protein sequences like that in other parts of the CSP that can help improve new vaccines, but also adopt new combinations of administering existing vaccines, for example with RH5 as a blood-state candidate, and explore potential vaccine targets other than parasite proteins. During the course of my DPhil, I have taken this into consideration by including junctional and cleavage epitopes of the CSP to generate new R21 variants, administering the R21 vaccine as a pre-formed immune complex with the monoclonal antibody 2A10, and looking into the potential of sporozoite surface glycan α- gal as a pre-erythrocytic vaccine candidate. Identification and quantification of levels of correlates of protection have been instrumental in understanding the basis of efficacy, but identifying a durable correlate of protection remains a challenge. With this aim, chapter 3 of this D.Phil thesis focuses on the design and production, and chapter 4 on immunogenicity and efficacy of 25 new R21 variants generated using parts of CSP

amino acid sequence. CSP is the major sporozoite surface protein and availability of new published data on immunogenicity of regions flanking the immunodominant NANP repeat region has driven the design and generation of these R21 variants. These regions include the junctional epitope antibodies to which have been found to induce sterile efficacy[147, 165, 166]. It was also interesting to investigate increasing and decreasing the number of NANP repeats from 18 as present in the R21 vaccine, and it was found that 3 repeats induced the same levels of anti-NANP response after two vaccinations, and a higher number - i.e. 36 repeats - did not demonstrate any significant increase in the response. But for highest efficacy, as seen with R21 variant G18, 18 NANP repeats did require strong CD4+ T cell help as seen when a PADRE sequence was introduced. It was quite intriguing to see the effect of C-terminus removal on efficacy which reduced markedly from R21 with the C-terminus to the new G8 without a C-terminus and consisting only of 18 NANP repeats. This shows that, although important, T cells help provided by the presence of C-terminus may be suboptimal when compared to presence of PADRE sequence, at least in a mouse model. The immune response and efficacy generated by JE and CE using a VLP platform was an interesting learning that both, despite being potent immune response generators, are not capable of conferring efficacy when present exclusively in the construct. It could be seen that the JE had an effect on levels of anti-NANP antibody levels and showed the best outcome when the NANP was present as 18 repeats. Comparing the efficacy data of all 25 R21 variants, it can be concluded that although JE and CE are capable of inducing high immune response, the presence of 18 NANP repeats with a PADRE sequence is sufficient to induce high efficacy. Also, with the longevity data from R21 variant G1 it was evident that JE antibodies do not

hold this property and it would be interesting to look into the longevity of protective antibodies generated by G18 and efficacy after a period of 12 months.

Chapter 4 also describes the results form co-delivery of two VLP vaccines, G1 + R21, G11 + R21 and G18 + G1. Two quite distinct results were obtained with increase in efficacy seen with G1 + R21 and reduction in efficacy for the other two combinations, despite no change in levels of antibody production observed. The possible increase in the G1 + R21 combination efficacy of 83.5% was not significant, which was ~6% higher than G1 alone and ~ 10% higher than R21 alone, but it may be attributable to the T cell help from C-terminus of R21 and antibodies from the JE and CE. It can thus be inferred that this combination worked synergistically in enhancing efficacy. As the combined sequence from both VLPs is present in the R21 variant construct G3 as a linear peptide failing to induce any efficacy, comparing the antibody isotype profile along with avidity of the antibodies generated might provide a better understanding of the underlying mechanism.

Another possible combination that can be tried including these sequences, is separating the G1 and R21 sequence so that they are at the N and C terminus respectively of the HBsAg sequence on the VLP surface. This would mean exploring these unique combination of sequences presented to the immune system in three different ways. With the outcome of two of them already known, the outcome with presentation of these sequences separated by the lipid layer embedded HBsAg sequence would be interesting to study.

This chapter also presents the data for the vaccine candidates' dose titration of R21, G1 and G18 from 1 μg to 1/100th that dose which is 0.01 μg. With these three vaccines, the efficacy change observed did not correlate clearly with the level of dose

reduction. Reduction in efficacy was seen with G1 and G18 when the dose was reduced from 1µg to 0.1µg, while it increased in comparison to the 0.1µg dose when reduced to 0.01µg. This was different in the case of R21 when the efficacy increased with reduction of the dose to $1/10^{th}$ from 1µg, but decreased when reduced further to 0.01µg. For both G1 and G18 efficacy remained above 80% at $1/100^{th}$ of 1µg dose. This is a promising indication that this dose reduction could be translatable to human trials that will help in dose sparing, making vaccination of a large population potentially more cost-effective. It has been well established for R21 in both pre-clinical [179] and clinical trials[151] that a lower dose can be associated with higher immunogenicity and even efficacy. It has been suggested that low dose is associated with more effective induction of long-lived memory cell populations and self-renewing memory cell populations [64]. Studying the longevity and durability of protective antibodies generated at low dose would provide a crucial insight into to what level the new vaccine can advance when planning an in-human trial. Despite high initial efficacy at low dose, if the longevity is reduced in comparison to a standard dose, it might not justify the dose sparing and cost effectiveness of the vaccine. Considering this, future plan would be to understand the avidity and affinity of the protective antibodies for the constructs with high efficacy G1, G18, G21 and G22 and planning efficacy studies with these in C57BL/6 inbred and CD1 outbred mice.

Looking into the limitations associated with these two chapters, while analytical techniques were employed to confirm the VLP sequence and formation, other quality control techniques for particle size and aggregation levels like the DLS or analytical SEC and sequence confirmation using Mass spectrometry would have provided additional confirmation. These were not possible due to inability to access due to COVID restrictions, but could be considered when available to re-access the variant

VLPs and for future constructs. Results from chapter 4 indicate G18 as the best variant candidate in comparison to R21 and G1. The limitation associated with this conclusion is that the efficacy of G18 from one experiment is being compared to that of R21 and G1 from 7 experiments. Hence, re- assessing them as an individual experiment, when G18 and G1 gave the same efficacy of 100% (Appendix, 7). This highlights that including G18 in more experiments and with larger group of mice would provide a better insight into the range of efficacy levels that can then be truly compared to that of G1. Also, including G18 in an antibody longevity and durability study, as performed on G1 an R21 would show differences in the performance of both candidates.

