Antigen-Specific IL-2 Secretion Correlates with NK Cell Responses after Immunization of Tanzanian Children with the RTS,S/AS01 Malaria Vaccine

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Antigen-Specific IL-2 Secretion Correlates with NK Cell Responses after Immunization of Tanzanian Children with the RTS,S/AS01 Malaria Vaccine

Amir Horowitz,*1,2 Julius C. R. Hafalla,*1 Elizabeth King,* John Lusingu,†‡§ Denise Dekker,‡3 Amanda Leach,§ Philippe Moris,§ Joe Cohen,§ Johan Vekemans,§ Tonya Villafana,§ Patrick H. Corran,* Philip Bejon,§⁎ Chris J. Drakeley,*⁎ and Eleanor M. Riley*  

RTS,S/AS01, a vaccine targeting pre-erythrocytic stages of Plasmodium falciparum, is undergoing clinical trials. We report an analysis of cellular immune response to component Ags of RTS,S—hepatitis B surface Ag (HBs) and P. falciparum circumsporozoite (CS) protein—among Tanzanian children in a phase Ib RTS,S/AS01E trial. RTS,S/AS01E vaccinees make stronger T cell IFN-γ, CD69, and CD25 responses to HBs peptides than do controls, indicating that RTS,S boosts pre-existing HBs responses. T cell CD69 and CD25 responses to CS and CS-specific secreted IL-2 were augmented by RTS,S vaccination. Importantly, more than 50% of peptide-induced IFN-γ+ lymphocytes were NK cells, and the magnitude of the NK cell CD69 response to HBs peptides correlated with secreted IL-2 concentration. CD69 and CD25 expression and IL-2 secretion may represent sensitive markers of RTS,S-induced, CS-specific T cells. The potential for T cell-derived IL-2 to augment NK cell activation in RTS,S-vaccinated individuals, and the relevance of this for protection, needs to be explored further. The Journal of Immunology, 2012, 188: 000–000.

Malaria remains a major cause of human disease in the developing world (1). Despite reports of declining malaria morbidity in several African countries (2), a safe and effective malaria vaccine would make a substantial contribution to malaria control programs. The most advanced vaccine, RTS,S/AS, has consistently been found to confer partial protection against malaria infection and clinical malaria episodes in phase II trials (3, 4). RTS,S, a chimeric recombinant protein expressed in Saccharomyces cerevisiae, comprises the central repetitive and C terminal portions of the Plasmodium falciparum circumsporozoite (CS) protein fused to hepatitis B virus surface Ag (HBs) combined with 90% in the first 8 mo after vaccination (6) and ~45% over a period of 15 mo (7).

Identifying immunological correlates of protection for RTS,S would facilitate evaluation of future vaccines and may allow vaccines to be specifically engineered to induce protective responses. Anti-CS Ab titres in excess of 20 μg/ml of CS-specific IgG seem to be required for protection against infection (8), whereas titres of ~40 EU/ml are associated with a significant step-change in the risk of clinical P. falciparum malaria by ~50% in the first 8 mo after vaccination (6) and ~45% over a period of 15 mo (7).

In this study, we report an analysis of postvaccination cellular immune responses of children enrolled, between 5 and 17 mo of age, in a phase Ib trial of RTS,S/AS01E in Korogwe, Tanzania. We have identified additional, sensitive markers of CS-specific T cell recall responses and a previously unrecognized response by NK cells. These newly identified markers of cellular immunity to RTS,S reveal a potential new pathway of vaccine-induced pre-erythrocytic immunity, namely rapid activation of NK cells by Ag-specific IL-2–secreting CD4+ T cells.

Materials and Methods

Study subjects and vaccinations
Four hundred forty-seven, 5- to 17-mo-old Tanzanian children were enrolled in a phase Ib, randomized, double-blind, controlled trial of RTS,S/AS01E
PBMC preparation and culture

PBMCs were isolated from heparinized blood by density gradient centrifugation (13), resuspended at 2 × 10⁶ cells/ml in growth medium (GM; RPMI 1640 containing heat-inactivated normal human AB serum [10%, L-glutamine [2 mM], and penicillin and streptomycin [each at 100 IU/ml]) in U-bottom, 96-well tissue culture plates (total volume = 200 µl) and incubated at 37°C in 5% CO₂ for 24 h. Brefeldin A (3 µM) was added 3 h prior to harvesting. PBMCs were stimulated with peptide pools (15 mers, overlapping by 11 aa, each peptide at 1 µg/ml) as a positive control. Negative controls contained GM alone. Culture supernatants were analyzed by Luminex for IL-2, IL-10, IFN-γ, sCD40-L, IL-12p70, IL-17, IL-15, and IFNa-R2.

