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A GUIDE TO...

A guide to investigating immune responses elicited by whole-sporozoite pre-erythrocytic vaccines against malaria

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In the last few decades, considerable efforts have been made toward the development of efficient vaccines against malaria. Whole-sporozoite (Wsp) vaccines, which induce efficient immune responses against the pre-erythrocytic (PE) stages (sporozoites and liver forms) of *Plasmodium* parasites, the causative agents of malaria, are among the most promising immunization strategies tested until present. Several Wsp PE vaccination approaches are currently under evaluation in the clinic, including radiation- or genetically-attenuated *Plasmodium* sporozoites, live parasites combined with chemoprophylaxis, or genetically modified rodent *Plasmodium* parasites. In addition to the assessment of their protective efficacy, clinical trials of Wsp PE vaccine candidates inevitably involve the thorough investigation of the immune responses elicited by vaccination, as well as the identification of correlates of protection. Here, we review the main methodologies employed to dissect the humoral and cellular immune responses observed in the context of Wsp PE vaccine clinical trials and discuss future strategies to further deepen the knowledge generated by these studies, providing a toolbox for the in-depth analysis of vaccine-induced immunogenicity.

Abbreviations
ACD, acid-citrate dextrose; AMA1, apical membrane antigen 1; APC, antigen-presenting cell; ASC, antibody-secreting cell; CelTOS, cell-traversal protein for ookinetes and sporozoites; CFSE, carboxyfluorescein succinimidyl ester; CHMI, controlled human malaria infection; CPS, chemoprophylaxis and sporozoites; CSP, circumsporozoite protein; CTL, cytotoxic T lymphocyte; CyTOF, cytometry by inductively coupled time of flight; EBA-175, erythrocyte binding antigen 175; ELISpot, enzyme-linked immunosorbent spot; EXP1, exported protein 1; FNA, fine-needle aspiration; FRG, $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$; GAP, genetically attenuated parasite; GIA, growth inhibition assay; GLURP, glutamate-rich protein; HIS, human immune system; ICS, intracellular cytokine staining; IFA, immunofluorescence assay; Ig, immunoglobulin; ILSDA, inhibition of liver stage infection and parasite development; LAMP-1, lysosome-associated membrane protein 1; LDH, lactate dehydrogenase; LSA, liver stage antigen; MHC, major histocompatibility complex; MSP-1, merozoite surface protein 1; PBMCs, peripheral blood mononuclear cells; PE, pre-erythrocytic; RAS, radiation-attenuated sporozoites; RBC, red blood cell; SSP2, sporozoite surface protein 2; STARP, sporozoite threonine-asparagine-rich protein; TCR, T-cell receptor; TRAP, thrombospondin-related adhesive protein; T_{RM}, tissue-resident memory T cells; Wsp, whole sporozoite.

Introduction

Malaria, caused by *Plasmodium* parasites, remains the most prevalent parasitic infection in the globe, featuring among the leading causes of mortality from infectious diseases worldwide, and particularly in Africa. In 2019 alone, a total of 229 million cases were reported by the World Health Organization, 409 000 of which led to death [1].

The widespread implementation of malaria control interventions, including the use of insecticide-treated bed nets, indoor residual spraying, and chemoprevention, as well as improved access to diagnostic tools and treatments, has led to a significant decrease in malaria incidence between 2000 and 2015 [2]. However, emerging drug and insecticide resistance over the last few years has stalled the progress towards the control and eventual elimination of malaria [3]. Furthermore, naturally acquired immunity depends on repeated infections, is nonsterilizing, and wanes over time in the absence of continuous exposure [4]. Therefore, the development of an effective and long-lasting vaccine against malaria is a key priority that remains a significant challenge, mainly due to the complex, multistage, and multiantigen life cycle of *Plasmodium* parasites [5].

Sporozoites, the liver-infective forms of malaria parasites, are injected into the skin and skin vasculature of a mammalian host by a female *Anopheles* mosquito, and home to the liver via the bloodstream, where they productively infect hepatocytes, initiating an asymptomatic phase of asexual reproduction and development [6]. Following intrahepatic replication, parasites are released into the bloodstream, where they invade, multiply asexually, egress, and reinvade host erythrocytes, in a continuous cycle that is responsible for malaria-associated clinical symptoms [7]. During the blood stage of infection, a small proportion of parasites differentiates into sexual forms, known as gametocytes. The cycle progresses as mosquitoes feed on an infected host, ingesting gametocytes and initiating the vector phase of the parasite's life cycle, which results in the formation of salivary gland-resident sporozoites [8].

While there have been crucial advances in the development of vaccines targeting different stages of the *Plasmodium* life cycle, the obligatory but asymptomatic nature of the PE stage of infection makes it the ideal target for immunization interventions. Vaccines targeting this stage have consistently led to protection against clinical malaria [9,10]. The subunit vaccine RTS,S, based on the PE *P. falciparum* circumsporozoite protein (CSP) immunogen, constitutes the most advanced malaria vaccine candidate to date. However, it affords relatively low sterile protection against disease, which wanes over time and is age-dependent [11].

In recent years, significant progress has been made in the development of whole-sporozoite (Wsp) vaccines, which presently constitute the most promising approach to PE malaria vaccination. Wsp vaccines rely on the generation of immune protection against PE stages following immunization with live but attenuated *Plasmodium* sporozoites, including radiation-attenuated sporozoites (RAS) [12], genetically-attenuated parasites (GAPs) [13], and immunization with fully infective sporozoites in combination with chemoprophylaxis (CPS) [14]. More recently, we developed an alternative Wsp vaccination approach based on the use of genetically modified rodent *Plasmodium berghei* sporozoites as a platform to deliver human *P. falciparum* antigens [15–18].

The increase in funding and the enhanced awareness of the crucial role of an effective malaria vaccine for controlling this disease over the last years have allowed a significant number of vaccine projects to transition from successful preclinical development into clinical trial evaluation [19]. Following a phase I trial, where safety and immunogenicity are demonstrated, the candidate vaccine moves to a phase II study, where volunteers are naturally exposed to infection or are subjected to controlled human malaria infection (CHMI). CHMI can be carried out either by inoculation of sporozoites via mosquito bites, or by direct venous injection of purified, cryopreserved sporozoites or *Plasmodium*-infected blood into malaria-naïve volunteers [20]. Although the primary objective of such trials is to estimate vaccine efficacy and safety, CHMI models provide a vital opportunity to study vaccine-induced immune responses at well-defined time-points and to identify relationships between immunogenicity and efficacy [21].

Antibodies and peripheral blood mononuclear cells (PBMCs) obtained from immunized volunteers can be analyzed by a plethora of assays, which are designed and optimized to evaluate the overall immune responses induced by the vaccination agent in a clinical setting. In this review, we will describe the methods that have been employed in the context of clinical trials to assess the immunogenicity of candidate Wsp PE malaria vaccines, as well as to find potential immune correlates of protection against infection. A set of recently developed techniques, hitherto not employed in malaria vaccine clinical trials, and which may provide valuable insights into antigen-specific immune responses, are also highlighted. Therefore, this review

constitutes a repository of information to guide and assist in future such studies, as well as in the rational development of vaccines with enhanced efficacy against this devastating disease.

Blood collection and processing

Clinical trials frequently involve several immunizations and time-points of analysis, as well as different institutions in distinct locations. The sample collection schedule usually includes time-points prior to the prime immunization (and likely before each boost immunization) and CHMI, but must be fine-tuned to the particular specificities and goals of each clinical trial. Collection, isolation, and cryopreservation of serum/-plasma and PBMCs are fundamental sample processing procedures that must be standardized in order to allow accurate comparison of immune profiles. In this section, we describe the techniques most commonly utilized to isolate and process the different blood components, broadly defining the immunological information sought by these analyses (Figure 1).

