Safety and efficacy after immunization with Plasmodium falciparum sporozoites in the controlled human malaria infection model

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Chad. April 2005. Visiting refugees in their tent in Forchana camp.

CHAPTER 7

Discussion

DISCUSSION

The development of a malaria vaccine is far from an easy path and should preferably fulfill several criteria before implementation in the field is acceptable. A malaria vaccine needs, firstly, to be highly efficacious and provide long term protection against a variety of strains. Secondly, this vaccine preferably should also have transmission blocking capacities and provide cross-species protection against *P. vivax*. Thirdly, this vaccine should be easy to administer with a minimum number of immunizations for optimal patient adherence.

Aside from a tremendous number of outstanding questions in current malaria vaccine research, two aspects of vaccine development remain of utter importance. Firstly, to develop a malaria vaccine that protects against a variety of *falciparum* strains. However, none of the current vaccine initiatives have proven to fully protect for years and, up to date, only very few whole parasite vaccine candidates have evaluated heterologous protection to other strains [1-3]. Secondly, progress needs to be made in further identification of immunological mechanisms and markers of sterile protection (and/or disease). Unravelling these mechanisms may enhance vaccine development.

Answers to these questions are still basically absent in current malaria vaccine research and this fundamental lack of knowledge hampers the development of an effective vaccine that could theoretically avoid 438.000 deaths and 214 million clinical cases of malaria annually.

Chloroquine Prophylaxis Sporozoites immunization is a highly efficient strategy to induce sterile protection

In malaria research there are several malaria vaccination models, one of these is the Chloroquine Prophylaxis Sporozoites (CPS) strategy. CPS has proven to effectively induce 100% homologous protection with a minimum of 3x15 mosquito bites [4], is highly reproducible (**chapter 2 and 3**), and has proven to last up to 28 months in two out of three volunteers as was shown by Controlled Human Malaria Infection (CHMI) [5]. Reducing the immunization dose from 3x15 infectious mosquito bites to 3x8 bites (**chapter 3**) or 3x5 bites (**chapter 2**) resulted in a clear dose-dependent profile.

This dose-dependent efficacy we found is remarkable given the breadth of approximately 50 to 200 sporozoites that are inoculated per mosquito bite. It is known that the inoculation dose is dependent on the duration of feeding of the mosquito in one bite session [6], and from animal models it is known that approximately half of the inoculated sporozoites remain in the skin [7]. But

how could this discordant finding between the variation of inoculated sporozoites per bite and the variation in numbers of parasites reaching the liver explain this striking dose-dependent efficacy? The most obvious reason could be that the variability in the number of inoculated sporozoites (within one batch of mosquitoes) may be smaller than postulated [8] and the percentage of parasites reaching the liver in relation to the parasites that remain in the skin is more constant than assumed.

After challenge with five infected mosquito bites and follow-up, volunteers can be grouped to either being sterilely protected to challenge, unprotected to challenge or being partially protected. The latter is characterised by a significantly prolonged pre-patent period compared to the unprotected control volunteers.

The prolonged pre-patent period in immunized subjects is most probably the result of killing of liver stage parasites and not by inhibition of the parasite multiplication during erythrocytic stages [9]. However, it is still unclear if this intrahepatic killing and subsequent reduced release of merozoites is a reflection of either (or a combination of) a reduction or a delayed development of parasites in the liver. This pre-(or intra) hepatic early killing of sporozoites by adaptive immune responses is characterized by inter-individual variation and may bias trial outcome especially when using small number of volunteers per study arm in trials [10].

Chloroquine and mefloquine equal as prophylaxis in the CPS model

Partly by unknown mechanisms [11], chloroquine (CQ) is able to kill the intra-erythrocytic parasite by blocking the transformation of haem into non-toxic haematoin crystals, resulting in the accumulation of a highly toxic haem. CQ also possesses immune-modulatory properties and is used in auto-immune diseases like rheumatoid arthritis or SLE diseases [12]. It is hypothesised that the efficient induction of sterile protection in CPS, found in **chapter 2 and 3**, might have been partially explained by these immune-modulating properties of CQ. CQ is known to enhance $CD8^+$ T cell responses by induction of cross-presentation in which malaria antigens are presented on MHC class I molecules to cytotoxic CD8 T-cells without the usual proteosomal processing and presentation in dendritic cells [13].

Due to widespread resistance of malaria parasites to CQ, other chemoprophylactic drugs need to be assessed for use in the CPS strategy. Mefloquine (MQ) is a registered chemoprophylactic drug that also acts on blood stages of *falciparum* and could in theory be used in the CPS model. In **chapter 3** we compared CQ to MQ which is not known for such immune-modulatory properties. Protective efficacy was expected to be reduced using MQ as prophylaxis. However, we were unable to demonstrate a difference in protective efficacy

between the use of CQ or MQ using an immunization dose of 3x8 mosquito bites (**chapter 3**). However, concerns about neuro-psychiatric side-effects of MQ, further fuelled by a FDA black-box warning, may limit its clinical use [14].