Chapter 5 of the thesis describes the results obtained from using an alternative immunization strategy with R21. With promising published evidence, immunization was performed using preformed immune complex consisting of R21 vaccine complexed to the Mab 2A10 in 1:1 ratio. It was evident from the first experiment that Matrix-M1 is crucial for induction of efficacy conferring protective antibodies. The results from the second experiment has provided a clear evidence that a particular immunization strategy might work for viruses, but not for highly complex parasites like the *Plasmodium.* This was clear from the efficacy results of IC + VLP co-administration group. On the other hand, encouraging efficacy results from IC groups has provided a basis for designing VLPs where the Fc region of the antibody can be fused to the VLP sequence and produced as fusion protein consisting of the immunodominant peptide and the antibody Fc region expressed on the surface of the VLPs. This might also remove the possibility of unstable complex formation that might lead to erroneous and inconclusive results. It has also opened up a possibility of exploring the SC route of immunization for R21 which hasn't been tried before. My

plan to follow up on this will be to repeat this study in a larger group of mice and different breed of mice like the C57BL/6 and CD1 for confirmation of results and also provide understanding on any differences if seen on the basis of genetic make up.

Chapter 6 describes the immunogenicity and efficacy results of a novel pre-erythrocytic vaccine candidate that is a glycan. The naturally- occurring antibodies to this glycan called the α- gal, are present at high levels in African population which might be not only due to the commensal bacteria, but also due to the continuous exposure to a variety of pathogens that express α- gal, including the *Plasmodium*. Despite being present at high levels, the levels of these antibodies do not reach protective levels and an α- gal vaccine might provide a solution for this. Although anti- α-gal IgE has been associated with tick and meat allergy, Yilmaz et al. showed that no circulating IgE was detected after immunization with α- gal [226]. Also, a detailed study conducted by Roman-Carrasco et al., provides a strong evidence that only α- gal linked to lipids and not proteins is linked to the associated delayed allergic reaction[239]. Moreover, Matrix-M is a Th-1 type adjuvant and is less likely to induce substantial IgE levels. This has provided further encouragement to explore its potential as a vaccine candidate.

From the two experiments that I conducted, it was clear that un-conjugated α- gal fails to induce an immune response to a level that can confer efficacy, while good immunogenicity results obtained from using α- gal conjugated to BSA has paved a path for planning further pre-clinical immunogenicity and efficacy studies. With the current study demonstrating high antibody levels after three immunizations, it would be worth to not only conduct a challenge experiment after three immunizations, but also after two immunizations. This will help in planning immunization schedule for

future experiments. My plan forward would be to perform an efficacy experiment and understand if the level of the antibodies induced via IM immunization have a protective role, and on the basis on the result future experiments can be planned. With the potential to induce efficacy-conferring antibodies, a VLP vaccine can be designed to have α- gal moieties naturally attached to the protein by choosing murine cell lines like NS0 and Sp2/0 that is α- gal transferase enzyme positive required for synthesis of α-gal or mammalian cell lines like HEK293 and CHO which require additional processing steps. α-gal being hydrophilic protrudes away from the protein or VLP core likely reducing the possibility of masking the immunogenic domains [240]. These positive attributes provide further encouragement for this molecule to be assessed for efficacy that might identify it as a potentially strong malaria vaccine candidate.

To summarize, the work described in this thesis is my contribution to the efforts of the team for making a better malaria vaccine by investigating previously unexplored regions of the sporozoite CSP on a VLP platform, a new way of immunizing with pre-formed VLP-Antibody immune complexes, and the potential of a highly immunogenic sporozoite surface glycan. My goal here is to highlight that for complex infectious diseases like malaria looking into new proteins and sequence is crucial, but thinking of other possible ways as explored here in the form of immune complex vaccination and glycans might help to combine and deliver already proven protective proteins differently opening a possibility of better performing vaccine, contributing to the malaria vaccine development field. The results obtained from each chapter provide pointers to the possibility of better vaccine candidates than R21 and identify a path to

plan future experiments that can help achieve this very important goal for malaria control.