Serum and plasma collection and processing

Serum or plasma are used in the quantification of components of both the humoral (circulating antibodies) and cellular (blood cytokine levels) immune responses. Isolation and storage procedures are common to those employed in most studies involving human peripheral blood, including in the context of other infectious diseases [22,23]. Serum and plasma are obtained upon centrifugation of blood samples collected by venipuncture in the presence of clot activators (such as silica particles) or anticoagulants (such as sodium citrate, EDTA or heparin) [22,24]. Serum or plasma should be separated from other blood components shortly after collection to reduce contamination,

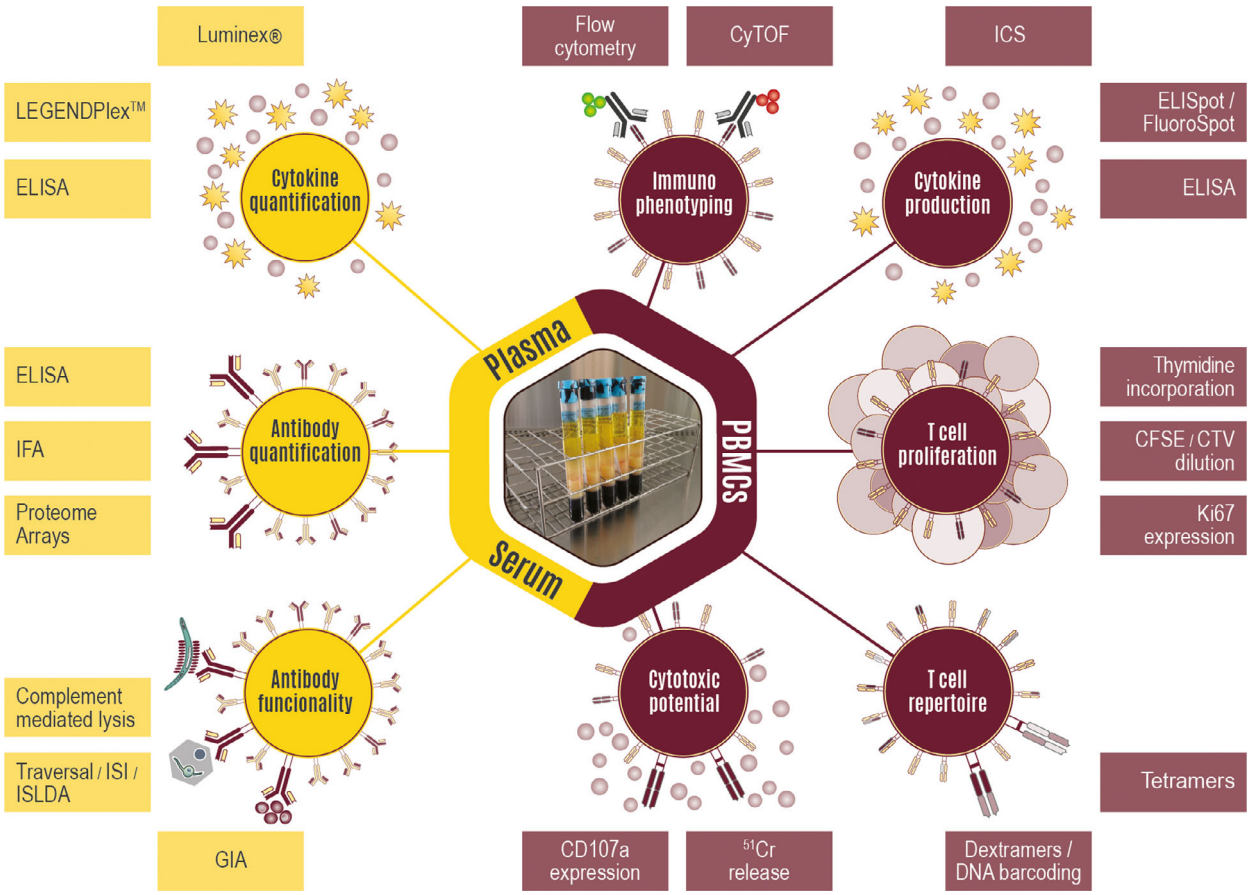

Fig. 1. Assays used for the evaluation of humoral and cellular responses induced by Wsp PE vaccines. Blood collected at defined time-points during the clinical evaluation of a vaccine candidate can be separated in its components and used for the quantitative and qualitative assessment of antibodies (serum/plasma) and to evaluate the phenotype and function of antigen-specific T cells (PBMCs) using the assays depicted in the figure (see text for further information).

and either used immediately or stored at −80 °C. Antibodies are relatively stable and sustain storage for several years at −80 °C, as well as a few freeze–thaw cycles [25,26].

Importantly, the specificity of *in vitro* immunoassays employing serum/plasma may be affected by the presence of antimalarial drugs or the occurrence of immune responses against other pathogens [27]. Therefore, the purification of immunoglobulins (Igs) from serum/plasma samples by affinity chromatography may be considered in order to reduce nonspecific background activity [27]. Although Protein G or HiTrap Protein G HP columns are commonly employed for this purpose in the context of Wsp PE vaccine candidate clinical trials [18,28–31], one study concluded that polyethylene glycol purification of human serum/-plasma samples is optimal for recovering functional antigen-specific antibodies [27].

PBMC isolation and processing

PBMCs are essential for the assessment of cellular immune responses elicited by vaccination. Since isolation, cryopreservation, and subsequent thawing of PBMCs may significantly impact cellular features, numerous studies from several fields have sought to identify conditions that ensure minimal loss of cell viability, phenotype, and function [32–43]. The results from such studies should be taken into consideration when selecting the procedures to implement in the context of Wsp PE vaccine clinical trials.

PBMC isolation

PBMCs are isolated from venous blood collected in the presence of an anticoagulant, such as sodium or lithium heparin, sodium citrate, acid–citrate–dextrose (ACD), or EDTA. Although, to the best of our knowledge, a direct comparison of all available anticoagulants has not been reported, no major differences have been found between them in terms of cell recovery, viability, or function, as measured by IFN-γ enzyme-linked immunosorbent spot (ELISpot) of $CD8^+$ T-cell responses of PBMCs when using sodium heparin, ACD, or EDTA [34].

Isolation of PBMCs is usually performed using a density gradient, such as Ficoll, in either standard or specific tubes, such as Accuspin™ (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), Leucosep® (Greiner Bio-One), or CPT™ (BD, Franklin Lakes, NJ, USA) tubes. Some studies have compared these methods and found discordant results for the yields of PBMCs recovered (reviewed in [37]). In our opinion, CPT™ tubes have the advantage of allowing blood collection and subsequent separation into plasma and PBMCs without further manipulation, thus representing an efficient and largely operator-independent alternative. They have been commonly used in Wsp PE malaria vaccine trials [14,18,44–47].

Importantly, the entire procedure, from blood collection to cryopreservation, should be performed within 8 h of venipuncture, as delays may impact on viability and recovery of PBMCs, as well as inhibit T-cell function [34,48,37].

PBMC cryopreservation

Following isolation, survival of PBMCs under poised metabolism is attained using cryoprotective solutions, such as DMSO-containing media, which prevent ice crystal formation and osmotic damage [37,49]. Commonly used cryopreservation media include 10% DMSO in fetal bovine serum, in 12.5% human serum albumin, or in human AB serum, although the latter has been reported to result in significantly lower PBMC viability [33,34]. No differences in viability were observed when cells were frozen at up to 30×10^6 cells·mL^{-1} [33]. Subsequent stepwise temperature drop, which avoids rapid intracellular freezing, is achieved using appropriate devices, such as isopropyl alcohol-based Mr. Frosty™ (Nalgene® Mr. Frosty®, Thermo Fisher Scientific, Waltham, MA, USA) or alcohol-free CoolCell™ (Corning™ CoolCell™, Thermo Fisher Scientific, Waltham, MA, USA) containers, which are placed at −80 °C. Cells should be transferred to liquid nitrogen (liquid or vapor phase) after 24–72 h, as PBMC recovery and viability are affected by long-term storage at −80 °C [34,37].

Peripheral blood mononuclear cell cryopreservation for up to 15 months was shown not to affect cell viability, recovery, or lymphocyte proliferation assays [36]. However, losses of IFN-γresponses of $CD4^+$ T and $CD8^+$ T cells may be more pronounced in long-term (more than 1 year) than in short-term (< 6 months) storage, possibly due to T-cell apoptosis and reduction in the number of antigen-presenting cells (APCs) [35]. Importantly, $CD4^+$ T-cell function appears to be particularly sensitive to cryopreservation, while $CD8^+$ T-cell responses are reportedly less affected [35,40,50]. This was observed in a study where immunogenicity to malaria thrombospondin-related adhesive protein (TRAP) and CSP peptide antigens was compared by ELISpot and intracellular cytokine staining (ICS) in fresh or frozen PBMCs from vaccinated individuals [40]. Thus, in order to minimize the impact of cryopreservation on T-cell function, short-

term (< 6 months) storage of cryopreserved PBMCs is recommended.

