Systems analysis of protective immune responses to RTS, S malaria vaccination in humans

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RTS,S is an advanced malaria vaccine candidate and confers significant protection against *Plasmodium falciparum* infection in humans. Little is known about the molecular mechanisms driving vaccine immunity. Here, we applied a systems biology approach to study immune responses in subjects receiving three consecutive immunizations with RTS,S (RRR), or in those receiving two immunizations of RTS,S/AS01 following a primary immunization with adenovirus 35 (Ad35) (ARR) vector expressing circumsporozoite protein. Subsequent controlled human malaria challenge (CHMI) of the vaccinees with *Plasmodium*-infected mosquitoes, 3 wk after the final immunization, resulted in ~50% protection in both groups of vaccinees. Circumsorozoite protein (CSP)-specific antibody titers, prechallenge, were associated with protection in the RRR group. In contrast, ARR-induced lower antibody responses, and protection was associated with polyfunctional CD4+ T-cell responses 2 wk after priming with Ad35. Molecular signatures of B and plasma cells detected in PBMCs were highly correlated with antibody titers prechallenge and protection in the ARR cohort. In early signs of innate immunity and dendritic cell activation were highly associated with protection in the ARR cohort. For both vaccine regimens, natural killer (NK) cell signatures negatively correlated with and predicted protection. These results suggest that protective immunity against *P. falciparum* can be achieved via multiple mechanisms and highlight the utility of systems approaches in defining molecular correlates of protection to vaccination.

**RTS,S malaria vaccine**

RTS,S is the most advanced malaria vaccine candidate to be tested in humans. Despite its promise, there is little understanding of its mechanism of action. In this work, we describe the use of a systems biological approach to identify "molecular signatures" that are induced rapidly after the standard RTS,S vaccination regimen, consisting of three RTS,S immunizations, or with a different regimen consisting of a primary immunization with recombinant adenovirus 35 (Ad35) expressing the circumsporozoite malaria antigen followed by two immunizations with RTS,S. These results reveal important insights about the innate and adaptive responses to vaccination and identify signatures of protective immunity against malaria.


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**Conflict of interest statement:** R.v.d.M., R.A.v.d.B., W.R.B., and E.I. are employees of the GSK group of companies. They report ownership of GSK shares and/or restricted GSK stock.

**Data deposition:** The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE89290).


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also been evaluated (21). Recently, an Ad35-CSP (AdVac)–RTS, S/AS01 prime-boost approach was tested in humans, and its efficacy and immunogenicity was compared with the RTS,S/AS01 vaccine candidate alone (18). Surprisingly, however, inclusion of the adenoviral prime immunization did not result in increased vaccine efficacy (18).

In this study, we sought to enhance our understanding of the mechanisms of vaccine-induced protection against malaria. In recent years, the tools of systems biology (22, 23) have been applied to identify signatures of immunogenicity to vaccination and have provided insights into the mechanisms of immune responses induced by vaccines such as the live attenuated yellow fever (YF-17D) and seasonal influenza vaccines (24–26). Here, we used systems approaches to trace the temporal variations of the transcriptional response elicited by the two vaccines and to identify transcriptional signatures associated with protection and immunogenicity.

Results

Challenge Model for the RTS,S/AS01 and AdVac Malaria Vaccines. The clinical trial (NCT01366534) was conducted at Walter Reed Army Institute of Research, as described (18). Forty-six healthy malaria-naïve volunteers, randomized to two study arms, participated in this study testing the efficacy of RTS,S and AdVac malaria vaccine candidates (Fig. 1), as described (18). Study arm 1 (hereafter referred to as ARR), comprised of 25 volunteers who received the AdVac vaccine composed of Ad35 vector expressing full-length CSP, as a primary immunization, was followed by two doses of RTS, S/AS01 vaccine. The subjects in the second arm, consisting of 21 volunteers, received three doses of RTS/AS01 (RRR regimen). Participants in both study arms were vaccinated at 28-d intervals, and subjected to CHMI 21 d following the final immunization. Parasitemia was monitored for 28 d, and immunomonitoring continued for 159 d following challenge. The study also included 12 nonvaccinated subjects as infectivity controls. Vaccine efficacy was 44% and 52% in ARR and RR arms, respectively, and was not statistically different between the two arms (18). All subjects in the control group developed parasitemia (18).

