Understanding drivers of experimental malaria sub-unit vaccine induced immunity in Tanzanian volunteers

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Catherine Gerald Mkindi

aus United Republic of Tanzania

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Prof. Dr. Claudia Daubenberger, Prof. Dr. Matthieu Perreau

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Prof. Dr. Martin Spiess

Dekan

Summary

Despite being a preventable and treatable disease, *Plasmodium falciparum* malaria remains a major threat, especially in children and pregnant women in sub-Saharan Africa. Considerable progress has been achieved during the past decade, however these positive trends have stalled in 2017. Efforts towards better disease control and focal elimination are hindered by development and spread of insecticide and drug resistance, leaving a malaria vaccine as a required tool to complement these approaches. RTS, S a subunit pre-erythrocyte stage vaccine is the only advanced malaria vaccine that has received an approval for pilot administration in three countries in sub Saharan Africa. This vaccine is however challenged by low efficacy and fast waning of protection. There is therefore an urgent need for development of more potent malaria vaccines. WHO targets malaria elimination by 2030 and achieving this goal will depend on stopping malaria transmission. This goal will largely depend on reducing asexual blood stage Plasmodium parasites – which are not only the cause of morbidity and mortality -but also responsible for development of gametocytes. Induction of parasite growth inhibitory antibodies has been shown to be key for protection following natural exposure and therefore, many vaccine development approaches try to follow this guidance from nature. In order to reach this goal of a highly protective vaccine targeting asexual blood stages with acceptable longevity of duration, more research is needed understand mechanisms of optimal induction of long lived antibody responses in a population that is also affected from other co-infections like helminths or HIV.
Therefore, this thesis aimed to 1) investigate a novel blood stage sub-unit malaria vaccine candidate, P27A, for its potential to induce long lasting antibody responses when formulated in the novel adjuvant GLA-SE in malaria pre-exposed populations, 2) understanding magnitude and cytokine production of the CD4 T cell responses induced by this novel vaccine formulation and the interaction with ongoing helminth co-infections, 3) shed more light on the mechanism of GLA-SE adjuvant being able to induce high and long-lasting antibody responses by studying follicular helper T cells in peripheral blood, 4) implement lymph node excision biopsy in rural Tanzania for detailed investigation of germinal center responses which are crucial for production of potent antibody response. These aims are detailed in the following manuscripts which build up the current thesis.

Manuscript 1: The Candidate Blood-stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase 1 Trial in Exposed and Non-exposed Volunteers.

In this chapter we evaluated safety profile and immunogenicity of the subunit vaccine candidate P27A when administered with adjuvant GLA-SE in comparison with the standard adjuvant, Alhydrogel. We analysed the magnitude and quality of antibody produced following vaccination and found that the antigen induced a robust humoral immunity, with enhanced production of cytophilic antibodies, IgG1 and IgG3 which are very important in parasite inhibition and protection.

Manuscript 2: GLA-SE improves quality and magnitude of cellular immunity to the blood stage malaria vaccine candidate P27A

CD4 T helper cells have been widely reported to enhance antibody response and memory cell development through production of cytokines. This is an ultimate goal of vaccination, which is to prepare the immune system to fight against a specific pathogen. In this study we sought to investigate the role of the adjuvant GLA-SE on the expansion of CD4 Th1 cells producing IL2, TNFa and IFNg and subsequent memory development. Volunteers vaccinated with the antigen formulated with GLA-SE, but not Alum, had improved Th1 expansion, which was skewed into polyfunctional TNFa and IL2, respectively. Volunteers who had concurrent helminth infection were seen to have reduced CD4 T cell response, raising an important consideration not only for malaria vaccine but also for other vaccination programmes in developing countries where cellular immune response is required for an efficacious antibody production.

Manuscript 3: The adjuvant GLA-SE promotes human Tfh cell expansion and emergence of public TCR clonotypes

Generation of high affinity-class switched antibodies, long lived plasma cells and memory B cells for enhanced antibody production requires formation of germinal centres in lymphoid follicles of the secondary lymphoid organs. Germinal centres provide an environment where follicular B and T cells interact after antigen priming following natural infection or after vaccination. We investigated the role of GLA-SE in activation and differentiation of circulating follicular helper T cells (cTfh). A population of cells expressing ICOS+ CD38+ PD1+ CXCR5+CD4 expanded on day 7 after the last vaccination and interestingly, this population

expressed a public T cell receptor clonotype which shows that GLA-SE promotes recruitment of T cells bearing common T cell receptors, a phenomenon which is desired for a vaccine intended for the general population.

Manuscript 4: Lymph node excision biopsy to study germinal centre residing T cell subsets in rural Tanzania

Peripheral blood has always been the source of immune cells for studying vaccine induced immune responses. However, priming, and differentiation of immune response takes place in secondary lymphoid organs. Follicular helper T cells, the critical CD4 T cell population involved in generation of long-lived antibody response are primarily found in germinal centres of the secondary lymphoid organs. The relationship between circulating follicular helper T cells and *bona fide* germinal center T follicular helper cells is not clear in humans. We isolated paired mononuclear cells from peripheral blood and lymph node biopsy in order to be able to better follow and evaluate immune response mechanisms following vaccination. We report here that lymph node excision biopsy is a safe procedure, that could be performed in the context of future vaccine trials – particularly in the field of HIV-1 vaccine development – in sub-Saharan Africa.

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Table of Contents

List of Abbreviations

ACT	Artemisinin combination therapy
AMA-1	Apical membrane antigen 1
ART	Anti-retroviral therapy
ASC	Antibody secreting cells
BCG	Bacillus Calmette-Guérin
BCR	B-cell receptor
cTfh	Circulating follicular helper T cell
DC	Dendritic cells
GC	Germinal centre
GLA-SE	Glucopyranosyl lipid A soluble emulsion
GLURP	Glutamate-rich protein
HbsAg	Hepatitis B virus surface antigen
HIV	Human immunodeficiency virus
IDoP	Infectious diseases of poverty
IFN	Interferon
IL	Interleukin
LLN	Long lasting bed nets
LLPCs	Long lived plasma cells
LN	Lymph node
LNMC	Lymph node mononuclear cells
LPS	Lipopolysaccharide
MBCs	Memory B cells
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
NTD	Neglected tropical diseases
PBMC	Peripheral blood mononuclear cells
PfCSP	*Plasmodium falciparum* circumsporozoite protein
PV	Parasitophorous vacuole
RESA	Ring-infected erythrocyte surface antigen
SERA	Serine-repeat antigen erythrocyte-binding antigen
SHM	Somatic hyper-mutation
SSA	Sub-Saharan Africa

TB	Tuberculosis
TCR	T-cell receptor
Tfh	Follicular helper T cell
Tfr	T follicular regulatory cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World Health Organization

Chapter 1

Introduction

1.1. Malaria disease burden

Malaria remains the major cause of morbidity and mortality especially in children under five years of age and in Primigravidae in sub-Saharan Africa (SSA) – despite being a preventable and treatable disease [1]. In 2017, an estimated 219 million (95% confidence interval [CI]: 203–262 million) cases and 435 000 deaths from malaria occurred worldwide. Most malaria cases were in the WHO African Region (200 million or 92%), followed by the WHO South-East Asia Region with 5% of the cases and the WHO Eastern Mediterranean Region with 2% (world malaria report, 2018). Currently deployed management tools for malaria include vector control by use of insecticide treated bed nets, indoor residual spraying, and pesticide spraying of larval habitats [2][3]. Recently, the building of mosquito-proofed houses has been proposed as additional prevention measure [4] which is however costly to most rural communities in low and middle income countries. In combination with improved disease diagnosis and rapid treatment using artemisinin combination therapy (ACT), a rapid decline of malaria deaths was observed between 2000 and 2015 [5]. Despite all the efforts, there is evidence of growing resistance of vector population to insecticides [6] as well as description of ACT resistance in south East Asia [7] which could easily spread to SSA [8][9]. In fact, the director general of WHO, Tedros Adhanom Ghebreyesus, has called for renewed efforts and focus on malaria control efforts if the current trend ought to be reversed and the goal of malaria epidemic control to be achieved by 2030 as formulated in the Sustainable Development Goal 3 [10]. A highly protective malaria vaccine would be a complementary tool to combat the disease [11]. However, efforts to obtain an effective malaria vaccine have not been realized, partly based on the complexity of the parasite life cycle in human host and the limited understanding of the immune effector mechanisms needed to be induced by vaccination.

1.2. Human malaria parasites and the *Plasmodium falciparum* life cycle

Malaria is caused by a protozoan parasite belonging to the genus *Plasmodium*. Five malaria species infect humans with differing prevalence and global distribution [12]. *Plasmodium falciparum* (*P. falciparum*) is the most pathogenic species and the major cause of clinical malaria and malaria morbidity in SSA [1]. *P. vivax* is found in tropic and temperate areas and has been considered less common in SSA because Africans lack of the Duffy blood group expression on red blood cells [13][14]. However, recent studies have confirmed that *P. vivax* is also present in SSA countries justifying further epidemiological studies to understand its contribution of clinical disease [15]. *P. malariae* is found mostly in West African countries but

also in South America [16][17]. *P. ovale* with its sympatric members *P.ovale curtisi* and *P.ovale wallikeri* have also been found in SSA countries [18][19] often as co-infection with *P. falciparum* and *P. malariae* [20]. *P. knowlesi* is an emerging zoonotic malaria species being transmitted between humans and non-human primates by sharing the mosquito vectors in jungle areas into which humans invaded recently [21]. In fact, *P. knowlesi* has become a dominant malaria species of clinical relevance in South East Asia, particularly in Malaysia [22]. If the goal of malaria elimination is to be achieved, all human infecting malaria species need to be included into control measures and elimination efforts [23].

1.2.1. Pre-erythrocytic life cycle stage

The life cycle of *P. falciparum* alternates between the vertebrate host, the humans, and the definitive host, the mosquito, where the sexual recombination takes place (Figure 1) [24]. The pre-erythrocytic liver stage is not associated with disease symptoms. About 41 species of female anopheles mosquitoes can transmit *P. falciparum* parasite [25] in which four species namely *Anopheles gambiae* (*An. gambiae*) complex *An. funestus* and *An. arabiensis* are wide spread in Africa [25]. During blood meal, mosquitoes inject sporozoites through human skin where they enter the blood and lymphatic vessel system. In the blood vessels, they travel to the liver, cross liver sinusoids and invade hepatocyte [26] and initiate multiplication [24]. Each single sporozoite reaching the liver multiplies in the liver cell to develop into thousands of merozoites [27] in a time span of around 6 days. Merozoites are released into the blood in the merosomes to start the next stage by invading red blood cells.

1.2.2. Asexual blood stage

Following invasion of red blood cells, repeated rounds of asexual blood stage multiplication occurs in cycles of 48 hours producing thousands of merozoites which infect new blood cells upon release. Released merozoites invade the fresh red blood cells in a multistage process involving specialized interaction of merozoites proteins and erythrocyte surface receptors [24]. The internalized parasites reside and develop into different stages (ring, trophozoites and schizonts) within the erythrocyte`s parasitophorous vacuole (PV) compartment. The ring stage parasites are known to induce active remodeling of the host cells. Also, they feed on host derived haemoglobin and plasma nutrients, which facilitate their development into trophozoites. The trophozoite stage parasites are marked with active glucose metabolism, ingestion of host cytoplasm and proteolysis of haemoglobin into amino acids [24]. However, toxic effects of heme on the parasite, forces the parasite to transform it into a byproduct known as haemozoin which is then stored in the food vacuole [24]. Several rounds of cell division that

occur at the end of trophozoite stage lead to the formation schizonts. Each of these schizonts contained in the infected erythrocytes carries approximately 16-32 merozoites that can infect other cells upon rupture [24]. This stage of parasite development is the cause of clinical malaria including anemia due to massive red blood cells destruction and severe malaria based on sequestration of infected red blood cells in microvasculature. During asexual blood cycle, some parasites will differentiate into gametocytes [24] making the transitions to the sexual stage of the parasite life cycle.

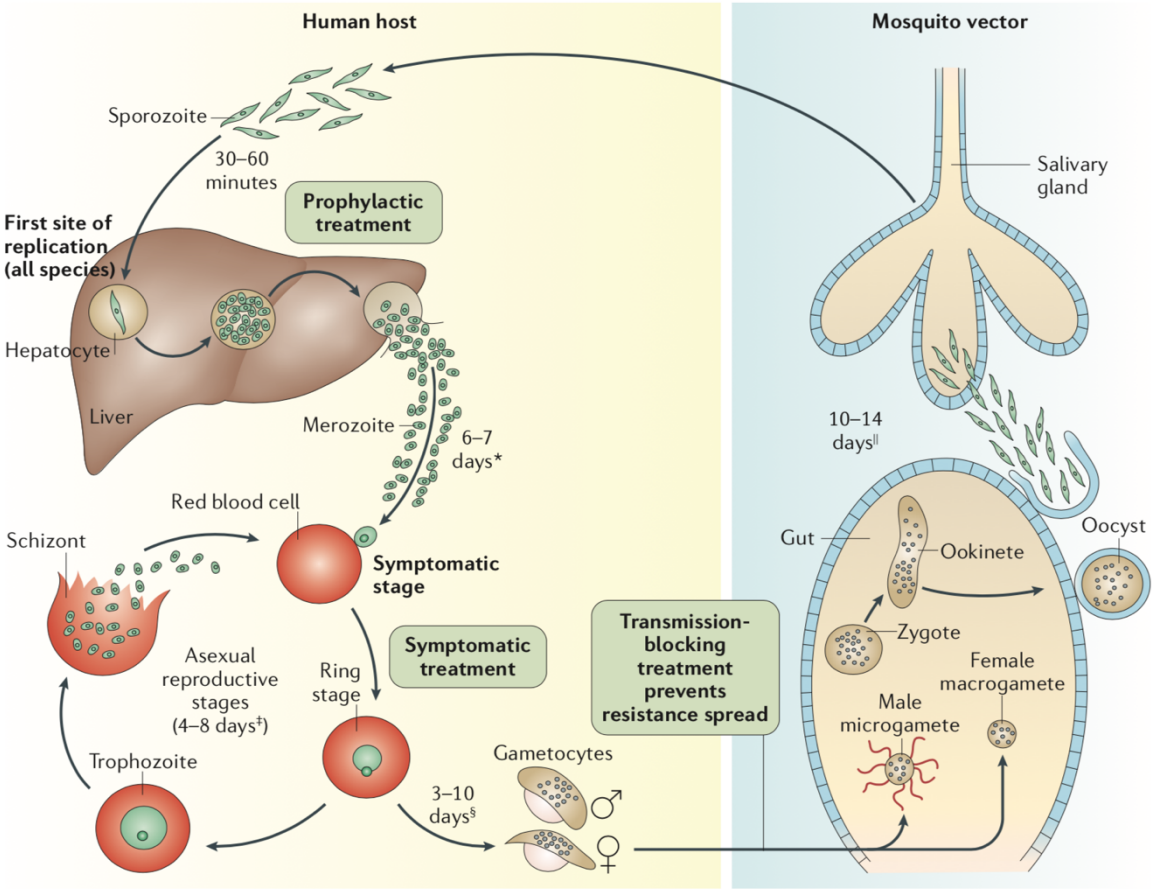

Figure 1: The life cycle of *Plasmodium* spp. [24].

1.2.3. Sexual stage and mosquito stage

Asexual parasite stage is critical for the successive transmission of parasite from human to mosquito host. During this stage a subpopulation of parasites commits into formation of male and female gametocytes [24]. Malaria parasite gametogenesis is known to occur in five morphological developmental stages and interestingly only the first and very last stage can be found in the peripheral blood. It is has been shown that the stages II, III and IV occur in the erythroid precursor cells in the bone marrow potentially to avoid immune recognition by the host and clearance through the spleen [24]. During blood meals the mosquito ingests blood containing male and female gametocytes; they fuse to form the zygote within the mosquito midgut. The environment (pH and temperature) in the mid-gut favors the development and differentiation of the parasites into ookinete. Ookinetes traverse to the epithelial layer of the midgut and mature further to form oocysts. These oocysts rupture and release sporozoites in the haemocoel that then travel to the mosquito salivary glands – ready to be transferred to another human host in a blood meal [24].

1.3. Vaccine approaches and adjuvants

Different types of vaccines exist, all having advantages and disadvantages in relation to production cost, safety profiles, temperature sensitive handling and storage conditions, and suitability for different human subpopulations (Table 1) [28]. First generation vaccines rely on attenuated whole organisms like yellow fever vaccine [29] or BCG [30] against *Mycobacterium tuberculosis*, a strategy that is also generally followed in recently conducted malaria vaccine studies based on metabolically active, purified *P. falciparum* sporozoites [31][32]. Live attenuated whole organisms as vaccines are however challenged by vaccine safety issues, particularly in immunocompromised individuals, cost of production, and applicability in resource limited areas based on the need for a cold chain [28].

Subunit or conjugate vaccines contain a defined component of the pathogen known to elicit protective immune responses [28]. When compared to live attenuated whole organism vaccines, subunit vaccines have limitations in relation to induction of long lived immunity providing protection [28]. With current development in system vaccinology, different strategies have been employed to develop next generation subunit vaccines ranging from conventional to reverse vaccinology and structural vaccinology [33].

Vaccine Type	Advantages	Disadvantages
Live attenuated	A single dose of this type of vaccine is more potent as infectious agent can replicate in host.	May cause disease itself.
	Multiple doses may not be required.	Since vaccine is composed of live organism, storage is very critical.
	Since micro-organism itself is used, immune response against all antigens is generated.	Cannot be given to immunosuppressed individuals.
Killed/Inactivated	Safe to use in immunosuppressed patients.	Less immunogenic than live attenuated vaccines.
	Can't cause disease state.	May require more booster doses to achieve desired immunity.
	Storage conditions are not critical compare to live attenuated vaccines.	
Recombinant/DNA	Better stability compare to traditional vaccines.	High production cost compare to other vaccine types.
	Storage conditions not critical.	Mutation in host DNA is possible in case of DNA vaccines.
	Better control on vaccine design as desired gene can be added or deleted.	
Subunit	Safe to use in immunosuppressed patients.	Less immunogenic than live attenuated vaccines.
	Cannot cause disease state.	Particular antigen or antigens should be identified causing the disease.
	Because of the purified antigenic component, less chances of side-effects.	
Conjugated	Safe to use in immunosuppressed patients.	Conjugation chemistry is difficult to control which could cause batch-wise variation.
	Cannot cause disease state.	Choice of carrier protein is crucial as they could be immunogenic causing suppression of antigenic immune response.
	Because of the purified antigenic component, less chances of side-effects.	

Table 1: Overview of different vaccine approaches in current use [28].

Vaccine candidates based on subunit proteins only are not very immunogenic when administered on their own and thus immunogenicity needs to be augmented by the use of adjuvants. The word adjuvant is derived from Latin word "adjuvare", meaning "to help or aid". Different adjuvants have been licensed recently for use in human research expanding the possibility of a rational design of subunit vaccines [34]. In general, adjuvants stimulate cells of the innate immune system such as dendritic cells and macrophages making them to mature, upregulate major histocompatibility complex (MHC) molecules and other co-stimulatory and chemokine receptors finally directing them to secondary lymphoid organs to stimulate naïve T cells and B cells for production of effector T cells and antibodies [34]. This activation of the innate immune system by adjuvants is schematically depicted in Figure 2.

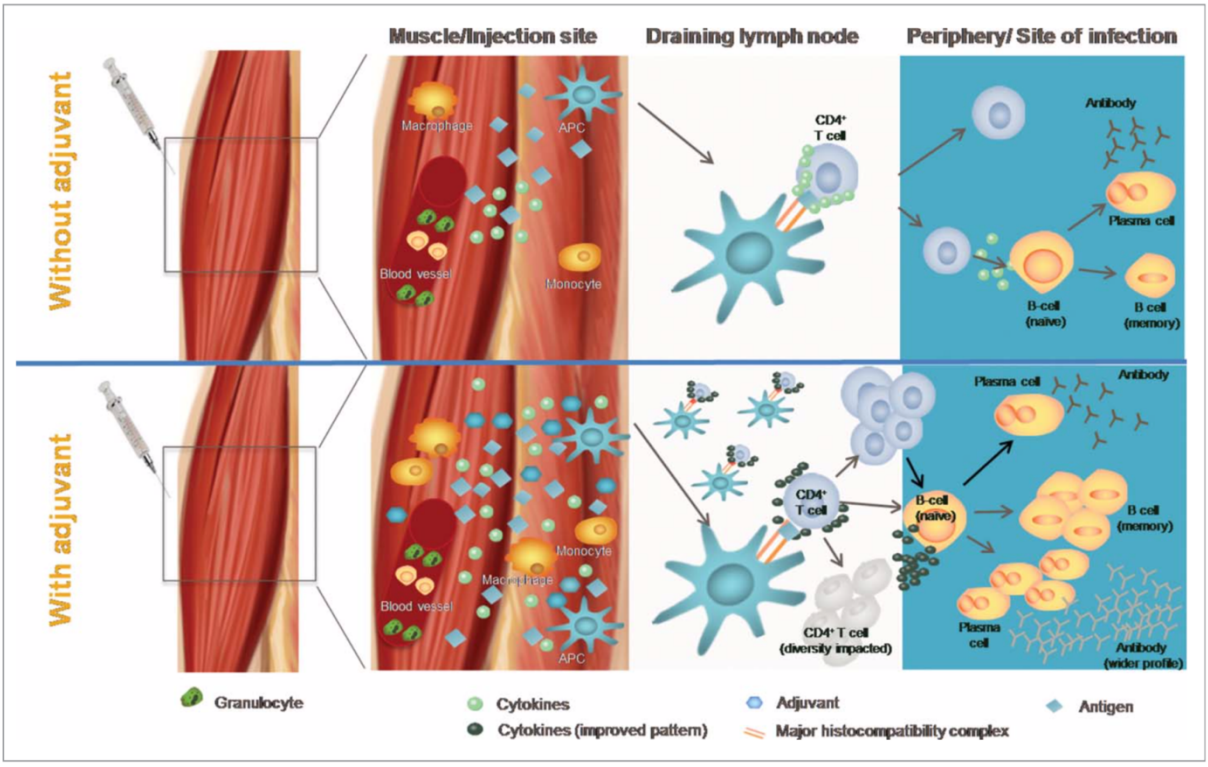

Figure 2: Activation of innate immune cells by vaccine adjuvants [35].

Alum (aluminium hydroxide) is one of the most widely used, safe adjuvants and is used for example in hepatitis B (Fendrix®) and human papillomavirus (Cervarix®) vaccines [36]. It is commonly accepted that alum functions through the activation of NALP3 inflammasome leading to the production of potent T cell stimulatory cytokine IL-1beta [37]. In mice, alum biases the cellular immune response towards a Th2 response – probably by suppressing the production of IL-12 in macrophages [38]. Overall, aluminium salts are poor inducer of T-cell responses when evaluated in humans, probably because of the lack of toll-like receptor (TLR) stimulation. The depot effect of alum might or might not contribute to its adjuvant activity and the exact mechanism of adjuvant activity remains elusive.

Since alum is not very efficient in induction of Th1 immune responses, next generation adjuvants have been developed including GLA-SE. Glycopyranosyl lipid adjuvant (GLA) signals through TLR4 and when formulated with SE, an oil-in-water emulsion, and mixed with antigen, the resulting Ag/GLA-SE induces potent Th1 immune response in mice [39]. In humans, GLA-SE has been tested first *in vitro* [40] and then in combination with subunit vaccines against influenza [41] tuberculosis [42] malaria [43][44][45], leishmaniosis [46] and

schistosomiasis [47]. Results support the idea that GLA-SE is a safe adjuvant that is able to skew the immune response towards Th1 and induces also strong antibody responses in humans.

1.4. Germinal centres and follicular helper T cells

Most effective vaccines available to date work by stimulating the production of protective humoral immunity. Vaccine induced, circulating immunoglobulins prevent disease after reinfection by binding to the surface of pathogens to block the intruder´s ability to establish an infection, prevent spread from point of entry to other body locations or inhibit the activity of toxins produced by bacterial or parasite infections.

Antibody production after vaccination or pathogen re-infection can occur via two cellular pathways. The first wave of antibody production comes from the extra-follicular plasmablast response, in which short-lived antibody secreting cells (ASC) produce immunoglobulin for a few days, which then die by apoptosis in secondary lymphoid tissues [48]. These antibodies are characterized by class switching but show few somatic hyper-mutations (SHM) in the hypervariable regions of the immunoglobulin genes [48]. These antibodies provide the first line of defense until the more matured second wave appears.

The source of ASC of this second wave are the germinal centre (GC) responses that forms in secondary lymphoid tissues like lymph nodes or spleen after immunization [49]. The GC is a specialized microenvironment where antigen-activated B cells clonally expand within the B cell follicle and undergo SHM of their immunoglobulin loci. The process of SHM, followed by affinity based selection of GC B cells by binding to antigen found on the surface of follicular dendritic cells and interaction with Tfh, results in the emergence of long-lived, high affinity, antibody-secreting plasma cells and memory B cells that provide protection against subsequent infection [49].

The successful GC response is dependent on a specialized subset of $CD4^+$ T cells, the T follicular helper (Tfh) cells [50][51]. The development of Tfh is a multistep process, with naïve CD4 T cells primed by dendritic cells (DC) during which the T cell receptor binds to peptides presented by MHC class II molecules on the surface of DC. The second co-stimulatory signal is the interaction of CD28 on the T cell with CD86/CD80 expressed on the surface of DC. The third signal is comprised of cytokines leading to upregulation of transcription factor Bcl6 and chemokine receptor CXCR5 and concomitant down-regulation of CCR7 [52]. Activated Tfh

migrate to the T:B cell boarder area of the GC where they interact with B cells to finalize their development [53].

Tfh cells together with follicular dendritic cells mediate the positive selection of B cell clones in the GC and thereby determine which B cells exit the GC as plasma cells or memory B cells [50]. During Tfh and B cell interactions in the GC there is a bidirectional exchange of signals: Tfh cells provide help in the form of CD40L, IL-21 and IL-4 to GC B cells, which supports proliferation and survival, while B cells provide inducible T cell co-stimulator ligand (ICOSL) to Tfh cells expressing ICOS on their surface. Thus, Tfh cells facilitate the preferential expansion and mutation of high-affinity GC B cell clones and are key regulators of the size and quality of the GC response. Because Tfh cells are key determinants of the long-lived humoral immunity that arises from the GC, they represent an exciting therapeutic target through which vaccine strategies could be improved [54]. This is particularly important for diseases for which so far no efficient vaccination exist, like malaria or HIV, and that depend on the development of long-lived highly matured antibody responses [55].

Most studies on the function of Tfh have been conducted in mice and hence knowledge gaps still exist for the biology of Tfh in humans. As a biomarker of the activity of GC Tfh responses, CD4 CXCR5 ICOS expressing Tfh that circulate in peripheral blood (cTfh) have been described [56]. The tendency to omit the analysis of Tfh cells in human vaccination studies may be due to difficulty in studying these cellular responses since they are located in secondary lymphoid tissue, which is not easily sampled during vaccine trials. To circumvent this issue, a population of circulating Tfh-like (cTfh) cells that are found in the blood and phenotypically and functionally resemble lymphoid tissue Tfh cells, can be used as a biomarker of ongoing Tfh cell responses during vaccination studies [57].

It has become increasingly clear that different subset of cTfh exist that are characterized by the surface expression of CD4, ICOS, PD1, CXCR5, CXCR3, CCR6 and CCR7 [58]. CXCR3 and CCR6 expression on cTfh enables identification of cTfh cells with Th1-like (cTfh1, CXCR3+CCR6−), Th2-like (cTfh2, CXCR3−CCR6−) and Th17-like (cTfh17, CXCR3−CCR6+) properties, including the expression of transcription factors and cytokines that define these T helper subsets [59]. Different cytokine environments drive the development of these subsets as exemplified by the observation that cTfh2 cell frequency increases in people with Th2-polarised *Schistosoma japonicum* infection [60] whereas cTfh1 cells are preferentially expanded during Th1-biased acute *P. falciparum* episodes or after seasonal influenza vaccination [57][61]. These different Tfh subsets presumably drive appropriate

humoral immune responses like isotype distribution adapted to the needs of fighting intracellular or large extracellular pathogens like helminths.

Despite Tfh cells being central for long-term humoral immunity, most human vaccine studies have not included these cells in their analysis, rather focusing on cytokine-producing CD4+ T cells [62], an approach that does not accurately capture vaccine-reactive Tfh cells [63]. Tfh bona fide residing in GC are not easily tractable in humans and therefore the biological relationship between lymph node resident Tfh and cTfh found in blood is not completely resolved. But there is mounting evidence that studying cTfh will provide a biomarker for GC responses and could be of great relevance in shedding light on the diverse mechanisms of stimulation of different Tfh subsets by using different antigen delivery systems and adjuvant combinations.

1.5. Malaria vaccine development

For over 50 years now, researchers have worked on developing different types of malaria vaccines, targeting different stages of the parasite life cycle [24]. Research for malaria vaccines is rooted from the evidence that malaria diseased children were treated and clinically cured by using purified immunoglobulins derived from semi immune individuals who have been exposed to malaria [64]. The possibility of a malaria vaccine is also supported by the fact that adults in malaria endemic regions develop naturally acquired immunity [65] that is associated with asymptomatic malaria infection and control of parasitaemia [66][67]. These examples provide proof that under natural conditions, parasite growth inhibitory immune mechanisms develop including growth inhibitory antibodies. The complexity of malaria is a major challenge for vaccine development with over 5000 genes, there are potentially hundreds of proteins that could serve as malaria vaccine candidates [68].