Thawing and resting of PBMCs

In order to minimize osmotic fluctuations and the duration of exposure to DMSO, which is toxic to human leukocytes, thawing of PBMCs should be performed in under 5 min and at 37 °C [33,37,41]. If severe cell clumping is observed, due to DNA release by unviable cells, DNase may be added to the washing medium to prevent cell adherence without affecting phenotypical and functional results [37,51].

Several studies have reported that allowing the cells to 'rest' for 6–18 h at 37 °C/5% CO_2 after thawing increases the sensitivity and magnitude of subsequent functional assays, as it reduces the unspecific activation of PBMCs and removes dead or dying cells [37,39,41,42,51–53]. In addition, it reestablishes the proportion of monocytes, which is reported to increase upon thawing, to levels similar to those found in fresh samples [42,43]. Of note, both cryopreservation and resting after thawing may also negatively affect the frequency and function of some myeloid-derived suppressor cell subsets [39,54]. Cell resting has also been correlated with the recovery of several surface and intracellular markers (such as the adhesion molecule CD62L, the chemokine receptor CCR7, the costimulation molecule CD28, the Treg markers CD25 and Foxp3, or the degranulation marker CD107a upon stimulation), restoring their associated cell subsets and/or function, including specific T-cell responses by IFN-γ ELISpot or IFN-γ/TNF-α/IL-2 detection through ICS (see below) [37,39,52,55]. Resting of PBMCs after thawing and before assessment of T-cell phenotype or function is thus favored, and is often performed for the evaluation of cellular responses in Wsp PE malaria vaccine clinical trials [18,30,56–59].

Conclusions

Clinical trial design should establish appropriate standardized blood sample collection and processing procedures, in order to ensure the quality of the materials obtained, maximizing the experimental throughput and the information obtained at each endpoint.

Evaluation of humoral immune responses elicited by Wsp PE vaccination

Humoral immune responses play a key role in vaccine-induced immunity. The assessment of the quantity and quality of antibodies elicited by immunization against the *Plasmodium* parasite, as well as against specific parasite antigens, constitutes a crucial step in the analysis of vaccine efficacy and may ultimately contribute to the identification of immune mechanisms underpinning protection [9,12,30,60–62]. Although *in vivo* assays can also be performed, humoral responses elicited by Wsp PE malaria vaccine candidates are commonly assessed through a plethora of *in vitro* assays, employing either purified Igs or whole serum/plasma isolated from the volunteers' venous blood. The classes of antibodies most commonly analyzed are IgG [18,28–31,63–66] and IgM [18,63,67]. Although the former bind more efficiently to the antigen and aid in opsonization, making them better neutralizing antibodies than IgM, the latter are the first antibodies to be produced during a primary immune response [68].

Plasmodium antigens targeted by Wsp PE vaccine-induced antibodies

Because Wsp PE vaccines mainly induce immunity to the sporozoite and liver stages of the *Plasmodium* life cycle, methods aimed at assessing humoral immunity generally target antigens expressed in sporozoites, early liver stages, and late liver/blood stages. CSP, a highly abundant protein present on the surface of sporozoite and early liver stages, constitutes the most commonly studied target of humoral immune responses in Wsp PE vaccine clinical trials [18,69,70]. However, a broader landscape of Wsp PE vaccine-induced antibodies can be identified by including in immunoassays antigens expressed throughout the *Plasmodium* life cycle, such as (a) in sporozoites [sporozoite surface protein 2/thrombospondin-related adhesion protein (SSP2/TRAP), cell-traversal protein for ookinetes and sporozoites (CelTOS), apical membrane antigen 1 (AMA1), and sporozoite threonine-asparagine-rich protein (STARP)] [9,63,71–73]; in early liver stages [exported protein 1 (EXP1), liver stage antigen 1 and liver stage antigen 3 (LSA-1, LSA-3), and glutamate-rich protein (GLURP)] [58,62,71,72]; or in late liver/blood stages (merozoite surface protein (MSP-1) and erythrocyte binding antigen 175 (EBA-175)] [12,58,63,73,74] (Table 1). Although the selection of antigens to be analyzed should depend on the specific objectives of each clinical trial, the target antigens MSP-1 and CelTOS are commonly added to immunoassays including CSP, to provide information on the development of antibodies against late liver stages and other *Plasmodium* species (cross-species immunity), respectively.

Table 1. Main protein targets for antibodies studied in the context of Wsp PE vaccine candidate trials.

Plasmodium life cycle stage	Antibody target	Cellular location and main function	Role in Wsp PE vaccination and possible correlates of protection reported
Sporozoite	CSP	Sporozoite membrane surface Essential for sporozoite formation and invasion of mosquito salivary glands and host hepatocytes	Antibodies targeting CSP have been detected in several human studies of Wsp PE vaccines Following vaccination of human volunteers with RAS, anti-CSP antibody titers were correlated with immunization dosage and vaccine protective efficacy [12] Following immunization with GAP or under chloroquine prophylaxis, anti-CSP antibodies were detected in most volunteers, albeit with no association with protection was reported [14,29,58,153] *Pb*Vac, the Wsp PE vaccine based on genetically modified rodent *Plasmodium* parasites, elicited functional anti-PfCSP antibodies capable of efficiently blocking sporozoite invasion *in vitro* [18]
	SSP2/ TRAP[a]	Transmembrane surface protein involved in protein–protein interactions, associated with cell adhesion Essential for locomotion, binding of sporozoites to hepatic cells, and subsequent invasion	Immunization of human volunteers with RAS induced anti-SSP2/TRAP antibodies capable of inhibiting sporozoite infection of hepatocytes *in vitro* [154] Antibodies directed against SSP2/TRAP have been detected in human volunteers following Wsp PE vaccination, although a correlation with protective immunity was not identified [153,155]
	CelTOS	Localized to the parasite micronemes Critical for ookinete traversal of the mosquito midgut and for sporozoite infectivity of hepatic cells	Human volunteers immunized with RAS elicit a strong humoral response to CelTOS [156] CelTOS is also reportedly involved in the mediation of cross-species protection [157]
	AMA1[a]	Localized in the rhoptries, but also detected on the surface of the merozoite after schizont rupture Essential for sporozoite invasion of hepatocytes [158] and merozoite invasion of erythrocytes [159]	High titers of naturally acquired antibodies against AMA1 are correlated with age [160] A series of *in vivo* immunization experiments suggested that AMA1 elicits immune responses against blood stage parasites, but a correlation between anti-AMA1 IgGs and liver stage protective immunity has not been described [161]
	STARP	Present on the surface of sporozoites, indicating a role in hepatocyte invasion [162]	Volunteers immunized with RAS produced anti-STARP antibodies that prevented *in vitro* infection of human hepatic cells [163] The presence of antibodies to STARP was associated with protection from clinical malaria in West African children [164]
Early liver stages	EXP1[a]	Localized on the surface of the parasitophorous vacuole (PV) and in the cytoplasm of the host cell in both the liver and the blood stages. It is required for intrahepatic parasite growth [165] and nutrient uptake through the PV membrane (PVM) [166]	EXP1 antibody titers were used for initial screening of volunteers for clinical trial as an indicator of recent *Plasmodium falciparum* exposure [73], although vaccine-induced EXP1 titers are reported to be very low [9,63,72]
	LSA-1	Expressed after parasites have invaded hepatocytes and localized within the PV. It may also adhere to the merozoite surface, suggesting a role during liver schizogony	Immunization of malaria-naïve volunteers with either RAS or under chloroquine prophylaxis induced antibodies to LSA-1, although these did not correlate with protection [62,167]
	LSA-3	Localized to internal organelles and the surface of *P. falciparum* sporozoites and is associated with LSA-1 in the liver stage PV. The exact role of LSA-3 is unknown, but it has been suggested that it could have a common function during parasite development in both hepatic and erythrocytic stages [168]	West African children develop antibodies against LSA-3, whose titers increase with age [164,169] Immunization of nonhuman primates with RAS elicits production of anti-LSA-3 antibodies, although their possible role in protective immunity remains unexplored [170]

Table 1. (Continued).