Adaptive Immune Responses. The RRR regimen induced significantly greater antibody titers against CSP than ARR regimen at all time points before or on the day of challenge (Fig. SI4 and ref. 18). Similar results were also seen for antibody titers against HBsAg, the protein fused to CSP, although the differences at later time points were modest (Fig. S1B). Two doses of RTS, S/AS01 following the ARR were not able to induce as high a magnitude of antibody titers as two doses of RTS,S/AS01 vaccine in the RRR arm (Fig. SL4). We also assessed the number of antibody secreting cells (ASCs) induced after immunization, using ELISPOT. Both vaccines induced similar frequencies of CSP-specific (Fig. S1C), or HBsAg-specific ASCs, 6 d after the second and third immunizations. This was surprising because RRR vaccination induced a greater magnitude of CSP-antibody titers compared with ARR vaccination (Fig. SL4). This discordance may reflect differences in kinetics of the ASC response induced by ARR versus RRR. Alternatively, a different population of ASC (which was not sampled in this study) may contribute to enhanced antibody response in the RRR group.

CSP-specific CD4+ and CD8+ T-cell responses to vaccination were also assessed (18). There was negligible induction of CD8+ T-cell responses by RRR and a modest induction by ARR. In contrast, there was a significant induction of CSP-specific CD4+ T-cell response by ARR, and to a much weaker degree by RRR (18). The functionality of T cells was monitored by FACS analysis using a panel including four markers: CD40L, IL-2, TNFα, and IFNγ. In the ARR vaccine group, there was a markedly enhanced frequency of polyfunctional (expressing three or four functions) CSP-specific CD4+ T cells at D14, D42, and D77, and postchallenge (Fig. S1D).

Immunologic Correlates of Protection. As reported (18), in the RRR vaccine arm, individuals who did not develop parasitemia within 28 d after challenge (referred hereafter as “protected”) had higher concentration of anti-CSP antibodies at the time of challenge than nonprotected individuals (Fig. 2A). In the ARR arm, the concentration of anti-CSP antibodies was substantially lower than that in the RRR arm (Fig. SL4), and there was no statistically significant difference in the titers on the day of challenge, between the protected versus nonprotected subjects. Anti-HBsAg antibody concentrations were not significantly different between protected and nonprotected individuals (Fig. 2B). In the ARR arm, it was in fact the frequency of CSP-specific polyfunctional CD4+ T cells at day 14 that significantly correlated with protection (Fig. 2C). The frequencies of CSP-specific polyfunctional T cells were similar between protected and nonprotected groups at all later time points (Fig. 2C). The frequency of polyfunctional CD4+ T cells did not correlate with protection in the RRR arm (Fig. 2C).

Transcriptional Signatures Induced by Vaccination. Vaccination with ARR or RRR induced potent transcriptional responses in PBMCs, with several thousands of genes being induced or repressed (Fig. 3A). The transcriptional responses at day 1 and day 6 after vaccinations, which include signatures of inflammation/TLR signaling and cell cycle genes in proliferating ASCs, correspond to the early innate and the later ASC responses (Fig. S2 A and B). ARR and RRR induced a small number of differentially expressed genes at D1 after primary vaccination (Fig. S2C). The genes more strongly induced by ARR included genes associated with the type I IFN antiviral response and innate immunity, such as IFI27, IFI44L, IFI6, and HESXI (27), consistent with previous studies (28). We then investigated the regulation of known IFN type I response-associated genes (27), as well as genes up-regulated in response to the live attenuated virus YF-17D vaccine (24). Both Ad35.CS and RTS,S/AS01 primary vaccinations induce potent expression of IFN type I and YF-17D signatures (Fig. S3), suggesting that the virus-like particles and the AS01 adjuvant contained in RTS,S/AS01 induces a potent antiviral type I IFN response, similar to that observed with viruses such as Ad35 or YF-17D. The identity of such genes induced by Ad35.CS and RTS,S/AS01 was largely overlapping (Fig. S3).

