Characterization of T-cell immune responses in clinical trials of the candidate RTS,S malaria vaccine

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ABSTRACT
The candidate malaria vaccine RTS,S has demonstrated 45.7% efficacy over 18 months against all clinical disease in a phase-III field study of African children. RTS,S targets the circumsporozoite protein (CSP), which is expressed on the Plasmodium sporozoite during the pre-erythrocyte stage of its life-cycle; the stage between mosquito bite and liver infection.

Early in the development of RTS,S, it was recognized that CSP-specific cell-mediated immunity (CMI) was required to complement CSP-specific antibody-mediated immunity. In reviewing RTS,S clinical studies, associations between protection and various types of CMI (CSP-specific CD4+ T cells and INF-γ ELISPOTs) have been identified, but not consistently. It is plausible that certain CD4+ T cells support antibody responses or co-operate with other immune-cell types to potentially elicit protection. However, the identities of vaccine correlates of protection, implicating either CSP-specific antibodies or T cells remain elusive, suggesting that RTS,S clinical trials may benefit from additional immunogenicity analyses that can be informed by the results of controlled human malaria infection studies.

Introduction

History of RTS,S development
The estimated 45.7% efficacy of the candidate subunit vaccine, RTS,S, against all episodes of malaria over the first eighteen-month period in the phase-III study of African children aged 5 to 17 months, has followed on from comparable efficacy estimates in smaller phase-II studies of both children and adults in the field, i.e., in malaria-endemic regions of Africa, and of malaria-naïve adults after experimental challenge.

Plasmodium is the mosquito-borne parasite that causes malaria, and RTS,S targets the pre-erythrocyte stage of the Plasmodium falciparum’s life cycle; the stage at which sporozoites pass from the mosquito bite via the blood to the liver. About 50–100 sporozoites are estimated to be injected in the skin during a blood meal by an infected female Anopheles mosquito (reviewed in Graewe et al. 2012). Over a couple of hours, about a third of inoculated sporozoites pass through the dermis, enter the blood stream and reach the liver. At the liver, the sporozoites traverse Kupffer cells, cross the liver sinusoidal endothelial cells barrier, and migrate through several hepatocytes before entering one in which they establish infection resulting in the production of thousands of merozoites which are packaged into membrane-bound structures termed merosomes. Within a period of one to two weeks, the erythrocyte stage begins with merozoites released into the blood stream. The merozoites then escape from the merozoite and rapidly invade erythrocytes giving rise to parasitemia and the first clinical symptoms.

In malaria-endemic areas, naturally-acquired immunity mainly against the blood stage of the parasite only develops after several years and after repeated rounds of infection; with these infections continuing into early adulthood. Although antibodies against parasite-encoded antigens on erythrocytes can restrict clinical symptoms, the mechanisms that support (non-sterile) acquired-immunity remain complex, and no clear correlates of protection have been identified for antibody-mediated or cell-mediated immunity (CMI).

The antigen in RTS,S is a recombinant protein derived from circumsporozoite protein (CSP) from Plasmodium falciparum and the hepatitis B surface antigen (HBsAg; see Fig. 1). CSP is expressed on the surface of sporozoites and mediates sporozoite entry into hepatocytes. The selection of CSP was also informed by the results of vaccination with inactivated sporozoites, in which sterile immunity could be achieved; i.e. the absence of parasitemia after sporozoite challenge. This sterile immunity was dependent on CSP-specific antibodies and CMI. CSP-based vaccines could also elicit CSP-specific antibodies able to block sporozoite entry into hepatocytes in vitro. However, CSP-specific antibodies alone were insufficient to achieve sterile immunity. Hence RTS,S was designed to include CSP T-cell epitopes in addition to the prominent B-cell epitope made up of the asparagine-alanine-asparagine-proline (NANP) amino acid repeat sequence (Fig. 1).
A clear association between CMI and protection was first identified in the proof-of-concept phase II clinical study of RTS,S formulated with three different Adjuvant Systems AS02, AS03 or AS04 (Table 1). Of these, the RTS,S/AS02 vaccine was the only RTS,S formulation that demonstrated substantial protection against experimental Plasmodium falciparum malaria challenge in malaria-naive adults. The CSP-specific antibody titers alone were not predictive of protection because both RTS,S/AS02 and RTS,S/AS03 elicited similarly high levels of CSP-specific antibodies. However, in addition to antibody levels, and potentially antibody quality, the degree of CSP-specific CMI could account for the difference between protection and non-protection for RTS,S/AS02 and RTS,S/AS03 (measured by a short-duration IFN-γ ELISPOT assay) (Table 1).