Plasmodium life cycle stage	Antibody target	Cellular location and main function	Role in Wsp PE vaccination and possible correlates of protection reported
	GLURP[a]	Localized to the PV of both liver and blood stage parasites	GLURP-specific IgGs are significantly correlated with clinical protection from *P. falciparum* malaria [171] An *in vitro* study revealed that human anti-GLURP IgGs mediate a strong monocyte-dependent parasite growth inhibition in a dose-dependent manner [172]
Late liver stages/ blood stages	MSP-1[a]	Localized on the surface of late liver stage and erythrocytic stage merozoites Essential for merozoite formation/budding [173] and erythrocyte invasion	The presence of antibodies against MSP-1 is correlated with protection from clinical *P. falciparum* malaria, potentially by blocking the entry of merozoites into erythrocytes [174]
	EBA-175	Essentially localized in the micronemes of blood stage parasites, although it is also expressed in infected hepatocytes and on the sporozoite surface [175] Serves as a merozoite ligand that binds to the erythrocyte receptor, glycophorin-A, through a receptor–ligand interaction [176]	High-titer antibodies to EBA-175 are associated with protection from clinical malaria in children in a malaria holoendemic area of Kenya [177]

[a]AMA1, EXP1, TRAP, MSP-1, and GLURP are cross-stage antigens.

Evaluation of antibody-mediated immunity

Evaluation of vaccine-induced humoral immune responses is usually performed by ELISA, which can be complemented with other types of analyses, such as *in situ* identification of *Plasmodium*-binding antibodies using an immunofluorescence assay (IFA). The functional capacity of the vaccine-induced antibodies to hinder overall parasite fitness (gliding motility, cell traversal and invasion, and intrahepatic development) can also be assessed *in vitro*, utilizing hepatic cell lines or primary human hepatocytes, or *in vivo*, employing humanized animal models.

ELISA

Antigen-specific humoral immune responses elicited by vaccination can be assessed by ELISA, through the use of recombinant parasite-derived proteins or peptides for specific, previously characterized antigens (Table 1) or of crude *Plasmodium* parasite lysates and whole *Plasmodium* sporozoites [70]. General ELISA procedures have been extensively described in previous reports [70,75]; however, special care should be taken when using whole or lysed parasites, favoring air drying for immobilization onto the plate over the use of fixatives such as paraformaldehyde, glutaraldehyde, or methanol, in order to preserve epitope conformation and ensure a stronger signal [65–70]. Serial dilutions of serum/plasma samples collected at several time-points should be included in the assay, as well as an adequate standard curve generated from positive control samples, and a negative control sample obtained prior to immunization [76]. Bound Ig are quantified by spectrophotometry using enzyme-conjugated anti-human Ig antibodies, followed by chromogenic detection with a corresponding substrate.

ELISA spot (ELISpot)

ELISpot assays can be employed to obtain a more comprehensive measurement of the antibodies secreted by B cells [77]. ELISpot differs from ELISA in that it allows the absolute quantification of the number of B cells producing antigen-specific antibodies. This is achieved through the culture of volunteers' PBMCs with a B-cell stimuli (e.g., for 5 days with lectin Pokeweed mitogen, *Staphylococcus aureus* protein A, CpG oligodeoxynucleotide ODN-2006, and recombinant human interleukin-10) to promote development of memory B cells into antibody-secreting cells (ASCs) [78]. Antigen-bound Ig is detected as quantifiable spots using an enzymatically labeled secondary antibody, upon deposition of the corresponding substrate [77,78]. Data from ELISpot quantification of ASCs and from serum/plasma titers of malaria antigen-specific antibodies, as determined by ELISA, can then be integrated [62].

Proteome arrays

Systemic profiling of anti-*Plasmodium* antibodies induced by the vaccine candidate can also be performed using proteome arrays, which enable a wide

characterization of the humoral responses in a high-throughput format. A whole-proteome microarray containing *in vitro*-translated *Plasmodium* full-length proteins and polypeptides, representing 4805 (~ 91%) of the proteome predicted for *P. falciparum*, was constructed by Antigen Discovery, Inc. (ADI, Technology Drive Irvine, CA, USA) and employed to characterize the global IgG response from the volunteers vaccinated with chemoattenuated PfSPZ [57]. Although this proteomic approach circumvents the difficulties in identifying and measuring vaccine-induced humoral immune responses, some limitations of this methodology have been highlighted [79]. These include a possible underestimation of the association of antigens to the serum antibodies or the possibility of false-negative hits emerging as a result of the absence of post-translational modifications in protein epitopes [79].

Immunofluorescence assay

The IFA uses anti-human Ig antibodies conjugated to fluorescent dyes to identify the presence of antibodies bound to specific antigens. This technique is commonly employed for the detection of vaccine-induced antibodies able to bind to native *Plasmodium* sporozoites. IFA involves an initial step of air-drying purified sporozoites on microscope slides or plates, followed by incubation with serial dilutions of the volunteers' serum/plasma samples [9,10,18,30,74] and with a fluorochrome-conjugated anti-human Ig [44,57,72–74]. As for ELISA, dilutions of positive and negative control samples should be included in the assay. Additionally, in the case of IFA for sporozoites, CSP staining can be used to facilitate their identification [44,66]. The fluorescent signal can be detected using a fluorescence microscope [10,44] or a laser scanning imaging cytometer [30,57,66,73]. Despite its simplicity and specificity, IFA is laborious and potentially difficult to standardize between different laboratories. Therefore, IFA and ELISA are commonly performed in a complementary manner, in order to increase reproducibility and sensitivity when evaluating humoral responses elicited by immunization [80].

Complement fixation and lysis assays

In addition to direct sporozoite neutralization, Wsp PE vaccines can also elicit the production of antibodies with complement-fixing capacities. Complement activation results in pathogen opsonization, recruitment of phagocytes, and pathogen lysis via C3b complement protein deposition [81]. In fact, it has been reported that antibodies naturally acquired after infection are able to promote complement deposition and activation, resulting in *in vitro* inhibition of hepatocyte traversal [82].

To assess the functional capacity of vaccine-induced antibodies to fix complement proteins on sporozoites and to compromise parasite membrane integrity, a flow cytometry-based complement lysis assay can be performed [82]. To this end, freshly dissected *Plasmodium* sporozoites can be incubated with heat-inactivated pre- or post-immunization serum, or purified Ig [18], in the presence of fresh human serum (containing active complement) or inactivated complement. The deposition of C3b on sporozoites and the percentage of membrane-compromised parasites can then be assessed by flow cytometry using an anti-C3b antibody and a viability dye, respectively. Alternatively, the ability of serum/plasma antibodies to fix complement can be assessed by C5a detection in the supernatant by ELISA [67].

Inhibition of sporozoite invasion assay

Assessing the functional role of vaccine-induced antibodies in protection is a crucial step in the characterization of the humoral immune responses elicited by vaccination and in the search for immune correlates of protection. The ability of antibodies to interfere with the parasite's normal function is most commonly assessed by inhibition of sporozoite invasion (ISI) assays [12,18,31,57,63,65,67,73,74,83]. In addition, the antibodies' ability to hamper sporozoite motility [28], hepatocyte traversal [28,31], and liver infection [10,31,47,84] can also be evaluated.

During an ISI assay, freshly dissected *P. falciparum* sporozoites are incubated with serially diluted serum/-plasma samples to allow antibody–antigen interactions to occur, prior to sporozoite addition to *P. falciparum* infection-susceptible HC-04 cells. All plates should include negative (sporozoites incubated with preimmunization serum/plasma, as well as in the absence of sample) and positive (infected cells incubated with an anti-*P. falciparum* sporozoite monoclonal antibody, such as anti-CSP) controls [12,18,57,67,73,74,83]. A differential immunostaining, based on the use of secondary antibodies labeled with different fluorochromes and a permeabilization agent, enables distinguishing intracellular *Plasmodium* sporozoites from those that did not invade the cells [9,12,57,63–66,73,85]. Plates can be scanned manually or using a laser scanning imaging cytometer, which counts the number and fluorescence intensity of infected cells. Alternatively, cells can be immediately permeabilized and stained with a fluorescently labeled anti-CSP monoclonal antibody to detect

intracellular (invading) sporozoites. Employing microscopy to manually scan the plates offers the possibility of visualizing the parasite, increasing the amount of information that can be obtained. However, flow cytometry-based ISI represents a faster alternative, enabling the high-throughput analysis of a larger number of samples [18,67,71]. The inhibitory activity of each serum/plasma dilution can be calculated by comparing the results with that of either negative control [9].