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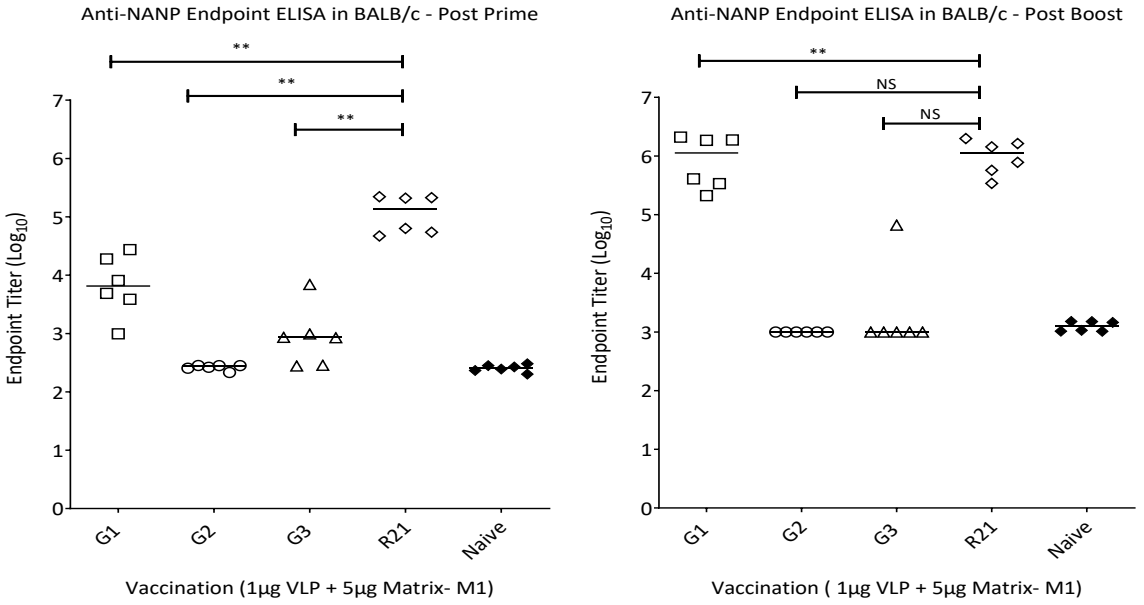
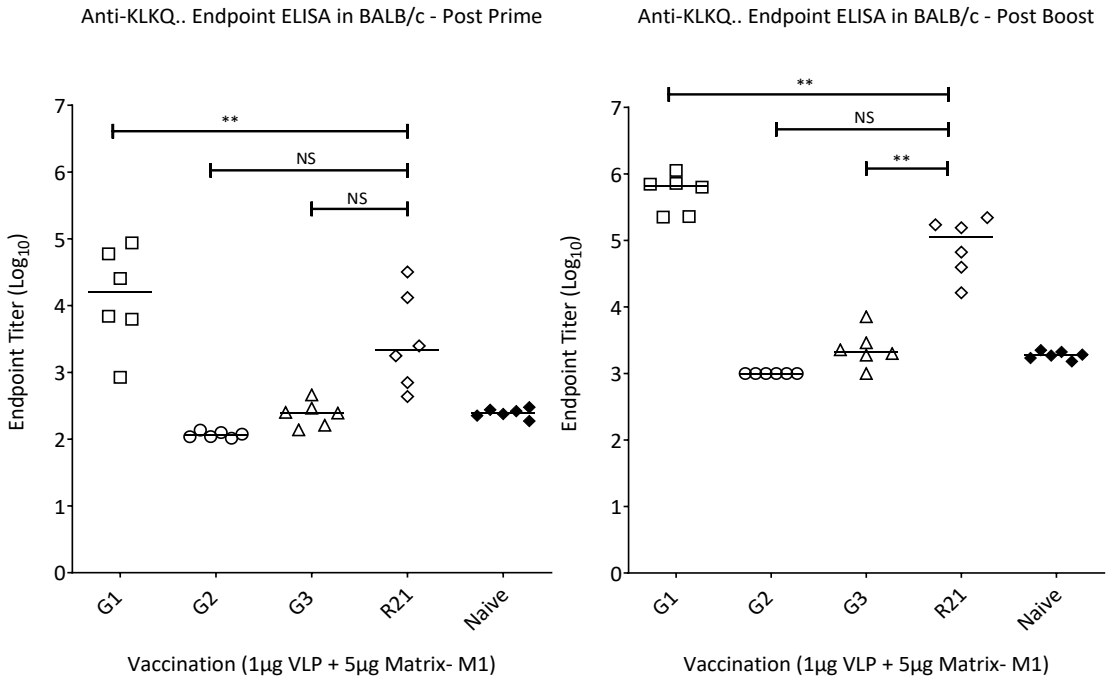
Appendix

Comparison of immunogenicity and efficacy of the R21v vaccine candidates in order of the experiment

1. R21v G1, G2 and G3

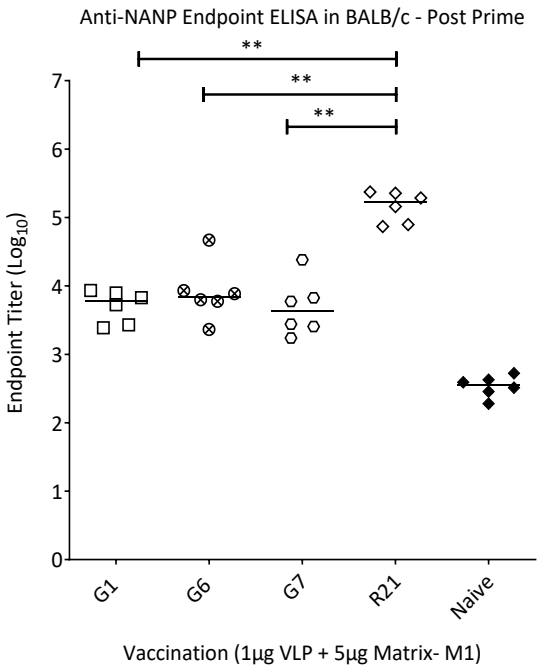
Construct	CE	JE	NANP	NVDP	C ter	Efficacy (%)
R21			+(18)		+	33
G1	+	+	+(3)	+(3)		67
G2	+	+	+(9)	+(3)		0
G3	+	+	+[53]	+(3)	+	0