After the initial demonstration of efficacy against infection, the RTS,S/AS02 vaccine was evaluated in field trials. Several years later, RTS,S formulated with the Adjuvant System AS01 was also evaluated and subsequently replaced RTS,S/AS02 based primarily on efficacy evidence, but also on immunogenicity and safety evidence. Both Adjuvant Systems contain the immunostimulants MPL and QS-21. AS01 differs from AS02 in that AS01 is liposome-based and AS02 is oil-in-water-based.

**Differences between CMI assays and interpretation**

The premise for CMI assays is that antigen recognition by a specific T-cell receptor results in changes in T-cell behavior, such as proliferation, the production and/or secretion of cytokines or other activation markers, and/or the capacity to mediate cytotoxicity. The selection and implementation of different analytical techniques was also shaped by the techniques which were available at the time of the studies. Different methods may detect different antigen-specific cell subsets. Short duration (~24 hours; *ex vivo*) of antigen (peptide) re-stimulation and the absence of stimulatory cytokine supplements in cultures of whole blood samples or peripheral-blood mononuclear cells (PBMCs) has been considered to favor the identification of effector or effector-memory T cells, whereas the long duration (10–14 days re-stimulation culture prior to the 24 hour assay) has been considered to favor the identification of central-memory T cells. Limited correlations have been observed between long-duration ELISPOT and lymphoproliferation and between long-duration ELISPOT and intracellular-cytokine staining combined with flow cytometry (ICS-FC).

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**Table 1. Efficacy and immunogenicity of RTS,S vaccines containing different adjuvant systems from first proof-of-concept efficacy trial.**

| Adjuvant System in RTS,S vaccine | Adjuvant System composition | Protection | CSP-specific IgG geometric mean concentration | Proportion of subjects with CSP-specific IgG concentrations above geometric mean | Proportion of subjects with IFN-γ ELISPOT above maximum pre-immune levels |
|---------------------------------|-----------------------------|------------|-----------------------------------------------|------------------------------------------------------------------|-------------------------------------------------------------------|
| AS02                            | QS-21 and MPL in oil-in-water emulsion | 6/7        | 53 μg/ml                                      | 3/6                 | 0/1                  | 5/6 | 0/1 |
| AS03                            | α-tocopherol in oil-in-water emulsion | 2/7        | 53 μg/ml                                      | 2/2                 | 1/5                  | 2/2 | 1/5 |
| AS04                            | MPL and aluminum salt        | 1/8        | 7.0 μg/ml                                     | 1/1                 | 2/7                  | 1/1 | 1/7 |

*Significant in ELISA against recombinant R32LR that contains circumsporozoite protein (CSP) tandem-repeat epitopes.*

*IFN-γ ELISPOT responses to CSP peptides in 11–15 day re-stimulation peripheral blood mononuclear cell cultures.*
With ICS-FC, different phenotypes and functionalities of antigen-specific CD4+ and CD8+ T cells have been assessed, typically through differences in the expression of the activation marker CD40L.60,61 and cytokines including IL-2, IFN-γ and TNF-α.10 Other activation markers have also been examined, including CD69 (a leukocyte-activation marker)62 and CD25 (IL-2 receptor).63 Furthermore, memory T cells have been characterized by the expression of CD45RO and subdivided into central memory and effector/effector memory subsets by the presence or absence, respectively, of the cell surface expression of the chemokine receptor CCR7.64,65