To identify functional pathways perturbed by the two regimens, we used Gene Set Enrichment Analysis (GSEA) using blood transcription modules (BTMs) (29) as gene sets. Transcripts were ranked according to a fold change differences relative to the D0 baseline. The functional responses elicited by the two vaccines were broadly similar (Fig. S4 and Dataset S1). Both vaccines induced strong innate responses (Fig. S4), including inflammatory/TLR/chemokines BTMs, following each vaccination (Fig. S4). Enrichment of cell cycle and plasma and B-cell–related
BTMs was observed 6 d after each vaccination (Fig. S4). A noticeable difference was the contraction of B-cell and plasma cell BTMs at D2 following prime immunization, observed in the ARR regimen, but absent in the RRR arm (Fig. S4). Interestingly BTMs related to cell cycle were enhanced even at D14 after primary vaccination, suggesting the persistence of cycling cells. Furthermore, in both arms, we observed a repression of BTMs related to NK cells at D1 following each vaccination (Fig. S4).

**Molecular Signatures of Immunogenicity.** We next analyzed the transcriptional signatures that correlated with immunogenicity of vaccination. In the case of RRR vaccination, we assessed transcriptional correlates of CSP-specific antibody titers on D77 (the day of challenge). Following RRR vaccination, the expression of BTMs related to plasmablasts at D1 after each vaccination was positively associated with the antibody titers on the day of challenge (Figs. S5A and S6A). This observation was surprising, given that the plasmblast response in humans to vaccination with other vaccines such as influenza (30) has been shown to peak at day 7 after vaccination, and with ARR and RRR vaccination robust, plasmabl ast responses were observed 6 d after each boost (Fig. S1C). The observed correlation between BTMs related to B cells and plasmablasts, at day 1 after each boost, and immunogenicity might reflect a transient burst of genes related to B-cell activation within a day of vaccination, but this hypothesis needs further exploration. Additionally, cell division BTMs showed positive correlation to the antibody titers even at later time points (D14, 28, and 56), suggesting the persistence of cycling cells (Figs. S5A and S6A). Furthermore, the expression of several innate immunity modules (antigen presentation M95, dendritic cell activation M165), including many antiviral and type I IFN-related modules at day 6 post primary and secondary vaccinations, were positively correlated with the antibody titers on the day of challenge (Figs. S5A and S6A and Dataset S2). Most strikingly, on the day of first and second boosts, genes modules relevant to NK cells showed strong negative correlation to the antibody titers at the day of challenge (Fig. S5A and Dataset S2). Indeed, we observed that the majority of genes included in these NK cell-related BTMs showed negative association with antibody titers.

In the case of ARR, the frequency of polyfunctional CD4+ T cells at day 14 was associated with protection (Fig. 2C). We thus assessed whether early transcriptional signatures correlated with the polyfunctional CD4+ T-cell response at day 14. At day 1, several modules representative of innate immune activation (antigen presentation M71, M95:1; activated dendritic cells and monocytes M168, M11; TLR and inflammatory responses M16, M25, M146) were strongly associated with polyfunctional CD4+ T-cell response at day 14 (Figs. S5B and S6B). Interestingly, modules representative of respiratory electron transport were strongly associated with the response. In contrast, modules representative of NK cells and T cells were negatively associated with the response. Similar, but weaker, associations were observed at D2 after prime Ad35.CS immunization. By day 6, the landscape of correlates changed, with many modules representing DC markers becoming negatively enriched, whereas NK cell modules continued to be negatively associated (Figs. S5B and S6B and Dataset S2).