Several factors could have hypothetically affected the protective efficacy we found in the CPS model. Remaining drug concentrations of CQ or MQ could have aided parasite clearance in conjunction with suboptimal (protective) immune responses and may have led to a longer pre-patent period, even up to day of treatment and in this way could have resulted in misclassification of partially protected individuals into protected individuals. However, in **chapter 3**, all volunteers had remaining plasma levels of CQ (and desethyl-chloroquine) between 7–10 µg/L or MQ (no active metabolites) between 5–116 µg/L on the day before challenge (C-1). These concentrations are well below therapeutic or prophylactic plasma levels of CQ (30 µg/L) [15] or MQ (406-603 µg/L) [16, 17] and therefore could not have biased the protective efficacy we found. In addition, the highest MQ levels at C-1 were present in two control subjects, and their pre-patent periods (by thick smear) were 9,5 and 12 days, similar to pre-patent periods of historical controls (ranging between 7 and 12,3 days). In addition, none of the protected subjects had a positive qPCR during the entire challenge period of 21 days. Also parasite multiplication rates after challenge in both CQ and MQ groups were similar to earlier infection studies without malaria prophylaxis, suggesting that blood-stage parasite multiplication was not significantly inhibited [4] and therefore excludes anti-parasitic effects of remaining anti-malaria drug concentrations.

Another factor that could have biased the striking efficacy of CPS is that in current trials, volunteers are only monitored up to 21 days after challenge after which all subjects are curatively treated with antimalarials. A combination of remaining drug levels and an extremely low inoculation dose and/or liver load could theoretically have delayed the thick smear pre-patent period beyond 21 days. In one CPS trial [9] one volunteer did become qPCR positive, retrospectively analyzed on day 21 after challenge. It should therefore be taken in consideration to further extend the observation period after challenge in future trials to detect volunteers who may have an extreme late pre-patent period.

Adverse events, parasitaemia and safety of volunteers in CHMIs

During and after CPS and CHMI safety and adverse events are constantly monitored. Part of these adverse events can be clinical manifestations of (immune-) reactions to parasites. We observed a declining or even absence of parasites and adverse events after each subsequent immunization, suggesting early acquisition of sterile protection in subjects (**chapter 2 and 3**). This could be explained by increasing pre-erythrocytic killing of parasites by the immune sys-

tem leading to absence of circulating blood stage parasites. Unfortunately, the absence of parasites during immunizations was not a very sensitive or specific marker of protection. Nine out of 25 protected subjects (**chapter 2 and 3**) and one unprotected subject (**chapter 3**) did not show any positive qPCR signal during the entire immunization period, not even after the first immunization. Two factors may have contributed to the absence of parasitaemia during immunizations in the unprotected subject. Firstly, it is possible that the early primary innate immune response (Interleukin 1 and 6), responsible for killing of pre-erythrocytic stages, may lead to a liver load that is insufficient to induce an adaptive immune responses and establish a sterile immune response [18]. Secondly, aside a reduced liver load, the chemoprophylaxis also might have reduced parasitaemia during immunizations, keeping the parasitaemia below the qPCR detection limit of approximately 20-50 parasites per millilitre.

The predictive values calculated from both the studies combined, taking either a positive or a negative qPCR after the first immunization as a predictor, the positive predictive value for protection (PPV) was 39% and the negative predictive value (NPV) is 79%. Conclusively, a negative qPCR in the days after the first immunization is a poor predictor of protection after CHMI. When taking respectively the second and the third immunization as a predictor the NPV (91%, 83%) and in particular the PPV (67%, 100%) becomes more reliable.

Using qPCR instead of thick smear leads to lower parasitaemia levels at day of treatment in the CHMI model

Volunteers in CHMIs are closely monitored for safety reasons, and adverse events are recorded during the entire study. Serious adverse are rare in CHMIs but cardiac complications have occurred. Up to date, several instances of cardiac complications in CHMIs have occurred: one case of suspected acute coronary syndrome after immunization with a recombinant vaccine (PfLSA3) and subsequent treatment with arthemeter/lumefantrine [19], one case of myocardial infarction [20] and one case of myocarditis [21] . Despite intensified cardiac screening and selection of volunteers in the trials conducted afterwards, another case of myocarditis occurred recently. This myocarditis occurred in a CPS study 12 days after CHMI infection on the second day treatment [21].