Vaccine strategies are currently grouped according to the malaria parasite life stage that is targeted. Pre-erythrocytic vaccines target sporozoites and prevent infection, clinical disease and block transmission. Blood stage vaccines generally target the infected red blood cell or merozoites aiming to prevent or reduce parasite multiplication – thereby reducing clinical disease and transmission. Targeting the sexual stages of the parasite either in the blood or in the mosquito is named transmission blocking vaccines [69].

During immune-epidemiological studies of malaria vaccine candidates, antigens are usually tested against sera from malaria endemic individuals which has shown existence of antibodies

against such candidates [70][71]. This naturally acquired immunity requires usually several malaria episodes to develop and is not sterile, thus people remain vulnerable to reinfection. Understanding the generation and partial protective mechanisms of these naturally acquired antibodies is important in order to develop a vaccine that is superior to naturally acquired immunity [72][73].

Many of the vaccine candidates which are now in different stages of development (preclinical or clinical) are targeted by antibodies [74]. Figure 3 provides an overview of vaccine candidates that are currently under development - highlighting that only few subunit vaccine candidates have progressed to the clinic.

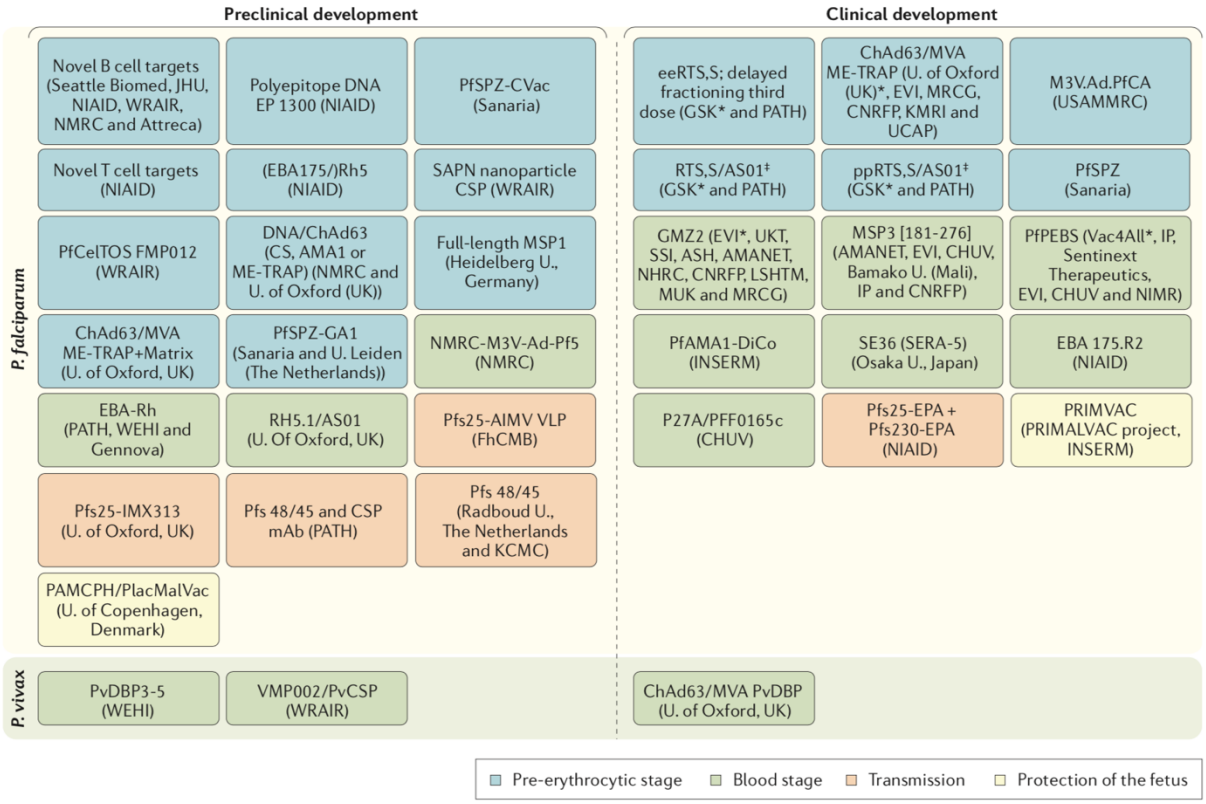

Figure 3: Overview of malaria vaccine candidates in preclinical and clinical development [24].

1.5.1. Pre-erythrocytic stage subunit malaria vaccine development

The most advanced malaria vaccine candidate is RTS,S/AS01 [75][76]. It is composed of 18 copies of the central repeat region and the C-terminal domain of the circumsporozoite protein fused to hepatitis B virus surface antigen (HbsAg) with free HbsAg as surplus in a 1:4 ratio. This vaccine is designed to induce high levels of antibodies against PfCSP, which is covering

the surface of the infective sporozoite. RTS,S, formulated with the potent liposomal adjuvant system AS01 from GlaxoSmithKline, is the only vaccine that has demonstrated protective efficacy against clinical malaria in a Phase III clinical trial [77]. Protection against clinical malaria is partial, wanes over time, and may be age dependent (protection was lower in infants 6–12 weeks of age than in young children 5–17 months old at time of first vaccination). In the latter, receiving three vaccinations in a 0-1-2 month schedule, the incidence of clinical malaria was reduced by 51% over the first year of follow-up post-dose three [95% CI 48%–55%]. Over 48 months of follow-up, efficacy was 26% [95% CI 21%–31%], and among children receiving a fourth booster dose at month 20 (18 months post-dose three), efficacy was 39% [95% CI 34%–43%].

1.5.2. Asexual blood stage vaccines

The main candidates developed against asexual blood stage candidates have been either expressed on the surface of infected red blood cells (VAR2CSA), on the surface of merozoites (MSP1, MSP2, MSP3) or in the apical complex organelles of the merozoites (AMA1). All of these candidates have so far struggled to achieve convincing efficacy levels in clinical studies although titers of vaccine induced antibodies have been reported. These candidates are usually immune-dominant merozoite proteins and suffer from the problem of lack of strain transcending immunity and the biology of the merozoite invasion into red blood cells that takes only seconds, requiring high levels of inhibitory antibody titers. Recently, several novel candidates have emerged that might circumvent some issues of sequence polymorphisms like the *P. falciparum* reticulocyte-binding protein homolog 5 (PfRH5), that binds to the basigin receptor on the red blood cell surface [78]. PfRH5 forms a functional complex with PfRipr and PfCyPRA and antibodies targeting these multiple components increases protective potential [79]. Figure 4 depicts the different vaccine candidates and formulations tested that are currently in clinical development, underlying that most vaccine candidates are supposedly to function via induction of inhibitory antibodies.

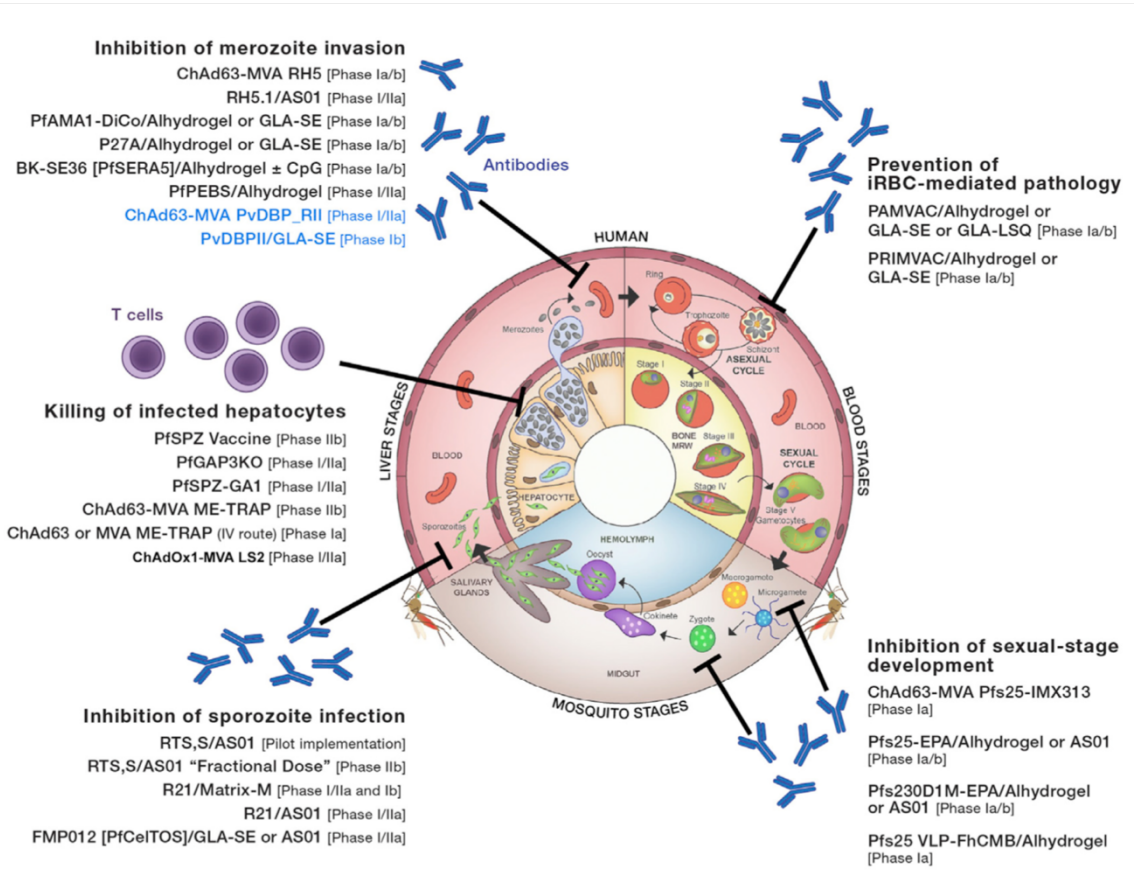

Figure 4: Malaria vaccine candidates currently in clinical development including P27A [74].

1.6. The P27A asexual blood stage vaccine candidate

A systematic genome-wide screen of *P. falciparum* for alpha-helical coiled coil motifs aiming at identification of structurally defined asexual blood stage vaccine candidates identified several novel candidates including PFF0165c [80]. One of the major goals of this thesis was to contribute with immunological studies to the assessment of the potential of this novel protein that is also known as MAL6P1.37 or Pf27, as a malaria blood-stage vaccine candidate in malaria pre-exposed populations.

Two Pf27 segments are known: i) the sequence predicted to contain an alpha helical coiled coil protein motif (Peptide 27; P27), that is 27 amino acids in length, and ii) the sequence predicted to assume a random coiled structure (Peptide 27A; P27A) which is 104 amino acids long [80]. Both peptides were synthesized and found to be the targets of human antibodies inhibiting parasite growth in an antibody-dependent cellular (ADCI) assay [80]. P27A was selected for clinical development because a) it was found to be highly antigenic in individuals living in malaria-endemic areas [80]; b) the antibodies developed by protected individuals were

predominantly the cytophilic IgG1 and IgG3 isotype [81]; c) human affinity-purified antibodies were able to inhibit parasite growth in an antibody-dependent cellular fashion [81]; d) analysis of immune response from both malaria protected and unprotected individuals following natural exposure showed that P27A elicited antibodies strongly associated with naturally occurring protection [82][81][83] and e) sequence analysis show an unusual degree of conservation with very minimal genetic variation among over 90 laboratory and field isolates of *P. falciparum* [84]. Pf27 has been found to be exported into the red blood cell cytosol during the trophozoite stage and co-localization experiments revealed that it is located to the Maurer's clefts facing the red blood cell cytosol [85]. It is hypothesized that induction of high antibody levels against P27A of IgG1 and IgG3 isotype will provide protection against asexual blood stage parasites by antibody dependent cellular cytotoxicity [85]. Therefore, a combined phase Ia/Ib clinical trial was initiated to evaluate P27A immunogenicity in malaria naïve and pre-exposed populations, when formulated with either the novel adjuvant GLA-SE or alum.

1.7. Aims of this thesis

The overall aim of this thesis is to monitor comprehensively the cellular and humoral immune responses induced by the malaria subunit vaccine P27A formulated with alum and GLA-SE in Tanzanian malaria pre-exposed volunteers and to understand underlying reasons of variations in immune responses elicited.

Objectives of this thesis are:

1. To compare P27A antibody dynamics, size, isotype distribution and duration between malaria naïve and pre-exposed volunteers inoculated with the identical vaccine formulations (Chapter 2).
2. To evaluate the role of adjuvants and helminth co-infections on the phenotype and cytokine production of elicited P27A specific CD4 T cell responses in Tanzanian volunteers (Chapter 3).
3. To describe the difference between alum and GLA-SE in induction of GC responses and identification of distinct circulating Tfh subsets as biomarkers of GC responses induced by vaccination (Chapter 4).
4. To implement lymph node excision biopsy in rural Tanzania for detailed investigation of GC responses and comparison with circulating Tfh (Chapter 5).

Chapter 2

The Candidate Blood-stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase 1 Trial in Exposed and Nonexposed Volunteers

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This chapter contains the following publication:

Steiner-Monard V, Kamaka K, Karoui O, Roethlisberger S, Audran R, Daubenberger C, Fayet-Mello A, Erdmann-Voisin A, Felger I, Geiger K, Govender L, Houard S, Huber E, Mayor C, **Mkindi C**, Portevin D, Rusch S, Schmidlin S, Tiendrebeogo RW, Theisen M, Thierry AC Vallotton L, Corradin G, Leroy O, Abdulla S, Shekalaghe S, Genton B, Spertini F, Jongo SA. *The Candidate Blood Stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase I Trial in Exposed and Non-Exposed Volunteers.* Clin Infect Dis. 2018 Jun 26. doi: 10.1093/cid/ciy514.

Clinical Infectious Diseases

MAJOR ARTICLE

The Candidate Blood-stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase 1 Trial in Exposed and Nonexposed Volunteers

Viviane Steiner-Monard,[1] Kassim Kamaka,[2] Olfa Karoui,[1] Samuel Roethlisberger,[1] Régine Audran,[1] Claudia Daubenberger,[3] Aurélie Fayet-Mello,[4] Aude Erdmann-Voisin,[4] Ingrid Felger,[3] Kristina Geiger,[5] Lerisa Govender,[1] Sophie Houard,[6] Eric Huber,[3] Carole Mayor,[1] Catherine Mkindi,[3] Damien Portevin,[3] Sebastian Rusch,[3] Sandro Schmidlin,[3] Regis W. Tiendrebeogo,[7,8,9] Michael Theisen,[7,8,9] Anne-Christine Thierry,[1] Laure Vallotton,[4] Giampietro Corradin,[5] Odile Leroy,[6] Salim Abdulla,[2] Seif Shekalaghe,[2] Blaise Genton,[3,10,11] François Spertini,[1] and Said A. Jongo[2]

[1]Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland; [2]Ifakara Health Institute, Bagamoyo, Tanzania; [3]Swiss Tropical and Public Health Institute, Basel, [4]Clinical Trial Unit, CHUV, Lausanne, and [5]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland; [6]European Vaccine Initiative, Heidelberg, Germany; [7]Department for Congenital Disorders, Statens Serum Institut, [8]Centre for Medical Parasitology, University of Copenhagen, and [9]Department of Infectious Diseases, Copenhagen University Hospital, Rigshospitalet, Denmark; and [10]Policlinique médicale universitaire, and [11]Infectious Disease Service, CHUV, Lausanne, Switzerland

Background. P27A is an unstructured 104mer synthetic peptide from *Plasmodium falciparum* trophozoite exported protein 1 (TEX1), the target of human antibodies inhibiting parasite growth. The present project aimed at evaluating the safety and immunogenicity of P27A peptide vaccine in malaria-nonexposed European and malaria-exposed African adults.

Methods. This study was designed as a staggered, fast-track, randomized, antigen and adjuvant dose-finding, multicenter phase 1a/1b trial, conducted in Switzerland and Tanzania. P27A antigen (10 or 50 μg), adjuvanted with Alhydrogel or glucopyranosil lipid adjuvant stable emulsion (GLA-SE; 2.5 or 5 μg), or control rabies vaccine (Verorab) were administered intramuscularly to 16 malaria-nonexposed and 40 malaria-exposed subjects on days 0, 28, and 56. Local and systemic adverse events (AEs) as well as humoral and cellular immune responses were assessed after each injection and during the 34-week follow-up.

Results. Most AEs were mild to moderate and resolved completely within 48 hours. Systemic AEs were more frequent in the formulation with alum as compared to GLA-SE, whereas local AEs were more frequent after GLA-SE. No serious AEs occurred. Supported by a mixed Th1/Th2 cell-mediated immunity, P27A induced a marked specific antibody response able to recognize TEX1 in infected erythrocytes and to inhibit parasite growth through an antibody-dependent cellular inhibition mechanism. Incidence of AEs and antibody responses were significantly lower in malaria-exposed Tanzanian subjects than in nonexposed European subjects.

Conclusions. The candidate vaccine P27A was safe and induced a particularly robust immunogenic response in combination with GLA-SE. This formulation should be considered for future efficacy trials.

Clinical Trials Registration. NCT01949909, PACTR201310000683408.

Keywords. malaria; vaccine; GLA-SE; blood-stage; ADCI.

Due to increasing drug resistance against antimalarial drugs, the development of a safe and effective vaccine would be an invaluable tool in the fight against malaria, and eventually eradication of malaria [1]. Opinions are diverse on how antibodies to blood-stage antigens achieve protection: inhibition of merozoite invasion into erythrocytes, triggering of the release by monocytes of parasitostatic and parasitocidal substances via antibody-dependent cellular inhibition (ADCI), or inhibition of cytoadherence of infected red blood cells to endothelial cells [2–5]. In our search for novel vaccine candidates through genome mining, we have addressed the first 2 paradigms in a systematic manner and identified trophozoite exported protein 1 (TEX1) (also known as PF3D7_0603400, PFF0165c, or MAL6P1.37, here referred to as *Pf*27) [6, 7]. A highly conserved segment of *Pf*27 corresponding to a sequence predicted to assume a random coiled structure of 104 amino acids (peptide 27A [P27A]) was synthesized and purified. P27A was found to be highly antigenic and the target, at high prevalence, of B- and T-cell responses in individuals living in malaria-endemic areas [7, 8]. The antibodies developed by protected individuals were predominantly cytophilic immunoglobulin G1 (IgG1) and immunoglobulin G3 (IgG3) able to inhibit parasite growth in an antibody-dependent cellular fashion (ADCI). Interestingly, in a parallel analysis of the response to a recombinant candidate vaccine including P27A, the antibody response of African volunteers was strongly associated with clinical protection (G. Corradin et al, submitted manuscript). Although the soluble factors, including tumor necrosis factor alpha (TNF-α), involved in ADCI are still largely uncharacterized, ADCI is the result of the overall functional effect of antibodies and monocyte collaboration on in vitro parasite growth [9]. Recent data

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Correspondence: F. Spertini, Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland (francois.spertini@chuv.ch).

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suggest a potential role of the ADCI assay as a correlate of protection.

The objective of this trial was to assess the safety and immunogenicity of a synthetic peptide vaccine candidate based on the P27A fragment of *Pf*27, with the aim to induce a protective antibody response that may complement immune responses induced by antigen(s) from the preerythrocytic stage in a multicomponent vaccine.

METHODS

Trial Design and Study Participants

The study was designed as a staggered, randomized, antigen and adjuvant dose-finding, multicenter phase 1a/1b clinical trial using the fast-track strategy set by the European Vaccine Initiative and its partners to accelerate malaria vaccine clinical development. Study was conducted in Switzerland for phase 1a and in Tanzania for phase 1b.

The phase 1a (Lausanne, Switzerland [CH]) was designed as a single-center, staggered randomized, volunteer- and laboratory-blinded trial. Healthy adult men and women aged 18–45 years were eligible to participate when human immunodeficiency virus (HIV) negative, without known exposure to malaria, and P27A or parasite antibody negative by enzyme-linked immunosorbent assay (ELISA). Phase 1b (Bagamoyo, Tanzania [TZ]) was designed as a single-center, staggered randomized, double-blind, controlled trial. Healthy adult volunteers aged 18–45 years were eligible to participate when HIV negative, having lived in areas of Tanzania with minimal malaria transmission (urban Dar es Salaam). As contraception is not always practiced by women of childbearing age in the study population, female subjects were not included. Participants were enrolled if they met the inclusion criteria (see Supplementary Materials for details) and, for the nonexposed group, were not pregnant or lactating. The phase 1a volunteers were allocated (on a 1:1 basis) into 2 groups of 8 volunteers each, who were injected intramuscularly with 50 μg of the P27A antigen adjuvanted with Alhydrogel (group CH-Alum/50) or 2.5 μg glucopyranosil lipid adjuvant stable emulsion (GLA-SE) (group CH-GLA2.5/50). The phase 1b volunteers were randomized and allocated (on a 4:1 basis) in a dose-escalating manner to 4 cohorts. Each cohort included 10 subjects, 8 of whom were injected intramuscularly with 50 μg P27A and Alhydrogel (group TZ-Alum/50), with 10 μg P27A and 2.5 μg GLA-SE (group TZ-GLA2.5/10), with 50 μg P27A and 2.5 μg GLA-SE (group TZ-GLA2.5/50), and with 50 μg P27A and 5 μg GLA-SE (group TZ-GLA5/50). Two subjects per cohort were injected with the rabies vaccine Verorab as control (group TZ-Ver). Injections were performed at days 0, 28, and 56 with a follow-up of 6 months (Figure 1).

The transition phase from the European to African trial population started after completion of the first injection series of each group sequentially in the European site and after evaluation of the safety data by an independent data and safety monitoring board (DSMB). A 2-week stagger was left to ensure safety and reactogenicity evaluation prior to transition to the next higher P27A dosage or change from Alhydrogel to GLA-SE in the same site, and a 4-week stagger for transition from European phase 1a to African phase 1b (Figure 1). Further information on study design, including the clinical trial protocol, is provided in the Supplementary Materials.

Study Vaccines and Procedures

The investigational vaccine antigen and adjuvants were produced under Good Manufacturing Practice (GMP) constraints according to relevant national regulations. The bulk P27A 104-residue synthetic peptide was manufactured by Almac (Craigavon, United Kingdom) and further diluted, filled in monodose vials by Nova Laboratories Ltd (Leicester, United Kingdom). The bulk Alhydrogel was manufactured by Brenntag (Frederikssund, Denmark) and diluted with water for injection, filled into vials by Nova Laboratories Ltd. P27A and Alhydrogel vials have been labeled, released, and shipped to the clinical sites by Nova Laboratories Ltd. The GLA-SE and the GMP EM060-SE used as diluent were manufactured by the Infectious Disease Research Institute (Seattle, Washington). The GLA-SE and SE diluent were labeled, released, and shipped by Output Pharma GmbH (Aachen, Germany). The P27A vaccine antigen was formulated with 1 of the 2 adjuvants at each site pharmacy prior to the injection.

Primary Outcome

Safety and tolerability of the vaccine were assessed based on the number and intensity of solicited and unsolicited adverse events (AEs). The safety profile included local and systemic AEs as well as the biological safety tests, based on clinically significant changes of the baseline value of the main biological criteria.

Further information on secondary and exploratory outcomes (immunogenicity) as well as statistical analysis is provided in the Supplementary Materials.

RESULTS

Participant clinical characteristics are provided in the Supplementary Materials and Supplementary Table 1.

Safety and Reactogenicity

Local Reactogenicity

Local AEs from day 0 to day 7 are summarized in Figure 2A, and are presented as the cumulative number of events occurring in each vaccination group. Local reactogenicity was reported by 100% and 82.5% of volunteers in phase 1a and 1b, respectively, and was mostly mild to moderate and self-limited. Local AE were more frequent after GLA-SE. Detailed description of local reactogenicity can be found in the Supplementary Materials.

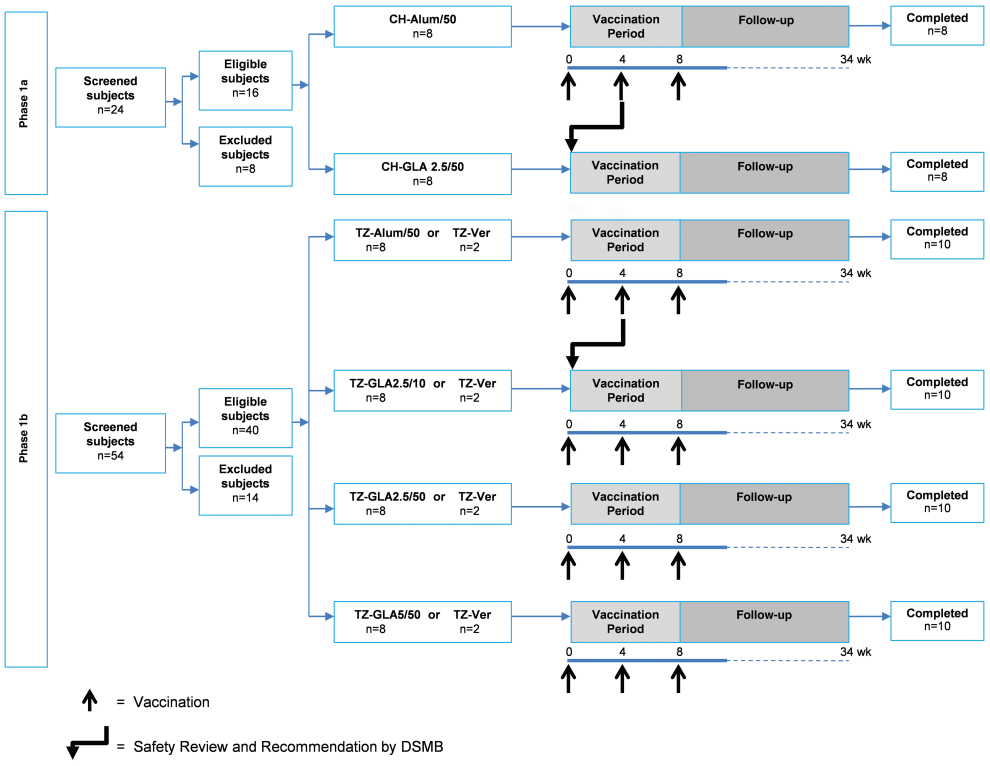

Figure 1. Trial profile, injection schedule, and safety follow-up. Abbreviations: CH, Lausanne, Switzerland; DSMB, data and safety monitoring board; GLA, glucopyranosil lipid adjuvant; TZ, Bagamoyo, Tanzania; Ver, Verorab.

Systemic Reactogenicity

Systemic AEs (solicited and unsolicited) from day 0 to day 7 are summarized in Figure 2B, and are presented as the cumulative number of events in each vaccination group, and described below in the text as number of AEs per injections.

In phase 1a, systemic AEs occurred in 39 of 48 injections (81.3%), most of them being considered as unrelated to the vaccination (76/125 events [60.8%]). The most frequent solicited systemic AEs were tiredness (reported after 11/24 injections [48.5%] for group CH-Alum/50 and 10/24 injections [41.7%] for group CH-GLA2.5/50) and headache (7/24 injections [29.2%] for group CH-Alum/50 and 7/24 injections [29.2%] for group CH-GLA2.5/50). Fever was not reported during the evaluation period. Up to 1 month after the third injection, a total of 53 unsolicited systemic AEs following 29 of 48 injections (60.4%) were reported from 15 subjects (8 subjects for group CH-Alum/50 and 7 subjects for group CH-GLA2.5/50) and resolved without sequelae. Forty-four were grade 1 AEs (3 related to vaccination), 5 were grade 2 (1 related), and 3 reached grade 3 with no relationship with vaccination. One was left ungraded. There were no significant abnormal vital signs and no relevant changes in physical examination. No clinically relevant variations in blood cell counts and biochemistry analysis were recorded.

In the phase 1b population, systemic AEs occurred after 25 of 120 injections (19/40 subjects) (47.5%) for a total of 39 events, mostly reported as related to vaccination (25/39 [64.1%]). The most frequent solicited systemic AEs were headache (reported after 3/24 injections [12.5%] for group TZ-Alum/50 and 1/24 injections [4.2%] for group TZ-GLA2.5/10, 2/24 injections [8.3%] for group TZ-GLA2.5/50, 1/24 injections [4.2%] for group TZ-GLA5/50, 1/24 injections [4.2%] for group TZ-Ver), and fatigue (2/24 injections [8.3%] for group TZ-Alum/50 and 0/24 injections [0%] for group TZ-GLA2.5/10, 2/24 injections [8.3%] for group TZ-GLA2.5/50, 1/24 injections [4.2%] for group TZ-GLA5/50, 0/24 injections [0%] for group TZ-Ver). At least 1 subject experienced systemic unsolicited AE during each vaccination; however, no grade 3 AE was reported. Fever was not reported during the evaluation period. There were no significant abnormal vital signs and no relevant changes in physical examination. No significant variations in blood cell counts and biochemistry analysis were recorded.