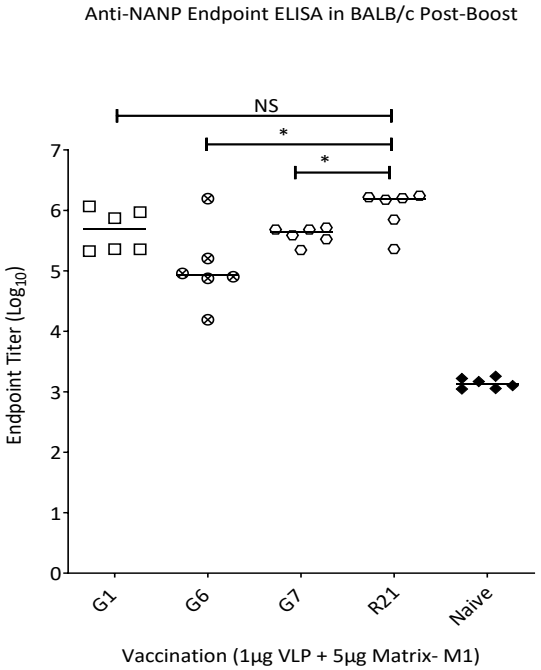


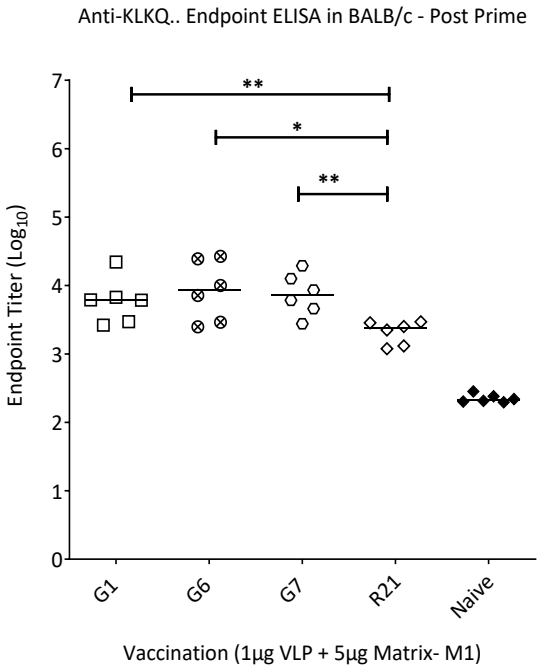


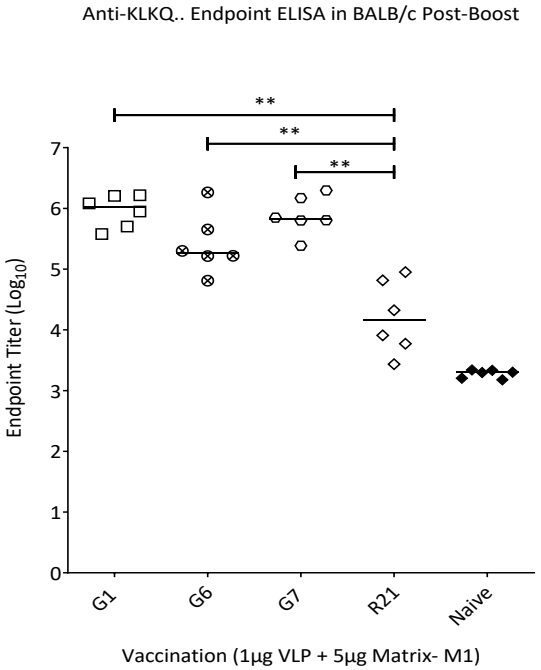
2. R21v G1, G6 and G7

Construct	CE	JE	NANP	NVDP	C ter	Efficacy (%)
R21			+(18)		+	16.7
G1	+	+	+(3)	+(3)		50
G6		+(5)				0
G7	+(5)	+(5)				0