Although parasite densities are already very low at initiation of treatment in CHMIs when compared to natural infections [22], parasite numbers on day of treatment can be considerably reduced further if treatment would be initiated based on qPCR detection of parasitaemia instead of thick smears. Although no causal relationship has been proven between cardiac complications and parasitaemia, a qPCR-based initiation of treatment, to further reduce parasitaemia in volunteers, was proposed to increase trial safety.

In **Chapter 5 and 6** we evaluated the advantages and disadvantages of using qPCR to initiate treatment. In **chapter 6** we calculated, in a retrospective analysis of nine trials, the reduction of the prepatent period and adverse events using different qPCR cut-offs.

Taking a positive qPCR as point in time to initiate treatment reduces the number of parasites a volunteer is exposed to by 90%, by shortening the pre-patent period and reducing the number of erythrocytic parasite multiplication cycles. Shortening of the prepatent period reduces the peak and the cumulative number of parasites during an infection and correspondingly reduces the number of adverse events in volunteers by approximately 70% because of earlier initiation of treatment compared to treatment after a positive thick smear. Using the studies in **chapter 2 and 3** as fictive test trials in **chapter 5**, the impact of qPCR-based initiation of treatment on adverse events and parasitaemia could only be evaluated up to day of treatment.

Unfortunately, it is unclear if this reduction in parasitaemia also will reduce the risk of cardiac events in CHMIs. For example, in the case of the myocardial infarction, no detectable parasitaemia was present and it raises the question whether reducing parasitaemia by using a low qPCR cut-off for initiation of treatment could effectively prevent cardiac complications in future trials. In **chapter 6** we evaluated what the most optimum cut-off for parasitaemia by qPCR should be and how frequent blood samples should be tested for optimal trial results. Using a qPCR threshold of 100 parasites per millilitre the prepatent period can theoretically be reduced by 3,5 days and, together with twice daily sampling, is the most optimal strategy to reduce costs and clinical burden for volunteers [23, 24].

Besides a clear benefit for subjects, the detection of immunological markers of disease, like for example cytokines or blood cells, may become more difficult to detect or can even missed because of limited immunological stimulation due to earlier treatment. Depending on the study objective, studies can be designed to either use thick smears or a positive qPCR for initiation of treatment.

qPCR based initiation of treatment most probably won't affect assessment of biomarkers for protection as they are per definition found in protected individuals without parasitaemia and before the pre-patent period of controls or unprotected individuals. Future trials have to confirm whether (early) qPCR-based treatment truly will enhance safety for volunteers and simultaneously does not (profoundly) hinder immunological assays by using different cut-offs for parasitaemia depending on the goal of the (immunological) study.

Heterologous protection in the CPS model

In malaria affected areas many different strains of *P. falciparum* exist. These strains are genetically diverse both in and between regions and are under constant selective pressure by the human immune system and anti-malaria drugs [25]. It is unclear to what extent this diversity in strains is immunologically relevant in malaria vaccine development as it is unknown which antigens of these strains are exactly involved for induction of full sterile (long lasting) heterologous protection [25]. The NF54 *P. falciparum* clone used in **chapter 2 and 3**, together with clones 3D7 and 7G8, have been extensively used in CHMI trials worldwide and were the only strains available for CHMI for a long time [10]. The NF135.C10 clone used in **chapter 4**, from a patient from Cambodia, is available for CHMI studies since a few years [7] and made testing of heterologous protection in our model feasible. New strains like NF166, originating from Ethiopia, were very scarcely used in CHMIs [26] before but were recently reintroduced in trials to study infectivity (Clinicaltrials.gov NCT01627951; McCall et al unpublished). Unfortunately, up to date, it is unknown which (combination of) strains should be used in malaria vaccine models to induce sufficient heterologous protection for future field application.

The reason why acquisition of natural immunity in malaria endemic areas (probably) takes years and years of repeated exposure is partly due the large variety of genetically different strains that hosts are exposed to in the field. It is hypothesised that each infection with a different strain creates its own unique immune response [27]. Repetitive small inoculation doses with (highly) antigenic different strains over time are insufficient to accomplish sufficiently high liver loads required for an adequate immune response and to generate subsequently sterile protection.

It is even hypothesised that each immunological different (sub)strain might need its own sufficient liver load to reach the threshold for sterile protection against that specific strain [27, 28].