The capacity of vaccine-elicited antibodies to inhibit sporozoite gliding motility can also be evaluated and, unlike ISI assays, does not require hepatic cell cultures. In gliding assays, sporozoites are incubated with the volunteers' serum/plasma samples and added to plates coated with a monoclonal anti-CSP antibody. CSP gliding trails are fixed and stained with a fluorochrome-labeled anti-CSP antibody [28]. Finally, hepatocyte traversal assays involve the infection of cultured hepatic cells in the presence of a membrane-impermeant dye, such as tetramethylrhodamine dextran [28,31]. Cells wounded during traversal by sporozoites incorporate dextran, and the percentage of dextran-positive cells can be quantified by microscopy or flow cytometry. By adding dextran to the ISI assay described above and selecting adequate fluorochrome-labeled anti-*Pf* sporozoite monoclonal antibodies, it is possible to simultaneously analyze the ability of antibodies to inhibit both sporozoite traversal and invasion [31].

The ISI assay can be further expanded to enable quantification of overall inhibition of liver stage infection and parasite development (ILSDA) in suitable cells, such as primary human hepatocytes. The assay follows procedures identical to those previously described, but infection and parasite development is allowed to progress for approximately 3 days prior to fixation and staining [10]. It is also possible to perform this assay *in vivo* employing humanized mouse models, such as the human liver chimeric mouse overexpressing urokinase-type plasminogen activator on a severe combined immunodeficiency background [28] or the FRG ($Fah^{-/-}Rag2^{-/-}$-$Il2rg^{-/-}$) huHep mouse [30]. To this end, mice are injected intraperitoneally with purified total IgG from the serum/plasma samples, or with anti-*Pf*CSP mouse monoclonal antibody as control, the day before challenge by the bites of *P. falciparum*-infected mosquitoes. Five to six days later, at the peak of liver infection, the liver stage burden is analyzed either by a bioluminescence assay (if a luciferase-expressing transgenic *P. falciparum* parasite is available) or through the isolation and quantification of *P. falciparum* and human hepatocyte DNA in liver samples [28,30]. This constitutes a biologically relevant setup to analyze the functional capacity of the vaccine-induced antibodies, although the number of samples that can be analyzed is highly limited by the costs of this methodology. Additionally, the passive transfer of human monoclonal antibodies, derived from B cells of vaccinated volunteers, into FRG huHep mice and their subsequent challenge by *P. falciparum*-infected mosquito bites can be employed to measure the protective capacity of vaccine-induced antibodies [86].

Growth inhibition assay

Wsp PE vaccines, particularly the ones employing late-arresting and chemoattenuated parasites, present a diverse antigenic repertoire that potentially elicits some level of cross-stage protection [87]. In fact, late liver stage-arresting parasites and blood stage parasites share similar transcriptomes, suggesting that many parasite proteins may be expressed during both stages of the parasite's life cycle and thus serve as antigenic targets of stage-transcending protective immune responses [88]. Thus, besides assessing the functionality of vaccine-induced antibodies during the liver stage of infection, their impact on invasion and development of asexual blood stage parasites can also be evaluated *in vitro* through a growth inhibition assay (GIA) [89]. To this end, dilutions of serum/plasma samples or purified IgGs are added to synchronized *P. falciparum* cultures for 40–42 h. The growth inhibitory activity of post-immunization antibodies can be assessed employing a biochemical assay that measures *Plasmodium* lactate dehydrogenase (LDH) levels in culture supernatants, and comparing them with those obtained for pre-immunization samples, used as controls [29].

Conclusion

Wsp PE malaria vaccine candidates can elicit a diverse repertoire of antigen-specific antibody responses. The titers and functionality of these antibodies can be assessed through a variety of methods that play a crucial role in the analysis of vaccine efficacy and in the identification of potential correlates of protection.

Evaluation of antigen-specific cellular immune responses elicited by Wsp PE vaccination

A comprehensive analysis of antigen-specific cellular responses after vaccination is essential to understand the potency and efficacy of vaccine candidates, and may contribute to the identification of immune correlates of protection [18,30,56,90,91]. In contrast to the thorough investigation of lymphoid and nonlymphoid tissue-

associated cellular responses that is possible in animal model studies, human T-cell function is largely assessed through peripheral blood collection. Although PBMCs may not mirror the full immune response to Wsp PE vaccination, they provide key information on vaccine-induced cell-mediated immunity, which can be evaluated employing an array of *ex vivo* phenotypic and functional immunoassays, as described in the following sections.

Core immune cell populations

The evaluation of the cellular immunity elicited by a Wsp PE malaria vaccine candidate is usually performed by flow cytometry and involves the assessment of specific memory responses elicited by various T-cell populations against the *Plasmodium* parasite or its antigens (extensively reviewed in [21,90,92]). The main *Plasmodium*-specific T-cell populations of interest in Wsp PE vaccine candidate clinical trials include helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells, in particular polyfunctional memory cells producing IFN-γ, TNF-α, and/or IL-2 cytokines, as well as γδ T cells, all of which are frequently expanded upon vaccination and have been reported to correlate with protection against CHMI (Table 2) [10,12,14,18,20,30,45–47,57–59,64,65,73,84,93–95]. Other circulating populations can inform on the kinetics of the immune response elicited by the vaccine candidate, and particularly on the impact of specific immunization doses or schedules (Table 2). The study of these cellular immune responses relies on the identification of combinations of appropriate markers that are either expressed by or absent from a specific cell population [38,96–98]. For example, T cells activated upon Wsp PE immunization can be identified *ex vivo* by the expression of markers such as HLA-DR and CD38 [30,57,65,73,93,99], which, in $CD4^+$ T cells, reportedly correlate with *P. falciparum*-specific responses [65]. Further analysis of vaccine-associated cell-mediated immunity can be achieved through *in vitro* stimulation of PBMCs and assessment of the ensuing responses.

In vitro stimulation

The evaluation of Wsp PE vaccine-induced immunogenicity involves the culture of volunteers' PBMCs with stimuli associated with the different stages of the *Plasmodium* parasite's life cycle (Table 3). These stimuli vary in complexity, and may include recombinant proteins or peptides (individual or pooled) [12,18,46,56,58,60], homologous [9,10,12,18,30,44,46,57,58,60,63–65,73,74,94] or heterologous [65] sporozoites, and homologous [14,20,30,44–47,57,64,73,74,93,95] or heterologous [20,47] *Plasmodium*-infected red blood cells (RBCs). Sporozoites and infected RBCs can be purified and cryopreserved prior to use in the *in vitro* assays (see references in Table 3). For techniques that rely on antigen processing and presentation mediated by APCs in the autologous PBMCs, such as ICS, the length of the culture usually depends on the type of antigen: 4–6 h for proteins and peptides and 16–24 h for sporozoites or RBCs (see references in Tables 2 and 3). Techniques that assess cell proliferation are more time-consuming and may require several days of culture (see references in Table 3).

Positive and negative controls of stimulation should be included to validate the assay and assess the basal and the maximum stimulation potential of the tested cells. Positive controls may include polyclonal activators such as the lectin phytohaemagglutinin, the superantigen staphylococcal enterotoxin B, or the protein kinase C activator phorbol 12-myristate 13-acetate, in combination with the calcium ionophore ionomycin [37,100,101]. Unstimulated or uninfected cells, as well as cells in the presence of either costimulation only (such as anti-CD28 and anti-CD49d) or the sporozoite diluent (e.g., human serum albumin for PfSPZ), can be used as negative controls. In addition, it is important to include a pre-immunization sample from each patient as an internal negative control in order to set the basal stimulation threshold.

Evaluation of cell-mediated immunity

Several types of assays can be performed to assess vaccine-induced responses. These typically rely on either the measurement of T-cell proliferation, of cytokine production, or of cytotoxicity (Table 4). These assays, albeit with varying levels of sensitivity and different throughputs, can be used individually or in combination (e.g., CD107a degranulation and ICS) to provide complementary information on T-cell phenotype and function [102–104]. ICS is currently the most widely used technique to assess T-cell function, allowing the identification and characterization of polyfunctional T-cell responses. Depending on the flow cytometry analyzer available, ICS may enable the simultaneous evaluation of up to tens of molecules per cell. This and other techniques useful for the assessment of cellular responses against Wsp vaccines will be described in the following sections.