Comparison Between Adjuvants or Populations

Statistical differences in frequency of AEs between CH and TZ groups having received the same vaccine formulations are shown in Figure 2 and Supplementary Tables 2 and 3 (Fisher exact test). With regard to adjuvants, cumulating all local AEs

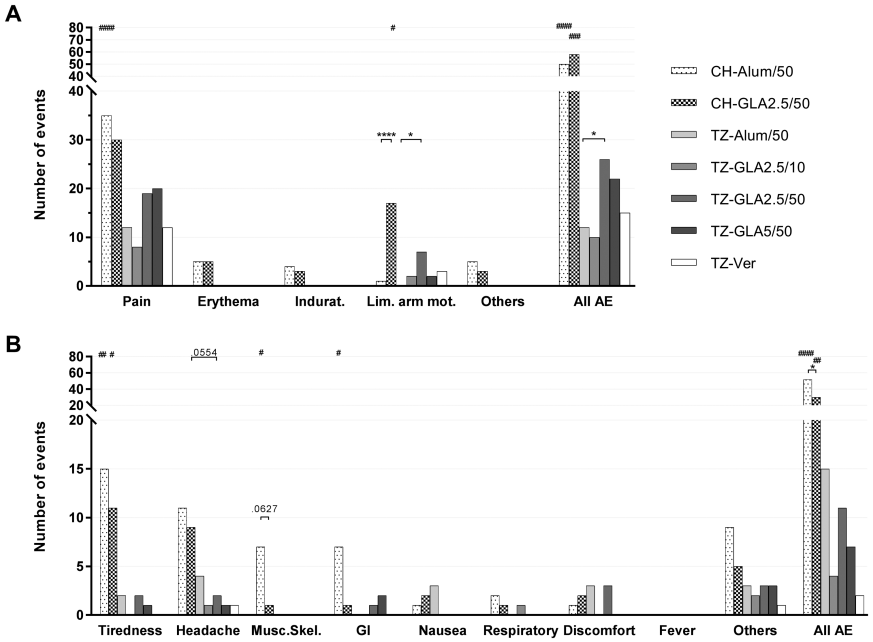

Figure 2. Local and systemic reactogenicity. Cumulative number of local (*A*) and systemic (*B*) adverse events (solicited and unsolicited) observed from day 0 to day 7 after each vaccination in each group are indicated. For each parameter, 72 reports were done per group, 8 subjects per group, 3 injections, and 3 time points per injection (60 minutes postvaccination, day 1 ± 12 hours, and day 7 ± 24 hours). Comparisons of numbers of events between the 4 groups that received Alum/50 or glucopyranosil lipid adjuvant (GLA) 2.5/50 were done using Fisher exact contingency tests, each group taken separately; *P* values are indicated as * or # for comparisons of adjuvants (alum vs GLA–stable emulsion) or populations (Lausanne, Switzerland vs Bagamoyo, Tanzania), respectively. Abbreviations: AE, adverse event; CH, Lausanne, Switzerland; GLA, glucopyranosil lipid adjuvant; Indurat., induration; Lim. arm mot., limitation in arm motion; Musc.Skel, musculoskeletal AEs; TZ, Bagamoyo, Tanzania; Ver, Verorab. $^{*,\#}P < .05$; $^{**,\#\#}P < .01$; $^{***,\#\#\#}P < .001$; $^{****,\#\#\#\#}P < .0001$.

from volunteers who received the same adjuvant at the same dose of peptide, there was no significant difference between alum and GLA-SE ($P = .066$), whatever the severity of the reaction. However, limitation in arm motion was more frequently induced after GLA-SE ($P < .0001$). Systemic AEs (all) were significantly more frequent after Alum ($P = .02$), with a trend toward more musculoskeletal AEs with alum ($P = .07$). When AEs (all) were compared according to sites, CH vs TZ, we observed significantly more frequent AEs in the Swiss volunteers than in the Tanzanian volunteers both for local (all AEs, $P < .0001$; odds ratio [OR], 3.167, as well as individual AEs) and systemic AEs (all, $P < .0001$; OR, 3.429, and tiredness, headaches, musculoskeletal, and gastrointestinal AEs). Grade 3 local AEs were also more frequent in the Swiss population ($P = .011$; OR, 10.13).

Immunogenicity

IgG Antibody Responses

Nonexposed phase 1a volunteers mounted a specific anti-P27A IgG antibody response that peaked at day 84 showing median titers of 3200 (range, 200–12 800) and 51 200 (range, 3200–204 800) in groups CH-Alum/50 and CH-GLA2.5/50, respectively (Figure 3). A difference in median titers of at least 10-fold between the 2 groups at day 84 persisted at day 238 (week 34), that is, 26 weeks after the last immunization. Titers were maintained at a high level in group CH-GLA2.5/50, with median titer of 9600 (range, 1600–51 200) at day 238 while group CH-Alum/50 displayed a median titer of 400 (range, 50–800).

In the exposed phase 1b volunteers, 9 of 40 volunteers already presented a positive humoral response to P27A at day 0 according to criteria defined for the screening of the phase 1b volunteers. IgG titers at day 84 in groups TZ-Alum/50, TZ-GLA2.5/10, and TZ-GLA2.5/50 reached median titers of 4800 (range, 100–9051), 2400 (range, 800–12 800) and 6400 (range, 1600–12 800), respectively (Figure 3). These responses were in the same range as those obtained at day 84 in group CH-Alum/50. Nevertheless, group TZ-GLA2.5/50 responses persisted significantly longer than in group TZ-Alum/50 (Kruskal-Wallis *P* value of .0008, with a posttest *P* value of .0103 at day 238). Volunteers from group TZ-GLA5/50 showed even stronger responses at day 84 with median titers of 13 577 (range, 9051–25 600), levels comparable to those obtained in group CH-GLA2.5/50 of 51 200 (range, 3200–204 800) ($P = .1290$). No variations in anti-P27A IgG titers were observed in the TZ-Ver control group.

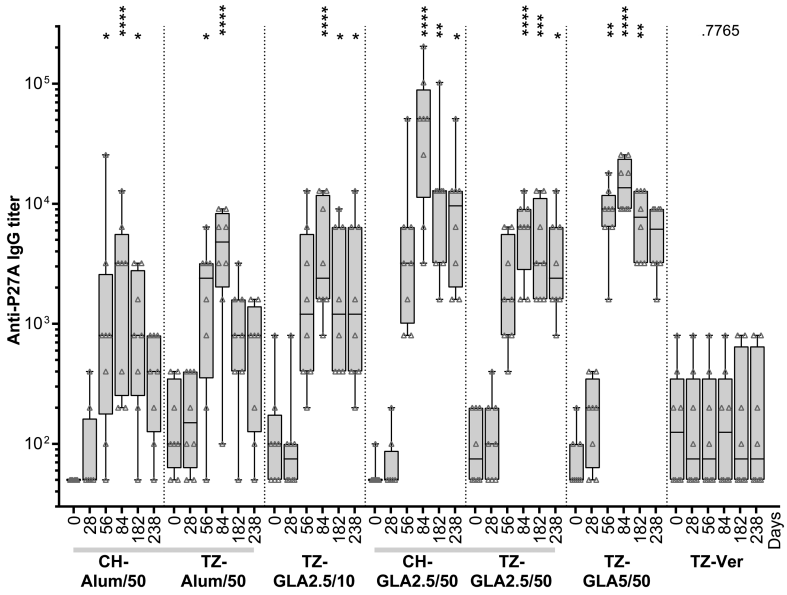

Figure 3. Anti-P27A immunoglobulin G responses. Kinetics of responses in 6 groups that received P27A formulated in Alhydrogel (Switzerland [CH]–Alum/50 and Tanzania [TZ]–Alum/50) or glucopyranosil lipid adjuvant (GLA) stable emulsion (TZ-GLA2.5/10, CH-GLA2.5/50, TZ-GLA2.5/50, and TZ-GLA5/50) or Verorab as control (TZ-Ver) are shown as box plots and whiskers (interquartile range, minimum and maximum). Comparisons intragroup were performed using Friedman test; *P* values of analysis of variance were at least < .001 for vaccinees; *P* values of Dunn posttest of comparison with day 0 are indicated. Abbreviations: CH, Lausanne, Switzerland; GLA, glucopyranosil lipid adjuvant; IgG, immunoglobulin G; TZ, Bagamoyo, Tanzania; Ver, Verorab. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

IgG Subclasses and IgM Responses

The levels of P27A specific IgG1, immunoglobulin G2 (IgG2), IgG3, immunoglobulin G4 (IgG4), and IgM were assessed by ELISA in all phase 1a and 1b volunteers at day 84 (peak response) (Figure 4). The vaccine formulation GLA2.5/50 induced higher IgG1, IgG2, IgG4, and immunoglobulin M (IgM) responses in the nonexposed Swiss group than in the semi-immune group TZ-GLA2.5/50, but no significant difference in IgG3 levels (Figure 4B–F). P27A in Alhydrogel induced a higher IgG3 response in group TZ-Alum/50 volunteers with a median titer of 800 (range, 100–6400) than in group CH-Alum/50 volunteers (median titer of 150 [range, 12.5–400]) (Mann-Whitney *P* value of .0458) with no difference with the other subclasses. Clearly Alhydrogel failed to induce IgM in both nonexposed and exposed volunteers (group CH-Alum/50 and TZ-Alum/50) (Figure 4B).

In Vitro Parasite Growth Inhibition Assay ADCI

Eleven of 16 subjects from group CH-Alum/50 and CH-GLA2.5/50 showed an increase in the inhibitory capacity of specific IgG with vaccination, without significant difference between the 2 groups (Figure 5A). IgM and IgG3 isotypes appeared to be associated with the strongest parasite growth inhibition, although the correlation was nonsignificant (Figure 5B and 5C).

Further information on other secondary endpoints (Western blot recognition of TEX1, antibody response to parasite by indirect fluorescence antibody test [IFAT], peripheral blood mononuclear cell [PBMC] cytokine profile) is provided in the Supplementary Materials. In brief, Western blotting based on affinity-purified IgG revealed 2 bands at 165 kDa and 130 kDa specifically recognized postvaccination in 5 volunteers from the CH-Alum and GLA-SE cohorts, compatible with previous descriptions of TEX1 migration [7] (Supplementary Figure 1*A*). IFAT at day 84 showed a positive immunofluorescence closely associated with intraerythrocytic trophozoites (Supplementary Figure 1*B*). P27A vaccination induced significant responses for interferon gamma (IFN-γ), interleukin (IL) 2, IL-5, IL-10, and TNF-α starting at day 56 and still present at day 238 (Supplementary Figure 2*A*–*J*).

DISCUSSION

Blood-stage vaccines are aiming to achieve nonsterile protective immunity with hopefully a good memory response, an aspect which has been so far one of the major weakness of the most advanced, partially effective vaccine tested in a phase 3 trial, the preerythrocytic vaccine RTS,S [10]. In this respect, this paper shows that an unstructured segment from the TEX1 of *P. falciparum* appears an ideal candidate for a synthetic peptide vaccine. Indeed, P27A vaccine was not only safe, but also induced a strong specific humoral response in a formulation with Alhydrogel, even reinforced (close to 10-fold) in a formulation with GLA-SE. Alhydrogel is a classical, well-tolerated vaccine adjuvant with a good capacity to induce a robust humoral,

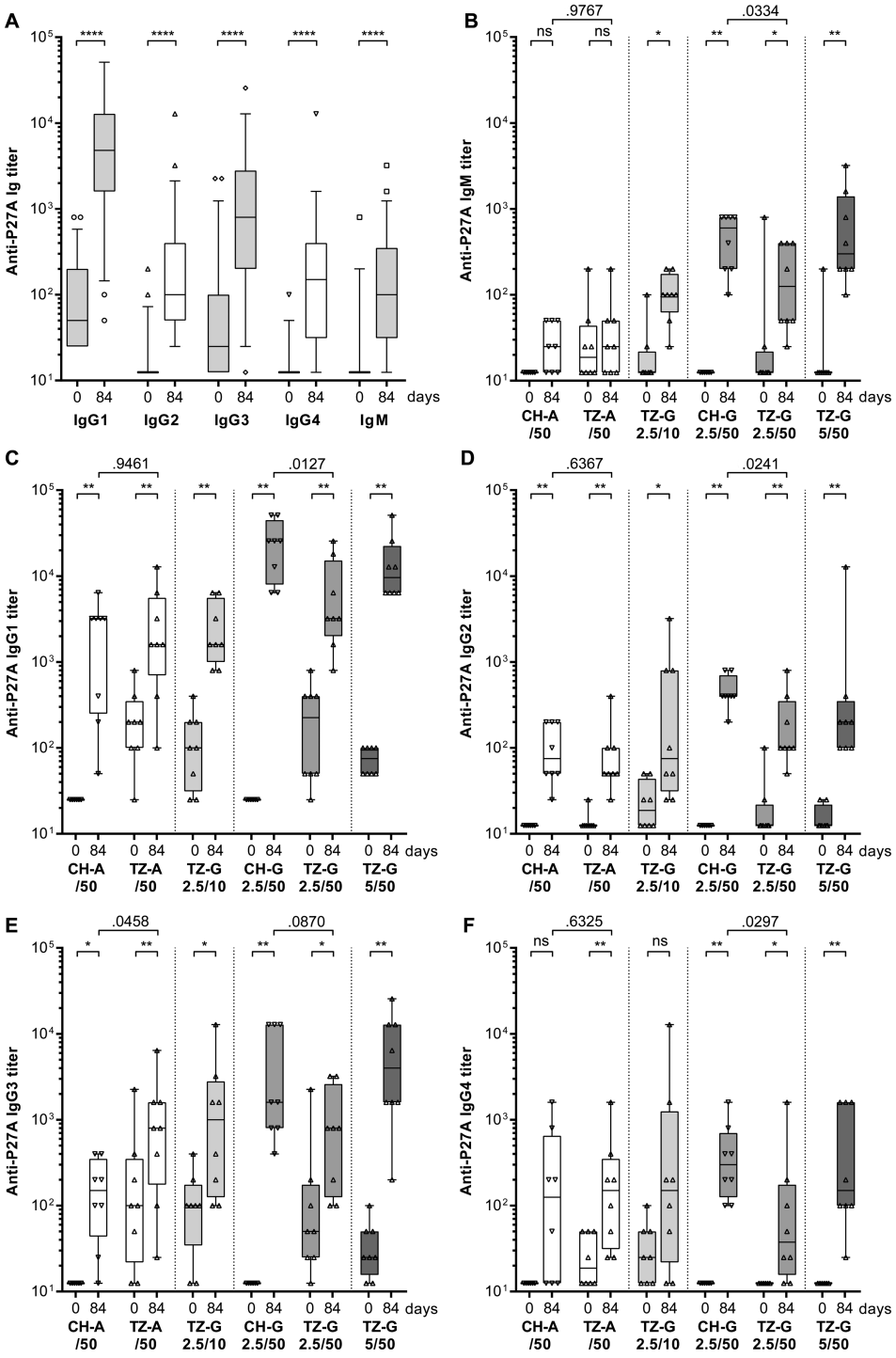

Figure 4. Anti-P27A isotypic responses. *A*, P27A-specific immunoglobulin G (IgG) 1, 2, 3, 4 and immunoglobulin M (IgM) titers at day 0 and day 84 in all volunteers who received P27A formulated in Alhydrogel (group Switzerland [CH]–Alum/50, Tanzania [TZ]–Alum/50) or glucopyranosil lipid adjuvant (GLA) stable emulsion (group TZ-GLA2.5/10, CH-GLA2.5/50, TZ-GLA2.5/50, and TZ-GLA5/50) as box plots and whiskers (interquartile range and 5th and 95th percentiles). Responses per group are shown for IgM (*B*), IgG1 (*C*), IgG2 (*D*), IgG3 (*E*), and IgG4 (*F*). Comparisons for day 84–day 0 were performed using Wilcoxon paired tests, comparisons within groups of identical formulations using Mann-Whitney tests. Abbreviations: A, alum; CH, Lausanne, Switzerland; G, glucopyranosil lipid adjuvant; IgG, immunoglobulin G; IgM, immunoglobulin M; ns, not significant; TZ, Bagamoyo, Tanzania. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

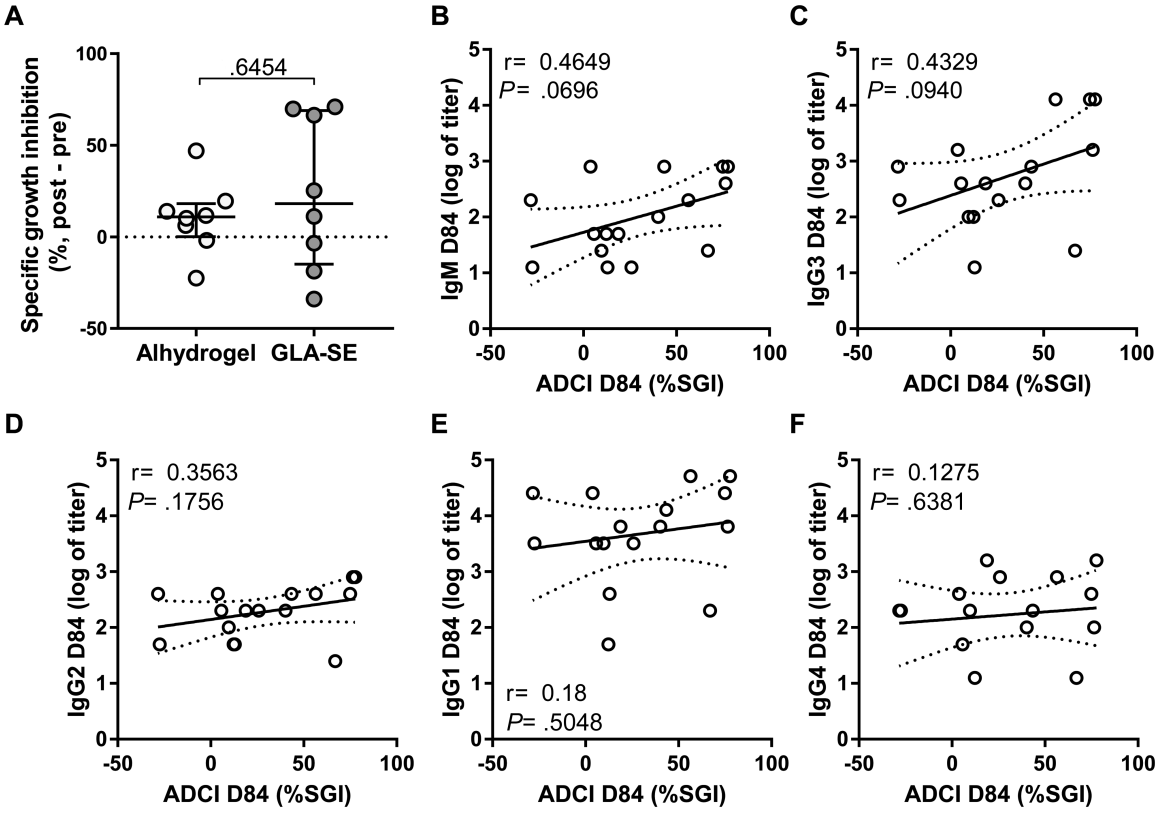

Figure 5. Antibody-dependent cellular inhibition (ADCI). Purified anti-P27A immunoglobulin from phase 1a volunteers (n = 8 per group) were evaluated in ADCI using a model of infection of human red blood cells by *Plasmodium falciparum* in presence of human monocytes. *A*, Results expressed as the delta post–pre (day 84–day 0) of percentage of inhibition of the infection. Lines represent medians and quartiles. A Mann-Whitney test was performed to compare adjuvant formulations (Alhydrogel or glucopyranosil lipid adjuvant stable emulsion) and the *P* value is indicated. *B–F*, Correlations between ADCI and anti-P27A immunoglobulin G isotypes at D84 with Pearson *r*, *P* value, and 95% confidence bands. Abbreviations: ADCI, antibody-dependent cellular inhibition; GLA-SE, glucopyranosil lipid adjuvant stable emulsion; IgG, immunoglobulin G; IgM, immunoglobulin M; SGI, sporozoite growth inhibition.

preferentially but not exclusively Th2 response, whereas GLA, a Toll-like receptor 4 (TLR4) agonist, is a strong T-helper 1 (Th1) cell inducer recently tested in various early phase vaccine trials in humans including tuberculosis, and malaria, among others [11–13]. In this trial, humoral response was supported by a mixed Th1/T-helper 2 (Th2) cell-mediated immunity, leading to preferentially IgG1 and IgG3 antibody subclass response. Importantly, P27A antibodies were able to recognize TEX1 in vitro in Western blot analysis as well as in vivo on the parasite itself, as shown by indirect immunofluorescence assays, and finally led to effective antibody-dependent growth inhibition of the parasite.

This study protocol was designed to spare as much time as possible in moving from phase 1a to phase 1b in endemic area. This design permitted quick achievement of the various study steps sequentially and in parallel from the phase 1a study site in Lausanne, Switzerland, to the African study site 2–4 weeks later. In <6 months, all volunteers from phase 1a and 1b had received at least their first injection of the investigational vaccine. This fast-track design was closely supervised by a single academic sponsor, the Centre Hospitalier Universitaire Vaudois, which was in constant contact with investigators from both sites to interact efficiently with ethics review boards and regulatory authorities both in Switzerland and in Tanzania. Safety was furthermore closely reviewed by a DSMB that had the responsibility to recommend to the sponsor the sequential progression from phase 1a to phase 1b. Overall, this type of design was not only administratively light and efficient, but also cost saving. It appeared ideally suited for a fast track to the field, accelerating development toward efficacy trials. Last, but not the least, it allowed head-to-head comparison of safety and immunogenicity data in 2 different populations.

This phase 1a/1b trial showed the vaccine formulations to be safe as no vaccine-related serious AE was observed. Reactogenicity was generally good. Local and systemic AEs were of low-grade severity, both in nonexposed and in exposed populations. Interestingly, nonexposed European

volunteers reported more frequent local and systemic complaints than malaria antigen–primed Tanzanian participants. These differences may be real or, rather, related to cultural perceptions. Interestingly, formulation of P27A with Alhydrogel vs GLA-SE, a TLR4 agonist, displayed rather different AEs profiles, with significantly more frequent limitation in arm motion in GLA-SE groups for local AEs. Despite this difference, arm motion limitation was mostly self-limited, benign to moderate (grade 1 or 2) and reached grade 3 in only 1 occasion. In contrast, comparing cumulative AEs from groups receiving the same dose of antigen and adjuvant, Alhydrogel appeared more reactogenic than GLA-SE in terms of systemic AEs. These comparisons have to be taken nonetheless with caution because of the sample size. This is in agreement with a previous first-in-human phase 1 study based on a GLA-SE formulated leishmaniasis vaccine [14]. The good tolerability of GLA-SE at the dosages of 2.5 and 5 μg was confirmed in the P27A trial as, irrespective of the P27A dosage (10 or 50 μg), we did not notice any evidence of induction of systemic inflammatory reaction.

The humoral immune response, the main secondary outcome, was particularly robust in phase 1a volunteers, especially after immunization with 50 μg P27A formulated in GLA-SE 2.5 μg, leading to specific antibody titers 10-fold higher than those obtained with Alhydrogel. Comparing both populations, it appears that antibody titers in Tanzanians required a higher adjuvant dosage (GLA-SE 5 μg) to reach titers comparable to the Europeans. These findings must be confirmed with larger number of volunteers, but several factors may explain this difference. First, the fact that most Tanzanian volunteers were naturally primed against P27A as demonstrated by higher day 0 anti-P27A and anti–*P. falciparum* IgG background levels, an observation also reported by others [15], but so far not fully understood. Second, parasite infestation such as helminth infections may have contributed to a reduced response [16]. Other genetic (human leukocyte antigen) or environmental factors may have played a role also, including exposure to malaria itself [17].

Importantly, as demonstrated with sera from group CH-Alum/50 and CH-GLA2.5/50 volunteers, the P27A vaccine induced IgG antibodies able to inhibit parasite growth in ADCI assay, a test performed in blinded manner. IgG1 and IgG3 subclass responses known to exhibit cytophilic activity [6, 8] predominated. Data from this trial are in line with parallel findings that antibody response to a recombinant vaccine candidate including P27A was strongly associated with clinical protection (G. Corradin et al, submitted manuscript). Furthermore, they are also in agreement with a recent observation that ADCI activity was significantly associated with reduced risk against malaria [18]. A key outcome of this trial was the demonstration that that anti-P27A antibodies induced by formulation with Alhydrogel as well as GLA-SE were able to recognize the natural TEX1 protein as expressed by the parasite in vivo as well as in vitro in Western blot analyses.

This humoral response was supported by a mixed Th1/Th2 cell-mediated immunity (see Supplementary Figure 2). Peaking between day 56 and day 84, IFN-γ, IL-2, and TNF-α responses from PBMCs were robust, both in combination with Alhydrogel as well as GLA-SE, a cytokine profile in agreement with previous vaccine trials with Alhydrogel or GLA-SE [14, 19]. IL-10 and IL-5 were also induced, at lower levels, underlining the mixed Th1/Th2 response to P27A, comparable in African and Swiss volunteers.

In summary, the candidate vaccine P27A appears to be safe and able to generate a robust antibody-specific response with parasite growth inhibitory capacity. This was particularly true when the vaccine was formulated with GLA-SE 5 μg and P27A 50 μg, the preferred formulation for the next developmental step of this candidate vaccine. The value of antigen P27A should now be challenged in preliminary efficacy trials using a controlled human malaria infection in nonexposed and exposed subjects.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contribution. V. S.-M., K. K., O. K., R. A., C. D., A. F.-M., S. H., L. V., G. C., O. L., S. A., S. Sh., B. G., F. S., and S. A. J. made substantial contributions to the conception and design of the study. V. S.-M., K. K., O. K., S. Ro., R. A., C. D., S. Sh., B. G., F. S., and S. A. J. performed data collection. A. F.-M., A. E.-V., L. V., E. H., and S. Sc. performed data monitoring and reporting. V. S.-M., K. K., O. K., S. Ro., S. H., L. V., S. Sh., B. G., F. S., and S. A. J. performed safety data analysis and interpretation. R. A., A.-C. T., C. M., C. D., C. Mk., D. P., S. Ru., I. F., R. W. T., M. T., G. C., F. S., and S. A. J. performed immunogenicity data analysis and interpretation. V. S.-M., S. Ro., R. A., C. D., S. H., G. C., B. G., F. S., and S. A. J. wrote the manuscript. All authors contributed to the revision of the manuscript and approved the final version. As corresponding author and principal investigator, F. S. had full access to all the data in the study and took responsibility for the decision to submit for publication.

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Chapter 3

GLA-SE improves quality and magnitude of cellular immunity to the blood stage malaria vaccine candidate P27A

This chapter contains the following manuscript

Catherine G Mkindi[1,2,3], Nina Orlova-Fink[1,2], Damien Portevin[1,2,] Anneth M N Tumbo[1,2], Julian Rothen[1,2], Isabelle Zenklusen[1,2], Tobias Rutishauser[1,2], Seif Shekalaghe[3], Salim Abdulla[3], Saidi Jongo[3], Kamaka Ramadhani[3], Sophie Houard[5], Régine Audran[4], Francois Spertini[4] and Claudia A. Daubenberger [1,2*]

[1]Swiss Tropical and Public Health Institute, Basel, Switzerland; [2]University Basel; [3]Ifakara Health Institute, Bagamoyo, Tanzania; [4]Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; [5]European Vaccine Initiative (EVI), Heidelberg, Germany

GLA-SE improves quality and magnitude of cellular immunity to the blood stage malaria vaccine candidate P27A. *To be submitted to the European Journal of Immunology*

GLA-SE improves quality and magnitude of cellular immunity to the blood stage malaria vaccine candidate P27A

Catherine G Mkindi[1,2,3], Nina Orlova-Fink[1,2], Damien Portevin[1,2,] Anneth M N Tumbo[1,2], Julian Rothen[1,2], Isabelle Zenklusen[1,2], Tobias Rutishauser[1,2], Seif Shekalaghe[3], Salim Abdulla[3], Saidi Jongo[3], Kamaka Ramadhani[3], Sophie Houard[5], Régine Audran[4], Francois Spertini[4] and Claudia A. Daubenberger [1,2*]

1. Swiss Tropical and Public Health Institute, Basel, Switzerland

2. University Basel

3. Ifakara Health Institute, Bagamoyo, Tanzania

4. Centre Hospitalier Universitaire Vaudois , Lausanne, Switzerland

5. European Vaccine Initiative (EVI), Heidelberg, Germany

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* Corresponding author

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Abstract

T-cell responses are hypothesized to be essential for longevity and affinity enhancement of antibodies. Recent malaria blood stage vaccine trail with novel antigen, P27A, conjugated to a novel adjuvant, GLA-SE, demonstrated high safety, tolerance and enhancement of antibody responses[1].

Here, we investigated the magnitude and quality of T-cell responses induced by the vaccine P27A candidate. The GLA-SE adjuvant promoted enhanced $CD4^+$ T-cell responsiveness in malaria pre-exposed Tanzanian volunteers. The response correlated with increased IgG1 titers and expansion of circulating Tfh cells after vaccination. $CD4^+$ T cells with polyfunctional characteristics, coexpressing IL-2 and TNFa, as well as long-living central memory phenotype dominated the response in participants, who received the vaccine containing GLA-SE. In addition, the study also revealed that the magnitude of $CD4^+$ T-cell responses is strongly impaired in the presence of on-going helminth infections during vaccination period.

In conclusion, this study demonstrated that the choice of vaccine adjuvant can largely contribute and enhance malaria vaccine induced Th1 response in humans. In addition, the presence of helminth infections can drastically alter the vaccine outcome by impairing T-cell expansion and should not be neglected in future vaccine studies.

Introduction

Vaccination is one of the most successful and cost-effective public health interventions worldwide [2]. It is essential for decreasing morbidity and mortality caused by infectious diseases especially in young children, who are yet to develop natural immunity.

Despite being a preventable and treatable disease, malaria remains a major threat, especially in children and pregnant women in sub-Saharan Africa. Many efforts towards vector control and disease treatment are hindered by the increase of drug resistance and ineffectiveness, leaving malaria vaccine as an ultimate tool to combat the disease [3] [4]. For over 50 years now, researchers have worked on developing an effective malaria vaccine, but only

RTS, S (a subunit pre -erythrocyte stage vaccine) has been advanced to a phase lll clinical level. This vaccine is however challenged by low efficacy [5], which has been reported to depend on specific, not highly prevalent HLA genotypes [6]. Thus, there is an urgent need for more potent malaria vaccine candidates.