Cell surface and intracellular staining for flow cytometry

Cell staining was performed as described (13) using mouse mAbs: anti-CD3 PerCP (SK7; BD Biosciences); anti-CD56 APC (N901; Beckman Coulter); anti-CD69 PE (CH-4; Serotec); anti-IFN-γ FITC (D9D10; Serotec); anti-CD4 FITC (RPA-T4; eBioscience) and anti-CD25 APC (BC96; eBioscience). Data were acquired on a Becton Dickinson FACSCalibur and analyzed with FlowJo (TreeStar).

Given the small blood volumes available and the limitations of four-color flow cytometry, CD69 was chosen as a marker of activation because it is upregulated within 24 h in both T cells and NK cells. It was not possible, within this four-color assay, to include CD69 and CD25 in the same staining panel.

Statistical analysis

Only data from children who were fully vaccinated according to protocol were presented for analysis. Numbers of samples tested varied among the assays performed because of insufficient PBMCs being obtained from some children. Data acquisition was performed on blinded samples. Data were imported into Stata 11.0 (Stata Statistical Software) or GraphPad Prism version 5.0c for analysis. For flow cytometry, responders are defined as those with a peptide-specific response twice their own negative control value or greater. For cytokine secretion (Luminex) assays, responders are defined as those with a peptide-specific cytokine response ≥ the mean + 2 SD of their own negative control value. Differences in the proportions of responding individuals were determined using Fisher exact test. Paired comparisons between groups were made using Wilcoxon matched-pair signed rank test. Unpaired comparisons were made using Mann–Whitney tests. Correlations between different cellular parameters were assessed using Spearman correlation coefficient. For comparison of Ab titres with cellular immune parameters, all data were log10 transformed, resulting in a normal distribution. Pearson correlation coefficients with p values were calculated.

Results

After exclusion of clotted (n = 4), small (n = 104), and low-viability (n = 61) samples, 185 samples (81 RTS,S, 104 control) were available for analysis (Table I). Of these, 178 samples (80 RTS,S, 98 control) generated data on CD69 expression and IFN-γ production, and 116 samples (55 RTS,S, 61 control) generated data on CD25 expression. Sixteen of these children (3 in the RTS,S group and 13 in the control group) developed a clinical episode of malaria during the follow up period for this study.

Vaccination with RTS,S/AS01E induces CS-specific T cell responses and boosts T cell responses to HBs

PBMCs were cultured for 24 h without stimulation (in GM), with recombinant human IL-12+IL-18 (each at 100 ng/ml) as a positive control. Negative controls contained GM alone. Culture supernatants were analyzed by Luminex for IL-2, IL-10, IFN-γ, sCD40-L, IL-12p70, IL-17, IL-15, and IFNa-R2.

Table I. Baseline characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>RTS,S/AS01E (n = 81)</th>
<th>Control (n = 104)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>11.7 ± 0.4</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>52.5</td>
<td>48.1</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>10.6 ± 1.1</td>
<td>10.7 ± 1.0</td>
</tr>
<tr>
<td>WBC(10⁹/l)</td>
<td>9.9 ± 3.3</td>
<td>9.6 ± 3.4</td>
</tr>
<tr>
<td>Plasmodium falciparum positive (n)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Anti-HBs Ab Ag titer (U/ml)</td>
<td>148 (43–382)</td>
<td>258 (87–643)</td>
</tr>
<tr>
<td>Prevacination Month 3</td>
<td>48,489 (4708–132,314)</td>
<td>213 (77–475)</td>
</tr>
<tr>
<td>Month 12</td>
<td>9357 (3937–16,269)</td>
<td>148 (33–436)</td>
</tr>
<tr>
<td>Anti-CS Ab titer (U/ml)</td>
<td>0.25 (0.25–0.25)</td>
<td>0.25 (0.25–0.25)</td>
</tr>
<tr>
<td>Prevacination Month 3</td>
<td>506 (314–814)</td>
<td>0.25 (0.25–0.25)</td>
</tr>
<tr>
<td>Month 12</td>
<td>37 (23–58)</td>
<td>0.25 (0.25–0.25)</td>
</tr>
</tbody>
</table>

aMean ± SD.

bMicroscopic analysis of Giemsa-stained blood smears.

cMedian values plus interquartile ranges (25–75%). The lower limit of detection of the assay is 0.25.

dTitres are significantly higher in RTS,S/AS01E vaccinees than in controls (p ≤ 0.01).