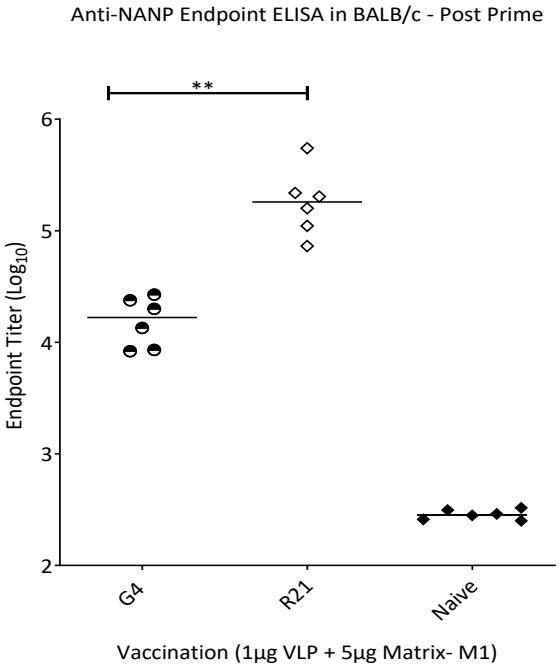


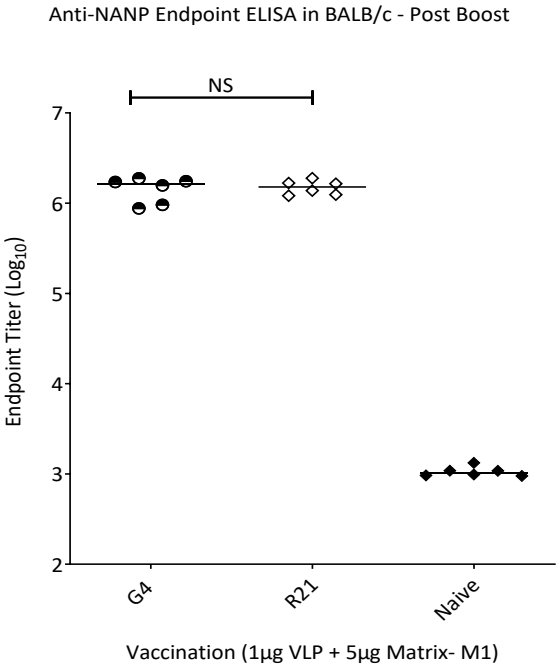


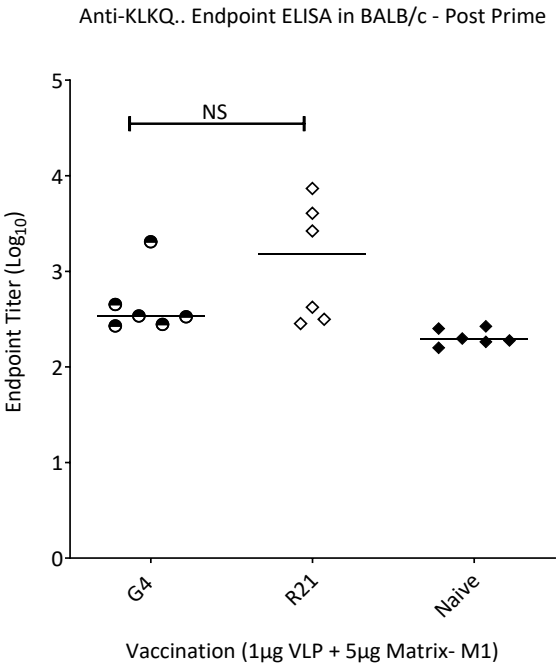


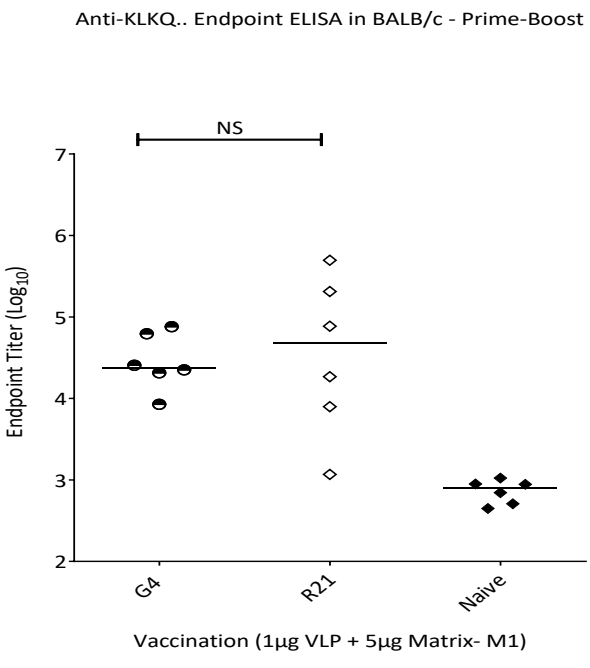
3. R21 v G4

Construct	N ter	NANP	C ter	Efficacy (%)
R21		+(18)	+	50
G4	+	+(18)	+	0



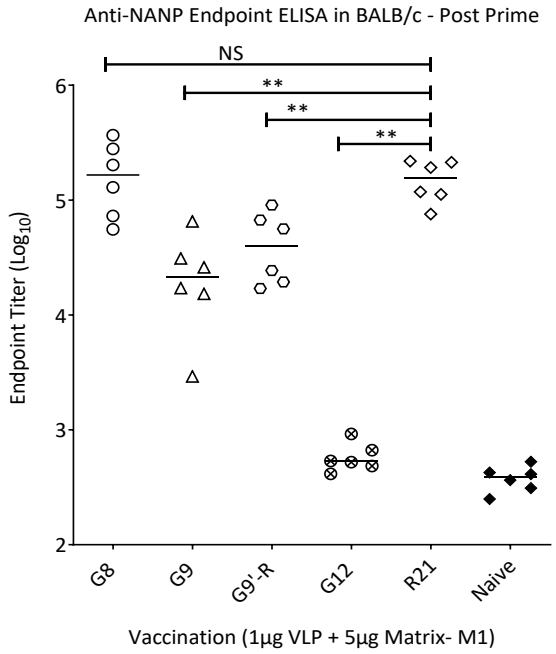


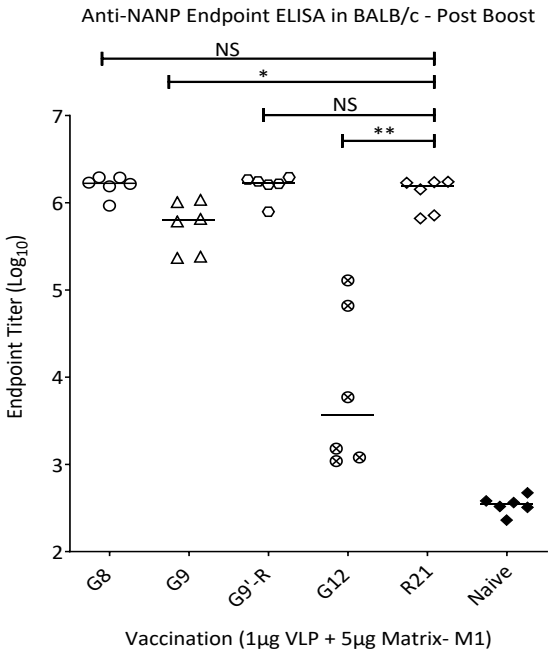


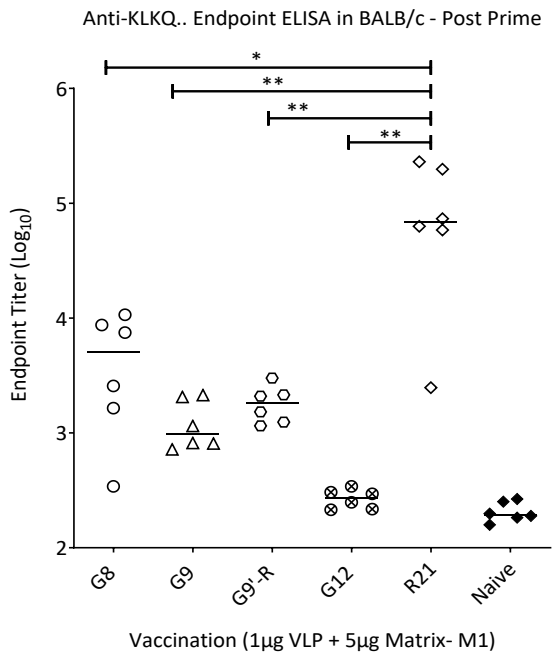


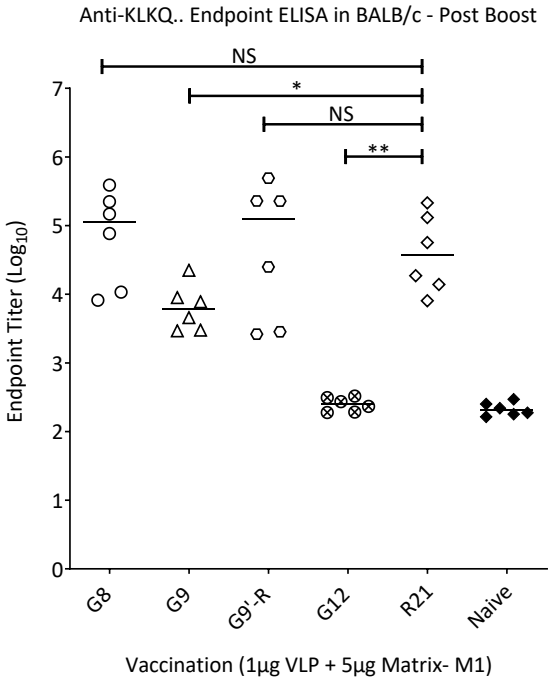
4. R21v G8, G9, G9'R and G12