One of the most appealing malaria vaccine candidates is one targeting the blood stage of the parasite, which is the clinical stage of the disease [7]. Previous blood stage vaccine candidates have been extensively evaluated [8, 9] for their ability to induce effective antibody responses against circulating asexual parasites, however no one proven a success [10]. Antibody response has long been known to be responsible for protection against blood stage infection [11, 12, 13], but all vaccine candidates so far were able to induce protection only against vaccine but not field parasites. Historically, effective vaccines are known to work based on the induction of potent neutralising antibodies. Potent and long-lived antibody responses have been proposed to rely on T-cell support; therefor evaluating T-cell responses following blood stage vaccination is an essential study component.

A novel subunit blood stage malaria vaccine, P27A, has been evaluated for its immunogenic potential[14]. This protein antigen is derived from exported protein in the Trophozoite stage of asexual plasmodium falciparum [15] and has shown to induce functional antibodies in pre-clinical and in phase one clinical trial conducted recently [1]. Although it is safe and less costly, like other subunit vaccines, it is generally less immunogenic when administered alone. Thus, it requires administration with an adjuvant to enhance immunogenicity [16]. Toll-like receptor agonists are recently proven to be potent and specific stimulants of the innate immune system [17], enhancing specifically Th1 responses [18]. In this study a novel TLR4 agonist adjuvant, GLA-SE (Glucopyranosyl Lipid A formulated in stable oil emulsion) is formulated with the novel P27A antigen. Aluminium hydroxide adjuvant (Alhydrogel or Alum) [19], which is a classical adjuvant that has been used in the majority of FDA approved vaccines, was used for a direct back-to-back comparison with GLA-SE. It has been shown that strong adjuvants induce Th1 immune responses, which are required for development of high affinity antibodies and long-lived memory cells [20]. It has been shown that Th1 responses following vaccination are down regulated by

helminth infection [21]. Since helminth infection is common in the study area [22], we evaluated also their associated effects following vaccination.

Thus, in the current study we focused on Th1 responses induced by the vaccine candidate (formulated with GLA-SE or Alhydrogel in direct comparison) and evaluated their magnitude, cytokine profiles and memory phenotypes. We also assessed the influence of helminth infection on vaccine induced $CD4^+$ T-cell responses, since trial participants live in malaria endemic areas, where co-infection with soil-transmitted helminths is very common.

Results

P27A induces robust $CD4^+$ T-cell responses in combination with GLA-SE

This study assessed longitudinally the magnitude and kinetics of T-cell responses ($CD4^+$ and $CD8^+$) against P27A peptide by ICS and flow cytometry for 3 major Th1/Tc1 cytokines: IFNg, IL-2 and TNFa (Fig.1 and Supp.Fig.1) in 40 trial participants classified in 4 groups receiving different dose of P27A conjugated to Alum or GLA-SE (described in detail in the original study [1]. At baseline, all participants had negative P27A-specific $CD4^+$ T-cell responses measured by all 3 cytokines individually. These responses increased significantly and peaked at day 28 post-vaccination. The highest magnitude was observed in GLA-SE 5/50 and Alum/50 groups for IL-2 (0.575% vs. 0.52%) followed by TNFa expression (0.485% vs. 0.4%). The magnitude of the responses slightly decreased, but persisted and remained robust at 126 day post-vaccination ($IL\text{-}2^+$ 0.37% vs. 0.406; $TNFa^+$ 0.26% vs. 0.3%) for the GLA-SE 5/50 and Alum/50 group respectively. The responsiveness (proportion of participants, which had a positive response after vaccination with 0.05% cut-off) was the highest (100%) in the GLA-SE group (8/8), followed by Alum50 (7/8) and decreased in participants receiving vaccines with less antigen and less adjuvant (5/8 for GLA-SE 2.5/50; 4/8 for GLA-SE 2.5/10). Surprisingly IFNg expression was nearly negative in all participants at the tested time points after vaccination. To validate that T cells were functionally capable of expressing IFNg, PBMC from each participant for every tested time point was stimulated with Staphylococcus enterotoxin B

(SEB) in parallel to P27A stimulation (Supp.Fig.2). The data showed that SEB-specific $CD4^+$ T cells can express highly robust levels of all 3 cytokines including IFNg, confirming that the lack of IFNg expression in P27A-specific T cells is not due to cell viability, but most likely due to the nature of the vaccine procedure.

Since magnitude of the P27A-specific $CD4^+$ T-cell responses was rigorously higher in the Alum/50 and GLA-SE 5/50 compared to the other two groups and included samples form baseline, they were further analysed more in depth in this manuscript. In addition, the groups' participants received exact the same amount of P27A antigen allowing a direct comparison of the immunogenicity of the GLA-SE and Alum adjuvants.

This study also evaluated magnitude and kinetics of P27A-specific $CD8^+$ T-cell responses (Supp.Fig.3), which fail to expand and were nearly absent in all participants after vaccination, a result, which was not surprising given the type of malaria antigen and adjuvants tested in this trial.

Majority of P27A-specific $CD4^+$ T- cells are coexpressing IL-2 and TNFa

Vaccination with P27A induced robust expression of two cytokines IL-2 and TNFa in $CD4^+$ T cells. We observed that trail participants, who developed high frequency of IL-2 expression also showed high proportion of TNFa and vice versa. To evaluated the correlation between the magnitude of IL-2 and TNFa, we plotted the proportion of P27A-specific $CD4^+$ T cells positive for IL-2 total or TNFa total expression (gated within the $CD3^+CD8^-CD4^+CD45RA^-$ subset; background levels from medium only controls were subtracted) (Fig.2A). T-cell response was measured from 2 groups: Alum/50 and GLA-SE 5/50, at 28 days and 126 days post-vaccination. Highly significant correlation was observed for both groups and time points ($r \geq 0.9458$, $p \leq 0.0001$). This suggested that P27A-specific $CD4^+$ T cells are coexpressing both cytokines, a phenotype, which is favourable for a quality immune response. Therefore, we evaluated the proportion of the double positive ($IL\text{-}2^+TNFa^+$) cells within the total cytokine expressing P27A-specific $CD4^+$ T cells. Figure 2B shows that half of the cytokine positive cells are simultaneously expressing IL-2 and TNFa at both time points post-vaccination. The proportion of $IL\text{-}2^+TNFa^+$ cells was even higher in the GLA-SE group (56% at d28post) and this

polyfunctional-like phenotype persisted (59% at d126post) for several months post-vaccination.

P27A-specific CD4$^+$ T-cell expansion is significantly impaired in the presence of helminth infection.

In order to examine the impact of helminth infection during vaccination period on T-cell development, we compared the magnitude of the P27A-specific T-cell response between helminth negative and helminth positive trial participants. The study considered testing for the most common worms in the region: schistosoma, strongyloides and hookworm infection. From 40 participants, 13 (2 were placebo and not considered for this analyses) were diagnosed positive at some point during the vaccination period. Most frequent were hookworm infections, followed by strongyloides and schistosoma infection. The majority of the cases were diagnosed at baseline, and several participants were positive at multiple time points during vaccination period. The frequency of P27A-specific CD4$^+$ T cells was overall significantly reduced in the helminth positive participants (0.11% vs. 0.33%) at 28 days post last vaccination (Fig.3A) and this negative effect persisted months later at d126_post (0.06% vs. 0.20%) (Fig.3C). Furthermore, we evaluated the impact of helminth infection in combination with different dose of antigen and type of adjuvant tested in this trial. Surprisingly, the T-cell magnitude was universally and severely impaired in all groups in the presence of helminth infection (Fig3.B), regardless of adjuvant type, peptide dose and time point, with fold - decrease ranging from 2.5 to 2.9 at d28_post and 2.7 to 3.4 at d126_post (Fig.3.B).

Reduced magnitude of P27A-specific IgG1 titer in the presence of helminth infection

In addition to T-cell frequency, we examined the impact of helminth infection on p27-specific antibody response (data provided from original study [1]). We compared the fold-increase of total IgG and subclass IgG 1, 2, 3 and 4 to baseline levels prior to vaccination between helminth negative and helminth positive trial participants. IgG1 was significantly reduced in helminthes positive participants at d28_post, which is partially reflected in slightly reduced

total IgG magnitude. This negative effect persisted months later at d182_post. Interestingly, other IgG subclasses and IgM were not affected by the presence of helminthes during vaccination period and showed similar levels of antibody titer increase at d28_post.

P27A CD4$^+$ T-cell magnitude strongly correlates with cTfh cell responses after vaccination with GLA-SE but not with Alum

Current study (Hill at el, unpublished) showed that only volunteers receiving the GLA-SE adjuvanted vaccine had an expansion of the circulating T follicular helper cells (cTfh) measured 7 days post last vaccination (original data was kindly provided for this analysis). Th1 and Tfh cells differentiate of naïve CD4$^+$ T cells into functionally distinct T helper subsets; however, their relationship is incompletely understood. Some data argues that Tfh and Th1 cells share a common transitional stage through a signal mediated by STAT4, which promotes differentiation of both phenotypes [23]. To explore the relationship of these two CD4$^+$ T-cell subpopulations we compared the frequency of the P27A-specific CD4$^+$ T cells (defined as IL2$^+$TNFa$^+$ within the CD3$^+$CD8$^-$CD4$^+$CD45RA$^-$ subset; background levels from medium only controls were subtracted) at 28 days post-vaccination to the expansion of cTfh cells measured 7 days post-vaccination (Fig.5). The comparison revealed that P27A-specific Th1 and cTfh-cell expansion strongly correlates only in participants receiving the GLA-SE vaccine (r=0.8201, p=0.0127), but not in combination with Alum (r=0.4947, p=0.3127). These data indicates that GLA-SE may promote simultaneous Th1 and cTfh expansion through a common signalling pathway leading potentially to a more effective immunity to P27A.

GLA-SE fostered P27A CD4$^+$ T-cell expansion correlates with IgG1 titer.

Production of high-affinity antibody responses is highly dependent on CD4$^+$ T-cell help, but is unclear to what extend magnitude of T-cell response supports or limits the magnitude and type of antibody response. In order to explore this relationship, we compared the frequency of the P27A-specific CD4$^+$ T cells and antibody titers tested at 28 days post-vaccination (Fig.6). The proportion of the P27A-specific CD4$^+$ T cells (defined as IL2$^+$TNFa$^+$ within the CD3$^+$CD8$^-$ CD4$^+$CD45RA$^-$ subset; background levels from medium only controls were

subtracted) was determined for each vaccine participant from the Alum50 (n=8) and GLA-SE 5/50 (n=8) group at 28 days post-vaccination. The T-cell responses were plotted against the antibody titers for total IgG and subclasses IgG1, 2, 3 and 4 (AB measurement was kindly provided by Steiner-Monard et al. [1]). As demonstrated in Figure 6A, there was a strongly significant correlation between $CD4^+$ T-cell expansion and total IgG titer (r=0.79, p=0.0197) but only after P27A-vaccination with GLA-SE and not with Alum adjuvant (r=0.2256, p=0.5912). Further more detailed analyses revealed that the magnitude of P27A-specific $CD4^+$ T cells induced by GLA-SE 5/50 correlates even more significantly with the titer of IgG1 (r=0.9102, p=0.0031) (Fig.6B), but any other IgG subclass (Fig.6C-D) or IgM responses (Supp.Fig.4B) do not correlate significantly with T-cell magnitude.

P27A-specific $CD4^+$ T cells have a predominant central memory phenotype after P27A vaccination with GLA-SE but not with Alum

The $CD4^+$ T-cell memory phenotype was determined by measuring surface expression of CD45RA and CD27 on P27A-specific cytokine-expressing ($IL\text{-}2^+$ and/or $TNFa^+$) $CD4^+$ T-cell subsets (Fig. 7A). The cells exhibited a predominant central memory phenotype ($CD45RA^-CD27^+$) in the GLA-SE 5/50 group (64%) 28 days post last vaccination and this phenotype persisted at a similarly high frequency (67%) even 126 days post, suggesting a long-living memory potential of the P27A-specific T cells (Fig. 7B). In parallel the proportion of the central memory P27A-specific T cells was strongly reduced in the Alum/50 group (48%) at d28post and even more (41%) at d126post. To insure that the development of CM cells is specific for P27A vaccination, we analysed the memory phenotype of the total $CD4^+$ T cells for the two vaccine groups and placebo as comparison, at 28 days and 126 days post vaccination and baseline in addition (Supp.Fig.6). The proportion of total CM $CD4^+$ T cells was very similar among the 3 groups and the different time points (ranging from 31% to 34%), confirming that the development of CM phenotype of T-cell subsets specific for P27A antigen is due to vaccination.

Discussion

In this study we evaluated the expansion of $CD4^+$ T cells, their cytokine expression profile and memory phenotype following vaccination with a novel blood stage malaria vaccine candidate. In addition we evaluated the impact of soil transmitted helminth infection on P27A-specific T-cell magnitude.

P27A vaccination demonstrated enhanced $CD4^+$ T-cell responses especially when administered with high dose GLA-SE. A pronounced expansion of IL-2 and TNFa specific $CD4^+$ T cells was observed. IFNg has been considered as a canonical cytokine defining Th1 $CD4^+$ T cells, but other cytokines such as IL-2, TNFa and IL-17 have been reported to be produced simultaneously. This observation was shown in other blood stage vaccine [24] and tuberculosis [25] vaccines, which were also formulated with GLA-SE adjuvant. More ever, the time at which IFNg is produced determines its protective or adverse outcome during malaria infection [26], the earlier production during infection the better [27]. Many studies have reported that early IFNg expression is associated with protection [28]. Secretion of IFNg at later time points during infection has been associated with severe malaria outcome, including activation of secretion of chemokines and adhesion molecules responsible for sequestration of infected red blood cells and infiltration of immune cells into the blood brain barrier [29]. Since IFNg has also been shown to be a key support for production of class switched antibodies by the B-cells and activation of macrophages to phagocytosis infected red blood cells, then we can hypothesize that modest expansion of IFNg producing $CD4^+$ T cells should be ideal. Due to existence of fine line between IFNg protection and immunopathology during malaria, it is important to understand the optimal level required, and be able to modulate it with suitable adjuvants. It is possible that vaccine induced $CD4^+$ T cells express moderate levels of IFNg at an earlier time point post vaccination and the current study missed their detection since the earliest T cell evaluation was done 28 days post vaccination. Future studies can address this question by performing more detail longitudinal kinetics.

In this study, T cell responses was characterised by Polyfunctional capacity shown by concurrent expansion of TNFa and IL2 cytokine producing CD4 T

cells. This was observed in the advanced malaria candidate vaccine RTS S [30], which was also formulated with a similar TLR agonist adjuvant AS01. There was no production of IFNg observed upon vaccination with Alum or GLA-SE, which was also seen in a blood stage antigen AMA1 when vaccinated with GLA SE in naïve and malaria exposed participants.

Presence of helminth infection in some participants showed decreased P27A specific $CD4^+$ T-cell responses. It is known, to what extend cells of innate immune system can phagocytize helminthes for antigen presentation, but they can excrete and secrete products, which can be recognised by the pattern-recognition receptors of the innate immune system [31]. However to evade the immune response, helminthes developed the ability to down regulate expression of the PRR Toll-like receptors [32] and use this as a survival strategy to persist in human hosts. This phenomenon could explain lower magnitude of T-cell responses in helminth positive trial participants as a result of hindered activity of the Toll-like receptor agonist GLA-SE adjuvant due to decreased TLR-surface levels.

In conclusion all together, the current study has demonstrated that choice of adjuvant should be carefully considered just as choice of antigen, since adjuvants are powerful modulators and attractive way of enhancing vaccines. More importantly this work also showed that presence of helminthes during vaccination significantly harms the development of immune responses. Therefor, helminth infections in trial participants should not be neglected but considered and evaluated in future studies, since treatment prior to vaccination with anthelmintics may drastically improve vaccine outcomes.

Materials and methods

Ethics Approval

Ethical and regulatory review boards in Tanzania, the Ifakara Health Institute review board (IHI-IRB, #30-2013), the National Institute for Medical Research (NIMR/HQ/R8a/Vol.IX/1742) and the Tanzanian Food and Drug Administration (TFDA13/CTR/004/03) approved this study protocol. The study was undertaken in accordance with the latest revision of the Declaration of

Helsinki (Fortaleza, Brazil, October 2013) and the ICH Good Clinical Practices (ICH-GCP Guidelines).

Study population

Adult male volunteers aged 18-45 years, with general good heath and having lived in areas of Tanzania with minimal malaria transmission were eligible to participate in the study. Subjects with Symptoms, physical signs or laboratory values suggestive of systemic disorders, including renal, hepatic, cardiovascular, pulmonary, skin, immunodeficiency, psychiatric and other conditions, which could interfere with the interpretation of the trial results or compromise the health of the volunteers were excluded from study . Females were not included due to risk of pregnancy since contraception is not a common practice in the region. Written consent was obtained from all participants before entering the study. Infection with helminth was not an inclusion criterion but participants were tested during trial period, and were not terminated from the study if found positive at and time point.

Study design

The study was designed as a single-center, staggered randomized, double blind, and controlled trial. Volunteers were randomized and allocated (on a 4:1 basis) in a dose-escalating manner to 4 groups. Each group included 10 subjects, 8 of whom were injected intramuscularly in the non-dominant arm with 50µg P27A + Alhydrogel® (group 1B), 10µg P27A **+** 2.5µg GLA-SE (group 2B), 50µg P27A **+** 2.5µg GLA-SE (group 3B), 50µg P27A**+** 5µg GLA-SE (group 4B) and 2 subjects injected with Verorab® as vaccine control respectively. All injections were performed at days 0, 28 and 56 with a follow-up of 6 months. The study was conducted from August 2014 to April 2016.

Blood Samples collection and processing

For immunological evaluations, peripheral blood was collected in tubes containing EDTA from all eligible and consenting volunteers before first vaccination (at baseline) 3 months after first vaccination and at six months after first vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation according to standard protocols and stored in liquid nitrogen in Fetal calf serum with 10%-

DMSO until analysis.

Measurement of cytokine responses following vaccination

To evaluate cytokine production following vaccination, PBMCs were thawed in complete medium (10% FCS in RPMI with Glutamine and Penicillin/ Streptomycin) containing benzonase (25KU, Novagen, 70664-3), washed and rested for 2 hours (5% CO_2 at 37*C). Trial samples from different time points for any given participant were analysed simultaneously in order to avoid experimental variability. A total of 1.5 million PBMC (per visit and participant) were stimulated with P27A peptide (50ug/ml) or Staphylococcus enterotoxin B antigen (SEB, 100ug/ml) as a positive control or incubated in medium only (background control) over night (14 hours) at 37*C. Brefeldin-A (Sigma, B7651) was added at 4 hours. Afterwards stimulated cells were washed and stained with live/dead fixable aqua fluorescent reactive dye, followed by surface staining with anti-CD3_PacBlue (UCHT-1, BD), anti-CD8_AF700 (HIT8a, Biolegend), anti-CD4_perCPCy5.5 (Oct4, Ebioscience), anti-CD45RA_PE-Cy7 (L48, BD) and anti-CD27_APC-H7 (Clone M-T271) BD. Cells were washed, fixed and permeabilized and intracellular stained with the following: anti-TNFα_FITC (MAb11, BD), anti-IL2_PE (MQ1-17H12, BD) and anti-IFNg_APC (B27, BD). Stained cells were acquired on a LSR II flow cytometer (BD Biosciences; San Jose, CA) and FACS data was analyzed by FlowJo software V10.1 (Tree Star). Color compensation was performed using beads stained for each fluorochrome.

Diagnosis of Helminth infection

For helminth evaluation, stool and urine samples were collected and tested at baseline and during second and third vaccinations. The study tested for: schistosoma, strongyloides and hookworm infection. Kato Katz method was used to test for the presence of schistosoma, hookworm and hymenolepis eggs, while Baermann method was used to test for strongyloides larva. Kato Katz method was used as described, briefly: the Kato template placed on a microscopic slide was filled with stool sample, which was sieved through a plastic screen paper. On removal of the template, a cellophane tape wetted on glycerol malachite green solution was put on sample and the slide was

inverted, pressed to evenly distribute the sample. The slide was incubated on a rack for 20 minutes and egg count was done on a compound microscope. For strongyloides larvae detection, by the Baermann technique, peanut size stool sample is placed on a piece of cotton gauze tied and put in the funnel connected to a tubing which is then filled with Luke warm water. It is incubated for 2 hours, collected through the tubing in a 50ml falcon tube, followed centrifugation. The sediment is placed on microscopic slide with cover slip and examined under light microscope.

Figures with legends

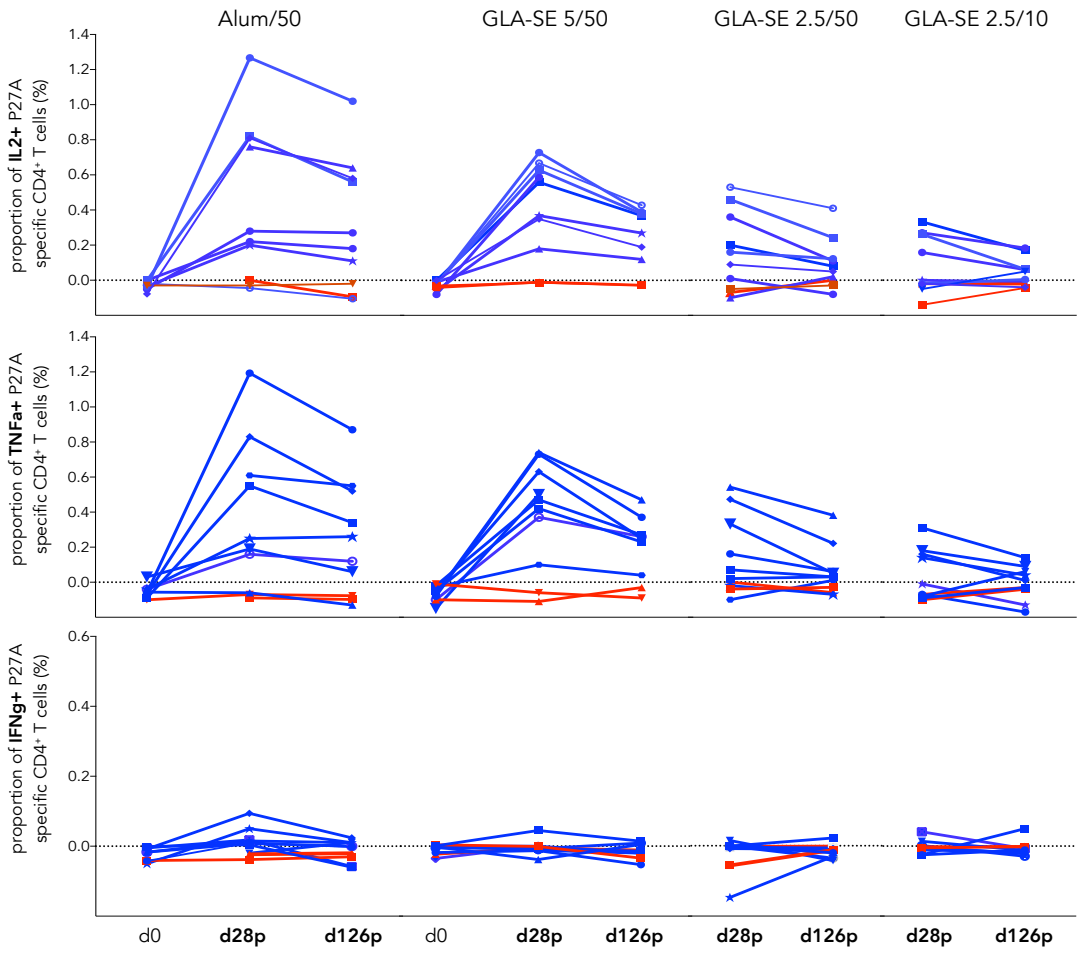


Figure 1. P27A-specific CD4$^+$ T-cell responses induced after vaccination

PBMC from 40 study participants (10 for each of the four trial groups indicated on top) collected at 3 different time points (indicated at the bottom; day 0 was assessed only for group Alum/50 and GLA-SE 5/50) were stimulated over night with P27A. CD4$^+$ T cells were identified by surface and intracellular cytokine staining as described in Supp. Figure 1. Responses were measured by flow cytometry and evaluated for IL-2, TNFa and IFNg cytokine expression, represented as a proportion (%) of the memory CD4$^+$ T cells (CD45RA$^-$). Background levels (from PMBC cultured with medium only in parallel) were subtracted. Each symbol represents a single participants, blue lines (P27A-vaccine participants) and red lines (placebo participants) connect measurements at baseline, 28 days post and 126 days post 3rd vaccination.

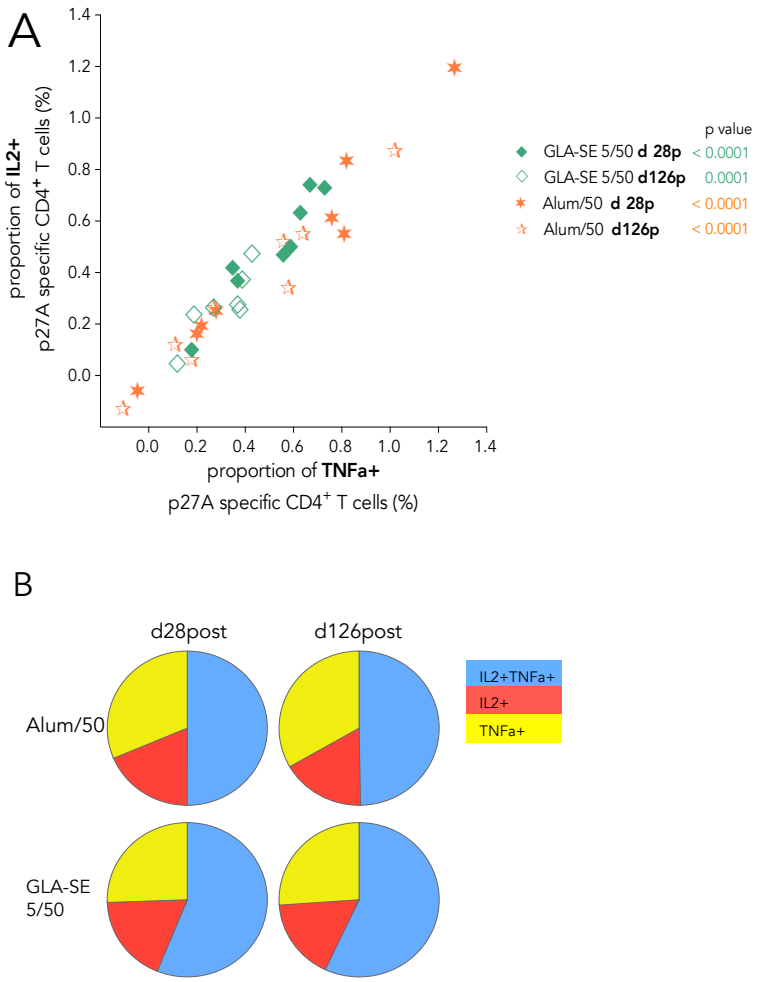


Figure 2. Coexpression of IL-2 and TNFa in P27A-specific CD4$^+$ T cells

Frequency of CD3$^+$CD4$^+$ T-cell cytokine responses were measured by flow cytometry in 16 participants (groups indicated on the graphs) after stimulation with P27A peptide. (**A)** Linear regression analysis of P27A-specific CD4$^+$ T cells detected by ICS for IL2 total or TNFa total and represented as a proportion of the memory CD4$^+$ T cells (CD45RA$^-$). Each symbol represents a participant either from the Alum/50 group (orange stars) or GLA-SE 5/50 group (green diamonds) tested at 28 days post (filled symbols) and 126 days (open symbols) post 3rd vaccination. Graphical representation and statistical analysis were performed with PRISM6, (p-values indicated on the plot) and calculated with nonparametric Spearman correlation. (**B**) SPICE analysis as pie charts representing the median proportions of cells expressing both IL2/TNFa or only one of the two cytokines among the memory CD4$^+$ T-cell subset expressing cytokines total.