Hb, Hemoglobin; WBC, white blood cell count.
peptides was observed in the RTS,S recipients (Fig. 1B), neither the magnitude (Fig. 1C) nor the frequency (Table II) of T cell IFN-γ responses to CS peptides differed significantly between RTS,S-vaccinated and control children.

To determine whether other markers of Ag-specific memory T cells might be more sensitive than IFN-γ secretion, T cell expression of CD69 and CD25 was examined. Expression of both CD69 and CD25—assessed as mean fluorescence intensity (MFI) (Fig. 1) or as the percentage of positive cells (data not shown)—was significantly upregulated on T cells from both RTS,S vaccinees and controls after restimulation with HBs peptides and on T cells from RTS,S vaccinees, but not controls, after CS peptide restimulation (Fig. 1B). The fold change in both CD69 (Fig. 1D) and CD25 (Fig. 1E) expression was significantly greater among RTS,S-vaccinees than among controls after restimulation with either HBs or CS peptides, and the proportion of RTS,S-vaccinees with positive CD69 and CD25 responses was significantly higher than the proportion of controls making such responses (Table II), indicating that CD69 and CD25 expression identify T cell memory responses to CS peptides that are not evident by IFN-γ secretion.

IL-2 is a sensitive marker of CS-specific responses in RTS,S/AS01E vaccinated individuals

Culture supernatants of 92 randomly selected individuals (38 of whom were subsequently revealed to be RTS,S recipients and 54 to be controls) were analyzed by Luminex. Concentrations of IFN-γ, IL-2, and IL-10 differed significantly between RTS,S/AS01E vaccinees and controls (Fig. 2).

Responses to HBs peptides. Concentrations of IFN-γ (Fig. 2A) and IL-2 (Fig. 2B) were significantly higher in HBs culture supernatants than in control (GM) supernatants among both RTS,S/AS01E recipients and controls, indicating that HBs vaccination in infancy had induced a durable effector memory cell response. However HBs-induced IFN-γ and IL-2 concentrations, and the proportion of subjects with a detectable IFN-γ or IL-2 response, were significantly higher among RTS,S/AS01E vaccinees than among controls (Table III), confirming that RTS,S/AS01E vaccination efficiently boosts HBs responses. IL-10 concentrations were significantly higher in HBs-stimulated cultures than in control cultures of RTS,S/AS01E recipients (Fig. 2C), but there was no significant IL-10 response among the controls, and significantly
more vaccinees than controls made a detectable IL-10 response (Table III), suggesting either that HBs-specific IL-10 is induced by RTS,S/AS01E but not by routine HBs vaccination or that IL-10 responses are short lived and had waned in RTS,S/AS01E vaccinees but had waned to undetectable levels in control subjects.

Responses to CS peptides. CS-induced IFN-γ concentrations were slightly higher than in control cultures, but neither the concentration (Fig. 2A) nor the proportion of responders (Table III) differed between RTS,S vaccinees and controls, suggesting that this response may result from natural exposure to malaria sporozoites. CS-induced IL-10 secretion did not differ significantly from background among RTS,S/AS01E vaccinees or controls (Fig. 2A) nor the proportion of responders (Table III) differed between RTS,S vaccinees and controls. CS-specific IL-2 concentrations were significantly higher among vaccinees than controls (p < 0.0001; Fig. 2B), and significantly more vaccinees than controls had a detectable CS-specific IL-2 response (p < 0.0001; Table III). Because Ag-specific T cells are the primary source of IL-2, these data suggest that CS-specific IL-2 may be a more sensitive marker of T cell responses to RTS,S than CS-specific IFN-γ production.