Construct	CE	NANP	C ter	Efficacy (%)
R21		+(18)	+	33.3
G8		+(18)		16.7
G9		+(18) @ c ter		0
G9'R		+(18) rev		0
G12	+(18)			0



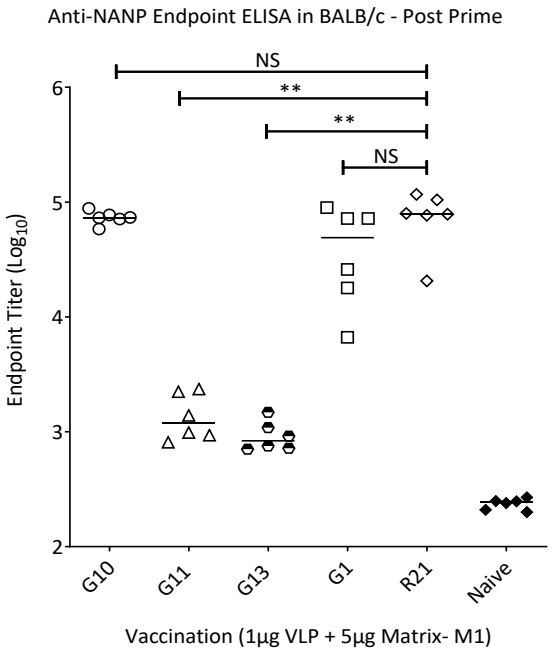


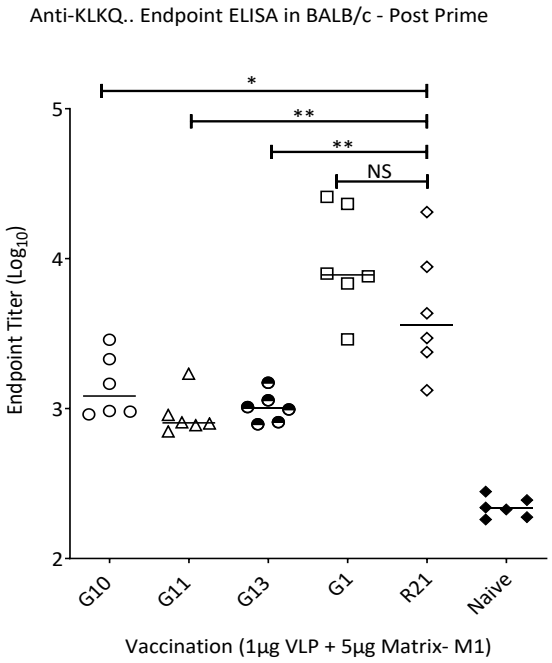


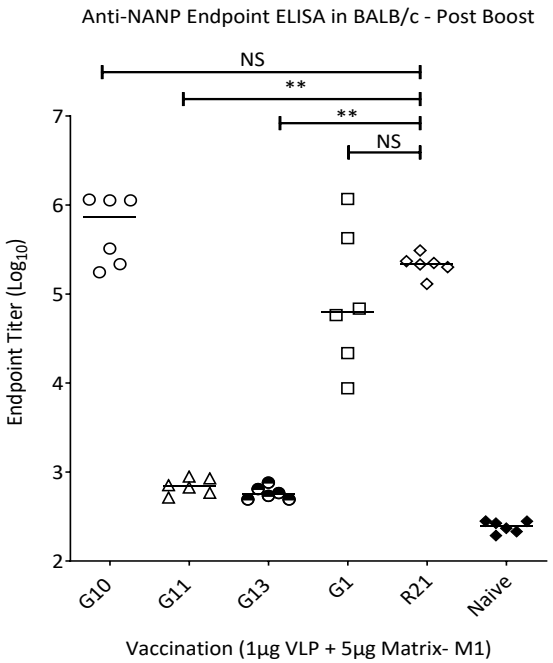


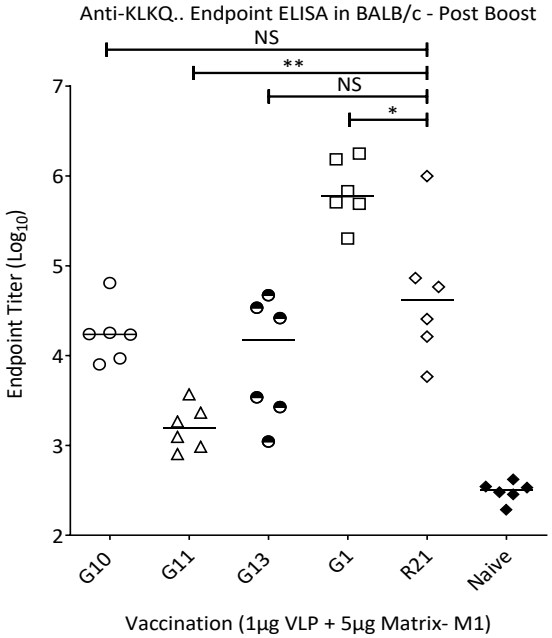
5. R21v G1, G10, G11 and G13

Construct	CE	JE	NANP	NVDP	C ter	NPDP	Efficacy (%)
R21			+(18)		+		66.6
G1	+	+	+(3)	+(3)			50
G10			+(36)				16.7
G11						+(18)	0
G13				+(18)			0