Cytokine production

Intracellular cytokine staining is a sensitive short-term (4–24 h) assay that enables the quantification and analysis of polyfunctional cytokine-producing T cells.

Table 2. Main circulating populations analyzed in the context of Wsp PE vaccine candidate clinical trials.

Cell population	Main functions and markers	Main populations analyzed and possible correlates of protection reported
CD4$^+$ T cells	a Recognize peptides presented on MHC class II on APCs. Have a wide range of important helper and regulatory functions in the immune system [178] b Naïve T cells (CCR7$^+$, CD45RA$^+$, CD45RO^{neg}) have not encountered specific antigen; central (TCM; CCR7$^+$, CD45RA^{neg}, CD45RO$^+$) and effector (TEM; CCR7^{neg}, CD45RA^{neg}, CD45RO$^+$) memory T cells mainly survey lymphoid and nonlymphoid tissues, respectively. Terminally differentiated EM-expressing CD45RA (TEMRA; CCR7^{neg}, CD45RA$^+$, CD45RO^{neg}) cells exert potent effector function after activation [38,98] c Upon antigen-mediated activation, they differentiate into several phenotypes, including Th1 cells (protect against intracellular pathogens; produce IFN-γ, TNF-α, IL-2), Th2 (protect against extracellular parasites; produce IL-4, IL-5, IL-13), or Th17 (protect against extracellular bacteria and fungi; produce IL-17). Treg (regulatory T cells; express Foxp3, CD25, CTLA-4 but neg/low CD127 levels; produce IL-10), which can be thymus-derived or peripherally induced, maintain immune homeostasis and tolerance through inhibition of pro-inflammatory T helper cells [178]	a *Plasmodium*-specific central or effector memory CD4$^+$ T cells, specifically those with a Th1-polarized phenotype-expressing IFN-γ, TNF-α, and/or IL-2 [10,12,14,18,20,30,45–47,57–59,64,64,65,73,84,93–95] or Th2 responses (IL-4 production) [57,59]; the frequency of Pf-specific polyfunctional CD4 memory T cells producing IFN-γ, TNF-α, and IL-2 [14,57,94] and of CD107a$^+$ CD4 T cells [84] have been reported to correlate with protection b Activated cells identified *ex vivo* by the expression of markers such as HLA-DR, CD38, or 4-1BB [30,65,93] c Treg [45,59,73,93]
CD8$^+$ T cells	a CTLs recognize pathogen-derived peptides bound to MHC class I on any nucleated cell and are responsible for the direct killing of infected, damaged, or dysfunctional cells [179] b Naïve/memory subsets: equivalent to those defined for CD4$^+$ T cells [38,98] c Mainly produce IFN-γ and TNF-α, but also IL-2 [98] d Produce and release cytotoxic granules that contain perforin and granzymes [98]	a *Plasmodium*-specific central or effector memory CD8$^+$ T cells expressing IFN-γ, TNF-α, and/or IL-2 [9,10,12,18,20,30,45–47,57,58,65,73,84,93,95,113,114]; increased frequency of Pf-specific CD8$^+$ IFN-γ-producing T cells has been associated with protection [12] or increased prepatent period [18] b *In vivo*-activated cells, identified *ex vivo* by the expression of markers such as HLA-DR, CD38, or 4-1BB [30,65,93] c Production of granzyme B by CD8 T cells was reported to associate with protection [84]
γδ T cells	a Unconventional T lymphocytes expressing TCRγδ, not restricted by classical MHC-mediated antigen presentation. Are important for mucosal immunity, response to microbial pathogens and tumor surveillance [180] b Vδ9$^+$Vδ2$^+$ cells, the major γδ T cell subset in human blood, recognize phosphoantigens that specifically and robustly activate them to proliferate, secrete cytokines, and display cytotoxic behavior [180] c Produce IFN-γ, TNF-α. Express granzyme B [180]	a γδ T cells, particularly the Vδ9$^+$Vδ2$^+$ subset [9,12,18,20,30,45,46,57,64,65,73,84,93]; the absolute frequency of Vδ2$^+$ γδ T cells has been correlated with CHMI outcome [30] b Activated cells identified *ex vivo* by the expression of markers such as HLA-DR, CD38, or 4-1BB [30,57,65,73,99]
NK cells	a Kill virally infected cells and detect and control transformed cells b Can be subdivided into different populations, such as CD56^{dim}CD16^{high} (cytotoxic subset; largest subset in the blood) and CD56^{high}CD16$^{neg/dim}$ (cytokine producers) [38,181] c Produce IFN-γ; express granzyme B [181]	a Total CD56$^+$ NK cells, as well as the CD56^{dim}CD16^{high} and CD56^{high}CD16$^{neg/dim}$ subsets [9,18,45,46,93]
NKT cells (or NKT-like)	a Important immunoregulatory cells, rapidly producing large amounts of cytokines, such as IFN-γ, that can influence other immune cells [182] b Express a restricted TCR repertoire [182]	a Increase in CD3$^+$ CD56$^+$ NKT cells has been described to associate with prepatent period and inversely with parasitemia in Wsp cross-species (PbVac) vaccination. [18,45,93,99]
Monocytes	a Myeloid mononuclear recirculating leukocytes that can act as precursors of tissue macrophages or dendritic cells [183]	a Decreased frequencies of circulating monocytes have been reported after Wsp cross-species (PbVac) vaccination [18]

Table 2. (Continued).

Cell population	Main functions and markers	Main populations analyzed and possible correlates of protection reported
	b Can be divided in classical monocytes ($CD14^{++}$, CD16neg; most abundant in the blood; mainly phagocytic), intermediate ($CD14^{++}$, $CD16^{+}$; produce reactive oxygen species, role in T-cell stimulation and inflammation), and nonclassical ($CD14^{+}$, $CD16^{+}$; inflammatory and antigen presentation characteristics on activation) [183]	b A systems biology approach identifying transcriptional signatures of protective immunity against malaria highlighted monocytes as potentially crucial [56]

Table 3. Stimuli used for determination of vaccine-induced immunogenicity of PBMCs.

Stimuli	Description	References
Whole sporozoites	PBMCs are cultured in the presence of homologous or heterologous sporozoites (e.g., 150 000 sporozoites per 1×10^6 cells) for 16–24 h (ICS) or for 24–40 h (ELISpot). Sporozoites may be purified and cryopreserved. For ICS, cells are incubated with Golgi inhibitors (brefeldin and/or monensin, for example) for the final 4–6 h. Technical negative controls: unstimulated cells, sporozoite diluent or salivary glands from the same number of uninfected mosquitoes (increased background may be observed in this case)	[9,10,12,18,30,44,46,57,58,60,63–65,73,74,94]
Asexual parasites, such as *Plasmodium*-infected RBCs (schizonts/mature trophozoites)	PBMCs are cultured in the presence of purified, infected, homologous, or heterologous RBCs (e.g., 2×10^5 schizonts per 1×10^6 cells) for 16–24 h (ICS) or for 24–40 h (ELISpot). *Plasmodium*-infected RBCs can be cryopreserved. For ICS, cells are incubated with Golgi inhibitors (brefeldin and/or monensin, for example) for the final 4–6 h. Technical negative controls: uninfected RBCs	[14,20,20,30,44–47,57,64,73,74,84,95]
Specific proteins or antigens, such as CSP (or repeats), SSP2/TRAP or MSP-5 for sporozoites; LSA-1 or EXP-1 for early liver stages; MSP-1 or EBA-175 for late liver stages; MSP-2, MSP-3 or GLURP for blood stage	PBMCs are cultured in the presence of recombinant proteins or peptides for 4–6 h (ICS) or for 24–36 h (ELISpot). Cryopreserved PBMCs should be thawed and rested overnight before short-term stimulation. Inclusion of costimulation (e.g., anti-CD28/CD49d) significantly increases measurable response, although it also increases the background. For ICS, cells are incubated with Golgi inhibitors (brefeldin and/or monensin, for example) for the final 4–6 h. Technical negative controls: unstimulated or costimulation only	[12,18,46,56,58,60]

ICS is commonly employed to elucidate the immunological profile of *Plasmodium*-specific T-cell responses to a variety of stimuli (Tables 2 and 3) [9,10,12,14,18,30,44–47,57,58,64,65,73,74,84,94,95,105]. Cell stimulation in the presence of Golgi inhibitors (such as brefeldin or monensin) prevents cytokine secretion and allows cytokine measurement after fixation, permeabilization, and staining with appropriate fluorochrome-conjugated antibodies [50–53,101,106,107]. Use of multiparameter flow cytometry enables the simultaneous inclusion of multiple cellular markers. In addition to the quantification of antigen-specific T-cell frequency, ICS also allows a comparison between the amounts of cytokines produced per cell through the determination of median (or geometric mean) fluorescence intensity [57,84].