**Association of Molecular Signatures with Protection.** We then analyzed transcriptional signatures associated with protection. In the RRR group, BTMs related to plasma cells, B cells, and cell cycle at D1, D29, and D57 (i.e., 1 d after each vaccination) were positively associated with protection (Fig. 4 and Fig. S7A), consistent with the correlations between the expression of such BTMs at 1 d after each partial permutation test (37) and indicate the statistical significance of the differences in frequencies of T-cell subsets in P vs. NP.

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**Fig. 2.** Serological and cellular associations with protection. (A) Serum anti-CS IgG antibody titers. Lines indicate median values, shaded areas indicate 25–75% interquartile range. *p = 0.05 level by Wilcoxon signed rank test. (B) Serum anti-HBsAg antibody titers in protected and nonprotected subjects. (C) SPICE plots indicate the functionality of CSP-specific CD4+ T cells. Inner sectors on SPICE plots indicate the number of markers expressed: Blue, green, orange, and red indicate 1, 2, 3, and 4 markers, respectively. Outside arches indicate the identity of expressed markers, per legend in the figure. Sum of χ² values was used as a test metric. P values were generated by
booster vaccination, and antibody titers (Figs. S5A and S6A). We also observed positive associations of multiple innate immunity modules at 6 days after primary and secondary vaccination, similar to the observed correlations with CSP-specific antibody titers (Fig. 4 and Fig. S7A). Additionally, several NK cell modules at D56 (day of the second boost) negatively associate with protection (Fig. 4 and Fig. S7A), consistent with their correlation with antibody titers (Figs. S5A and S6A). Strikingly, there were negative correlations of the expression of almost all of the genes contained within the NK cell-related BTMs, at D56, and protection (Fig. 5).

The transcriptional signatures of protection for the ARR arm were different from those for RRR. Here, multiple innate immunity modules positively associate with protection at D1 and 2 after the prime, and D28 (day of the first boost) (Fig. S7B and C), similar to the transcriptional correlates of polyfunctional CD4+ T cells described above (Figs. S5B and S6B). Again, NK modules display strong negative association with protection at multiple time points (D2, D28, D56) (Fig. S7B and C and Dataset S5).

We then determined the overlap between the molecular signatures of protection and immunogenicity. For the ARR arm, at D1 and 2 BTMs related to antigen presentation, TLR signaling and dendritic cells were associated with both immunogenicity (i.e., polyfunctional CD4+ T cells at D14) and protection (Fig. S7C). For RRR, there was considerable overlap between signatures of protection and immunogenicity. Several BTMs that correlate with protection were also associated with immunogenicity (Fig. 4). BTMs related to plasma and B cells, and the cell cycle were correlated with both protection and immunogenicity at 1 day after the primary and secondary vaccinations (Fig. 4). In contrast, several innate immunity modules, at day 6 after prime and day 6 after boost (D34), were correlated with both anti-CSP-specific antibody response and protection (Fig. 4). Strikingly, at D56 (the day of the final boost), we observed negative correlations of several NK cell-related BTMs with protection and immunogenicity (i.e., CSP-specific antibody titers at D77) (Fig. 4). A full description of all common associations with immunogenicity and protection is provided in Dataset S4.