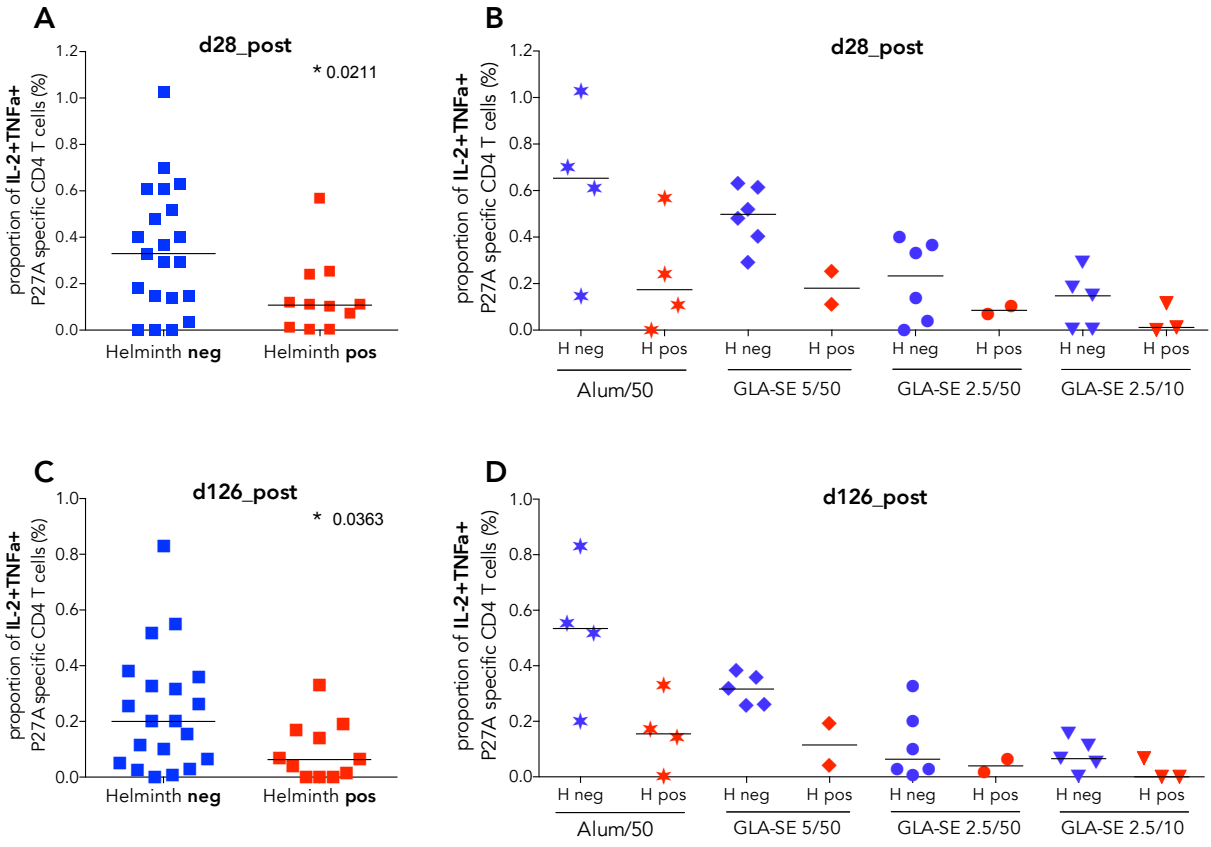


Figure 3. Comparison of P27A-specific CD4$^+$ T-cell expansion in trial participants positive and negative for helminth infection

Responses were measured by ICS and flow cytometry after PBMC stimulation with P27A antigen, and analysed for double IL-2$^+$TNFa$^+$ cytokine expression as a proportion of the memory CD4$^+$ T-cell subsets (CD45RA-). (A,C) Direct comparison of P27A-specific CD4$^+$ T-cell responses between all 32 trial participants (8 placebo participants were excluded), grouped depending on helminth status: positive (red) and negative (blue), measured 28 days and 126 days after the 3rd vaccination respectively. (B, D) Comparisons of the P27A-specific CD4$^+$ T-cell responses between helminth positive and negative participants within each of the 4 vaccine groups (indicated at the bottom), middle lines represent medians. Graphical representation and statistical analysis were performed with Prism6, differences (p-values indicated with * on the plots) were calculated with Mann Whitney test.

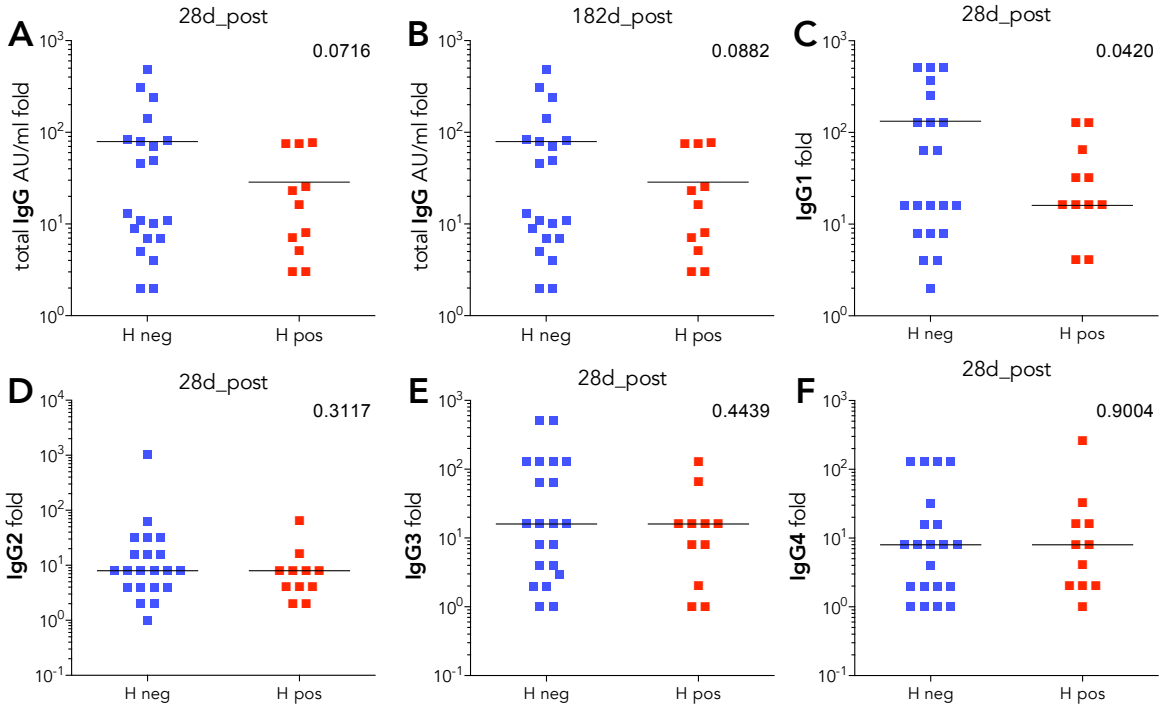


Figure 4. Comparison of P27A-specific antibody responses in trial participants positive and negative for helminth infection

Total IgG (A,B) and IgG subclasses 1-4 (C-D) were measured at baseline, 28 days post and 182 days post 3rd vaccination (data provided by Steiner-Monard et al. [1]). Each symbol represents a vaccine participant (32 total) positive (red) or negative (blue) for helminthes. Responses are presented as fold increase to baseline levels, middle lines represent medians. Graphical representation and statistical analysis were performed with Prism6, differences (p-values are indicated on the plots) were calculated by unpaired t test with Welch's correction.

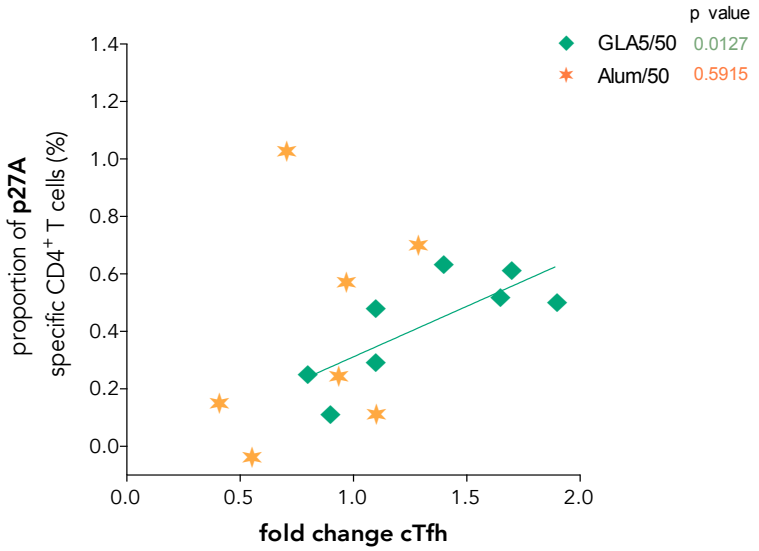


Figure 5. Correlation analysis between P27A-specific CD4$^+$ T-cell frequency and cTfh expansion after vaccination

Frequency of CD3$^+$CD4$^+$ T-cell cytokine responses in PBMC were detected by ICS and measured by flow cytometry after stimulation with P27A peptide. Background cytokine levels (PBMC in medium only) were subtracted. P27A-specific CD4$^+$ T cells were detected by coexpression of IL2$^+$ TNFa$^+$ and represented as a proportion of the total memory CD4$^+$ T cells (CD45RA$^-$). Each symbol represents a participant either from the Alum/50 group (orange stars, n=8) or GLA-SE 5/50 group (green diamonds, n=8) tested at 28 days post or 7 days post 3rd vaccination for CD4$^+$ T-cell or cTfh expansion respectively. Data for the cTfh was kindly provided by Hill et al. (unpublished) and plotted as fold change to baseline cTfh frequency. Linear regression analysis was performed for each group with Prism6 (correlation p-values: *0.0127* for GLA-SE 5/50; *0.5915* for Alum/50 group).

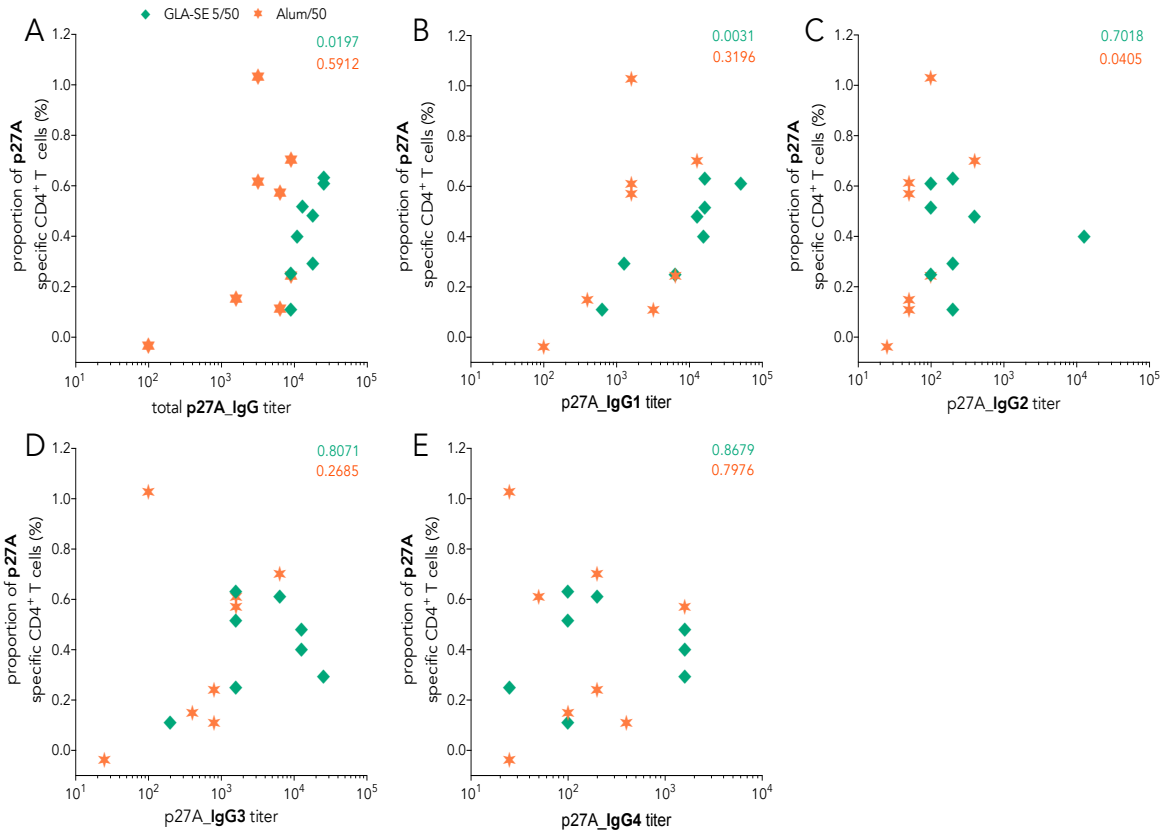


Figure 6. Correlation analysis between P27A-specific CD4$^+$ T-cell frequency and antibody titer after vaccination

Frequency of CD3$^+$CD4$^+$ T-cell cytokine responses in PBMC were detected by ICS and measured by flow cytometry after stimulation with P27A peptide. Background cytokine levels (PBMC in medium only) were subtracted. Response frequencies were detected by coexpression of IL2$^+$ TNFa$^+$ and represented as a proportion of the total memory CD4$^+$ T cells (CD45RA$^-$). Each symbol represents a participant either from the Alum/50 group (orange stars, n=8) or GLA-SE 5/50 group (green diamonds, n=8) tested at 28 days post 3rd vaccination. Data on antibody titers for P27A-specific total IgG (A) and IgG 1, 2, 3 and 4 (B-E) from the same time point was from the original publication [1]. Graphical and correlation analysis was performed with Prism6 for each group (GLA-SE 5/50 green diamantes n=8, Alum/50 orange stars n=8). Color-coded p-values are shown for each plot, determined by the Spearman rank correlation test.

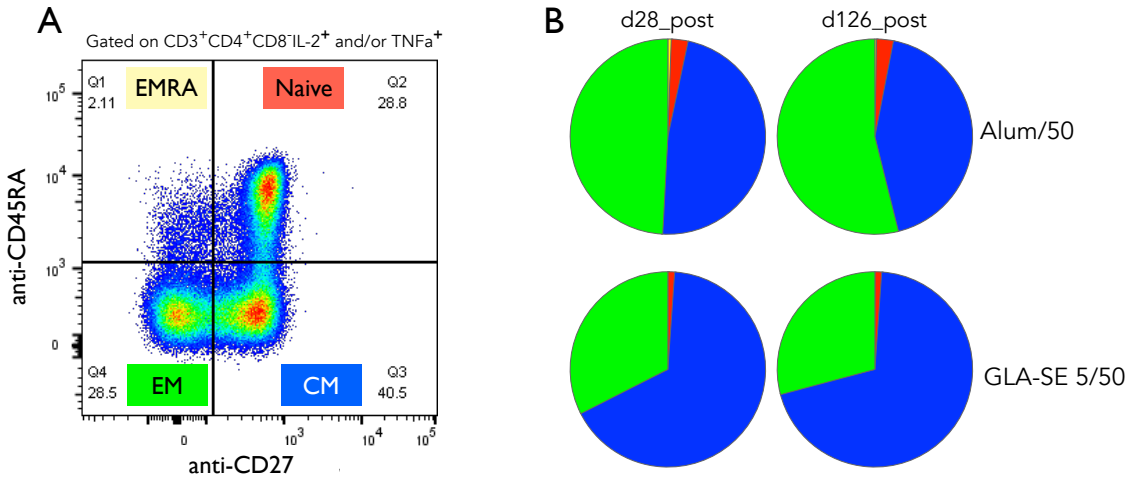
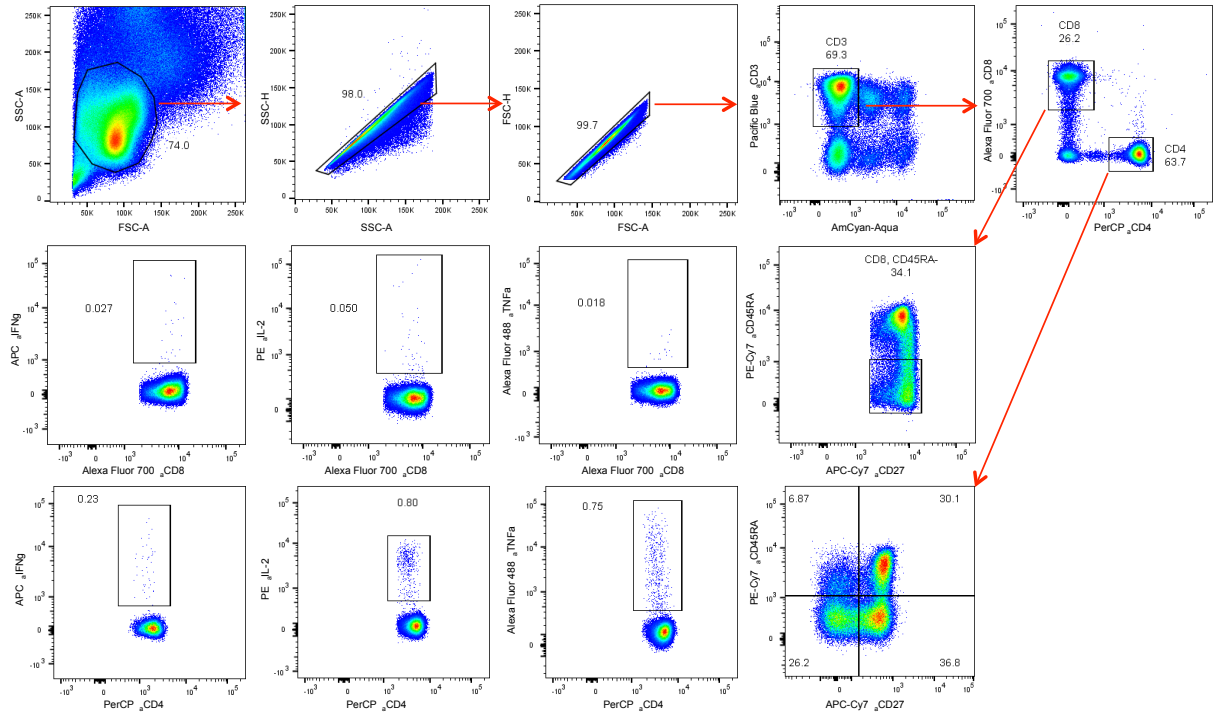


Figure 7. Memory phenotype of P27A-specific CD4$^+$ T cells after vaccination

Frequency of CD3$^+$CD4$^+$ T-cell cytokine responses were measured by flow cytometry in 16 participants (n=8 in each group) after stimulation with P27A peptide. (**A)** A representative dotplot, from a single participant at 28 days post-vaccination, CD4 memory phenotypes were identified on CD27 and CD45RA gating, defining 4 subsets: naïve (CD45RA$^+$CD27$^+$), central memory (CM; CD45RA$^-$CD27$^+$), effector memory (EM; CD45RA$^-$CD27$^-$) and effector memory CD45RA$^+$ (EMRA; CD45RA$^+$CD27$^-$), gated on the IL2$^+$ and/or TNFa$^+$ P27A-specific subpopulation.

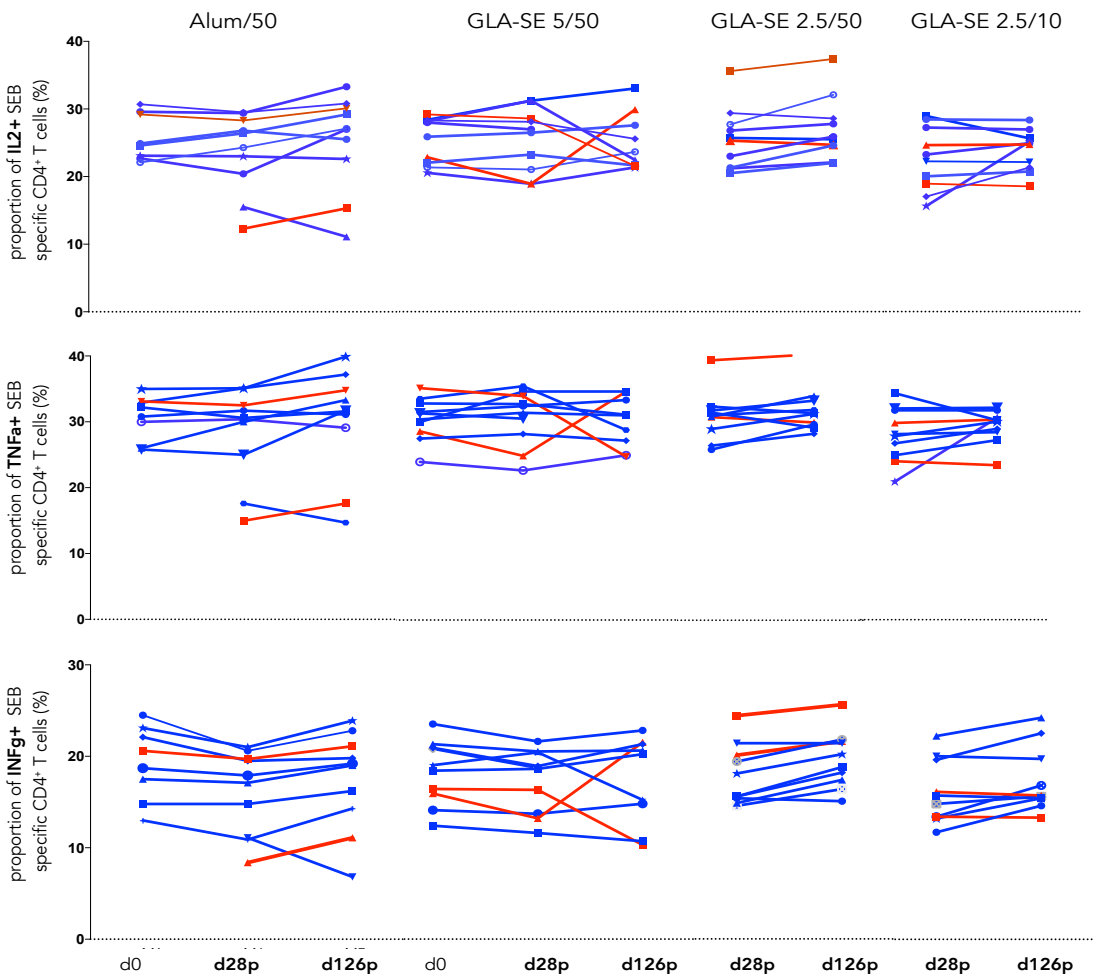
(**B**) SPICE analysis as pie charts representing the median proportions of cells within each of the four CD4$^+$ T-cell subsets (Naive-red, CM-blue, EM-green or EMRA-yellow) in two vaccine groups at 28 and 126 days post 3rd vaccination.

Supplementary Figures



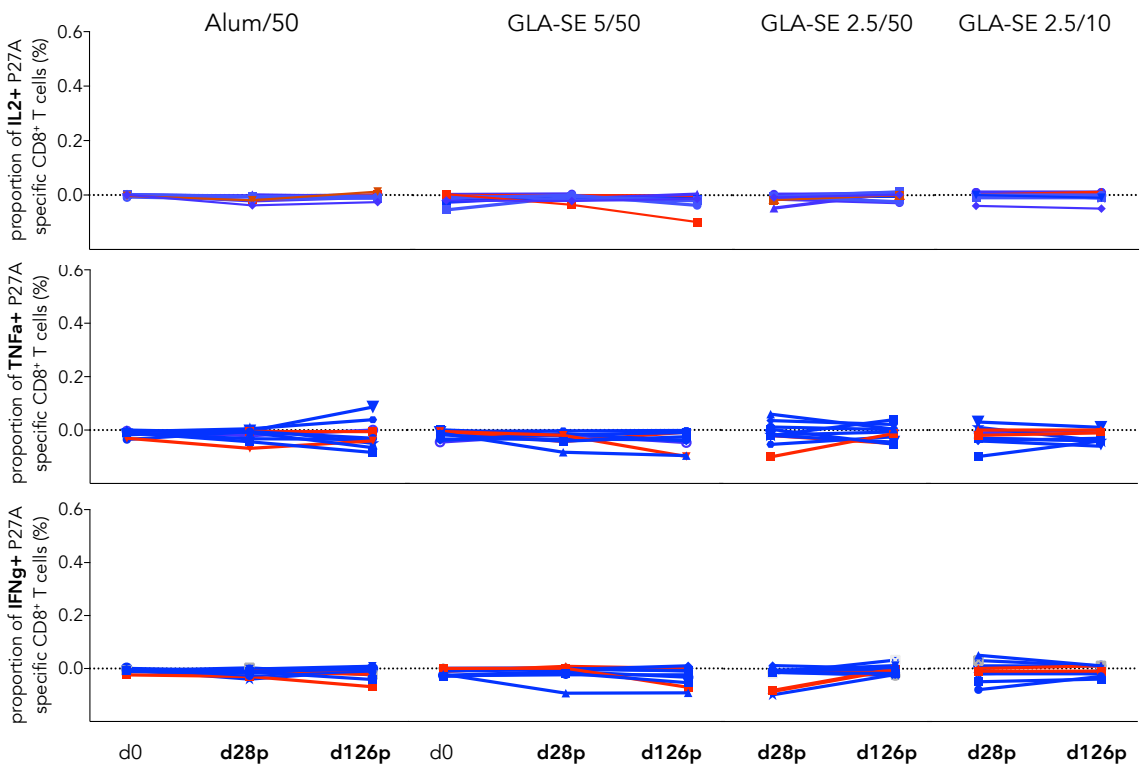
Supp. Figure 1. Gating strategy for flow cytometric analysis of human PBMC used to identify CD4+ and CD8+ T cells and their subpopulations of interest

Representative dotplots, from a single participant at 28 days post-vaccination. PBMC from whole blood were cultured in the presence of antigen (P27A peptide or SEB) or in medium only (background control) and stimulated over night. Cells were surface stained for CD3 (Pacific Blue), CD4 (PerCP), CD8 (Alexa Fluor 700), CD27 (APC-Cy7) and CD45RA (PE-Cy7) and intracellular stained for IFNg (APC), IL-2 (PE) and TNFa (Alexa Fluor 488). Data was collected on BD LSR II flow cytometer and analysed with FlowJo v10 software. Lymphocytes are identified by their scatter properties (FSC-A x SSC-A plot), then doublets were excluded by gating on SSC-A x SSC-H and FSC-A x FSC-H sequentially. The viable $CD3^+$ T-cell population was identified by gating on CD3 x AmCyan-Aqua. $CD4^+$ T cells and $CD8^+$ T cells were identified by gating on CD4 and CD8 respectively and further characterized for cytokine profiles on IFNg, IL-2 and TNFa by Boolean combination gates. In addition, memory phenotypes were assed by gating on CD27 and CD45RA.



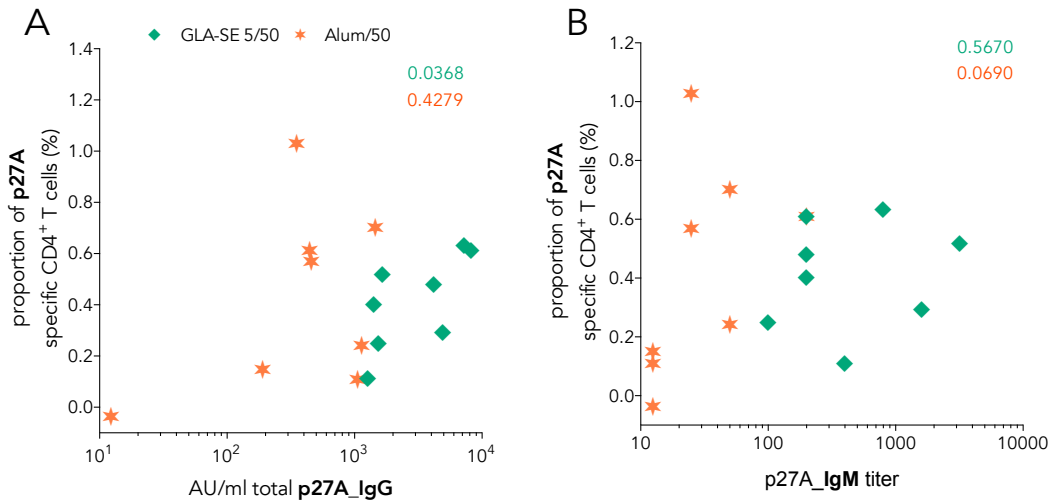
Supp. Figure 2. Longitudinal evaluation of SEB-specific CD4$^+$ T cells expressing IL-2, TNFa and IFNg.

PBMC form 40 study participants collected at 3 different time points (indicated below) were stimulated over night with SEB. CD4$^+$ T cells were identified by surface and intracellular cytokine staining as described in Supp. Figure 1. Responses were measured by flow cytometry and examined for IL-2, TNFa and IFNg cytokine expression, represented as a proportion of total memory CD4$^+$ T cells (CD45RA$^-$). Each symbol represents a single participants (10 for each of the four trial groups indicated on top), blue lines connected measurements for 3 different time points during vaccine trial, indicated at the bottom (day 0 was assessed only for group Alum/50 and GLA-SE 5/50)



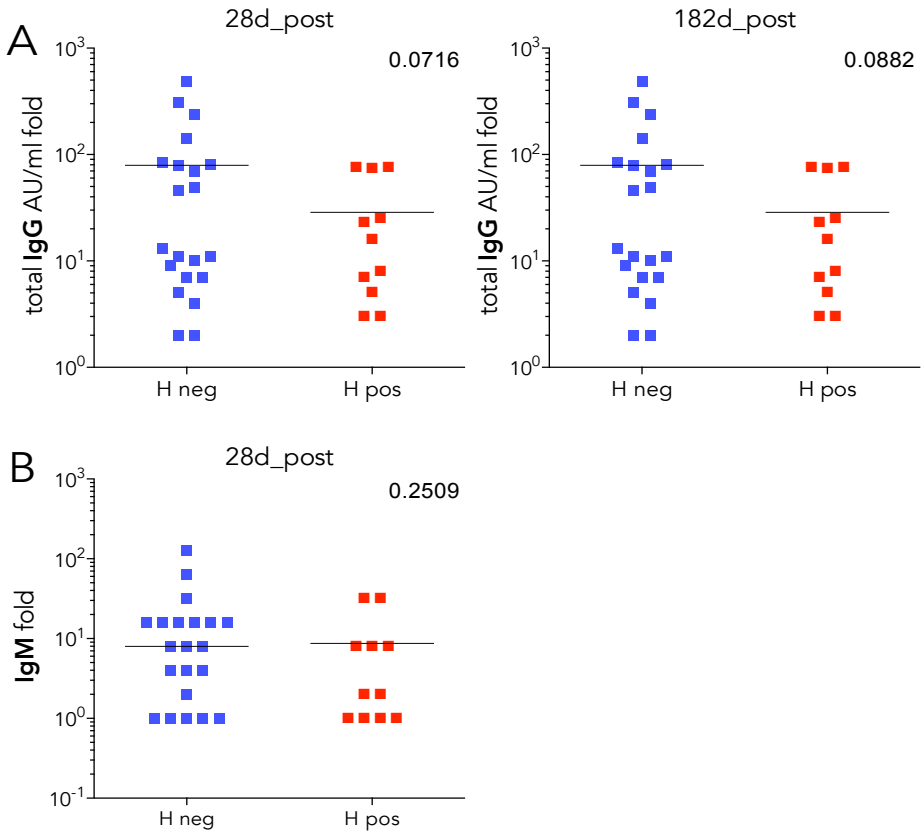
Supp. Figure 3. Longitudinal evaluation of P27A-specific CD8$^+$ T cells expressing IL-2, TNFa and IFNg.