### CMI in clinical studies

**CSP-specific T-cell responses to RTS,S**

CSP-specific CD4+ T-cell responses to RTS,S/AS01 and RTS,S/AS02, measured directly by ICS-FC or indirectly with ELISPOT assays, are more prevalent than CD8+ T-cell responses. Indeed, targeted CD4+ T-cell depletion, but not CD8+ T-cell depletion, has been shown to reduce the number of spot-forming units (SFUs) in the ELISPOT assay.50,66 And where CSP-specific CD8+ T cells have been identified by ICS-FC, they are at low levels,47,56,67 or are only detected in cell cultures subjected to long-duration antigen re-stimulation.49 The relatively high prevalence of antigen-specific CD4+ T cells versus antigen-specific CD8+ T cells in response to vaccination is likely to reflect the nature of the adjuvant systems used in the vaccine composition because the relationship has been observed with other AS01- or AS02-adjuvanted subunit vaccines.68-72

In some studies, the specificities of CD4+ T cells have been mapped to the epitopes of CSP, and include Th2R, Region II, Th3R and CS-T3 (Fig. 1, Table 2). One of the conserved CSP epitopes to which T-cell responses have been identified is also associated with protection to natural *Plasmodium falciparum* infection and disease.50

### Malaria-naive adults and controlled human malaria infection studies

In malaria-naive adults challenged two weeks after vaccination with *Plasmodium falciparum* parasites in a controlled human malaria infection (CHMI) setting, higher levels of short- and long-duration CSP-specific IFN-γ ELISPOTs on the day of challenge have been associated with protection against parasitemia.10,49 Protected vaccine recipients had higher levels of CSP-specific CD4+ T cells (identified by ICS-FC as expressing at least two markers among CD40L, IL-2, IFN-γ or TNF-α after short-term in vitro stimulation) than those from non-protected vaccine recipients.10 The differences were the most distinct on the day of challenge, and IL-2/CD40L was the most frequently identified phenotype of CSP-specific CD4+ T cells. A further investigation of the T-cell phenotypes of the same cohort also found that on the day of challenge, protection was associated with CSP-specific IL-2+ effector/effector-memory (CD45RO+CCR7+) and CSP-specific IL-2+ central memory (CD45RO+CCR7+) CD4+ T cells.65

Gene-expression profiling (of transcriptomes) was also applied to PBMCs taken from this CHMI study and suggested potential insights into CMI and protection.10,73 Using a statistical approach driven by knowledge of gene networks, the genes of the immunoproteasome pathway were associated with protection; and the differences in the expression of these genes were dependent on vaccination. In another investigation of the same CHMI study, a multiway partial least squares data analysis (N-PLS-DA) was used.10,74 This approach took into account the kinetics of gene expression prior to challenge and identified 110 genes that could be used in models to predict protection outcome. Of these genes, 42 were known immune-related genes, including 29 associated with the NF-κB pathway and 14 with the IFN-γ pathway. Moreover, the application of N-PLS-DA to the expression data of 45 genes in the IFN-γ pathway identified 44 genes that could predict protection. These
analyses, coupled with the observation that serum IFN-γ levels were higher in protected group than in non-protected group, most distinctly one day after the final (third) dose suggested that the IFN-γ pathway may have a role in protection against parasitemia. It is also plausible that IFN-γ can affect the differential expression of the immunoproteasome and HLA-A genes,73-75 supporting a putative role of the IFN-γ pathway.