NK cells of RTS,S/AS01E vaccinees are activated in HBs- and CS-stimulated cultures

We have reported that T cell IL-2 responses to malaria (14) and viral vaccine Ags (15) are associated with markedly enhanced NK cell responses to the same organisms. To explore whether RTS,S/AS01E vaccination enhances NK cell responses to HBs or CS, we compared IFN-γ and CD69 expression in CD3−CD56+ NK cells between the two groups of children. Representative data for one RTS,S vaccinee are shown in Fig. 3A; aggregate data are shown in Fig. 3B–D and Table II.

Responses to HBs peptides. Both IFN-γ secretion and CD69 expression were significantly upregulated in NK cells of RTS,S/AS01E-vaccinees after restimulation of PBMCs with HBs peptides.

Responses to CS peptides. Statistically significant NK cell IFN-γ and CD69 responses were observed among CS-stimulated PBMCs of RTS,S-vaccinated children (CS versus GM; p = 0.0002 and p < 0.0001, for IFN-γ and CD69 respectively) but not control children (Fig. 3B). NK cell IFN-γ and CD69 responses in CS-stimulated cultures were significantly higher among RTS,S vaccinees than controls (Fig. 3C, 3D), and the frequency (Table II) of NK cell responses may be indicative of an Ag-specific memory response and can be boosted by revaccination.

NK cell activation correlates with IL-2 secretion

We have previously observed that NK cell “recall” responses are mediated by IL-2 from memory T cells (14, 15); therefore, we...
looked for associations between IL-2 secretion and NK cell responses to HBs or CS peptides (Fig. 4). We observed statistically significant associations between IL-2 concentration and NK cell CD69 expression in response to both HBs ($p = 0.0001$; Fig. 4B) and CS ($p = 0.035$; Fig. 4F) among RTS,S/AS01E-vaccinees, and in response to HBs ($p = 0.02$, Fig. 4D) but not CS among controls (Fig. 4H), indicating that IL-2 may be sufficient to up-regulate NK CD69 expression. However, NK cell IFN-γ responses were not significantly correlated with IL-2 concentration for either HBs- or CS-stimulated cells in either vaccinated or control subjects (Fig. 4A, 4C, 4E, 4G), consistent with the notion that other signals—in addition to IL-2—are required for activation of NK cells to full effector status (14, 15). (Although it may appear that NK cell IFN-γ and IL-2 responses to CS peptides in the RTS,S vaccinees are mutually exclusive [Fig. 4E], this relationship was not statistically significant [$\chi^2 = 0.73; p = 0.39$]).

**NK cells contribute substantially to postvaccination IFN-γ responses**

Although the percentage of NK cells secreting IFN-γ after RTS,S/AS01E vaccination was greater (HBs: median 1.23%, range 0.08–16.55%; CS: median 1.00%, range 0.04–7.48%) than the percentage of T cells making IFN-γ (HBs: median 0.31%, range 0.04–2.56%; CS: median 0.46%, range 0.07–4.39%), T cells are far more numerous in peripheral blood than are NK cells. To determine which population was the major contributor to the early IFN-γ response, the data were reanalyzed by gating on all IFN-γ–positive cells and analyzing them for expression of CD3 and CD56.

| Table III. Proportion of individuals with a secreted cytokine response to HBs or CS peptides |
|-----------------|---------------|-------|-------|-------|
| Ag Group | IFN-γ | IL-2 | IL-10 |
|-----------------|---------------|-------|-------|-------|
| HBs RTS,S/AS01E (n = 38) | n (%) | 20 (52.6) | 37 (97.4) | 18 (47.4) |
| Control (n = 54) | 9 (16.7) | 32 (59.3) | 8 (14.8) |
| p-value | 0.0005 | <0.0001 | 0.0009 |
| CS RTS,S/AS01E (n = 38) | n (%) | 6 (15.8) | 26 (68.4) | 4 (10.5) |
| Control (n = 54) | 13 (24.1) | 3 (5.6) | 3 (5.6) |
| p-value | 0.44 | <0.0001 | 0.44 |

Responders are defined as those with a peptide-specific cytokine response $\pm$ mean $\pm$ 2 SD of their own negative control (GM) values.
CD56 (Fig. 5A). Among RTS,S/AS01E-vaccinees, NK cells were the largest population of cells making IFN-\(\gamma\) in response to both HBs (51.8%; SEM 2.3%) and CS (52.4%; SEM 2.6%) peptides (Fig. 5B).