PBMC from 40 study participants collected at 3 different time points (indicated at the bottom; day 0 was assessed only for group Alum/50 and GLA-SE 5/50) were stimulated over night with P27A. CD8$^+$ T cells were identified by surface and intracellular cytokine staining as described in Supp.Fig.1. Responses were measured by flow cytometry and evaluated for IL-2, TNFa and IFNg cytokine expression, represented as a proportion (%) of the memory CD4$^+$ T-cell subset (CD45RA$^-$). Each symbol represents a single participant (10 for each of the four trial groups indicated on top), blue lines (P27A vaccines) and red lines (placebo participants) connect measurements at different time points during vaccine trial.



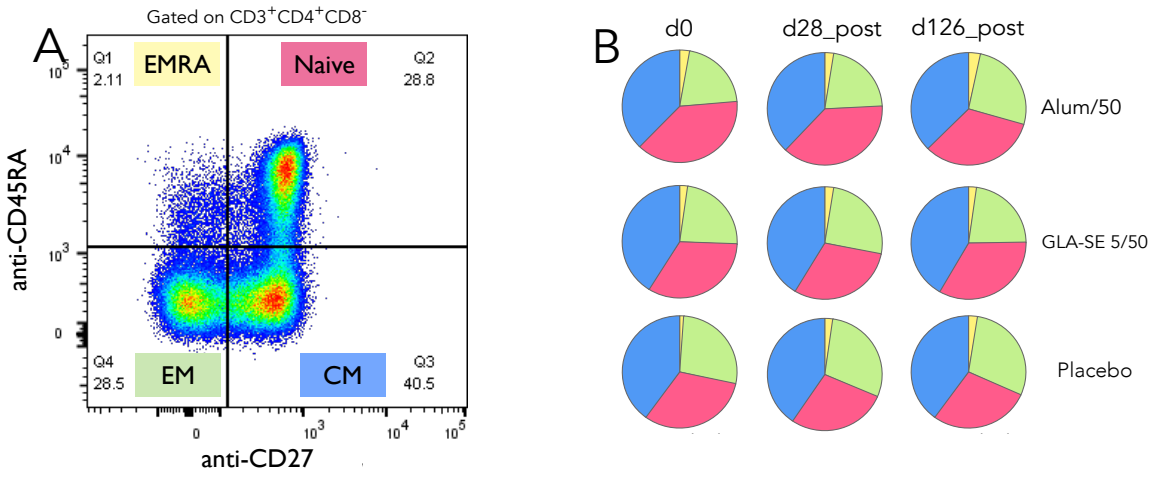
Supp. Figure 4. Correlation analysis between P27A-specific CD4$^+$ T-cell frequency and IgG and IgM titers after vaccination

CD4$^+$ T-cell frequencies were detected by coexpression of IL2$^+$ TNFa$^+$ and represented as a proportion of the total memory CD4$^+$ T cells (CD45RA$^-$). Cellular and humoral responses were evaluated at 28 days post 3rd vaccination. Data on antibody titers for total IgG (A) and IgM (B) was kindly provided by Steiner-Monard et al.[1]. Correlation analysis was performed with Prism6, color-coded p-values are shown for each plot.



Supp. Figure 5. Comparison of P27A-specific IgG and IgM responses in trial participants positive and negative for helminth infection

Total IgG (A) and IgM (B) were measured at baseline, 28 days post and 182 days post 3rd vaccination (data provided by Steiner-Monard et al. [1]). Each symbol represents a vaccine participant (32 total) positive (red) or negative (blue) for helminthes. Responses are presented as fold-increase to baseline levels, middle lines represent medians. Graphical representation and statistical analysis were performed with Prism6; differences (p-values are indicated on the plots) were calculated by unpaired t test with Welch's correction.



Supp. Figure 6. Comparison of Memory phenotype of total CD4+ T cells

Frequency of $CD3^+CD4^+$ T-cell cytokine responses were measured by flow cytometry in 20 participants (1 placebo and 2 vaccine groups, indicated on the graph) after stimulation with P27A peptide. (**A)** Representative dotplot, from a single participant at d28 post-vaccination, $CD4^+$ memory phenotypes were identified on CD27 and CD45RA gating. (**B**) SPICE analysis as pie charts representing the median proportions of memory subsets, after PBMC stimulation with P27A; measurement at 3 different time points as indicated on top

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Chapter 4

The adjuvant GLA-SE promotes human Tfh cell expansion and emergence of public TCR clonotypes

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This chapter contains the following manuscript:

Danika L. Hill, Wim Pierson, Daniel J. Bolland, **Catherine Mkindi**, Edward J. Carr, Peter Chovanec, Jiong Wang, Sophie Houard, Steven W. Wingett, Regine Audran, Said A. Jongo, Kassim Kamaka, Martin Zand, Francois Spertini, Claudia Daubenberger, Anne E. Corcoran, Michelle A. Linterman
The adjuvant GLA-SE promotes human Tfh cell expansion and emergence of public TCR clonotypes
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The adjuvant GLA-SE promotes human Tfh cell expansion and emergence of public TCR clonotypes

Authors: Danika L. Hill[1]†, Wim Pierson[1]†, Daniel J. Bolland[1], Catherine Mkindi[2], Edward J. Carr[1], Peter Chovanec[1], Jiong Wang[3], Sophie Houard[4], Steven W. Wingett[5], Regine Audran[6], Said A. Jongo[2], Kassim Kamaka[2], Martin Zand[3], Francois Spertini[6], Claudia Daubenberger[7,8], Anne E. Corcoran[1], Michelle A. Linterman[1*]

Affiliations:

[1] Laboratory of Lymphocyte Signalling and Development, Babraham Institute, Cambridge, UK.

[2] Ifakara Health Institute (IHI), Bagamoyo, Tanzania.

[3] Division of Nephrology, Department of Medicine and Clinical and Translational Science Institute, University of Rochester Medical Center, Rochester NY, USA.

[4] European Vaccine Initiative (EVI), Heidelberg, Germany.

[5] Babraham Bioinformatics Facility, Babraham Institute, Cambridge, UK.

[6] Division of Immunology and Allergy, CHUV, Lausanne, Switzerland.

[7] Swiss Tropical and Public Health Institute, Basel, Switzerland.

[8] University of Basel, Switzerland.

†These authors contributed equally to this work

*Correspondence to Michelle Linterman (michelle.linterman@babraham.ac.uk, ORCID: 0000-0001-6047-1996). Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Cambridge, UK.

Short title: The adjuvant GLA-SE enhances human Tfh responses

One Sentence Summary: The experimental adjuvant GLA-SE induces the expansion of human T follicular helper cells with common TCR clonotypes after vaccination, indicating this adjuvant is a rational choice to enhance long-lived humoral immunity in humans.

Abstract

Long-lasting humoral immunity is the basis of successful vaccination. This immunity derives from the germinal centre response, where T follicular helper (Tfh) cells support the production of long-lived plasma cells and memory B cells that provide protection against subsequent infection. Despite our understanding of the central role of Tfh cells in vaccine responses, there is currently no validated way to enhance the production or function of these cells in humans. Here, we show that the adjuvant GLA-SE enhances the extrafollicular plasmablast response and the magnitude of the circulating Tfh cell response in healthy Tanzanian volunteers inoculated with an experimental malaria vaccine. This correlates with increased antibody titres after vaccination and the emergence of public TCRβ clonotypes in cTfh cells in different individuals. This study demonstrates that altering vaccine adjuvants is a rational approach for enhancing the Tfh cell response in humans, thereby supporting the long-lived humoral immunity that is required for effective vaccines.

Introduction

Vaccination is one of the most powerful interventions for reducing the disability and death caused by infectious disease worldwide (*1*). Despite its success there are still numerous pathogens that are not controlled by current vaccination strategies, including HIV and *Plasmodium spp*, the parasites that cause malaria (*2, 3*). The majority of effective vaccines work by stimulating the production of antibodies that bind the surface of the pathogen to either block the pathogen's ability to establish an infection, or recruit and stimulate other immune cells, resulting in the pathogen's destruction. Antibody production after vaccination can occur from one of two cellular pathways, which are separated in time and anatomical space. The first wave of antibody production comes from the extrafollicular plasmablast response, in which short-lived antibody secreting cells (ASC) produce immunoglobulin for a few days, then die *in situ* in secondary lymphoid tissues (*4*). The second source of ASC is the germinal centre (GC) response. The GC is a specialised microenvironment that forms in secondary lymphoid tissues after immunisation, where antigen-activated B cells clonally expand within the B cell follicle and undergo somatic hypermutation (SHM) of their immunoglobulin loci. The process of SHM, followed by selection of GC B cells, results in the emergence of long-lived, high affinity, antibody-secreting plasma cells and memory B cells that are able to provide protection against subsequent infection (*5*). The GC response is absolutely dependent on a specialised subset of $CD4^+$ T cells, T follicular helper (Tfh) cells, that provide growth and differentiation signals to GC B cells. Tfh cells mediate the positive selection of high-affinity B cell clones in the GC and thereby determine which B cells exit the GC as plasma cells or memory B cells (*5*). Because Tfh cells are key determinants of the long-lived humoral immunity that arises from the GC, they represent an exciting therapeutic target through which vaccine strategies could be improved (*6*).

Adjuvants are an attractive way to improve vaccine responses in humans. This is reflected in the licencing of four new adjuvants in recent years: MF59, AS01, AS02 and AS04 (*7*). Nevertheless, the number of adjuvants that are in use in current vaccines is still very limited, as is our understanding of how adjuvants boost a specific cellular immune response in humans. While studies in animals indicate that adjuvants are a logical way to boost the GC and Tfh cell response (*8-10*), translational work is needed to determine if using novel adjuvants is able to boost GC and Tfh cell responses in humans. Despite Tfh cells being central for long-term humoral immunity, most human vaccine studies have not included these cells in their analysis, rather focusing on

cytokine-producing $CD4^+$ T cells (*11*), an approach that does not accurately capture vaccine-reactive Tfh cells (*12*). The tendency to omit the analysis of Tfh cells in human vaccination studies may be due to difficulty in studying these cellular responses. Tfh cells are located in secondary lymphoid tissue, which is not easily sampled during vaccine trials. To circumvent this issue, a population of circulating Tfh-like (cTfh) cells that are found in the blood and phenotypically and functionally resemble lymphoid tissue Tfh cells, can be used as a biomarker of ongoing Tfh cell responses (*13-18*). Here, we have developed a method to study the cellular and transcriptomic cTfh cell response in humans after vaccination, using influenza as a model vaccine. We have then applied this method to determine whether the toll-like receptor (TLR)4-stimulating adjuvant Glucopyranosyl Lipid Adjuvant-Stable Emulsion (GLA-SE, developed by the Infectious Disease Research Institute, Seattle, USA) can augment cTfh cell responses. In an experimental malaria vaccine phase 1b clinical trial in Tanzania, we show that a GLA-SE-formulated vaccine is superior to one formulated with Aluminium Hydroxide (Alum) in the induction of both the extrafollicular antibody response and the formation of cTfh cells. Whilst the different adjuvants did not induce differential gene expression profiles in cTfh cells, multiple GLA-SE vaccinated individuals had cTfh cells expressing public TCRβ clonotypes, indicating that GLA-SE may support the recruitment of T cells bearing specific TCRs to the Tfh cell compartment or promote their subsequent expansion. This demonstrates that experimental vaccine adjuvants offer a viable strategy to enhance Tfh responses and long-lived humoral immunity in humans.

Results

cTfh cells clonally expand after vaccination

The aim of our study was to determine whether the adjuvant GLA-SE promotes a cTfh cell response in humans. The location of the GC response, within secondary lymphoid tissues, is one of the major barriers to understanding how different vaccines, or their adjuvants, affect GC biology in humans. For this reason, circulating cells that can act as biomarkers of the GC response are an area of intense interest. In particular, it is well established that there is a population of blood $CXCR5^+CD4^+$ cells that expands after vaccination (*14, 17, 18*), and that these cTfh cells phenotypically and functionally resemble lymphoid tissue Tfh cells (*6*). There is considerable heterogeneity in the $CXCR5^+CD4^+$ T cell compartment, and multiple subsets have been described within this population (*19*). Because of this heterogeneity, we first sought to identify the subpopulation of $CXCR5^+CD4^+$ cells that were activated by vaccination to enable us to refine our analyses. Inducible COStimulator (ICOS) and CD38 are cell surface receptors expressed by tonsillar Tfh cells (Supp. Fig. 1), suggesting that cTfh cells expressing these markers may represent a circulating surrogate of activated lymphoid tissue Tfh cells. We first tested expression of these cell surface receptors after seasonal subunit influenza vaccination, a routine inoculation in which cTfh cell expansion has been well described (*17*). We observed an expansion of $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cells in healthy UK volunteers (n=41) seven days after vaccination (Fig. 1A, B), the peak of the cTfh cell response (*17, 20*). This expansion of $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cells correlated positively with the increase in influenza-specific antibodies seven days after vaccination (Fig. 1C, D). These data indicate that $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cells are a good biomarker of tissue Tfh cells.

To gain insight into how vaccines qualitatively alter the Tfh cell response, we performed RNA sequencing of 200 $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cells immediately prior to, and seven days after, influenza vaccination in four individuals. Differential gene expression analysis of these cTfh cells indicated upregulation of *CXCR3*, and downregulation of *CCR6* transcripts in cTfh cells isolated seven days after vaccination (Fig. 1E). This difference in mRNA was also reflected at the protein level, with the proportion of $CXCR3^+$ cTfh cells increasing after influenza vaccination, with a reciprocal decrease in $CCR6^+$ cTfh cells (Fig. 1F). This is consistent with previous work demonstrating that influenza vaccination results in a Th1-skewing of the Tfh cell population (*17*). Transcriptomic analysis also enabled an assessment of the T cell receptor (TCR) usage within cTfh cells. Assessment of the nucleotide sequence encoded by the CDR3 region of the TCRβ chain revealed ~160 clonotypes per person that were unique at the amino acid level prior to vaccination (Fig. 1G), a number that is consistent with the 200 cells sequenced. After vaccination, the number of unique clonotypes identified per person was reduced, consistent with clonal expansion after vaccination (Fig. 1G). This reduction in diversity was driven by the expansion of a handful of clones: prior to vaccination, most clonotypes represented less than 2% of the total CDR3 reads, whereas seven days after vaccination, 6-10 unique CDR3 sequences per individual were present at higher frequencies (Fig. 1H). This indicates that the cTfh cell response to influenza vaccination is dominated by the expansion of a small number of T cell clones. The flow cytometric profiling and RNA sequencing identifies the $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cell population as a biomarker of Tfh cell responses, and provides a quantitative and qualitative way to assess the biology of these cells during human vaccine trials.

GLA-SE promotes long-lasting IgG responses in humans

The P27A antigen is a 104 amino acid peptide of the PFF065c protein from the malaria-causing protozoan *Plasmodium falciparum*. Because seroreactivity to P27A is high in populations naturally exposed to malaria, and anti-P27A IgG can inhibit parasite growth *in vitro* (*21*), P27A offers a rational vaccine target to prevent malaria disease. In a phase Ib clinical trial in malaria pre-exposed adults from Tanzania, the P27A peptide (50 microgram) was formulated with one of two adjuvants: the widely used Alum (Alhydrogel®), or the experimental adjuvant GLA-SE (5 microgram), a synthetic TLR4 agonist, enabling side-by side comparisons (*22*). The volunteers were given three doses of the vaccine, each one month apart, with blood samples taken for serology at multiple time points over a 34-week period (*22*) (sampling schedule in Fig. 2A). We first determined the antibody response to each vaccine and found that P27A induced a higher anti-P27A IgG response when in GLA-SE than when in Alum (Fig. 2B). Moreover, in contrast to Alum, a significant increase in serum anti-P27A IgG titres was observed 28 days after the third vaccination in the GLA-SE group (Fig. 2C), a time when vaccine specific antibodies are derived from both extrafollicular and GC-derived ASC. Despite this increase in antibody titre in the GLA-SE adjuvanted group, there was not a significant increase in the frequency of circulating $CD38^{++}CD20^-CD19^+$ ASC seven days after the third vaccination in either group relative to baseline (Fig 2D, E).

GLA-SE stimulates the extrafollicular ASC response

Antibody is derived from two cellular sources after vaccination. The first is an extrafollicular antibody response from ASC that typically have not undergone SHM and die a few days after their formation, generating a short burst of antibody. The second source of antibody is ASC that

are generated in the GC response. The GC-derived ASC have B cell receptors (BCRs) that have undergone somatic mutation and bind antigen with increased affinity. After differentiation in the GC, ASC home to the bone marrow where they persist. To investigate the characteristics of vaccine-induced ASC, we performed V(D)J RNA sequencing of antibody repertoires in blood ASC from all individuals seven days after the third vaccination. The analysis revealed that individuals with high titre antibody responses (anti-P27A IgG >500 AU) had greater clonal expansion of ASC seven days after the third vaccination (Fig. 3A, B and Supp. Fig. 2). Strikingly, these responding individuals had an enrichment for clonotypes with fewer somatic mutations in their variable region (including CDR1, FR2 and CDR2) seven days after the third vaccination, compared to the blood sample obtained prior to vaccination (Fig. 3C-E). This suggests that high titre responders are initiating an extrafollicular antibody response more robustly than low responders. This decreased frequency of mutations occurred more frequently in GLA-SE vaccinated individuals than in those that received the Alum-formulated peptide (Fig. 3F), suggesting that GLA-SE stimulates extrafollicular ASC responses more efficiently than Alum.

GLA-SE enhances cTfh cell responses

Early antibody production after vaccination provides short term protection against subsequent infection, whereas long-lived GC-derived ASC can produce protective antibodies for years. Tfh cells regulate GC size and are essential for the emergence of long-lived plasma cells and memory B cells from the GC, therefore boosting Tfh cell number is a rational strategy to enhance vaccine responses (*6*). In this trial, only volunteers receiving the vaccine containing GLA-SE had an expansion of the $ICOS^+CD38^+CXCR5^+PD\text{-}1^+$ cTfh cell population seven days after the third vaccination (Fig. 4A-C), and the increase in cTfh cell frequency correlated positively with an increase in anti-P27A IgG 28 days after the third vaccination (Fig. 4D). This demonstrates that altering the vaccine adjuvant is a rational approach to enhance the magnitude of the cTfh cell response in humans. These data show that GLA-SE can provoke a robust immune response in the context of the very high pre-existing antibody titres induced by the previous two P27A vaccinations. This finding is critical in cases where pre-existing serological immunity may impact on vaccine responses, such as in malaria endemic regions.

GLA-SE promotes shared TCRβ clonotypes in cTfh cells

As GLA-SE quantitatively altered the cTfh cell response in humans, we wanted to determine if it also has a different qualitative effect compared to Alum on this cell population. As established in our UK influenza vaccination study (Fig. 1), we performed total RNA-sequencing on 150-200 $ICOS^+CD38^+$ cTfh cells per person prior to vaccination, and seven (d63) and 28 (d84) days after the third vaccination. There was no significant alteration in the cTfh cell transcriptome between adjuvant groups (Alum n=7, GLA-SE n=8) seven days after the third vaccination (Fig. 4E). This suggests that while this adjuvant can impact the magnitude of the Tfh cell response, it is not likely to alter Tfh cell function. To determine whether adjuvant had an effect on the clonality of the cTfh response, we examined TCRβ sequences in our RNA sequencing dataset. Within the Alum group, we identified ten TCRβ clonotype sequences that were in common between samples (five distinct clones, each present in two separate samples), however these were typically only shared within individuals at different time points (Fig. 4F). In the GLA-SE-vaccinated group, of the nine distinct clones that were common between two different samples, six were public clonotypes, shared between different individuals (Fig. 4G). Strikingly, these

shared clones were not typically present prior to vaccination, or twenty-eight days after the third vaccination, but rather were found at the peak of the Tfh response in five out of the eight GLA-SE-vaccinated individuals (seven days after the third vaccination: Fig. 4H, G, Supplementary Table 1). These data indicate that GLA-SE may promote the recruitment of T cells bearing common TCR clonotypes into the cTfh cell compartment or promote their subsequent expansion.

Discussion

Adjuvants are currently the most tractable way of altering how the human immune system responds to vaccination. The majority of vaccines on the market provide protection against subsequent infection by generating long-lived antibody responses. Therefore, adjuvants that enhance the magnitude of the GC response are a rational approach to enhancing humoral immunity. The size and the quality of the GC response depends on the number and function of Tfh cells that are induced by vaccination. While animal studies clearly show that different adjuvants can enhance the Tfh cell response (*8, 9*), these findings have not yet been translated into *in vivo* human studies. Here, we show, consistent with work on mice (*8*), that one of the next generation adjuvants can be used to enhance the magnitude of the Tfh cell response in humans. The GLA-SE adjuvant not only increases Tfh cell number, which will enhance the GC response, but it also enhances the extrafollicular ASC response. The stimulation of both these ASC sources in parallel maximises the production of vaccine-specific antibodies. This demonstrates that GLA-SE can be used to make vaccines that potentiate Tfh cell and antibody responses in humans.

Rapid antibody production after vaccination is typically provided by the extrafollicular plasmablast response. These cells normally do not exhibit SHM in their antibody variable (V) genes because they do not derive from GCs. In this study, individuals who had detectable ASC expansion seven days after the third vaccination had fewer mutations in the FR1-FR3 regions of the antibody V genes, indicating these ASC are probably of extrafollicular origin. This is consistent with mouse studies showing that GLA-SE augments the formation of early ASC in the draining lymph node (*8*). Although these ASC (obtained 7 days after the third vaccination) contained a lower frequency of mutations than ASC isolated before vaccination, they still had some mutations present, indicating that the precursors of these ASC have probably previously participated in the GC response. In this study, malaria pre-exposed volunteers received three P27A vaccinations at monthly intervals. Thus, it is likely that the extrafollicular ASC sequenced at seven days after the final vaccination differentiated from memory B cells with low levels of mutation derived either from a GC reaction during previous P27A immunisation, or from natural malaria infection. These memory B cell derived ASC would be expected to have fewer somatic mutations than GC-derived ASC because memory B cells emerge from the GC before long-lived plasma cells (*23*).

Analysis of the transcriptome of cTfh cells has the potential to yield information about how vaccination changes the phenotype of Tfh cells. We observed changes in the transcriptome of these cells after seasonal influenza vaccination in UK adults. These included alterations in CXCR3 and CCR6 expression which are chemokine receptors known to be affected by this vaccination (*17*), demonstrating that RNA sequencing is a viable approach to detect phenotypic changes in cTfh cells induced by vaccination. However, our study detected few differences in the transcriptomes of cTfh cells induced by Alum compared to GLA-SE, suggesting that the main

difference in Tfh cells driven by adjuvants is quantitative rather than qualitative. The difference may come about from the distinct mechanisms of action of the two adjuvants – while GLA-SE is thought to exert its effect on subcapsular sinus macrophages via IL-18 (*8*), Alum does not act through TLRs at all but rather via the inflammasome (*24*). Our results indicate that despite different mechanisms of innate immune cell activation by these two adjuvants, they do not affect innate immune cells in a way that results in a qualitatively different Tfh cell response. Importantly, transcriptomic analysis revealed that GLA-SE also promotes the emergence of common TCRβ clonotypes that are shared between different individuals. The emergence of so-called public clonotypes is informative for vaccine design as it indicates that there may be a common epitope that could be used in vaccine formulations to enhance responses in numerous individuals. The recent discoveries of public antibody clonotypes has revolutionised whole-parasite malaria vaccine strategies (*25, 26*) and here we extend this to the identification of public Tfh cell TCRβ clonotypes induced after vaccination. The identification of these common clonotypes in cTfh cells from participants that received the GLA-SE formulated vaccine could simply be a consequence of the greater magnitude of the response, indicating that more T cell clones are recruited into the response. Nevertheless, using the knowledge of key antigenic peptides to which multiple individuals respond, combined with adjuvants that can better recruit these T cells into the response, is a rational way to enhance vaccine responses at the population level. Together, this study demonstrates that adjuvant should be a key consideration in vaccine design to maximise the generation of protective T-cell dependent humoral immunity in humans.

Materials and methods

Study Design

The main research objective of this study was to characterise the cTfh and ASC cell responses to vaccination in humans at the cellular and molecular level. To that end, two related studies were performed: 1) Peripheral blood was tested from 41 healthy UK adults (18-98 years of age), who were vaccinated with the trivalent influenza vaccine (2016); and 2) 15 healthy HIV-negative Tanzanian male adults (18-45 years old) with minimal malaria exposure (urban Dar-es-Salaam), who were vaccinated with 50μg of P27A peptide formulated in either Alhydrogel® (n =7) or 5μg GLA-SE (n = 8) (*22*). Circulating Tfh cells and ASC were identified by flow cytometry in samples before and after vaccination and were flow-sorted for mRNA or BCR repertoire sequencing, respectively. Researchers remained blinded to adjuvant group throughout sample processing and data acquisition. All human blood and tissue was collected in accordance with the latest revision of the Declaration of Helsinki and the Guidelines for Good Clinical Practice (ICH-GCP). The seasonal UK influenza vaccination cohort was collected with UK local research ethics committee approval (REC reference 14/SC/1077), using the facilities of the Cambridge Bioresource (REC reference 04/Q0108/44). The P27A vaccine phase 1b trial (ClinicalTrials.gov Identifier: NCT01949909, Pan African Clinical Trial Registry identifier:PACTR201310000683408) was conducted with approval from the Tanzanian Food and Drug Administration (TFDA, Dar-es-Salaam, TFDA13/CTR/004/03), National Institute for Medical Research (NIMR, Dar-es-Salaam, NIMR/HQ/R8a/Vol.IX/1742), Swiss Agency for Therapeutic Products (Swissmedic, Bern, Switzerland, ref.#2013DR1165), and ethical review boards at Ifakara Health Institute and the University of Lausanne. Use of P27A trial samples in the UK was approved by the UK Health Research Authority (REC reference 17/EE/0063) and

Babraham Institute Human Ethics Committee. Tonsil tissues were used to compare the phenotypes of circulating and germinal centre resident Tfh cells and were collected from UK adults undergoing tonsillectomy, under ethical approval from UK Health Research Authority (REC reference 16/LO/0453), at Cambridge University Hospitals, and processed at the Babraham Institute. Written informed consent was received from all volunteers.

Isolation and sequencing of cTfh cells and ASC

Cryopreserved peripheral blood mononuclear cells were thawed and rested for 1 hour at 37℃. Cell types were pre-enriched using MagniSort® CD19 Positive Selection followed by CD4 memory T cell enrichment (eBioscience). Fc receptors on all cells were blocked using human IgG, followed by staining with panels outlined in Supplementary Table 2 and separation on BD Aria Fusion or Influx cell sorters. A dump channel consisting of viability dye and antibodies to CD14, CD16, and either CD19 or CD3 was used to exclude unwanted cell types from cTfh cell and ASC sorts, respectively. mRNA was isolated from sorted circulating Tfh cells ($CD4^+CD45RA^-CXCR5^+PD1^+ICOS^+CD38^+$ cells) using the Smart-seqV4 Ultra-low input RNA kit (Takara-Clontech) and sorting 200 cells directly into lysis buffer. cDNA libraries were subsequently generated using the NexteraXT DNA kit (Illumina), followed by sequencing on the Illumina Hi-Seq 2000 with approximately 50 million 100bp single-end reads per sample. ASC ($CD19^+IgD^-CD27^+CD71^+CD20^-CD38^{++}$) were sorted into RNAlater (500-10,000 cells), and RNA isolated using RNeasy Micro kit (Qiagen). In all samples for which sufficient RNA was extracted, immunoglobulin heavy (IgG, IgM) chains were amplified using 5'RACE with unique molecular identifiers (UMI) as previously reported (*27*) with some modifications (D. Chudakov, personal communication), using Q5® High-Fidelity DNA Polymerase (New England BioLabs) and sequencing on the Illumina MiSeq (340x280bp paired-end).

Serology

IgG to influenza HA proteins was measured before and after vaccination by Luminex using magnetic beads coated with full length recombinant haemagglutinin proteins from A/California/07/2009 (Cali09) and B/Brisbane/60/2008 (Bris08), as previously reported (*28*). IgG to P27A was measured by ELISA as previously reported (*22*). Titres are represented as arbitrary units per mL and, where indicated, pre-existing IgG titres were subtracted to calculate vaccination-induced IgG responses.