The hypothesis that CMI contributes to protection was further examined in a subsequent CHMI study in which two vaccination regimens were compared. In that study, a regimen of three doses administered 28-days apart, a regimen of three doses of RTS,S/AS01 (RRR regimen) was compared with a regimen of one dose of an CSP-expressing replication-deficient recombinant human adenovirus 35 (Ad35.CS.01) followed by two consecutive doses of RTS,S/AS01 (ARR regimen).47 As anticipated from a preceding preclinical study,76 the ARR regimen induced higher levels of CSP-specific IFN-γ ELISPOTs and CD4+ T cells than the RRR regimen. By contrast, the ARR regimen induced lower levels of CSP-specific antibodies. Nevertheless the higher degree of CSP-specific CMI with the ARR regimen did not translate into an increased level of protection against parasitemia compared with the RRR regimen. Overall, CSP-specific antibody levels were most associated with protection. Yet, antibody levels in the non-protected RRR group were similar to those in the ARR protected group. So antibody levels, were associated with protection. 56,79 These TNF-α T cells, the numbers of CSP-specific CD4+ T cells, were less frequent in the non-protected RTS,S/AS01 group than in the protected RTS,S/AS01 group. 

Potential roles of CMI in RTS,S-mediated protection?

In both the sporozoite-challenge studies and the field studies, associations with CMI endpoints and protection against parasitemia or clinical disease have been identified (summarized in Table 3). However, stronger associations with protection have been typically identified with CSP-specific antibody levels rather than CSP-specific CMI.10,47,63 Although fewer in number, CSP-specific TNF-α+ CD4+ T cells and IFN-γ+ CD4+ T cells were also induced at one month post-vaccination.55,56,79 CSP-specific CD4+ T cells expressing the markers CD69, or CD25 have also been detected in vaccinated children with RTS,S/AS01.63 The phenotype of CSP-specific CD4+ T cells that has been associated with vaccine-induced protection against clinical episodes of malaria is TNF-α+, but not IL-2+ or IFN-γ+.56,79 and in part, TNF-α+ CD4+ T cells may also be induced by natural exposure to malaria parasites.79

Field studies

The clinical field studies of malaria-exposed adults have suggested that CSP-specific long-duration IFN-γ ELISPOT levels, rather than CSP-specific short-duration IFN-γ ELISPOT levels, are associated with protection against parasitemia and clinical disease, such as over one malaria season of five months.80 However, an association between CSP-specific (long or short term) IFN-γ ELISPOT levels and protection was not identified in the recipients of three RTS,S/AS02 doses even though these ELISPOT levels were higher than in the control (rabies) vaccine recipients.50,51 In RTS,S-vaccinated children living in a malaria-endemic region, no association was identified between protection and short- or long-duration, CSP-specific IFN-γ or IL-2, ELISPOT levels,56 even though long-duration IFN-γ ELISPOT levels and short-duration IL-2 ELISPOT levels were higher after than before RTS,S vaccination.

CSP-specific CD4+ T cells have also been characterized by short-duration ICS-FC in the field studies of young children vaccinated with RTS,S/AS02 or RTS,S/AS01. The most prominent CSP-specific CD4+ T-cell phenotype induced at one month post-vaccination was IL-2+.55,56,78,79 Although fewer in number, CSP-specific TNF-α+ CD4+ T cells and IFN-γ+ CD4+ T cells were also induced at one month post-vaccination.55,56,79 CSP-specific CD4+ T cells expressing the markers CD69, or CD25 have also been detected in children vaccinated with RTS,S/AS01.63 The phenotype of CSP-specific CD4+ T cells that has been associated with vaccine-induced protection against clinical episodes of malaria is TNF-α+, but not IL-2+ or IFN-γ+.56,79 and in part, TNF-α+ CD4+ T cells may also be induced by natural exposure to malaria parasites.79

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**Table 3. CMI conclusions from clinical studies.**