Table 4. Methodologies that have been used for the evaluation of *Plasmodium*-specific cellular responses. CTV, CellTrace Violet; CBA, Cytometric Bead Array; CSA, cytokine-secretion assay; 7-AAD, 7-aminoactinomycin D

Basis	Methods	Description	Major strengths and limitations	Malaria studies
Cytokine production	ICS	Detection of multiple cytokines, as well as of phenotypical and functional markers, upon antigen stimulation in the presence of protein secretion inhibitors, by intracellular staining and multiparametric flow cytometry	Allows the phenotyping of cells producing multiple cytokines at the single-cell level, as well as conjugation with the analysis of proliferation (e.g., Ki67, CFSE), transcription factors (e.g., Tbet, Foxp3), and cytotoxic/ degranulation markers (e.g., surface CD107a, granzyme B, perforin) Very sensitive method	[9,10,12,14,18, 30,44–47,57,58,64, 65,73,74,84,94,95,105]
	ELISpot/ FLUOROSPOT	Quantification of specific responses by incubating cells in the presence of antigen in a plate coated with anticytokine antibody; individual cytokine-producing cells are detected as spots by colorimetric (ELISpot) or fluorescent (FLUOROSPOT) methods	Most sensitive method for the detection of very low T-cell responses Does not require cell fixation Does not allow simultaneous cell population phenotyping FLUOROSPOT but not ELISpot is suitable for multicytokine detection	[10,58,60,63,12]
	ELISA Multiplex bead arrays (e.g., BD CBA, LEGENDplex™, Luminex®)	Quantification of total cytokines, *ex vivo* (plasma or serum) or upon stimulation with antigen (cell culture supernatant), using anticytokine antibodies attached to a plate (ELISA) or to beads (multiplex assays), by colorimetric, fluorescent, or luminescence methods	One analyte per assay in ELISA and several in multiplex CBA or Luminex® (high efficiency and throughput) Low sensitivity for low T-cell responses Does not provide information on the identity of cytokine-producing cells	[184,108,20,18,94]
Proliferation	[^{3}H]-thymidine incorporation	Lymphoproliferative assay that quantifies *de novo* DNA synthesis through incorporation of thymidine or analogs, in the presence of cognate antigen	[^{3}H]-thymidine has low sensitivity for reduced T-cell responses, involves radioactive reagents, and does not provide information on the identity of proliferating cells	[20,184]
	Ki67	Detection of Ki67, a nuclear antigen expressed during the proliferative phase of the cell cycle that can be used as a marker of recent or ongoing cell division, *ex vivo* or upon stimulation with antigen, by flow cytometry	Allows the simultaneous identification of recently proliferating cell types and cytokines produced (in combination with ICS)	[30,45,93,57]
	Permeable fluorescent cell staining dye dilution assay	Cell labeling with a membrane-permeant dye (such as CFSE or CTV), followed by assessment of antigen-induced proliferation through fluorescence dilution using flow cytometry	Allows the simultaneous identification of proliferating cell types and cytokines produced Requires long stimulation (days) Dyes may be toxic at high concentrations or after long exposure	[59]
Cytotoxicity	Target cell viability/ apoptosis assays	Evaluation of CTL function through direct measurement of target cell lysis. Target cell viability may be quantified by chromium ^{51}Cr release or staining with cell	Chromium release assay involves radioactivity Does not allow cell phenotyping	[113,114]

Table 4. (Continued).

Basis	Methods	Description	Major strengths and limitations	Malaria studies
		viability dyes (7-AAD, propidium iodide, fixable viability dye) or apoptosis markers (Annexin V, active caspase 3)		
	Effector cell degranulation assays	Quantification of the degranulation marker CD107a (LAMP-1) on the surface of effector CTLs or of their granule content (such as granzyme B or perforin) upon antigen stimulation, by flow cytometry	Allows simultaneous immunophenotyping and identification of cytokines produced (in combination with ICS)	[47,84]

ELISpot is another very sensitive technique for the quantification of cellular responses, which has been employed for the determination of cytokine-producing *Plasmodium*-specific T cells in several Wsp PE vaccine clinical trials (Table 3) [10,12,58,60,63]. In this setting, ELISpot involves the stimulation of PBMCs with appropriate stimuli in wells coated with anticytokine antibodies, and the subsequent detection of the captured product with an enzymatically labeled antibody that, upon addition of a chromogenic substrate, allows the identification of cytokine-producing cells as spots. The assessment of the number and the size of spots permits the quantification of antigen-specific cells and of the amount of cytokine produced, respectively, even at low specific T-cell frequencies [10,58,60]. It is, however, a labor-intensive method, which does not enable the phenotypic identification of antigen-specific cells and which only allows the assessment of one cytokine per assay. Production of more than one cytokine can be assessed using FLUOROSPOT, a technique in which each anticytokine antibody is labeled with a different fluorochrome, allowing the simultaneous quantification of several cytokines [63]. Alternatively, cytokine production can be quantified *ex vivo* (in the serum of clinical trial volunteers) or in the supernatant of cultures after stimulation of PBMCs by ELISA or by methods that enable multiplex determination of cytokines, such as Luminex® or LEGENDplex™ [18,20,94,108].

Cell proliferation

Cell proliferation assays take advantage of the ability of T cells to divide upon recognition of their cognate antigen. Although more time-consuming than those assessing cytokine production, these assays can be combined with other methodologies (such as ICS) to assess the phenotype and function of antigen-specific T cells. Cell proliferation assays rely on the incorporation of [^{3}H]-thymidine [20,109] into nascent DNA, expression of cell cycle-associated antigen Ki67 [45,93,110], or dilution of fluorescent membrane-permeant dyes, such as carboxyfluorescein succinimidyl ester (CFSE), during cell division [59,111] (Table 4). These assays have been used to identify *Plasmodium*-specific T cells in Wsp PE vaccine candidate trials, as well as to confirm their *bona fide* proliferation potential [30,45,57,93].

Cytotoxicity

Infected hepatocytes presenting peptide/major histocompatibility complex (MHC) class I complexes on their surface can be recognized and killed by $CD8^+$ cytotoxic T lymphocytes (CTLs) [112]. CTL activity in response to antigens can be measured using *in vitro* cytotoxicity assays that evaluate indicators of apoptosis or necrosis on target cells, or markers of degranulation on effector CTL (Table 1) [103,104]. Target cell assays include the radioactive chromium (^{51}Cr) release assay or the colorimetric measurement of LDH activity in the medium of a culture of CTL with target cells (usually tumor cells, lymphoblasts, or tissue culture cells) loaded with the antigen of interest [103,104]. CTL activity may also be evaluated through the assessment of viability and/or expression of apoptosis markers (Annexin V, active caspase 3) by target cells [103,104]. Alternatively, transient expression of CD107a (lysosome-associated membrane protein 1, LAMP-1) on the surface of CTLs can be used as a marker of CTL degranulation, and hence of cytolytic activity [47,84,100]. Simultaneous analysis of surface CD107a, of the cytotoxic pore-forming glycoprotein perforin or of the serine protease granzyme B, stored in secretory granules of CTLs or NK cells, can be combined with ICS to provide information on antigen-

specific CTL activity. This methodology has been employed in various Wsp PE malaria vaccine trials [100,107,84,93]. Although not many clinical trial studies have directly addressed CTL function [113,114], this type of assays could be important for validating the cytotoxic potential of $CD8^+$ T cells, as well as their response to novel epitopes [reviewed in [115]].

Conclusion

Wsp PE malaria vaccine development can involve several types of immunoassays for simultaneous evaluation of antigen-specific phenotype and function in blood immune cell populations (Fig. 1). Multifactor analysis of antigen-specific humoral and cellular immune responses may provide key information, such as potential correlates of protection, guiding future vaccine design strategies.