### Predictive Modeling of Protection

We then developed predictive signatures of protection based on the transcriptional response to RRR vaccination. To achieve this goal, a discriminant analysis via mixed integer programming (DAMIP) (31) was used to generate the candidate predictive signatures. The two groups to classify are group 0 (protected) versus group 1 (not protected). For signature validation, we used a transcriptional dataset from an independent malaria challenge study with RTS,S/AS01 (32), NCT00075049, hereby referred to as “Vahey data set.” Responses in the two studies were broadly similar (Fig. S8 and Datasets S5 and S6). The baseline-normalized expression values for the RRR cohort in the present study was used as a training set, and candidate signatures that passed a minimum accuracy threshold in the training set were then applied to the independent validation set for blind prediction. The outline of the predictive modeling experiments is provided in Fig. 6A. A full list of signatures and their performance metrics is provided in Datasets S7 and S8. Analysis of the transcripts included in the successful predictive signatures revealed a high prevalence of transcripts that were commonly found in a large number of signatures at D56 (Table S1). One such transcript, KIR2DS1 (an NK cell marker), was found in 57 of 99 successful predictive signatures. This observation is consistent with the fact that NK cell-related BTMs are negatively associated with protection in both datasets at D56 (Fig. 6B). To validate these signatures, we used the set of transcripts identified in RRR to generate and train signatures in the Vahey data set (Dataset S9).

Many of the mRNAs that were highly represented in signatures trained in RRR arm of this study are also highly represented in signatures trained in the Vahey data set (Datasets S10 and S11). Up-regulated mRNA common to both RRR- and Vahey-generated signatures include several NK markers KIR2DS1, KIR2DL2, and KIR3DL1. Notably, many of the mRNAs that were included in the predictive signatures in 10-fold cross validation (10× CV) in RRR and the Vahey data set individually were also included in predictive signatures that were trained by using RRR, and were shown to blind predict outcome in Vahey data set (Dataset S11). Therefore, we conclude that a small number of mRNAs with high prevalence in predictive signatures are likely to be determining factors that distinguish protected versus nonprotected individuals.

Finally, we illustrated the ability of the generated signatures of protection to segregate the samples in their respective protection...
Expression of genes in NK cell-related modules inversely correlates with protection induced by RRR. Cytoscape plots illustrating correlations of individual genes contained within three representative “NK cell” BTMs. Color corresponds to the values of Spearman rho. Vertices indicate interaction of genes within the same subnetwork.

Fig. 5. Expression of genes in NK cell-related modules inversely correlates with protection induced by RRR. Cytoscape plots illustrating correlations of individual genes contained within three representative “NK cell” BTMs. Color corresponds to the values of Spearman rho. Vertices indicate interaction of genes within the same subnetwork.

groups. For this analysis, we used signatures consisting of features that include the high-prevalence genes noted above and plotted the distribution of protected and nonprotected subjects as a function of baseline normalized expression values of genes contained in these signatures. The results for three representative signatures are shown in Fig. 6C. Notably, although the overall accuracy of prediction was 80% or higher in both protected and unprotected groups, there were specific individuals in the Vahey validation set that were consistently misclassified. One of these individuals was misclassified by more than 90% of signatures, whereas four others were classified into one or the other group with nearly random probability. These subjects are indicated by black dots in Fig. 6C. In summary, we confirmed that expression of genes included in representative predictive signatures is sufficient to segregate protected and nonprotected subjects.

Discussion

Despite the fact that RTSS/AS01 is the most advanced malaria vaccine candidate under development, little is understood about the mechanisms by which it induces protective immunity. In this study, we performed a systems biology analysis of samples obtained from a clinical study involving 3xRTSS/AS01 (RRR) and Ad35-CS-prime followed by 2xRTSS/AS01 (ARR) vaccination regimens, with a view to identifying molecular correlates of immunogenicity and protection, and exploring the molecular mechanisms of protective immunity.

The two vaccination regimens elicited potent transcriptional responses, with several thousand genes differentially induced or repressed in response to each vaccination. Surprisingly, the transcriptional responses induced by the two vaccines were similar at the BTM level. However, the signatures of immunogenicity were different. For RRR, the correlates of antibody titers were expression of BTMs containing genes associated with cell cycle and several B-cell activation genes and some genes expressed in plasma cells, as early as 1 or 2 d after the second and third immunizations. This observation was surprising because the peak of the plasmablast response and B-cell activation has been shown to occur at ~7 d after vaccination (25, 30). This difference suggests that the signatures of cell cycle, and B and plasma cell activation observed at days 1 and 2, may reflect some of the earliest events in B-cell activation that precedes plasmablast generation. There was a noticeable increase in the numbers of antigen-specific plasmablasts at 6 d after vaccination, but unlike what was previously observed with other vaccines, the frequency of such cells were not observed to correlate with the magnitude of the antibody response, suggesting potential differences in the kinetics of the plasmablast responses between the two vaccines, or that other populations of antibody-producing cells, not detected in the present analysis, may contribute toward antibody production.