Differential gene expression analysis and V(D)J sequencing

cTfh cell transcriptomic analysis was performed using the Seqmonk software package (Babraham Institute, https://github.com/s-andrews/SeqMonk/), after alignment of reads to the reference human genome GRCh38 using hisat2 (*29*). Differentially expressed genes were determined by DESeq2 (adjusted p-value cut-off $p<0.05$) (*30*). TCRβ clonotypes were called from adaptor-trimmed RNA sequencing fastq files, using mixcr (version 2.1.9) (*31*) run in 'RNA-Seq mode' (mixcr align -p rna-seq -c TRB -s hsa -OallowPartialAlignments=true) with rescuing of partial alignments, set to collate clonotypes at the amino acid level, rather than the nucleotide level and requiring >15 reads to identify a clonotype. Analysis of the V(D)J sequencing reads of ASCs was done using Vdjtools (version 1.1.7) (*32*) was used for summarizing and visualizing results. Immunoglobulin heavy chain sequences were analysed using 'mixcr' and 'IMGT high V-quest'(*33*).

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Figure 1

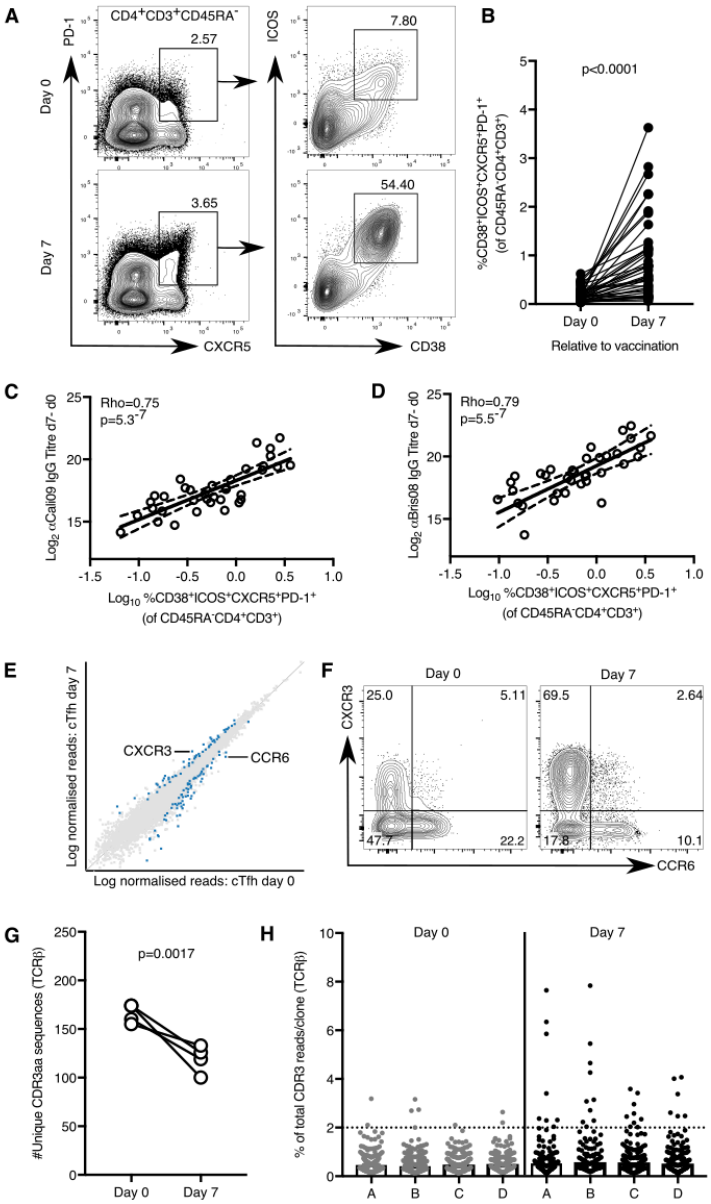


Figure 1. ICOS and CD38 mark cTfh cells following seasonal influenza vaccination.
Flow cytometric contour plots **(A)** and quantification **(B)** of the frequency of CD38$^+$ICOS$^+$CXCR5$^+$PD-1$^+$ cells amongst CD45RA$^-$CD4$^+$CD3$^+$ cells in the peripheral blood of healthy UK donors at days zero and seven relative to seasonal influenza vaccination, n = 41; in (B), each symbol represents a volunteer, and an individual donor is connected by a line at the two time points. Correlation of the frequency of CD38$^+$ICOS$^+$CXCR5$^+$PD-1$^+$ cTfh cells seven days after vaccination with the change in antibody titre of anti-Cal09 IgG **(C)** and anti-Bris08 IgG **(D)** seven days after vaccination, statistical analysis by Spearman's correlation (Rho = coefficient). **(E)** Scatterplot of whole transcriptome RNAseq data comparing expression of all genes expressed in CD38$^+$ICOS$^+$CXCR5$^+$PD-1$^+$ cells prior to, and seven days following, seasonal influenza vaccination. Differentially expressed genes (DEseq2) are indicated in blue. **(F)** Flow cytometric contour plots of CXCR3 and CCR6 expression on ICOS$^+$CXCR5$^+$PD-1$^+$ Tfh cells at the indicated time points relative to vaccination, a representative example of n=36 individuals **(G).** The number of unique TCRβ CDR3 amino acid sequences identified in RNA sequencing libraries from CD38$^+$ICOS$^+$CXCR5$^+$PD-1$^+$ cTfh cells from four volunteers were analysed at days zero and seven relative to vaccination. **(H)** Quantification of the percentage of total TCRβ CDR3 reads per unique clone: each symbol represents a unique CDR3 amino acid sequence. In (B) and (G), p-values are generated with a paired Student's t-test. In (H), each letter on the x-axis corresponds to a different volunteer.

Figure 2

A

	Day 0	Day 28	Day 56	Day 63	Day 84	Day 182	Day 238
Vaccination	Vac 1	Vac 2	Vac 3				
Anti-P27A IgG titre	X	X	X		X	X	X
Ex vivo flow cytometry	X			X	X		

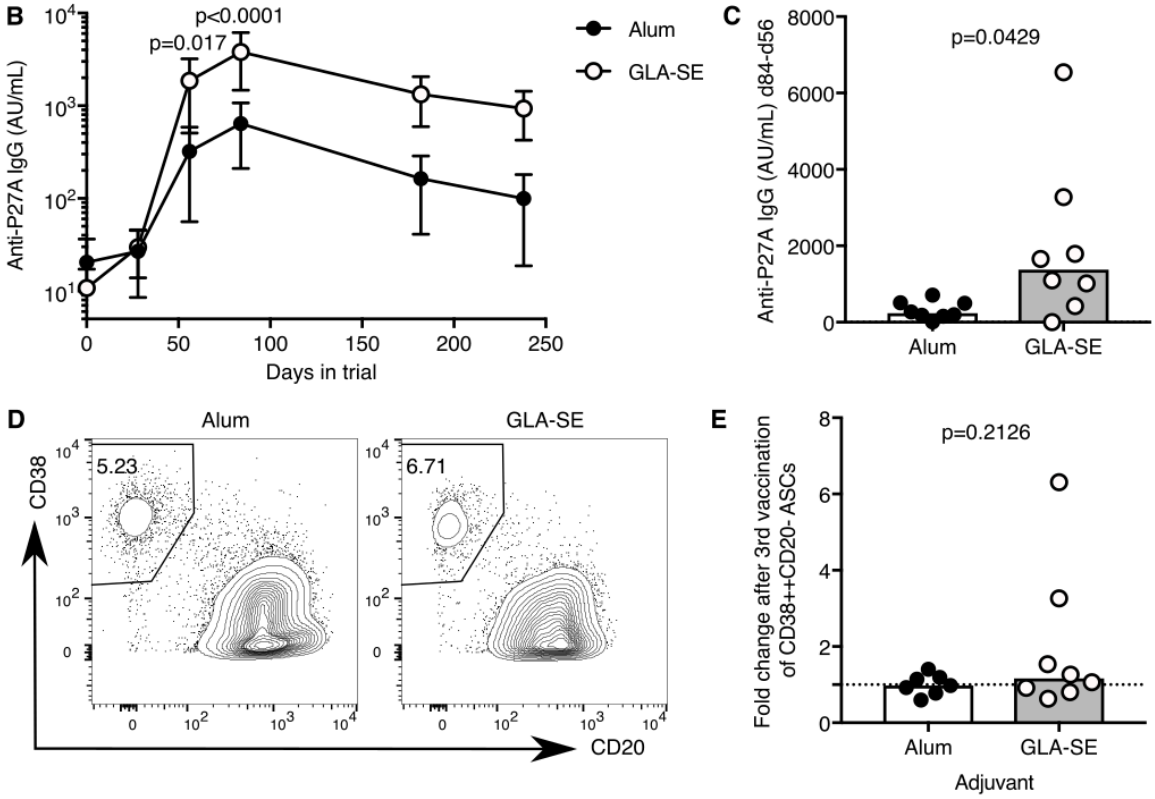


Figure 2. The adjuvant GLA-SE enhances antibody production but not early ASC expansion
(A) Vaccination and bleed schedule for the clinical trial. **(B)** Anti-P27A IgG antibody titres in volunteers vaccinated with 50μg P27A peptide in either Alum (White) or GLA-SE (Black); error bars represent the 95% confidence interval; p-values are calculated using a two-way ANOVA with Sidak's multiple comparisons test. **(C)** Change in anti-P27A IgG antibody titre 28 days after the third vaccination in the trial. Flow cytometric contour plots **(D)** and quantitation **(E)** of peripheral blood $CD38^{++}CD20^{-}$ cells of total $CD19^{+}$ cells seven days after the third vaccination. In (C) and (E) p-values are calculated using an unpaired t-test. Each symbol represents one individual: those that received Alum are in black, those that received GLA-SE are in white.

Figure 3

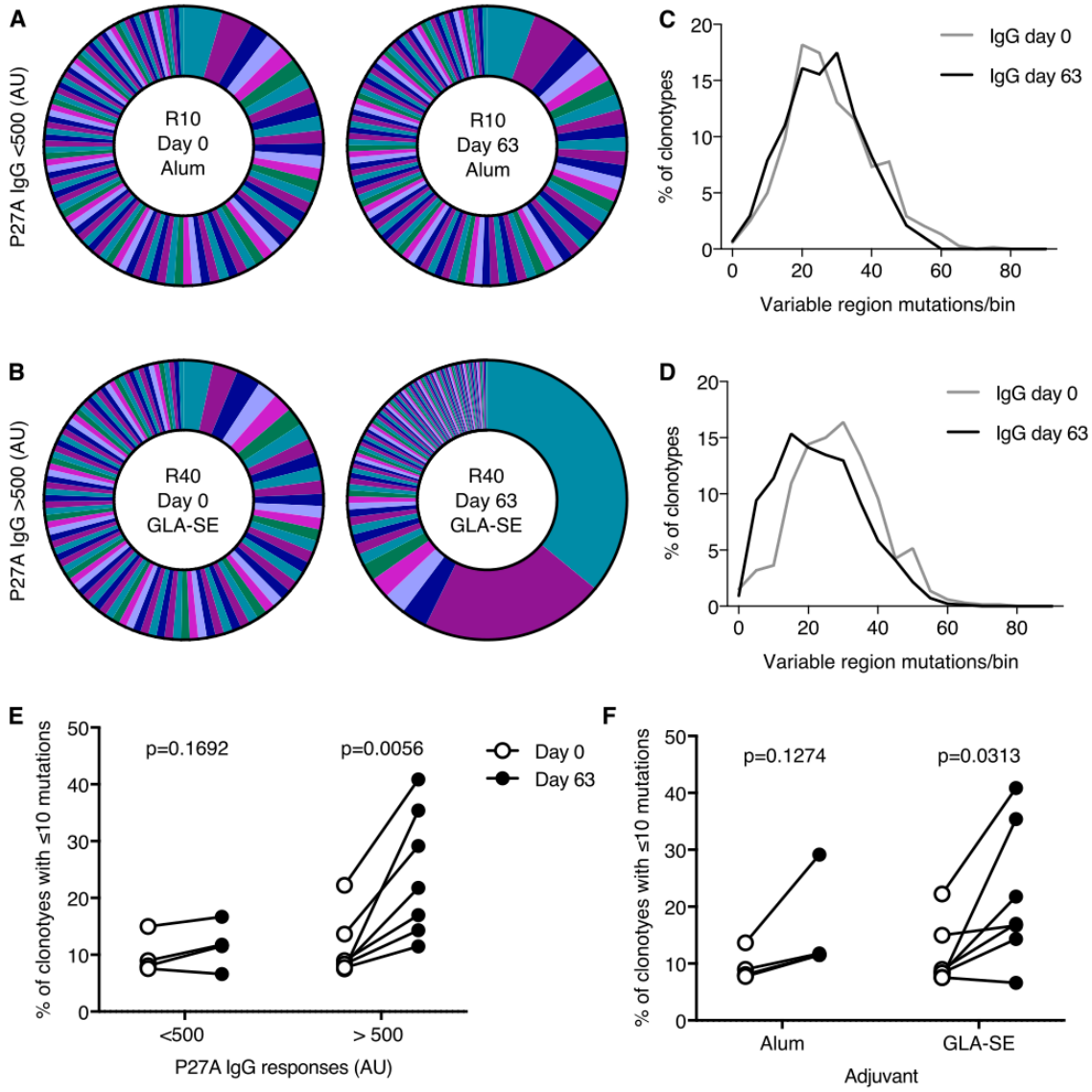


Figure 3. V(D)J sequencing shows low BCR mutation frequency in individuals with high titre antibody responses
Pie charts of the proportions of the 100 most abundant BCR clonotypes in $CD38^{++}CD20^{-}CD19^{+}$ ASC from **(A)** a representative individual that does not increase their anti-P27A IgG more than 500 AU after the third vaccination and **(B)** a representative individual that has a high anti-P27A titre after the third vaccination. Each segment of the pie chart represents a unique BCR clonotype. **(C)** and **(D)** Line graphs of the number of mutations in the V region of each clonotype (FR1-FR3, excluding CDR3, binned into 5 mutation bins) for the individuals shown in (A) and (B) respectively, at the indicated time points relative to vaccination. The percentage of clonotypes with ≤10 mutations in high and low antibody responders **(E)** and in the different adjuvant groups **(F)**. Each individual is connected with a line between their d0 and d63 sample; p-values are from a paired Student's t-test. Individual participants' data for all other samples that passed quality control are included as Supplementary Figure 2.

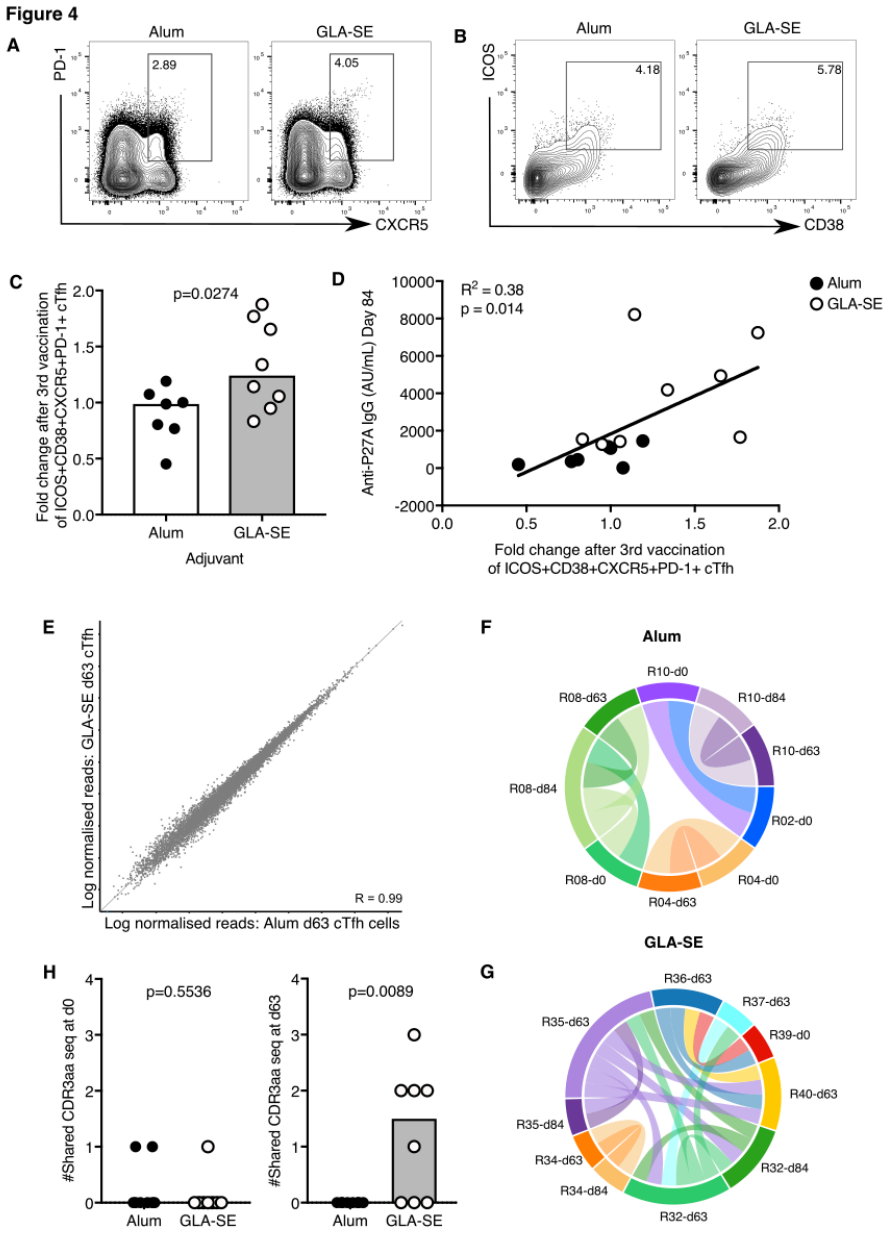
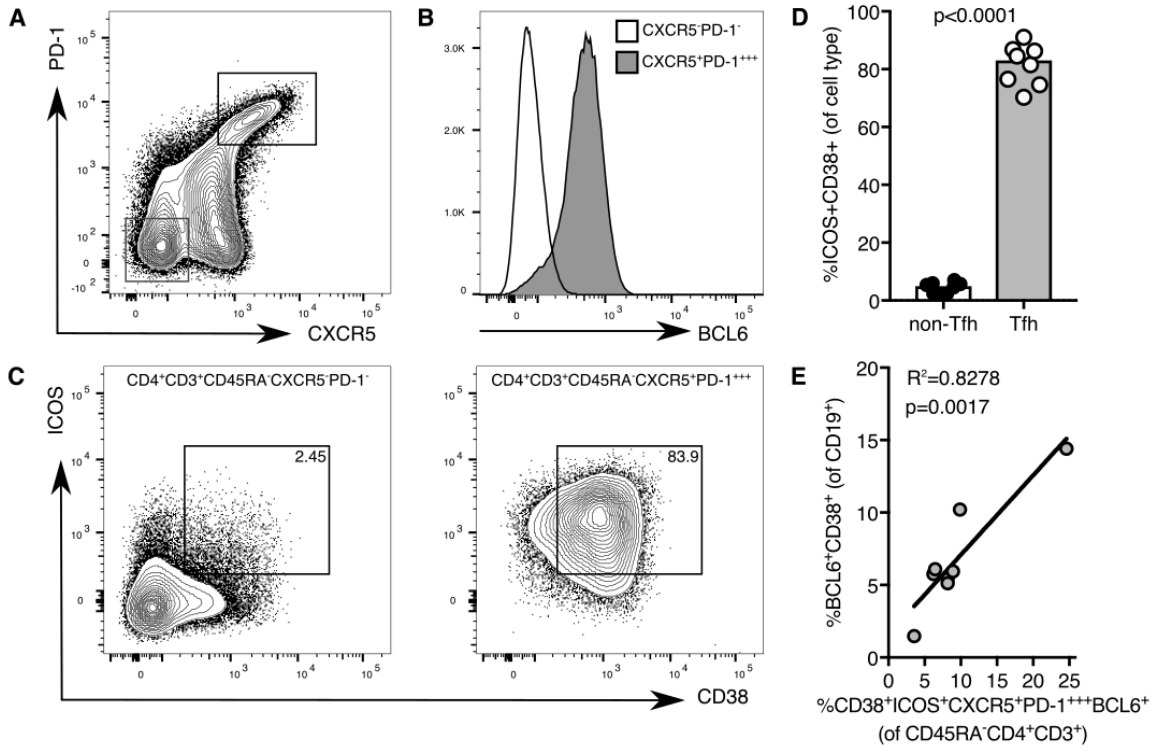
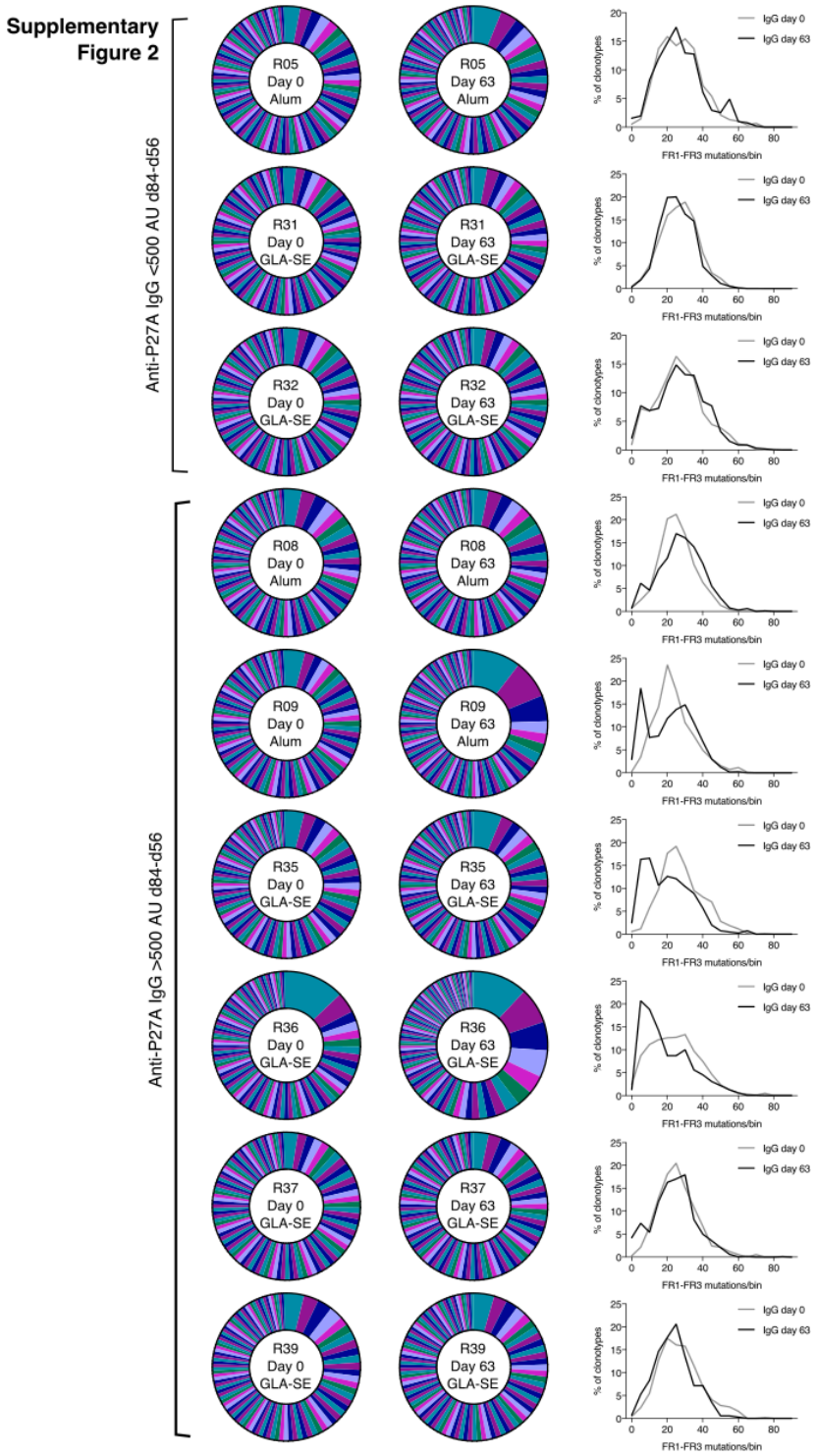


Figure 4. GLA-SE adjuvanted vaccines promote cTfh expansion and common TCR clonotype usage
Flow cytometric contour plots of **(A)** PD-1 and CXCR5 on total $CD45RA^{-}CD4^{+}CD3^{+}$ cells and **(B)** ICOS and CD38 on the population gated in (A) on peripheral blood cells from Tanzanian individuals seven days after the third P27A vaccination. **(C)** Fold change in the frequency of in $CD38^{+}ICOS^{+}CXCR5^{+}PD\text{-}1^{+}$ cTfh cells seven days after the third P27A vaccination. **(D)** Linear regression analysis of anti-P27A IgG antibody titre 28 days after the third vaccination and fold change in $CD38^{+}ICOS^{+}CXCR5^{+}PD\text{-}1^{+}$ cTfh cells seven days after the third vaccination. **(E)** Scatterplot of RNAseq data comparing all genes expressed in $CD38^{+}ICOS^{+}CXCR5^{+}PD\text{-}1^{+}$ cTfh cells seven days after the third P27A vaccination in either Alum (x-axis) or GLA-SE (y-axis) vaccinated groups. **(F)** and **(G)** Circos plots of shared TCRβ CDR3 amino acid sequences in $CD38^{+}ICOS^{+}CXCR5^{+}PD\text{-}1^{+}$ cTfh cells from donors who received the P27A vaccine in **(F)** Alum or **(G)** GLA-SE. **(H)** Quantification of the number of shared $CD38^{+}ICOS^{+}CXCR5^{+}PD\text{-}1^{+}$ cTfh TCRβ CDR3 amino acid sequences per donor either prior to vaccination (left, sharing between day 0 samples) or seven days after the third vaccination (right, sharing between day 63 samples). P-values are calculated using an unpaired t-test. Each symbol represents one individual; those that received Alum are in black, those that received GLA-SE are in white.

Supplementary Figure 1



Supplementary Figure 1. Tonsil Tfh cells express CD38 and ICOS
Flow cytometric identification of **(A)** $CXCR5^+PD\text{-}1^{+++}$ Tfh cells and $CXCR5^-PD\text{-}1^-$ non-Tfh cells amongst $CD45RA^-CD4^+CD3^+$ tonsil cells, and their expression of BCL6 **(B)**. Contour plots **(C)** and quantification **(D)** of ICOS and CD38 expression on tonsil non-Tfh (left) and Tfh cells (right). **(E)** Linear regression analysis of tonsil $BCL6^+CD38^+CD19^+$ germinal centre B cells and $BCL6^+CD38^+ICOS^+CXCR5^+PD\text{-}1^{+++}$ Tfh cells: each symbol represents one individual.



Supplementary Figure 2. V(D)J sequencing of circulating plasmablasts
Pie charts of the proportion of the 100 most abundant BCR clonotypes in $CD38^{++}CD20^{-}CD19^{+}$ ASC of individual P27A study participants at the indicated study time points. Each segment of the pie chart represents a unique BCR clonotype. The "R" number is the unique participant identifier. Line graphs report the number of mutations in the V region per clonotype (FR1-FR3, excluding CDR3, binned into 5 mutation bins) in each individual before vaccination and seven days after the third vaccination.

TRVB	*TRBJ*	*TRBC*	CDR3 aa sequence
TRBV10-3	*TRBJ1-1*	*TRBC1*	CAISEKKNTEAFF
TRBV12-3, TRBV12-4	*TRBJ1-1*	*TRBC1*	CASSLGGSMNTEAFF
TRBV12-3, TRBV12-4	*TRBJ1-1*	*TRBC1*	CASIKNTEAFF
TRBV2	*TRBJ2-5*	*TRBC2*	CASSADGQETQYF

Supplementary Table 1. Characteristics of TCRβ clonotypes shared at d63 in GLA-SE vaccinated individuals.

Supplementary Table 1. TCR gene usage and CDR3 amino acid sequence of the TCRβ CDR3 clonotypes shared between GLA-SE vaccinated individuals at day 63.

Marker	Clone	Fluorochrome	Company	Catalog no.
viability dye	n/a	eFluor780	eBioscience	65-0865-14
CD14	61D3	APC-eF780	eBioscience	47-0149-42
CD16	eBioCB16	APC-eF780	eBioscience	47-0168-42
CD3	UCHT1	APC-eF780	eBioscience	47-0038-42
CD19	HIB19	BB515	BD	564456
CD20	2H7	PECY7	Biolegend	302312
CD71	CY1G4	PE	Biolegend	334106
IgD	IA6-2	BV421	BD	563813
CD27	M-T271	BV510	Biolegend	356420
CD38	HIT2	APC	eBioscience	17-0389-42
CD19	HIB19	APC-eF780	eBioscience	47-0199-42
ICOS	ISA-3	APC	eBioscience	17-9948-42
CD3	UCHT1	BUV 395	BD	563546
CD45RA	HI100	BUV737	BD	564442
CXCR5	RF8B2	BB515	BD	564624
CD4	RPA-T4	PercpCy5.5	BD	560650
PD1	eBioJ105	PECy7	eBioscience	25-2799-42
CD38	HIT2	BV421	Biolegend	303525
CD127	A019D5	BV650	Biolegend	351325
BCL6	K112-91	PE	BD	561522
CXCR3	1C6/CXCR3	BV421	BD	562558
CCR6	11A9	BV786	BD	563704

Supplementary Table 2. Antibodies used for flow cytometry.