| Vaccination schedule       | Location  | Vaccines          | No. of subjects / samples analysed | CMI conclusion                                                                 | Reference |
|----------------------------|-----------|-------------------|-----------------------------------|-------------------------------------------------------------------------------|-----------|
| **Malaria naive adults**   |           |                   |                                   |                                                                               |           |
| 0, 1, 6 month Belgium      |           | RTS,S/AS02        | 10                                | CSP-specific IFN-γ ELISPOTs were induced in 8/10 subjects. RTS,S-specific IFN-γ production was induced in all subjects. Lymphoproliferative responses to CSP were induced in all subjects. CSP-specific CD8+ CTL responses were not detected. | Lalvani et al. |
| 0, 1, 2 month Belgium      |           | RTS,S/AS01        | 11                                | CS-specific CD4+ T-cell responses (i.e. cells expressing at least 2 markers among CD40L, IL-2, TNF-α, and IFN-γ) were detected in all vaccine groups with a trend for higher responses in the RTS,S/AS01 and RTS,S/AS02 groups versus the RTS,S group. | Leroux-Roels et al. |
| Adults in the field        |           |                   |                                   |                                                                               |           |
| 0, 2, 6 month USA          |           | RTS,S/Alum        | 10                                | One of two protected subjects had RTS,S and CSP-specific lymphoproliferative and cytotoxic T-cell activity. | Gordon et al. |
| 0, 1, 6 month USA          |           | RTS,S/AS02        | 7                                 | Highest rate of protection with RTS,S/AS02 although CMI results inconclusive | Stoute et al. |
| 0, 1, 7 month USA          |           | RTS,S/AS02        | 8                                 | Inconclusive due to small sample size.                                        | Stoute et al. |
| 0, 1, 2 month USA          |           | RTS,S/AS01        | 36                                | Association between CSP-specific CD4+ T cells and protection, 2 weeks after Dose 3 and on DOC. Association between short duration IFN-γ ELISPOTs and protection. Higher frequency of CSP-specific CD4+ T cells with RTS,S/AS01 vs RTS,S/AS02. | Kester et al. |
| 0, 1, 2 month USA          |           | RTS,S/AS01 (group RRR) | 21                              | Association between CSP-specific IL-2+ CD4+ T-cell central-memory and effector-memory populations and protection. | Lumsden et al. |
| 0, 1, 2 month USA          |           | Ad35.CS.01 (dose 1) & RTS,S/AS01 (doses 2 & 3; group ARR) |                      | No evidence of independent association between CSP-specific CD4+ T cells or IFN-γ ELISPOTs and protection. No difference in protection between groups. CMI responses significantly greater in AAR group than in RRR group. | Ockenhouse et al. |
| **Children in the field**  |           |                   |                                   |                                                                               |           |
| 0, 1, 2 month Mozambique   |           | RTS,S/AS02 HBsAg  | ≤63; ≤69                          | Significant induction of IL-2 secretion in CSP re-stimulation cultures in 24% of RTS,S vaccine recipients. IL-2 secretion was detected in CSP-re-stimulation cultures from 32% of individuals without a malaria episode whereas IL-2 secretion was detected in only 6% of individuals with malaria episodes (p = 0.053). | Barbosa et al. |
| 0, 1, 2 month Gabon        |           | RTS,S/AS01        | ≤31; ≤32                          | The frequencies of IL-2+ CD4+ T cells were higher than pre-immune levels in both RTS,S vaccine groups. CD40L+ CD4+ T cells were not detected. | Agnandi et al. |
| 0, 1, 2 month Ghana        |           | RTS,S/AS01        | ≤77; ≤73; ≤80; ≤73                | The frequencies of IL-2+ CD4+ T cells were higher than other marker positive CD4+ T cells (and responder rate of 76% 1 month after dose 3 with 0, 1, 7 month schedule). CD40L+ CD4+ T cells were detected in 0, 1, 7, 7 schedule. | Ansong et al. |
| 0, 1, 2 month Tanzania     |           | RTS,S/AS01        | ≤182; ≤197                        | The frequency of RTS,S-induced CSP-specific IFN(γ)-IL-2+TNF-α+ CD4+ T cells was associated with protection, and CSP-specific TNF-α+ CD4+ T-cell responses and anti-CSP antibody responses were synergistically associated with protection. | Otolu et al. |
| 0, 1, 2 month Kenya/Ghana  |           | RTS,S/AS01        | ≤80; ≤98                          | Evidence that IL-2+ secreting CSP-stimulated memory CD4+ T cells can activate NK cells to secrete IFN-γ. IFN-γ ELISPOTs may include IFN-γ-secreting activated NK cells. No CMI data on protection. | Horwitz et al. |