For ARR, several innate immune modules of gene expression within the first 6 days of Ad35.CS prime correlated with the frequency of polyfunctional CD4+ T cells 2 weeks after the first immunization. Additionally, in the case of RRR, it was the expression of BTMs related to innate immunity at 6 days after the first and second immunization that correlated with immunogenicity. We observed that multiple innate immunity modules were associated with the day of challenge antibody titers at day 6 after the primary immunization, and day 6 after the first boost (D34 of the study) (Figs. S5A and S6A). This observation is similar to the pattern of more persistent innate response observed in YF-17D vaccination compared with seasonal influenza, which may contribute to the exceptional vaccine efficacy of YF-17D (24, 28). Of interest, although we observed strong induction of multiple innate immunity modules within 1 or 2 days after each vaccination (Figs. S2A and S5), these responses were not associated with immunogenicity (Figs. S5A and S6A) or protection (Fig. 4). In contrast, expression of multiple innate immunity modules following Ad35.CS vaccination in the ARR arm was strongly associated with immunogenicity (Figs. S5B and S6B) and protection (Fig. S7 B and C).
A surprising result is that at D56 (the day of the final boost), we observed consistent signatures of immunogenicity and protection. In particular, the NK cell modules in peripheral blood at D56 (day of the third immunization) correlate negatively with high levels of anti-CS antibodies. In contrast, vaccination with ADR did not induce a high magnitude of antibody response, but rather enhanced frequencies of polyfunctional CD4⁺ T cells. This inverse correlation between the expression of NK cell-related genes and antibody titers and protection reflects the migration of NK cells from the blood to the liver, where they may help orchestrate antibody-mediated effector mechanisms such as antibody-dependent cell-mediated cytotoxicity against infected cells in the liver. In this context, several previous studies have implicated NK cells in immunity against malaria (34–36).

Together, our findings indicate that the RTS, S/AS01 vaccine candidate elicits protective immunity against infection primarily through rapid accumulation of high levels of anti-CS antibodies. In contrast, vaccination with ADR did not induce a high magnitude of antibody response, but rather enhanced frequencies of polyfunctional CD4⁺ T cells. Given the critical importance of CD4⁺ T cells induced by the Ad35 prime may have altered the quality of the antibody response, leading, for example, to higher-affinity antibodies as a result of enhanced germinal center response in the ARR vaccine compared with the RRV vaccine. Thus, the ARR and RRV vaccine might have conferred protection against malaria via two distinct mechanisms, involving the magnitude (RRR) and the affinity (ARR) of the antibody response. Furthermore, preclinical and sustained transcriptional response induced by this vaccine, and delineate several unappreciated molecular correlates of immunogenicity and protection (Fig. S9).

Importantly, these results provide candidate molecular signatures that may have potential as biomarkers of protective efficacy of vaccine-induced immunity against malaria.

Methods

This study was conducted at the Walter Reed Army Institute of Research (WRAIR) between August 2011 and July 2012, and was approved by the WRAIR Institutional Review Board (IRB) and Program for Appropriate Technology in Health-Malaria Vaccine Initiative’s Western IRB. The trial was undertaken in accordance with the provisions of the International Conference on Harmonization and Good Clinical Practice guidelines. Written informed consent was obtained from each subject before study procedures were initiated. All laboratories received deidentified samples and performed tests according to protocol, and therefore their work was IRB exempt.

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