Obviously, for a malaria vaccine to be efficacious, it is essential to cover this variety of strains present in an endemic area. Up to now, studies assessing heterologous protection after whole radiation- or chemically attenuated sporozoite vaccination are scarce [1-3]. In **chapter 4** we re-challenged volunteers previously immunized with the West-African NF54 strain, using the Cambodian NF135.C10 strain, and found a rather low heterologous protective efficacy of 15%. Several factors could have contributed to this relative low efficacy. Waning immunity over time could have resulted in lower efficacy as subjects were challenged 14 months after the last immunization. In addition, subjects previously received different and maybe suboptimal immunization doses which could have added to the relative low efficacy. Moreover, it is hypothesised that

NF135 has a higher infectivity and leads to a subsequent higher liver loads compared to NF54. This is supported by the finding that the NF135.C10 controls had an extremely short pre-patent period suggesting a higher liver load compared to NF54 controls. This is further underpinned by a higher first peak of NF135.C10 (2871 *Pf*/ml) parasitaemia compared to NF54 (456 *Pf*/ml) in previous controls [29]. This could implicate that the dose of five infectious bites with the NF135.C10 strain might equal ten or even fifteen infectious bites with NF54. Because of the small number of subjects, we could not include a NF54 control group in our re-challenge study by which we could have compared the heterologous efficacy we found with NF135.C10. Therefore, to assess the optimal dose for challenge, dose-escalating infection studies with NF135.C10 have been performed (Clinicaltrials.gov NCT02149550; Wammes et al unpublished).

It is hypothesised that for the induction of full homologous or even heterologous protective immunity a certain antigen magnitude is needed to overcome an immunological threshold. The required immunization dose could be further increased to amplify the (pluriform) antigen exposure and increase the immune response to the parasite. It is known that the malaria parasite exhibits profound immuno-evasive techniques preventing maximum exposure to the immune system. Genetic variability between individual parasites and cross-stage variability of antigen exposure during the parasite's lifecycle are important factors in the immuno-evasive techniques of parasites [28]. By increasing both the quality and quantity of antigens exposed to the immune-system (effectively the liver load), a more effective immune response could be mounted and overcome the induced immune-evasive capacity by the parasite. This in turn could lead to an increased (long lasting) heterologous efficacy or even cross-species protection against for example *P. vivax*. Currently, studies are ongoing assessing heterologous protection against three different strains after full effective CPS NF54 immunizations (Clinicaltrials.gov NCT02098590). In addition, recent work showed that 33 weeks after immunizations with 3 times $9x10^5$ irradiated P*f*SPZ (3D7 clone) i.v. injections, 5 out of 6 of these previously fully homologous protected individuals were also heterologously protected with strain 7G8, a clone of Brazilian origin [3].

And albeit with very limited evidence, it is hypothesised that vaccination efficacy found in malaria naïve volunteers could predict similar results in field settings [10] as the homologous NF54 challenge, used commonly in CHMIs, might be even too stringent compared to a 'natural challenge' in endemic areas with far less sporozoites inoculated by mosquito bites compared to the number of sporozoites in trials [30].

Field application of the whole-sporozoites model

The current most important and relevant phase 3 vaccine initiatives are whole sporozoite vaccines and the RTS,S subunit vaccine. The RTS,S vaccine has already been scheduled for field implementation although it has a known low efficacy. Despite the fact that the whole parasite model (e.g. CPS) is safe and is able to efficiently induce long-lasting homologous protection, several hurdles need to be taken before field application becomes suitable. Obviously, immunizing communities through mosquito bites lacks full applicability. Nonetheless, whole sporozoites vaccine candidates could be further optimized in several ways for field use. These whole sporozoite vaccine candidates could be further altered using irradiated (*Pf*SPZ), chemically attenuated (*Pf*SPZ-CVac) or genetically attenuated (GAP) parasites and could be either injected intravenously, intramuscularly of subcutaneously.

Recently progress has been made with intravenous injection of irradiated sporozoites (*Pf*SPZ), and is currently tested in several African countries [31, 32]. In this model infected mosquitoes are irradiated, dissected and sporozoites are harvested. These extracted sporozoites are purified and cryopreserved, and injected in humans after reconstitution. The irradiation dose needs to be carefully chosen to limit the development of these parasites during the liver stage, and to subsequently prevent breakthrough to blood stages, and simultaneously allow these live parasites to develop as long as possible in the liver stage to mount an adequate immune response in the human host.

The *Pf*SPZ-CVac method uses aseptic, purified, cryopreserved, non-irradiated P*f*SPZ injected intravenously whilst taking (for example) chloroquine as a chemo-prophylactic to prevent full erythrocytic multiplication and subsequent progress to disease of malaria. Also other chemo-prophylactic anti-malaria drugs can be used like mefloquine or for example ferroquine; a new drug still under phase IIb research. A single 800mg dose of ferroquine is able to provide for more than 8 days of erythrocytic parasite killing [33] and could be the ideal partner-drug for *Pf*SPZ-CVac to secure adequate serum drug concentrations while mass-vaccinating communities.