CMI, cell-mediated immunity; CSP, circumsporozoite protein; DOC, day of challenge; and HBsAg, hepatitis B surface antigen.
by secreting IL-2. In turn, the activated NK cells secrete IFN-γ, perhaps also in response to an additional signal. During an infection, this additional signal may come from activated CSP-presenting antigen-presenting cells (APCs) that are secreting cytokines such as IL-12 or IL-18 (Fig. 2B). As well as mediating cytotoxicity, IFN-γ may signal to the APC to produce more IL-12 or IL-18, thus establishing a positive feedback loop for its production. Therefore the CSP-specific IL-2+ CD4+ T cells would dictate the localized nature of the IFN-γ response by their direct interaction with the APCs in a similar mechanism to what has been proposed for CD8+ T-cell interactions with APCs (i.e. Kupffer cells). Since 35–50% of all liver-resident lymphocytes are NK cells, a parallel mechanism involving NK-cell activation and antibody-dependent cell-mediated cytotoxicity (ADCC) is attractive (Fig. 2C). In this mechanism, CD4+ T cells expressing IL-2 recognize CSP-fragments presented by local APCs and activate NK cells. These NK cells are further activated through the binding of their FcgRIII receptors with CSP-specific antibodies bound to CSP shed on the surface of infected hepatocytes. Hence ADCC may explain why the combination of CD4+ T-cell and antibody responses to RTS,S can be associated with protection.

A putative role for NKT-cell derived IFN-γ has been shown in a mouse model of primary Plasmodium infection. In this model, the control of infection in the liver was dependent on IFN-γ and on NKT cells but not NK cells, and the authors speculated that NKT cell could potentially recognize Plasmodium-derived lipids. However, it is not clear how this mechanism would translate in humans because the recognition of lipid antigens and production of IFN-γ may be a property restricted to invariant NKT (iNKT) cells rather than all NKT cells. Although, in human liver, the frequency of iNKTs is high, the relative proportion of iNKT cells to all NKT cells is much lower than in the mouse liver. Moreover, after CHMI in humans, the level of iNKT cells in peripheral blood appeared unaffected unlike that of NK cells, suggesting iNKT cells, at least in peripheral blood, were unresponsive to Plasmodium infection. Nevertheless, we speculate that NK cells are relevant to controlling Plasmodium infection in humans after RTS,S vaccination, and they adopt a function similar to those NK cells in the mouse model, except, as hypothesized above, the recognition of Plasmodium-infected cells by IFN-γ-producing NK cells is driven in by CSP-specific CD4+ T cells and antibodies.

Figure 2. Models for the initiation of NK-cell activation and the interactions between a CSP-specific CD4+ T cell, an antigen-presenting cell (APC) and an NK cell. Direct interactions are marked by cognate receptor-ligand interactions, indirect interactions via the production of cytokines are marked by black arrows, and effector mechanisms due to IFN-γ or cytotoxic molecules are marked by large grey-shaded arrows. (A) After vaccination, APCs take up RTS,S antigen and, in the draining lymph node, present processed RTS,S-derived peptides via HLA-II T-cell receptor (TCR) interactions. From these interactions and from CD40-CD40L interactions, CD4+ T cells are stimulated to produce IL-2. This IL-2 then activates NK cells and helps B cells to proliferate and produce antibodies, as well as inducing T-cell proliferation through a positive feedback loop. (B) Upon re-encounter with CSP in the draining lymph nodes, derived from RTS,S or sporozoites, APC present CSP derived peptides to CS-specific T cells. From these interactions and from CD40-CD40L interactions, CD4+ T cells would dictate the localized nature of the IFN-γ response by their direct interaction with the APCs in a similar mechanism to what has been proposed for CD8+ T-cell interactions with APCs (i.e. Kupffer cells). Since 35–50% of all liver-resident lymphocytes are NK cells, a parallel mechanism involving NK-cell activation and antibody-dependent cell-mediated cytotoxicity (ADCC) is attractive (Fig. 2C). In this mechanism, CD4+ T cells expressing IL-2 recognize CSP-fragments presented by local APCs and activate NK cells. These NK cells are further activated through the binding of their FcgRIII receptors with CSP-specific antibodies bound to CSP shed on the surface of infected hepatocytes. Hence ADCC may explain why the combination of CD4+ T-cell and antibody responses to RTS,S can be associated with protection.