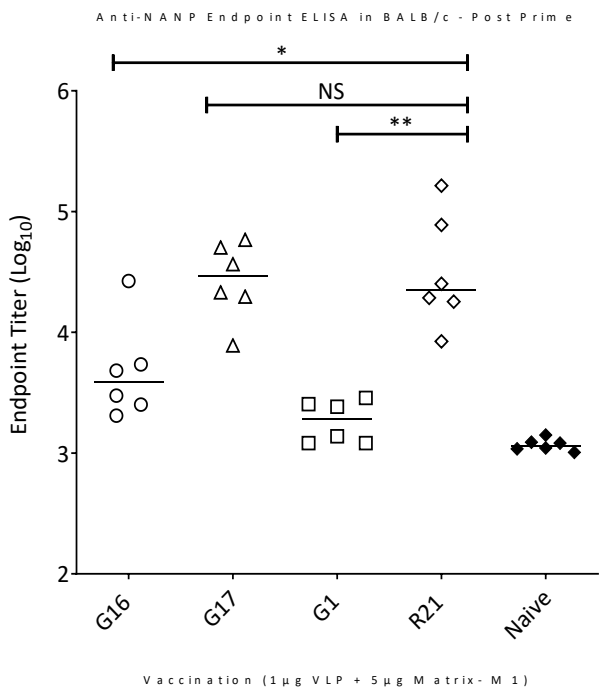


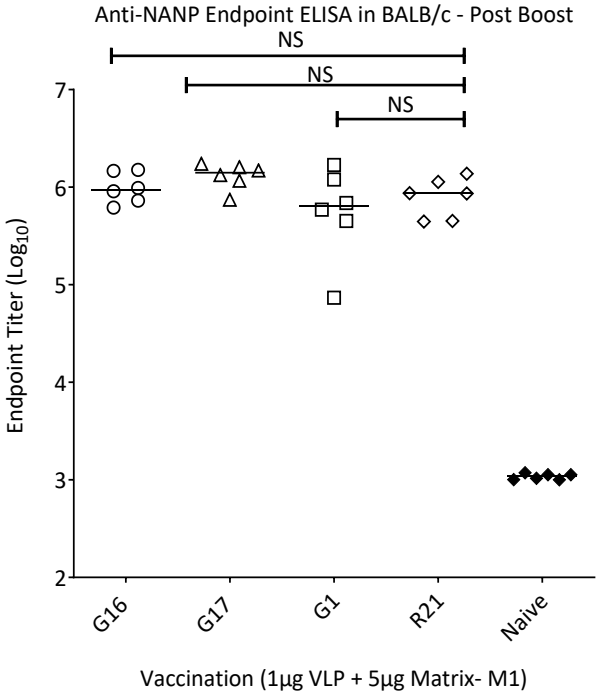


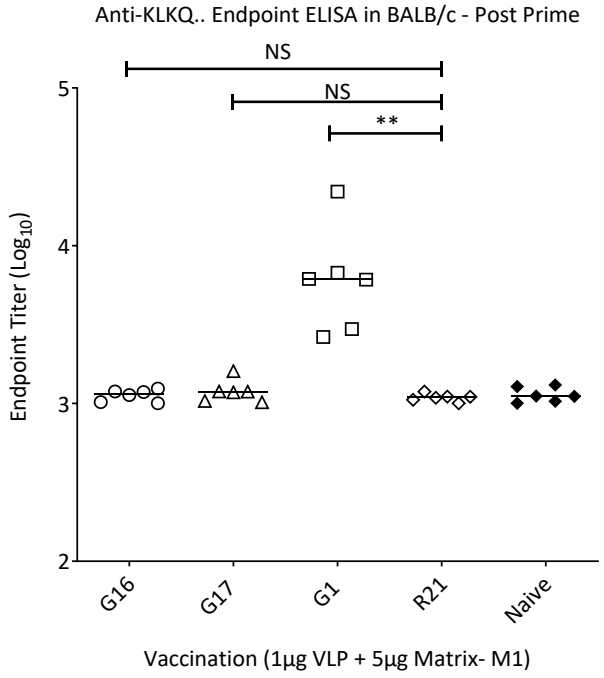


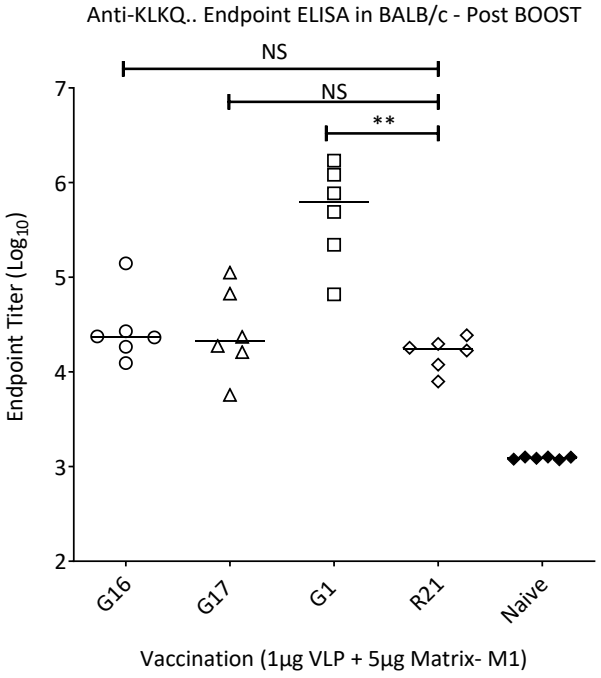
6. R21v G1, G16 and G17

Construct	CE	JE	NANP	NVDP	C ter	PADRE	Efficacy (%)
R21			+(18)		+		67
G1	+	+	+(3)	+(3)			83
G16		+	+[53]			+	67
G17			+(18)		+	+	67