Cell-mediated immune responses correlate with humoral responses to RTS,S. Finally, we looked for associations between anti-HBs and anti-CS Ab titres, measured at the 3 mo time point (i.e., 4 wk after the third dose of vaccine; Table I), and cellular immune parameters. In the control group, there were no significant correlations between anti-HBs titre and any of the cellular parameters. The number of individuals in the control group with a positive anti-CS titer was too small (\(n = 2\)) to allow meaningful comparisons with cellular parameters in this group.

In contrast, in the RTS,S vaccinated group, anti-HBs titres were significantly correlated with percentages of both T cells and NK cells secreting IFN-\(\gamma\) (Pearson correlation: \(r = 0.29, p = 0.01\); \(r = 0.26, p = 0.02\), respectively) whereas anti-CS titres were not correlated with T cell or IL-2 responses (\(r \leq 0.13, p \geq 0.27\) in all cases), but showed a tendency to correlate with NK cell IFN-\(\gamma\) and NK cell CD69 expression, although this was of borderline significance (Pearson correlation: \(r = 0.21, p = 0.08\), in both cases).

**Discussion**

RTS,S/AS01 is the first malaria vaccine to enter phase III clinical trials (16). Associations have been observed between resistance to infection and CS-specific Ab titres (17–20) and between resistance to clinical malaria, CS-specific IFN-\(\gamma\) (10, 11, 21) and secreted IL-2 (21) responses, but these associations are not correlates of protection at the individual level. Although the number of children in this study who experienced at least one episode of clinical malaria was too small to draw any legitimate conclusions regarding individual correlates of protection, our primary purpose was to look for additional markers of RTS,S-induced cellular immune responses that could be evaluated as correlates of immunity in future trials.
This study demonstrates that RTS,S/AS01 effectively boosts cellular responses induced by prior hepatitis B virus vaccination; this can be detected as upregulation of CD69, CD25, and IFN-γ in CD4+ T cells, upregulation of CD69 and IFN-γ amongst NK cells, and enhanced PBMC secretion of IL-2, IFN-γ, and IL-10. These results are consistent with previous observations that IL-2 emanating from memory T cells can activate NK cells, although we were surprised that restimulation of PBMCs with peptide alone was sufficient for NK cell activation, because we have previously observed that optimal NK cell responses also require cytokine-mediated and contact-dependent signals from accessory cells, and that this depends on preserving the pattern recognition pathways initiated by whole pathogens. However, in a separate study, we have observed that recombinant HBs is sufficient to activate NK cells in individuals whose cells produced large amounts of IL-2 (A. Horowitz, S.E. Moore, and E.M. Riley, unpublished observations), suggesting that the need for accessory cell signaling for NK activation may be partially overridden in the presence of high levels of IL-2. If so, then T cell-derived IL-2 may synergize with accessory cell-derived signals induced by whole pathogens, leading to even more potent NK cell responses than we have observed, but additional experiments are required to test this hypothesis. Importantly, the frequency and magnitude of the NK cell IFN-γ response was such that NK cells were found to contribute more than half of the HBs-specific IFN-γ response, raising the distinct possibility that NK cells may be important effectors of the immediate response to hepatitis B virus after vaccination.

RTS,S vaccination also induces CD4+ T cell and NK cell responses to CS peptides. Although we did not observe significant CS-specific T cell IFN-γ responses among RTS,S/AS01 recipients when compared with controls, we did observe clear induction of IFN-γ from NK cells, enhancement of CD69 expression in T cells and NK cells, upregulation of CD25 on CD4+ T cells, and secretion of IL-2 (but not IFN-γ or IL-10) after in vitro restimulation of PBMCs with CS peptides. Because CD8+ T cells have been shown to inhibit parasite development within hepatocytes in an IFN-γ-dependent manner, the observation that NK cells can secrete IFN-γ within 24 h of restimulation with CS Ag raises the possibility that NK cells may also be effective against malaria-infected liver cells. It would also be of interest to explore whether NK cell cytotoxicity, which can also be enhanced by vaccination, contributes to killing of malaria liver stages.