Although previous studies showed a relative low protective efficacy [34], recently, a study using three intravenous doses of 5.12×10^4 *Pf*SPZ, with an interval of 28 days, conferred short-term sterile homologous protection in 100% of subjects ten weeks after immunizations [35].

The reason previous studies using *Pf*SPZ-CVac were less effective is most probably due to both the quality and quantity of injected sporozoites, as well as the inoculation route that were suboptimal for sufficient numbers of viable parasites to reach the liver and mount an adequate immune stimulation needed for sterile protection. It is known that the route of inoculation is crucial for

the number of sporozoites that are able to reach the liver and the resulting parasite liver load is known to correlate with protective efficacy [36]. In the murine model it has been shown that injection of whole sporozoites by intravenous (i.v.) injection results in 2 to 50 fold higher liver loads compared to intramuscular (i.m.), subcutaneous (s.c.) or intradermal (i.d.) inoculation [36, 37]. The use of smaller volumes by multiple intradermal injections can increase the liver load further and might mimic probing and injection of saliva and sporozoites in the skin by *Anopheles* mosquitoes.

However, aside from the laborious process of extracting of these parasites from mosquitoes both in the *Pf*SPZ-CVac and in the *Pf*SPZ model, vialling and delivering cryo-preserved parasites to field settings remain a challenge. In addition, intravenous injection is far more time-consuming and risky than intramuscular or subcutaneous injection. And last but not least, only short-term homologous protection has been evaluated and no heterologous protection.

An alternative to the whole sporozoite *Pf*SPZ-CVac or *Pf*SPZ model is the use of genetically attenuated sporozoites (GAP) and this might be the most attractive option for future field application [38]. With GAP as a vaccine, a new field of research is entered, and it could be an alternative to the other whole parasite vaccines being either impractical because of the use of chemoprophylaxis during immunizations or because of using injection of large numbers of (irradiated) sporozoites [39]. Recently, intensive research in the production of genetically attenuated sporozoites has been performed [40-42] and has already proven a superior efficacy compared to irradiated *Pf*SPZ immunizations in humans [43]. However, the genetic alterations in the parasite, resulting in arrest of parasites in the liver, must be carefully chosen [44]. Essential genes for parasite survival in the liver are altered to arrest development and proliferation while still allowing exposure of antigens to the host during the liver stage. These genetic alterations leading to arrest of parasites could be either early or late during the development in the liver. Early liver arrest of parasites could be safer because of lower risk of breakthrough to blood-stages but might be inadequate to mount an immune response needed for sterile protection. Alternatively, late liver stage arrest of parasites might induce sufficient sterile protection but could implicate breakthrough to blood-stages and therefore being unsafe for vaccinees. This delicate balance between full arrest of parasites in the liver, allowing maximum antigen exposure, and acquisition of protective immunity but without breakthroughs to blood-stages, are critical for safety of vaccinees and vaccine efficacy. To improve efficacy and to enhance immune stimulation, adjuvants can be added, either separately or embedded in het parasites genome. Despite all current research, the production and implementation of a GAP vaccine, as any other candidate whole sporozoite vaccine, is still

a major challenge. A first-in-human trial of *Pf*Δp52Δp36GAP failed because of lack of safety due to break through to blood-stages [45]. Recently, trials in the mouse model show promising results with a double knockout of genes p52 and p36 (*Py*Δp52Δp36GAP) [46] or the genes Slarp and B9 (PbΔb9ΔslarpGAP) [47] and showed protective immunity without break through to blood-stages. In addition, in vitro studies using *Pf*Δb9ΔslarpGAP were able to infect humanized mice hepatocytes. Currently, *Pf*Δb9ΔslarpGAP is evaluated in humans for safety, immunogenicity and efficacy of protection (Clinical trial NCT03163121). Other revolutionary novel techniques using alterations in the genome of the parasite are attenuations in the CRISPR-CAS9 gene [48] and form a complete new field for both drug targets and well as for vaccine purposes [49] but have not been evaluated in humans yet.

Immunology in Malaria

Despite decades of research, up to date, it is still unknown what exactly contributes to natural or (artificial) sterile protection against *P. falciparum* in the human host. In the CPS model, the host immune system is exposed to all stages of the parasite, including early blood stages after which parasites are killed by the prophylactic drug. In whole sporozoite CPS vaccination, it is shown both in mice and humans, that sterile protection against *P. falciparum* is induced in the liver and is T-cell mediated [9, 50-52]. In line, challenge with i.v. asexual blood stages after CPS immunizations did not lead to protection. Instead, immunized subjects showed earlier fever and higher inflammation markers like IFNγ compared to controls and indicates a response sufficient for immune recognition but insufficient for killing of parasites [9]. Together these data suggest that protection is mediated by pre-erythrocytic immunity and next, raises the question how immunity is acquired during this clinically silent liver phase. *Plasmodium* can infect and replicate undetected in hepatocytes. In absence of clinical symptoms, presentation of parasite RNA in liver cells by the cytosolic pattern recognition receptor Melanoma differentiation-associated (Mda) protein, which acts as a Pathogen-associated Molecular Pattern (PAMP), induces Interferon (IFN) cytokines and triggers the recruitment of cytotoxic $CD8^+$ T cells for later killing [53].