Chapter 5

Lymph node excision biopsy to study germinal center responses upon infection or vaccination in rural Tanzania

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This chapter contains the following manuscript:

Catherine Gerald Mkindi, Elias Antony Marandu, Ngisi Masawa, Farida Bani, Amina Nyuri, Theonestina Byakuzana, Song Ding, Guiseppe Pantaleo, Nina Orlova-Fink, Manuel Battegay, Maja Weisser-Rohacek, MD, Claudia A. Daubenberger. *Lymph node excision biopsy to study germinal center responses upon infection or vaccination in rural Tanzania*
To be submitted to the Journal of Acquired immune deficiency syndrome

Lymph node excision biopsy to study germinal centre residing T cell subsets in rural Tanzania

Catherine Gerald Mkindi, DVM[1,2,3], Elias Antony Marandu, MD[5], Ngisi Masawa, MD[3], Farida Bani, BSc[3], Amina Nyuri, BSc[3] ,Theonestina Byakuzana, BSc[3], Song Ding, PhD [6], Guiseppe Pantaleo, MD [7,8], Manuel Battegay MD[4], Nina Orlova-Fink, PhD[1,3], Maja Weisser-Rohacek, MD, [1.3,4]*, Claudia A. Daubenberger, DVM [1,3]*

Running title: **Inguinal lymph node biopsy in rural Tanzania**

1. Swiss Tropical and Public Health Institute,
2. University of Basel
3. Ifakara Health Institute, Bagamoyo, Tanzania
4. Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Switzerland
5. St Francis Referral Hospital, Ifakara, Tanzania
6. EuroVacc Foundation, Lausanne, Switzerland
7. Service of Immunology and Allergy, Lausanne University Hospital, University of Lausanne, Switzerland
8. Swiss Vaccine Research Institute, Lausanne, Switzerland,

* Corresponding authors

Key words: HIV-1, lymph node biopsy, Tanzania,

Abstract:

Background: HIV-1 establishes rapidly a persistent infection that can be contained under life-long antiretroviral therapy (ART) but not cured. One of the preferred tissues for viral persistence are secondary lymphoid tissues including peripheral lymph nodes. Studying the impact of novel HIV-1 treatment and vaccination approaches on cells residing in germinal centers is essential for rapid progress towards HIV-1 prevention and cure.

Methods: Between June and August 2018 we included consenting adult patients enrolled in the Kilombero and Ulanga Antiretroviral Cohort (KIULARCO) with a newly diagnosed HIV infection (WHO stage I or II) and CD4 cell counts ≥ 350/ul, who were ART naïve. Consenting individuals underwent venous blood collection and inguinal lymph node excision surgery. Lymph nodes were used to extract mononuclear cells, which were stored in liquid nitrogen for further evaluation. Participants were followed up regularly for two weeks until complete healing of the surgical wound.

Results: Nine participants completed the lymph node excision surgery without clinical sequelae. Among the volunteers, one long-term elite controller could be identified. The number of mononuclear cells recovered ranged from 68 million to 206 million and correlated positively with lymph node size extracted.

Conclusion: Inguinal lymph node excision can be safely performed in a rural sub-Saharan African setting, if precautions such as infection prevention are taken care of. Thus, lymph node extraction is a feasible procedure in the framework of monitoring novel intervention studies in HIV-1 clinical research. The use of KIULARCO cohort as a platform for clinical research supports the evaluation of novel interventions in a population that is particularly affected by HIV-1 but rather neglected in relation of testing novel interventions.

Key words: lymph node biopsy, KIULARCO cohort, Tanzania

Introduction

HIV-1 is an infectious disease caused by human immunodeficiency virus and transmitted from human to human [1]. In 2017, of the estimated 36.9 million people living with HIV-1 (PLWHIV) worldwide, 19.6 million resided in Eastern and southern Africa (UNAIDS.

Global AIDS updated 2018). The UNAIDS goals of 90-90-90 by 2020 (90% of people know their HIV-status, 90% are enrolled in care, 90% are virally suppressed), are not yet met in Tanzania: 66 % of PLWHIV are on ART and 48 % of these have suppressed viral loads, while no numbers are published for the first 90 (UNAIDS 2018).

Strongly neutralizing antibodies develop in up to 25% of HIV-1 infected individuals over several years of infection [2]. Inducing these highly protective antibodies by active immunization has been the focus for many years of HIV-1 vaccine developers albeit with limited success so far [3].

Most current vaccines provide protection by generating antibodies that block pathogen establishment or spread [4]. Generation of robust, matured and long-lived vaccine induced antibody responses takes place in germinal centres (GC) [5]. GC develop in secondary lymphoid organs and major cell subsets identified are GC B cells, T follicular helper cells (Tfh), T follicular regulatory cells (Tfr), macrophages and follicular dendritic cells [6]. In humans, several markers for Tfh circulating in peripheral blood have been established, including ICOS, PD1 and CXCR5 expression [7] enabling the investigation of vaccine induced responses against vaccination in peripheral blood [8,9] [Hill et al., unpublished]. However, the functional and biological relationship between circulating Tfh and cells residing in secondary lymphoid organs is unclear and requires further investigations [10].

The Kilombero and Ulanga Antiretroviral Cohort (KIULARCO) is a single-site, open and ongoing prospective cohort of PLWHIV-1 established in 2005 at the Chronic Diseases Clinic of Ifakara (CDCI), located at the premises of the Saint Francis Referral Hospital (SFRH) in Ifakara, Tanzania [11]. One of the objectives of KIULARCO is to provide a platform for clinical studies on improving HIV-1 care and treatment [11]. Here, we performed as a proof of concept a lymph node excision study from asymptomatic, ART naive HIV-1 infected volunteers in this rural setting in Tanzania.

Methods

Ethical approval

This study was approved in Tanzania by the Ifakara Health Institute Institutional Review Board (IHI-IRB) Reference: **IHI/IRB/No: 029-2016** and the National Medical Review Board of Tanzania (NIMR) Reference: **NIMR /HQ/R.8a /Vol.IX/2374**. In Switzerland, the protocol was approved by the Commission Cantonale D'éthique De La Recherche

Sur L'être Humain (Cer-Vd).

Study site

This study was conducted in the rural communities of Morogoro region, Tanzania, involving the two districts Kilombero and Ulanga. The Chronic Diseases Clinic of the Ifakara Health Institute (CDCI) located at the St Francis Referral Hospital (SFRH) runs an Antiretroviral cohort (Kilombero and Ulanga Antiretroviral Cohort, KIULARCO), KIULARCO includes all consenting HIV-1-positive patients enrolled in care. SFRH has in- and outpatient services and specialized clinics including theatres with facilities needed for patients' recruitment and lymph node extraction. The Ifakara Health Institute runs an advanced laboratory infrastructure for immunological and virological sample processing and long-term storage.

Volunteer recruitment

At the SFRH, all patients seen for any health condition are offered an HIV-1 test following the national strategic plan [11]. In 2014, SFRH adopted the WHO strategy for universal HIV-1 testing. Antibody testing for HIV-1 diagnosis since then is done by the SD Bioline rapid diagnostic test (RDT), confirmed by the RDT Unigold. For this study HIV-1 viral loads in plasma samples were quantified by using GenXpert. A secondary RDT testing reconfirmed the HIV-1 positive status of volunteer V3, after HIV-1 viral load was found to be undetectable by GenXpert.

ART-naïve volunteers between 18-55 years of age, HIV-1 positive with CD4 T cell count above 350 cells/ml, no active co-morbidities and negative for hepatitis B (HBsAg), Syphilis, malaria were eligible for enrolment. Volunteers were explained study procedures and asked to sign a study-specific informed consent form describing paired blood and lymph node sampling. All volunteers were recruited into the KIULARCO on the same day after signing an informed consent form.

Blood sample collection and PBMC isolation

Whole blood was collected and serum, plasma and peripheral blood mononuclear cells (PBMCs) were prepared following established procedures [12]. Plasma and serum samples were separated and stored at -80°C. PBMCs were isolated from whole blood by Ficoll-Hypaque gradient density centrifugation and stored in Fetal calf serum (FCS) with 10% DMSO in liquid nitrogen until further analysis.

Lymph node extraction and LNMC isolation

A surgical procedure to excise inguinal lymph node was performed aseptically at the SFRH theatre following local anaesthesia, 2% lidocaine 5cc. An incision of 1-2 cm was made at the right or left inguinal area to take out one lymph node, which was immediately preserved in R10 medium (RPMI with 1% Penicillin/Streptomycin and 10% FCS) for transport and processing. The incision was sutured and patients prescribed with analgesics before being discharged home. Patients were asked to come for three visits within 14 days of surgery for postoperative wound care.

Lymph node mononuclear cells (LNMC) were then extracted in R10 medium by mechanical disruption of the tissue, followed by filtering on a 100un filter. Cell counts and viability were determined by microscopiy using 0.4% trypan blue solution staining.

Results

Nine ART naïve HIV-1 positive volunteers were enrolled into the study between June and August 2018. At enrolment, all participants were clinically healthy, with no fever, chills, headache recorded. Volunteers reported that they have come to SFRH for reasons other than acute sickness or suspicion of HIV-1 infection. This confirms their possible chronic nature of the HIV-1 infection, but durations of infection are unknown. All volunteers tested negatively for hepatitis B, syphilis and malaria. The female to male ratio was 3:1 and the average age of the volunteers was 34 years (range 23-55 years). CD4 T cell counts were on average 698 cells/mm^3 (range 434-1302) and the HIV-1 viral load measured on average 35,500 (range <50-133,200) copies /mL (Table 1**).** Interestingly, one of the volunteers (V3) showed undetectable HIV-1 viral load (< 50 copies/mL) and high CD4 T cell count (1301 cells/mm^3), the highest in this cohort suggesting a rare long-term non-progressor phenotype.

Lymph node biopsies were successfully conducted from all 9 volunteers. The size ranged from 3 to 15 mm (Figure 1A**).** After extraction, LMNC were immediately isolated. Viability of LMNC after purification was between 97 % to 99 % and the number of LMNC recovered ranged from 68 million to 206 million total. The LNMC count correlated positively with the lymph node size (Figure 1B). This correlation strongly supports that a healthy secondary lymphoid organ was extracted. LNMC were aliquoted and frozen down within an hour after surgical excision. Except for two patients who developed self-resolving hyperaemia around the incision site, no volunteer experienced

any adverse events in connection to the lymph node biopsy. The wounds healed as expected and all volunteers were discharged from attending regular hospital visits latest by day 14. Volunteers started HIV-1 treatment at the day of enrolment following the national guidelines of Tanzania.

Discussion

Lymph node biopsies of inguinal lymph nodes together with a paired venous blood sample were collected from 9 HIV-1 infected patients in rural settings of Tanzania. To our knowledge, this is the first study of this kind in Tanzania and we demonstrate that this procedure is safe with minimal adverse events that resolved within 14 days after surgery. This short recruitment period of volunteers into the study underscores that the KIULARCO cohort serves as an elegant platform for clinical research in HIV-1. Cure of HIV-1 is not possible using the currently available treatment approaches resulting in a growing number of PLWHIV needing to adhere to a life-long ART treatment [13]. One of the preferred compartments for viral persistence are Tfh cells making them an important target for experimental HIV-1 cure approaches [14].

A recent report by D´Souza provides an excellent overview of the value of lymph node biopsies in the context of experimental HIV-1 vaccine trials [15]. Lymph node biopsies have been shown to be safe in HIV-1 positive volunteers and even up to 4 lymph nodes could be consecutively removed from participants of an HIV-1 vaccine study in Thailand [16] The bulk of information generated about GC development and responses has been derived from studying the secondary lymphoid tissues in mice. It is assumed that heterogeneity of GC Tfh cells impacts antibody isotypes produced by plasma blasts and long-lived memory B cells [17] In humans, GC Tfh are functionally and phenotypically heterogeneous population based on expression of PD1, CXCR3, CCR6, CCR7 and ICOS markers [18]. People living in SSA are exposed to co-infections like malaria and helminths - potentially impacting on GC Tfh cell function and cytokine secretion [19]. Studying the environmental drivers of the functional heterogeneity of GC Tfh in a rural African population will provide essential information on HIV-1 pathogenesis, treatment and routine and experimental vaccine monitoring in a population highly affected by the HIV-1 epidemic [20].

Conclusion

Lymph node biopsy is a safe procedure and could be undertaken in rural settings. This is the first study to show successful lymph node excision biopsy with local experts in rural settings and has laid a foundation for detailed immune response investigations during clinical trials.

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We acknowledge the CDCI clinic for incorporating our study protocol in their cohort and the participation of all study volunteers. We are thankful to Dr. Alessandra Noto who offered training on LNMC isolation from lymph node biopsy and Prof. Jean-Marc Corpataux for training on inguinal lymph node surgical excision. Both are with the University Hospital, Lausanne, Switzerland.

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	Volunteer ID								
	V1	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**V9**
Age	40	55	42	29	23	23	37	26	30
Gender	F	F	F	F	F	M	F	M	M
BMI	18.7	22.2	28.4	24.9	21.3	23	31.6	18.8	29.7
Marital status	divorced	divorced	divorced	single	single	married	married	single	single
CD4 cells (cells/mm3)	538	662	1302	846	679	594	434	665	566
HIV-1 RNA (copies/mL)	1670	68300	< 50	8150	725	113200	72200	53900	1210
LN size (mm)	4	15	7	6	3	14	n.d.	n.d.	10
Hb (g/L)	13.9	12.0	12.1	12.7	10.2	14.1	13.8	15.0	13.7
Clinical presentation	healthy	healthy	healthy	healthy	healthy	healthy	healthy	healthy	healthy
Reason for HIV-1 testing	Ear injury	History of Herpes zoster lesions in 2015	Chronic body malaise	Marital requirement	Employer request	Wife's antenatal clinic visit	Referred	Voluntary Testing	Voluntary Testing

Table 1. Demographic and clinical characteristics of study volunteers

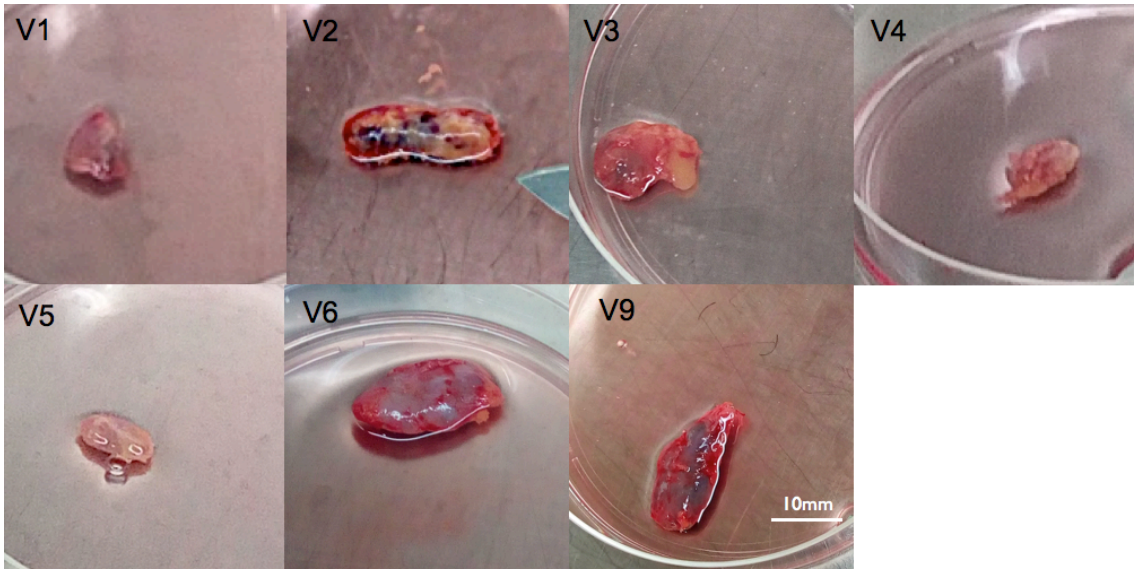

Figure 1A: Pictures of lymph node biopsies taken from volunteers

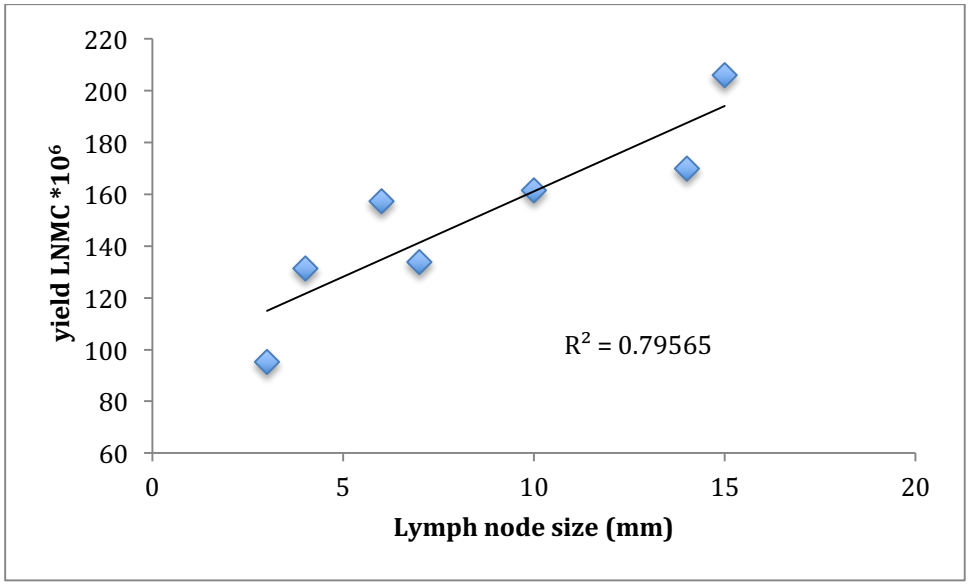

Figure 1B: Positive correlation between the size of the lymph node and isolated LNMC

Chapter 6

General Discussion

The overall goal of vaccination is to safely induce protective, long-lived immune responses to fight against the pathogen on the subsequent encounter. Vaccine induced humoral immune responses against infectious diseases are currently the best approach for prevention. This has been shown in all licensed vaccines to date, including the child hood vaccines which offer long term protection, except BCG [86]. This PhD thesis aimed at investigating cellular and humoral immune responses induced against P27A, a synthetic peptide of 104 amino acids in length and to develop novel monitoring approaches to better understand the cellular subsets driving successful antibody responses after vaccination. In this general discussion, I will highlight for each manuscript the key findings and their implication of malaria vaccine development.

6.1. The Candidate Blood-stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase 1 Trial in Exposed and Non-exposed Volunteers

The clinical trial described in this chapter was designed as a staggered, randomized, antigen and adjuvant dose-finding, multicenter phase 1a/1b clinical trial using the fast-track strategy set by the European Vaccine Initiative to accelerate malaria vaccine clinical development. Study was conducted in Switzerland for phase 1a and in Tanzania for phase 1b (NCT01949909). P27A, an unstructured segment of the trophozoite exported protein 1, was identified in a series of immune-epidemiological studies as an interesting vaccine candidate. In order to test P27A in malaria naïve and pre-exposed volunteers, it was formulated in alum and GLA-SE to compare its safety, tolerability and immunogenicity side by side in two different populations. This new trial design was found to be faster than conducting two independent phase Ia and phase Ib studies, without compromising on safety monitoring or study oversight. Both vaccine formulations were found to be safe and no vaccine-related serious adverse event was observed. The P27A antibody titers were around 10-fold higher in the GLA-Se formulated vaccine compared to alum confirming its superior adjuvant activity. Importantly, anti-P27A antibodies bound to the native protein in asexual blood stage parasites when tested by immunofluorescence assays. The main P27A antibody isotypes induced were IgG1 and IgG3, both of which are known to mediate opsonisation by interacting with phagocytes. *In vitro* assays confirmed that P27A vaccine induced antibodies derived from the European volunteers showed parasite growth-inhibitory activity. Comparing the antibody titers between malaria naïve and pre-exposed volunteers strongly indicated that in malaria pre-exposed populations a higher dose of GLA-SE was needed in order to arrive at comparable titers measured in Europeans. A similar study was conducted in France and Burkina Faso evaluating the safety and immunogenicity of AMA-1 in combination with alum and GLA-SE [87]. The outcome of this study confirmed the

good safety profile of GLA-SE as adjuvant in malaria pre-exposed populations and it higher potency in inducing antibodies when compared to alum. GLA-SE as suitable adjuvant has now been confirmed with other malaria vaccine candidates including the recombinant fragment of VAR2CSA, the *P. falciparum* protein responsible for binding to the placenta via chondroitin sulfate A [88] and the *P. vivax* encoded Duffy-binding protein [89]. Combined, these and our study support the use of GLA-SE as adjuvant in humans that is safe and induces potent, functionally active and long-lasting humoral immune responses against malaria.

6.2. GLA-SE improves quality and magnitude of cellular immunity to blood stage malaria vaccine candidate P27A

Understanding the interplay between humoral and cellular immune responses elicited after vaccination is critical for optimizing vaccine approaches. Therefore, we investigated the magnitude and quality of T-cell responses induced by P27A candidate. The GLA-SE adjuvant promoted enhanced CD4+ T-cell responsiveness in malaria pre-exposed Tanzanian volunteers. The response correlated with increased IgG1 titers and expansion of circulating Tfh cells after vaccination. CD4+ T-cells with poly-functional characteristics, co-expressing IL-2 and TNFa, as well as long-living central memory phenotype dominated the response in participants, who received the vaccine containing GLA-SE. Importantly, this study revealed that the magnitude of CD4+ T-cell responses was strongly impaired in the presence of active helminth infections during first vaccination. Helminth infections are known to evade clearance and become chronic by down regulation the host response and this immunomodulation extends also to other pathogens co-infecting the same host [90] [91]. It has been shown that one mechanism of helminths deployed to evade immune responses is based on specifically targeting and down regulating TLR mediated innate immune stimulation [90]. Esen et al., reported in Gabon also the reduced antibody responses in volunteers vaccinated against a subunit malaria vaccine candidate, in the presence of ongoing *Trichuris trichiura* infections [91]. Interestingly, *Trichuris suis* suppresses TLR-4 induced inflammatory responses in human macrophages, leading to a down-regulation of pro-inflammatory mediators including IL-12B, CCL1 and CXCL9 [92] GLA-SE adjuvant activity is based on its TLR4 stimulatory activity [93]. The fact that in helminth infected volunteers both, the CD4+ T cells and antibody responses were significantly impaired, supports the idea that the helminth mediated immune suppression was interceded via changed TLR4 signaling or expression. However, we need to conduct further *ex vivo* studies with antigen presenting cells known to express TLR4, like macrophages, that are recovered from helminth infected and non-infected volunteers to analyse the TLR4 expression

levels and signaling cascades to substantiate this hypothesis. Highly interesting was the finding that the P27A specific CD4+ T cells were dominated by poly-functional TNFa and IL2 producing T cells with no IFNg detection. This outcome was also observed also in other clinical studies testing GLA-SE and was not restricted to Tanzanian volunteers [94]. In summary, this study highlighted that the helminth co-infection status of individual volunteers is one important driver of inter individual variation in P27A vaccine induced cellular and humoral immune responses.

6.3. The adjuvant GLA-SE promotes human Tfh cell expansion and emergence of public TCR clonotypes

The location of the GC response, within secondary lymphoid tissues, is one of the major barriers to understanding how different vaccines, or their adjuvants, affect GC biology in humans. For this reason, circulating cells that can act as biomarkers of the GC response are an area of intense interest. In particular, it is well established that there exists a population of blood CXCR5+CD4+ cells expanding after vaccination, named cTfh [95][96]. These cTfh cells phenotypically and functionally resemble secondary lymphoid tissue resident Tfh cells [97]. Since GLA-SE was superior to alum in induction of humoral immune responses, we next aimed to determine whether the adjuvant GLA-SE promotes a cTfh cell response in humans making use of PBMC collected at baseline and 7 days past last vaccination and polychromatic flow cytometry and whole cell transcriptomic analysis of *ex vivo* sorted cTfh. We defined as cTfh cells that are co-expressing ICOS+, PD1+ and CXCR5+ and measured their frequencies before and after vaccination demonstrating that significantly higher numbers of cTfh could be found in GLA-SE immunized volunteers compared to alum. The increase in cTfh correlated with the increase in P27A antibody titers confirming that these cells are biomarkers for GC responses. Interestingly, in the GLA-SE vaccinated volunteers, higher numbers of antibody secreting cells with lower levels of SHM by V(D)J sequencing of the BCR were observed, supportive of a recall response from naturally primed P27A memory B cells that takes place in extra-follicular structures. Our study showed that the GLA-SE adjuvant increases the ICOS+CD38+CXCR5+PD-1+ total cTfh cell population and the GC-Tfh-like CXCR5+PD-1+++ cell after P27A vaccination, indicative that this adjuvant enhances the GC response. Importantly, transcriptomic analysis revealed that GLA-SE also promotes the emergence of common TCRβ clonotypes in cTfh cells that are shared between different individuals. The emergence of so-called public clonotypes is informative for vaccine design as it indicates that there may be a common epitope that could be used in vaccine formulations to enhance responses

in numerous individuals. The recent discoveries of public antibody clonotypes has revolutionized whole-parasite malaria vaccine strategies [98][99] and here we extend this to the identification of public Tfh cell TCRβ clonotypes induced after vaccination. The identification of these common clonotypes in cTfh cells from participants that received the GLA-SE formulated vaccine could simply be a consequence of the greater magnitude of the response, indicating that more T cell clones are recruited into the response. Using the knowledge of key antigenic peptides to which Tfh clonotypes from multiple individuals respond, combined with adjuvants that improve T cell recruitment into GC is a rational way to enhance humoral vaccine responses and duration at the population level.

6.4. Lymph node excision biopsy to study germinal centre residing T cell subsets in rural Tanzania

Peripheral blood is conveniently used as a source of cells and serum to study quality and quantity of innate and adaptive immune responses following natural infection or vaccination. However, the size and quality of ASC is determined by the interactions of cells residing in GC, including follicular dendritic cells, B cells, Tfr and Tfh. GC are found in secondary lymphoid organs like lymph nodes and the spleen and both organs are not usually accessible in humans and the bulk of information available about GC development and responses has been derived from studying the secondary lymphoid tissues in mice. The use of cTfh as biomarkers of GC responses is commonly accepted but they do not represent GC Tfh *bona fide* since they for example to not express transcription factor Bcl6 [97] Therefore, analyzing GC Tfh from lymph node tissue will enable us to study the biology of these important cells that are at the center of interest in vaccinology, HIV pathogenesis, and regulation of autoimmune diseases [100]. Lymph node biopsies of inguinal lymph nodes together with a paired whole venous blood sample were collected from 9 HIV-1 infected patients in rural settings of Tanzania. To our knowledge, this is the first study of this kind in Tanzania and we demonstrate that this procedure is safe with minimal adverse events that resolved within 14 days after surgery. We have conducted this pilot study for purification of LNMC from ART naïve HIV -1 positive volunteers as ideal starting material for isolation of bNabs since the density of antigen-specific ASC after antigen challenge is far higher than in peripheral blood [101]. Using LMNC will enable to understand the impact of HIV-1 and other co-infections on the diversity of GC Tfh subsets driving humoral immune responses [102]. GC Tfh are a reservoir for HIV even when the HIV-1 viraemia is suppressed by successful treatment with ART [103] and understanding the size and turnover of this reservoir is essential for testing novel interventions and approaches towards

HIV-1 cure. In summary, establishing the human resource for collecting lymph node biopsies will enable incorporation of these procedures in future immunological intervention studies in Tanzania.

Chapter 7

Outlook

In an effort towards reducing malaria cases and progress to malaria elimination, WHO published a strategic road map for 2030 to develop and license a malaria vaccine with a protective efficacy of more than 75% that can last for at least two years [104]. Although RTS,S /AS01 the advanced malaria vaccine is under pilot administration in three sub-Saharan Africa countries, it showed an average efficacy of 35.9%, which fell to 2.5 % after four years of follow up period [105], requiring additional vaccine development efforts. Malaria elimination is thought to be achieved following interruption of parasite transmission [106] through removal of asexual parasites which develop into gametocytes. This makes pursuing blood stage malaria vaccine is necessary. Development of subunit vaccines has been found a rational strategy to follow, especially for the countries in SSA where the population is challenged by limited cold chain facilities and lack of technical expertise to handle fragile live attenuated vaccines. In this population, the number of immunocompromised patients suffering from co-infections like HIV-1 are also particularly prevalent, raising additional concerns over safety of live vaccines [107]. Recently, malaria blood stage sub-unit vaccines including the merozoite surface proteins 1, 2, and 3 (MSP1, MSP2, and MSP3), serine-repeat antigen erythrocyte-binding antigen (SERA), ring-infected erythrocyte surface antigen (RESA), glutamate-rich protein (GLURP), and apical membrane antigen 1 (AMA1) showed reasonable safety and tolerability profiles and induced antibody responses in malaria naïve and pre-exposed individuals [45] but protection observed was limited [108]. Antigenic variation has been demonstrated to contribute into immune evasion of the parasite [109]. Since the peptide P27A has been shown to induce antibody and cellular immune responses in malaria pre-exposed volunteers, further development of this antigen in follow up studies could provide information on protection induced against field malaria strains. Also, combining P27A with other subunit vaccine candidates to be used in a multi-stage multi-antigen vaccine approach could mitigate the problem of immune evasion based on antigenic polymorphisms [110][111].

The role of antibodies in protection against malaria has been emphasized [112] but there are remarkable differences in the level of humoral immune responses observed between naturally exposed individuals probably contributing to variations of naturally acquired immunity. Following the promising results in the HIV [113] and Ebola [114] vaccine development field, a next generation vaccine approach in malaria could be based on the use of human monoclonal antibodies [115][116][117].

These novel tools in malaria research could be used to protect the most vulnerable populations, namely travelers, children and pregnant women by passive immunization deployed as long acting, injectable small molecules [118][119]. Additionally, detailed understanding of the three-

dimensional structure recognized by these highly potent antibodies could lead to the identification of the third-generation subunit vaccine candidates [120].

Chapter 8

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