Future perspectives

Besides the well-established methods described in this review, novel approaches to evaluate immune responses in the context of Wsp PE malaria vaccine development are likely to emerge, increasing the amount of information generated, as well as their predictive capacity. Among these, we highlight four areas in which we expect further developments in a relatively near future.

Detailed analyses of cell-mediated immunity and the search for correlates of protection

The pivotal role of cellular immunity in the protection conferred by Wsp PE immunization has been established in a wide variety of animal model and human studies, as reviewed in [15]. The use of increasingly sophisticated flow cytometry-based methods has enabled much progress in the assessment of cellular immune responses in immunized individuals, as thoroughly described above. Nevertheless, the incessant search for immune correlates of protection is only starting to deliver a clear picture of the immune factors that accurately associate with the protective efficacy of a Wsp PE vaccine candidate [15,56]. New methods contributing to a more thorough profiling of the cellular immunity elicited by immunization might prove invaluable in this regard. Such is the case of high-dimensional cytometry by inductively coupled time-of-flight (CyTOF) mass spectroscopy, where antibodies are labeled with heavy metal isotopes, allowing the single-cell characterization of up to 60 parameters [116]. This method enables the simultaneous analysis of a high number of cell markers, as well as the high-throughput unsupervised analysis of the data and their integration in a systems immunology approach toward the determination of correlates of protection.

The identification of novel *Plasmodium*-specific epitopes associated with protection could represent a relevant tool to guide the rationale design of new Wsp PE vaccines. Analysis and isolation of antigen-specific T cells based on direct T-cell receptor (TCR) specificity recognition can be performed using magnetic- or fluorescence-conjugated multimeric synthetic complexes of four (tetramers) or more HLA molecules loaded with antigen-derived peptide [115,117]. MHC tetramer-based specificity assays have the disadvantage of requiring the knowledge of the HLA type of each individual, and of allowing the recognition of only one HLA-peptide pair per tetramer, requiring previous pathogen epitope mapping and screening [104,115,118]. Nevertheless, MHC multimers provide key information on the phenotype and function of antigen-specific T cells [66,115,119,120]. More recently, the DNA barcoding-based dCODE^{TM} Dextramer technology has emerged, enabling high-throughput genomic profiling of antigen-specific T cells [121]. We envisage that the future application of this methodology in PE malaria vaccine research will enable linking antigen recognition to the T-cell immune repertoire and phenotype at the single-cell level, providing uniquely detailed insights into the cellular immunity elicited by Wsp PE vaccination.

Assessment of liver-resident immune responses

Based on several murine studies, T cells are thought to play a more dominant role in the protection afforded by Wsp PE malaria vaccines, with a particular contribution from tissue-resident memory T cells (T_{RM}) in the liver [122–124]. It has also been shown that PfSPZ immunization of nonhuman primates led to robust $CD8^+$ T cells responses in the liver, whereas such responses were undetectable in the PBMCs of the same animals [10], highlighting the importance of direct analyses of liver samples. However, a T-cell correlate of protection, or even the identification of malaria-specific T_{RM}, has yet to be seen in human trials, leaving much unknown with regard to cellular Wsp PE immunity in humans. The evaluation of liver-resident immunity in humans poses obvious challenges, as it requires direct access to liver samples of immunized individuals. Fine-needle aspiration (FNA) biopsy of the liver in patients with mononucleosis has been described as early as 1945 [125], and for analysis of liver enzymes in healthy adults from the late 1960s

[126]. Since then, the procedures employed in collection of these biopsies have evolved significantly, including the use of endoscopic ultrasound-guided procedures that increase patient comfort and shorten recovery time after the biopsy [127]. Repeated FNAs of the liver have now been employed to track the kinetics of intrahepatic immune responses in patients with chronic liver disease or HCV infection [128]. More recently, liver FNAs have been employed to perform a comprehensive analysis of intrahepatic immunity in the context of HBV infection, enabling the identification of several immune cells, including locally resident sentinel HBV-specific T cells and NK cells [129]. Nevertheless, FNA is not a trivial medical procedure, and its risks must be balanced with the potential benefits they may accrue to malaria vaccine research. However, as FNA procedures become less invasive and more rapid, it can be expected that this procedure may be employed for the analysis of liver samples from Wsp-immunized individuals. Such an achievement would enable a broad profiling of their hepatic immune responses and provide pivotal information about tissue-resident responses to Wsp PE vaccination.

Functional evaluation of cellular immune responses

Human immune system (HIS) mice, possessing functional human CD4, CD8, and B-cell responses, have been employed to evaluate protective humoral [130] and cellular [131] responses *in vivo*, following vaccination with *Pf*CSP and challenge with transgenic *P. berghei* parasites encoding this protein. HLA-expressing humanized DRAGA (HLA-A2.HLA-DR4.Rag1KO.IL2RγcKO.NOD) mice have also been shown to elicit autologous human CD4 and CD8 T-cell responses to *P. falciparum* antigens following immunization with live *P. falciparum* sporozoites under chloroquine chemoprophylaxis [132]. On the other hand, liver-humanized mice can be employed to evaluate the functionality of heterologous humoral immune responses against *P. falciparum* liver infection following passive transfer of antibodies from immunized volunteers [28,86,133]. However, liver-humanized mouse models that enable the adoptive transfer of immune cells from vaccinated human subjects, which would permit the functional evaluation of heterologous cellular immune responses elicited by immunization with Wsp PE malaria vaccine candidates, remain unavailable. Such studies are currently precluded by the fact that they would require the use of mice that are HLA-matched to each donor, as occurs in cancer immunotherapy studies, where tumor cells and tumor-infiltrating T cells from the same patient are transplanted sequentially into the same animal [134]. Nevertheless, much progress is being made on the development of HIS mouse models, as recently reviewed in [135]. Thus, while HLA matching remains a severe limitation for adoptive transfer experiments of human immune cells, future developments in this regard might open the door to investigate the functionality of the cellular immune responses elicited by Wsp PE human vaccination.

Systems vaccinology

Vaccinology is now transitioning into a new era that builds upon the powerful new tools of modern biology. The success of this new vaccinomics requires an understanding of the biology of the immune response and the application of this knowledge to the clinical trial process [136]. Systems-level analyses of omics data can contribute to a better understanding of the molecular changes in humans upon exposure to a vaccine and to rational vaccine design [137,138]. Omics methods employed in the context of systems vaccinology include genomics, transcriptomics, proteomics, metabolomics, epigenomics, and microbiomics, among others (reviewed in [139–141]). 'Big data' sets obtained from systems-based studies can be employed throughout the different stages of vaccine development to facilitate their rational design, decrease the risk of late-stage failure, and ensure their effectiveness and safety [140,142]. A comprehensive analysis of various levels of individual variations in response to vaccination might help to explain and mitigate differences in vaccine efficacy sometimes observed between phase I/IIa trials and subsequent phases of development [143]. Systems-based studies have been conducted in humans to investigate vaccine candidates against, for example, Ebola [144], HIV-1 [145], *Mycobacterium tuberculosis* [146,147], and *Francisella tularensis* [148]. Systems analysis and transcriptomics have also been employed to analyze protective immune responses following immunization of human subjects with the RTS,S subunit PE malaria vaccine and CHMI, identifying new molecular signatures and signaling pathways associated with protection [56,149,150]. This type of analysis could be further extended to simultaneously include high-parameter protein detection and deep mRNA characterization at the single-cell level, using novel single-cell multiomics technologies, such as AbSeq [151,152], linking particular immune targets to specific molecular signatures that can be exploited in future malaria vaccine design. However, to the best of our knowledge, systems vaccinology studies are only now

starting to be performed in the context of Wsp PE vaccination and CHMI protection [56,149,150]. We envisage that such studies will likely be performed in the future, with great potential for unveiling useful information about human responses to Wsp PE vaccines and establishing new correlates of immunization and protective efficacy.

Conclusion

Huge progress has been made over the last couple of decades on the development of Wsp PE vaccines against malaria and on the understanding of their mode of action in humans. As novel technologies emerge, they will likely become an integral part of the toolbox available for researchers to investigate human responses to Wsp PE vaccination and CHMI. Collectively, these methods will continue to contribute to our understanding of the immunity elicited by Wsp PE vaccination and to the identification of much-sought correlates of protection. This knowledge will undoubtedly play a pivotal role in guiding and informing the development of increasingly effective vaccines against one of the deadliest infectious diseases in the world.

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Author contributions

All authors contributed to writing and reviewing the text. All authors read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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