A putative role for NKT-cell derived IFN-γ has been shown in a mouse model of primary Plasmodium infection. In this model, the control of infection in the liver was dependent on IFN-γ and on NKT cells but not NK cells, and the authors speculated that NKT cell could potentially recognize Plasmodium-derived lipids. However, it is not clear how this mechanism would translate in humans because the recognition of lipid antigens and production of IFN-γ may be a property restricted to invariant NKT (iNKT) cells rather than all NKT cells. Although, in human liver, the frequency of iNKTs is high, the relative proportion of iNKT cells to all NKT cells is much lower than in the mouse liver. Moreover, after CHMI in humans, the level of iNKT cells in peripheral blood appeared unaffected unlike that of NK cells, suggesting iNKT cells, at least in peripheral blood, were unresponsive to Plasmodium infection. Nevertheless, we speculate that NK cells are relevant to controlling Plasmodium infection in humans after RTS,S vaccination, and they adopt a function similar to those NK cells in the mouse model, except, as hypothesized above, the recognition of Plasmodium-infected cells by IFN-γ-producing NK cells is driven in by CSP-specific CD4+ T cells and antibodies.
Perspectives for analyzing CMI in future clinical studies

So far, the most informative CMI results in clinical studies have been obtained from ELISPOT and ICS-FC analyses of re-stimulation cultures. The use of peripheral blood as the sampling material imposes certain logistical constraints as well as caveats on the interpretation of the results. T-cell frequencies in peripheral blood may only reflect patrolling populations of T cells and may not capture T cells that have a more localized activity such as the site of infection or secondary lymphoid organs. Nevertheless, the capture of antigen-specific CD4⁺ T cells using HLA class II tetramers and flow cytometry has the potential to allow a more relevant functional characterization of those cells because an ex vivo activation step can be avoided.¹⁰²,¹⁰³ Technical improvements in ICS-FC and the development of cytometry by time-of-flight (CyTOF) are expanding the range of markers that can be examined and therefore increasing the range of CD4⁺ T-cell phenotypes that can be measured in a single run.¹⁰⁴-¹⁰⁶ These improvements are coupled with new sensitive statistical approaches that consider the heterogeneity CD4⁺ T cell populations in the identification of correlations with clinical outcomes.¹⁰⁹,¹¹⁰

The co-operative relationship between different immune-cell populations even within an ELISPOT assay is illustrative of the idea that the association of CMI with protection may be difficult to identify with a single CMI endpoint and could therefore explain, in part, some of the inconsistent findings between different studies. Hence a more global appreciation of the relationships between CSP-specific antibodies, CSP-specific CMI and innate-immunity with protection may come with sophisticated systems-biology analyses of omics data in conjunction with data from more conventional immunology endpoints.²⁴,⁷³-⁷⁷,¹¹¹-¹¹⁴

Abbreviations

ADCC antibody-dependent cell-mediated cytotoxicity
APC antigen-presenting cell
CMI cell-mediated immunity
CSP circumsporozoite protein
HBsAg hepatitis B surface antigen
ICS-FC intracellular cytokine staining-flow cytometry
NANP asparagine-alanine-asparagine-proline
PBMC peripheral-blood mononuclear cell
SFU spot-forming unit

Disclosure of potential conflicts of interest

All authors are employees of the GSK group of companies. PM, EJ and RvdM report ownership of GSK shares and/or restricted GSK shares.

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Author contributions

All authors were involved in drafting the manuscript or revising it critically for important intellectual content.

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