CD8 T-cells play an important role in sterile protection

In the CPS model we were only able to assess the peripheral blood compartment for immunology taken as a reflection for the liver compartment. Taking the peripheral blood compartment as a proxy we found sterile pre-erythrocytic

protection to be likely mediated by cytotoxic $CD8^+$ T-cells, in conjunction with Th1 lineage effector mediators like IFNγ, IL2, TNF and other cytotoxic mediators (like Granzyme B and perforin) produced by innate and adaptive immune cells like NK-cells, $CD8^+$ and $CD4^+$ T-cells and γδ CD3+T-cells (**chapter 2 and 3**). These Th1 effector mediators have been assessed in several platforms: the murine model [54], non-human primates [55] and humans [56, 57].

Two mechanisms of $CD8^+$ T-cell mediated killing of infected liver cells are currently proposed, but mouse models show contradictory results. One method of killing is mediated by $CD8^+$ T-cells releasing perforin and granzyme B. The second method is mediated by the Fas receptor and its ligand on the activated effector T-cells (T_{eff}). However, granzyme$^{-/-}$, perforin pore protein (ppo)$^{-/-}$ and apoptosis ligand FasL/CD95L$^{-/-}$ deficient mice were fully protected after immunizations with irradiated sporozoites (either *P.yoelii* or *P.berghei*) [58]. This suggests that, in the mouse model, induction of sterile protection is indirectly mediated by a $CD8^+$ T-cell associated cytokine cascade and suggests to act independent of granzyme B. However, the mechanism of sterile protection might be dependent on the strain used, and additionally, it remains unclear if these findings can be extrapolated to humans.

The role of $CD4^+$ T cells in the CPS model remains unclear

In addition to $CD8^+$ T-cells, also $CD3^+$ γδ T-cells [59], NK-cells [58] and cytotoxic $CD4^+$ T-cells (**chapter 2 and 3**) [50] may play a role in pre-erythrocytic immunity but their exact contribution remains unclear. It is known that $CD4^+$ T-cells are needed to control blood-stage (natural) infections by IFN-γ production in assisting B-cells for antibody production [50]. In **chapter 2** we found cytotoxic CD4+ T-cells to be correlated in the induction of sterile protection and indirect killing of hepatocytes might take place via effector mediators despite hepatocytes lack MHCII receptors for antigen presentation.

B-cells in the CPS model correlate poorly with sterile protection

Aside from T-cell involvement, also B-cells and several antigens like AMA, MSP1-3, GLURP and CSP may play a role in the induction of sterile immunity in malaria. Unfortunately, levels of these antibodies appear to correlate poorly with sterile protection both in malaria naïve subjects [60] and in field trials [61], and show large intra-individual variation making clear that avidity of antibodies in general appear to be more important than the quantity of antibodies in sterile protection from malaria [62]. Recent immuno-epidemiological work showed antibodies (like AMA, MSP and GLURP) are associated with protection against clinical malaria in Malian [63, 64] and in Gabonese children [65]. Blood-stage parasites are able to alter both the number and function of B-cells in clinically

immune adults and further inhibit mounting of sterile protection [66]. This lack of number and function of B cells might be the reason of the insufficient association with sterile protection.

The role of regulatory T-cells in sterile protection

It is known that during natural infection T-cell responses are reduced [67, 68]. Studies in mice have demonstrated that after natural infection, $CD8^+$ T-cell responses against liver stage antigens were lower compared to immunization with irradiated sporozoites, even after repeated infections [69]. Also liver-stage specific T-cells in mice were reduced after infection [70]. Clearly, $CD8^+$ T-cell responses are down-regulated by blood-stages of malaria.

In natural infections, clinical immunity is slowly acquired but without effectively killing of all parasites leading to sterile immunity. Nonetheless, death by malaria can prevented by just one or two clinical infections [71]. In high endemic areas, when humans are repeatedly exposed to parasites, a delicate balance exists between: *i* controlling the infection and simultaneously acquisition of clinical protection and *ii* limiting collateral immunological damage whilst combatting parasites. This balance is partly effectuated by regulatory T cells (T_{reg}) which control the damage by down-regulating the force of the inflammatory response caused by T_{eff}.