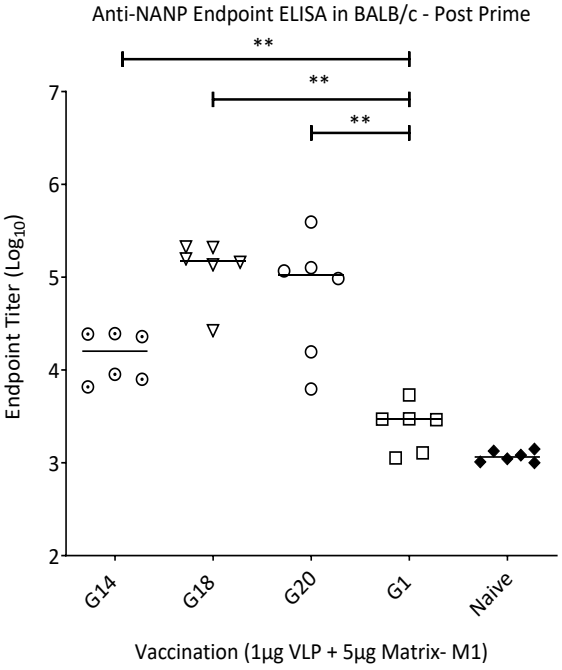


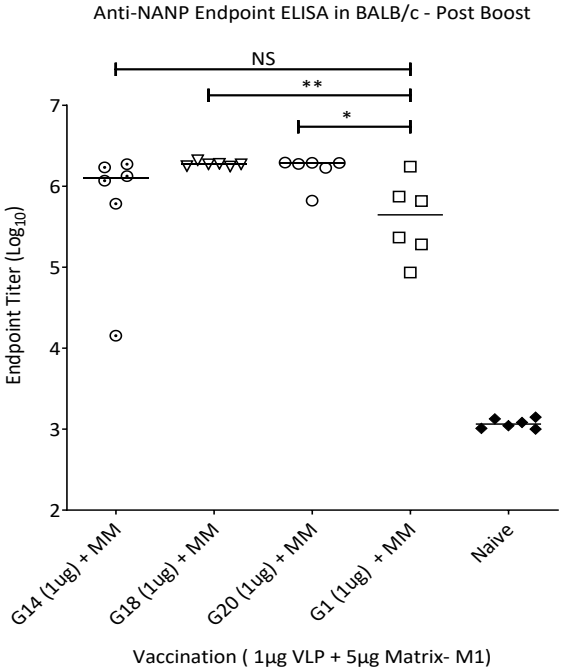


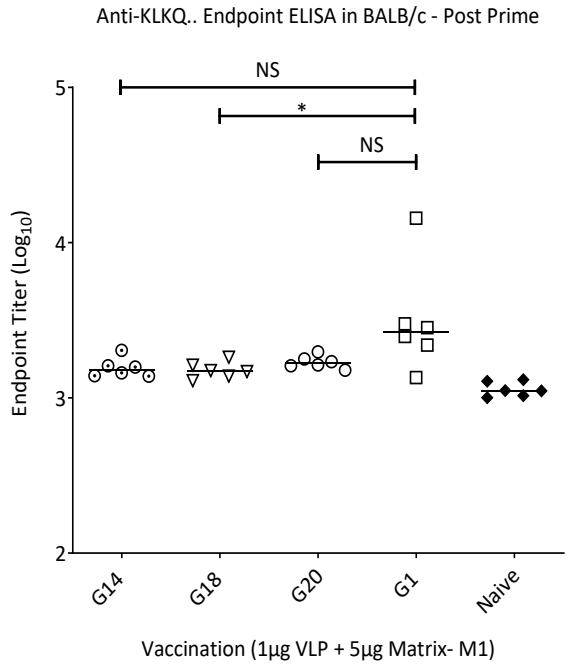


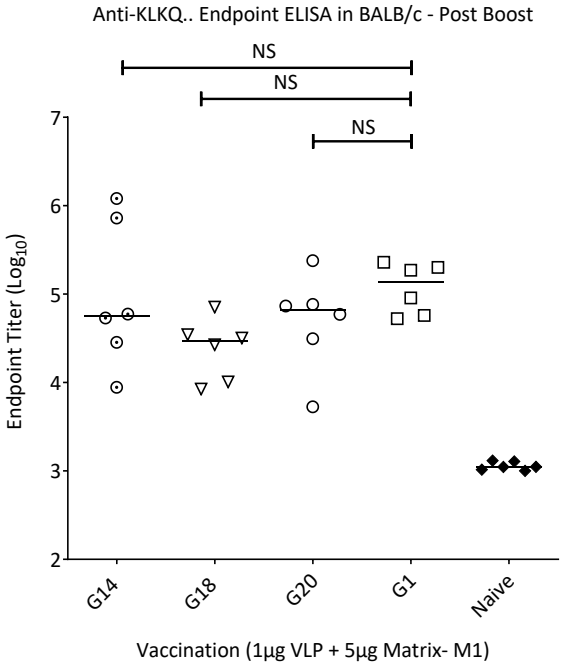
7. R21v G1, G14, G18, G20

Construct	CE	JE	NANP	NVDP	C ter	PADRE	Efficacy (%)
G1	+	+	+(3)	+(3)			100
G14	+	+	+[53]				67
G18			+(18)			+	100
G20	+	+	+(3) + PNAN (18)				67









8. R21v G1, G21, G22, G23, G24

Construct	CE	JE	NANP	NVDP	C ter	NPDP	PADRE	Efficacy (%)
R21			+(18)		+			67
G1	+	+	+(3)	+(3)				50
G21		+	+(18)		+			80
G22		+	+(18)		+		+	83
G23			+[53]	+[53]		+[53]		0
G24			+(12)	(12)		(12)		33