During combat against malaria parasites, pro-inflammatory cytokines and chemokines, to a large part produced by Th1 $CD4^+$ T-cells, recruit inflammatory cells to the site of malaria infection. After recruitment, cytotoxic T-cells (CTL or Effector T cells (T_{eff})) and Natural Killer (NK) cells kill intracellular malaria parasites in the liver. However, the timing and degree of the response and the ratio between T_{reg} and T_{eff} [72] and subsequent inflammatory response, is crucial in successfully combatting malaria infections. Both pro-inflammatory (IFN-γ, TNF-α, IL-12) and anti-inflammatory/regulating (IL-10 and TGF-β) responses need to be carefully orchestrated and timed, and, unless tightly controlled, unlimited pro-inflammatory cytokine responses can lead to severe immune-pathology and eventually to death [73, 74]. Alternatively, too early activation of T_{reg} responses can induce immune-suppression by inhibiting Th1 responses and subsequently increase of parasitaemia [74]. It is known from malaria infections studies in humans that a high parasitaemia correlates with induction of T_{reg} and lower inflammatory responses [75] resulting in a persisting blood stage infection. On the contrary, data in mice regarding the role of T_{regs} in malaria infection are contradictory, depending on the mouse–parasite strain combination used, and large differences in T_{reg} immunological responses exist between murine and human model [76].

The necessity of (early) T_{eff} induction and IFN-γ production by immune cells has been repeatedly proven to be related with sterile immunity in malaria, both in the murine model [50] as in humans [56] [77], but could not be proven to correlate with protection and subsequently be used as a biomarker in our CPS model (**chapter 2**; [52]). It is hypothesised that *because of* the very low parasitaemia in CPS during immunizations, the high T_{eff} / T_{reg} ratio is able to induce sterile protection [67] and shape memory responses [78]. Alternatively, prolonged parasitaemia during blood-stages can suppress T cell responses and IFN-γ production both by vaccination and by natural exposure [79] and can inhibit acquisition of protection through the activation of T_{reg} [57, 80-82]. However, it remains unclear how regulatory T-cells exactly control pro-inflammatory and anti-inflammatory responses in vaccine-induced responses [82].

The importance of biomarkers in the malaria vaccine model

Molecular techniques like transcriptomics, metabolomics and proteomics can be of assistance in finding biomarkers in malaria by elucidating the immunological processes that form the basis of protection against malaria [83]. Simultaneously, new software and internet-based integrated analysis (e.g. Ingenuity or Cytobank) provide researchers in systems biology and systems immunology [84] powerful information to solve complex multi-dimensional cellular and molecular interactions that underlie malaria pathogenesis and protection. Already key gene-expression signatures have been found for licensed vaccines against for example yellow fever [85] and for other infectious diseases like tuberculosis [86]. Similar approaches for malaria could be of benefit. Up to date, no markers are known that unequivocally correlate with sterile protection in any malaria vaccine model.

Nonetheless, we found in **chapter 3** markers that are associated with parasite exposure. Merozoite Surface Protein 1 (MSP-1) antibodies, a marker of parasite exposure, were elevated in all unprotected volunteers but not in protected individuals and therefore can't be used a predictor of sterile protection [62]. A recent proof-of-principle study, comparing RNA-seq profiles before and after malaria infection between malaria-experienced (Malian) individuals and malaria-naïve (CHMI) individuals showed that activation of pro-inflammatory, interferon-mediated, immune responses were highest in the malaria naïve individuals and lowest in malaria-experienced individuals from Mali [87] showing a reduced inflammatory response which suggests both reduced manifestations of clinical malaria and simultaneously increased B-cell receptor signaling demonstrating build-up of adaptive immunity. Differences in acquisition of clinical protection are considered caused by an inflammatory ('pyrogenic') threshold. The absence of fever and concomitantly low activation of pro-in-

flammatory responses in the malaria-experienced individuals, and lack of sterile protection, might be caused by the co-infection of helminths down-regulating these CD8+ T-cell inflammatory responses.

The role of parasitic infections in malaria vaccination

A limitation of the CPS model or CHMIs in humans is that these trials can only perform immunological assays in the blood-compartment and this may not reflect processes in important sites like the liver or the spleen. Additionally, assessing T-cell responses and efficacy of vaccination in malaria endemic areas might even be more difficult than in malaria naïve subjects in hyper-controlled trial settings in the western hemisphere. Repeated exposure of individuals to malaria parasites leads generally to naturally acquired immunity (NAI), and the level of acquired immunity depends on the combination of the individuals' specific immune-system and the previous level of exposure to parasites [88]. A complicating factor of assessing efficacy after vaccination in a field setting might be the lack of tools to assess the degree of (immunological) magnitude of pre-existing NAI in subjects [89] and assessment of for example the influence on the level of T_{reg} induction and IFNγ production [90]. Also co-infections with other parasites like for example helminths could dampen the vaccine efficacy in vaccinees. A quarter of the world population is infected with helminths, of which most infections are in highly endemic low income countries [91]. Helminth infections are known to induce strong regulatory mechanisms for survival in its host and have proven to inhibit Th1 responses to infections [92, 93] and can reduce protection after vaccinations [94, 95].

Future

Given the tremendous suffering of communities of malaria it might seem that quick deployment of any malaria vaccine is necessary, nonetheless, several aspects might need to be taken into consideration. RTS,S is the first licensed, and soon mass scale distributed, vaccine against malaria. However, it is questionable if the deployment of this vaccine is justifiable at this moment. And although the vaccine averted clinical episodes of malaria shortly after vaccination in children, the short and long term clinical protection has been proven to be poor, and this vaccine does not significantly protect against severe malaria or malaria hospitalization as being demonstrated after a trial conducted in eleven African sites [96]. Most positively seen this vaccine will avert clinical malaria cases, and will be a try-out and form a base for future vaccines to be rolled out in endemic areas. Additionally, this vaccine could assist in reducing (clinical) malaria to-

gether with other existing tools like the use of bednets, insecticide spraying, adequate diagnosis and treatment of malaria. Given the poor immunogenicity and protection of RTS,S, implementation of this vaccine in endemic areas could have other effects. If vaccines do not fully protect, resistance can develop as a result of selection of the remaining resistant parasite population. Additionally, (partial) clinical vaccine-induced protection might reduce the existing natural clinical immunity of the population over time. This waning natural clinical immunity might lead to more severe malaria infections in former vaccinees and this could theoretically intensify the already existing malaria burden even more. In contrast, once-yearly administration of a malaria vaccine that is only effective short-term to communities living in areas with seasonal short malaria episodes might be considered being useful [97].

What needs to be done while in the meantime while Africa waits for an effective vaccine? One option is to further improve the effectiveness of the two most promising vaccine model: the whole parasite vaccines like *Pf*SPZ, *Pf*-SPZ-CVac or GAP, and the subunit vaccine RTS,S. The whole parasite vaccines could be further optimised by improving longevity, heterologous protection and applicability: the use of cryopreserved parasites in combination with an adjuvans or the further development of a genetically attenuated plasmodium (GAP) vaccine. The laborious manual harvesting of (genetically, (non) irradiated parasites used in whole sporozoite vaccines is in the process of being automated and new parasite culturing techniques may improve mass-scale applicability in the future. Additionally, new injection techniques like the use of multiple small volume intradermal inoculations [98] might overcome the impracticability of repeated intravenous injections.

The subunit vaccine RTS,S could be made more effective by adding different (cocktail) multi-stage immuno-potent antigens, development of new adjuvants or combination with other vaccines like ChAd63/MVA ME-TRAP [99, 100]. These multi-antigen, multi-stage or even cross-species subunit vaccines (NCT01883609, NCT02252640) using a combination of carefully selected (but yet unknown) antigens. These highly immunogenic antigens at different - including sexual - stages, might be the key solution in malaria vaccine development [101]. It is even possible that it may be necessary to produce a vaccine for different regions or continents each containing a different cocktail (of antigens) of strains. The current deployment of the RTS,S vaccine in Africa could be taken as a platform for further deployment of this vaccine or others.

Malaria vaccine community needs to combine knowledge and strengths

It is under debate whether the international malaria vaccine community should proceed on the current research route [102]. Even when new antigens are found, new delivery systems or cocktails of vaccines are used, without basic knowledge of the immune biology of malaria and without correlates of protection this may not work in the case of finding the first in-human anti-parasite vaccine. Maybe other directions need to be explored, given the current status of vaccine research and the availability of a field vaccine of only limited efficacy, even after decades of research. Malaria vaccine development could benefit maybe more from the use of genome-based research to find important immunogenic antigens and further explore immunological pathways that are responsible for protective immunity [103]. In addition, with the current highly heterogeneous landscape of vaccine research, studies need to be further harmonized worldwide to combine strengths and to further facilitate comparability between studies [104]. The success of malaria research lies in multi-disciplinary approach where disciplines like malariology, epidemiology, bio-informatics, immunology and clinicians bundle expertise and enable efficient research in conjunction with next-generation molecular and cellular techniques.

Despite all existing shortcomings in current malaria vaccine research, eradication of malaria using all available anti-malaria tools, including a highly efficacious vaccine [102], might be feasible in the coming decades as declared by Bill Gates in 2007 [104]. However, the availability of sufficient funds for now and for the future, both for vaccine-research as well as for further implementation of current malaria tools, remains a tremendous additional challenge.

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