Overall, cellular responses to CS peptides were lower in magnitude and prevalence than those to HBs peptides. This finding may, in part, be explained by the fact that RTS,S recipients had received six doses of HBs (three doses of HBV vaccine in infancy plus three doses of RTS,S) but only three doses of CS. It might also suggest that the circulating CS-specific T cells differ qualitatively from HBs-specific T cells, secreting predominantly IL-2. This conclusion is supported by recent studies of RTS,S in the United States (26), Gabon (27), Ghana (28), and Kenya (29). In each of these studies, CS-responsive IL-2+ CD4+ T cells were significantly more frequent after vaccination, whereas CS-specific IFN-γ– or TNF-producing T cells were less frequently observed. A marginally significant association between CS-specific IL-2 secretion and protection from infection has been observed in Mozambican infants immunized with RTS,S/AS02 (21). In the Kenyan (29) and American studies (26), CS-responsive IL-2+ CD4+ T cells and TNF+ CD4+ T cells were associated with protection against clinical malaria and malaria infection, respectively. Thus, a picture is emerging that RTS,S induces a CS-specific CD4+ T cell response that is characterized by production of IL-2 and TNF but not IFN-γ, and that IL-2 or TNF production may be causally associated with vaccine efficacy. It remains to be determined whether IL-2 is a reliable correlate of protection and, if so, whether it is mediating its protective function through NK cell activation (as suggested from this study), by promoting longevity and renewal of memory T cells, or by acting as a growth factor for B cells or follicular Th cells. Although an association between the frequency of IL-2+ CD4+ T cells and anti-CS Ab titers has been described in RTS,S vaccinees (26), we did not observe any correlation between secreted IL-2 concentration, or T cell activation of IFN-γ–secretion, and anti-CS titer in the current study. Instead, we observed a tendency for NK cell responses to correlate with Ab titers. It remains to be determined whether this is a genuine causal association, or whether NK cells are simply a highly sensitive marker of APC or T cell functions that were not directly measured.

The discrepancy between the very low levels of CS-specific IFN-γ–producing T cells detected in this study, and in other recent studies (26–28), and the higher numbers of such cells detected previously (10, 11, 21) is likely explained by differences in experimental protocol. Exogenous T cell costimulation with anti-CD28 and anti-CD49d (11, 21) or recombinant IL-2 and IL-7 (10), detection of IFN-γ–producing cells by ELISPOT (9, 11) (which does not differentiate T cells from NK cells) or restimulation of cells with peptide and exogenous IL-2 for 14 d (30) are all likely to amplify estimates of T cell responsiveness. Nevertheless, it would be interesting to explore whether additional doses of RTS,S or repeated exposure to sporozoites would change the T cell response from an IL-2/TNF dominant to an IFN-γ–dominant profile, and if so whether it should not be automatically assumed that this would be clinically beneficial. T cell IL-2 production declines progressively with each episode of TCR stimulation, whereas IFN-γ production is maintained or increases (31, 32). IL-2 secretory capacity endows T cells with long-term memory potential (31, 32), but the possibility that IL-2 is an effector molecule in its own right is rarely considered (33). Our data suggest that maintaining IL-2 secretion may sustain NK cell responses and markedly increase the pool of early effector cells available to control pathogen colonization, invasion, or replication.
The observation that a substantial proportion of the IFN-γ produced by PBMCs in vaccinated individuals is produced not by T cells but by NK cells suggests that some data on correlates of protection may need to be reappraised. ELISPOT analysis of mixed PBMCs (9, 29, 34) or quantification of secreted cytokines from PBMCs or whole blood (21) has typically been interpreted as evidence for Ag-specific T cell responses. Flow cytometry with intracellular cytokine staining suggested (appropriately) that CD4+ and CD8+ T cells make IFN-γ after 24–48 h restimulation with CS peptides (10, 11), but conclusions regarding the phenotype of IFN-γ–producing cells in mixed PBMC ELISPOTS based on T cell depletion (9, 34) are less robust because we know that CD4+ T cell-derived IFN-γ is essential for NK cell IFN-γ production (14, 15). There is a distinct possibility that some IFN-γ–producing cells in these ELISPOT assays were NK cells.

This study also raises questions about the current focus on using numbers of IFN-γ–producing T cells as a primary measure of vaccine immunogenicity (35). It is possible that optimizing numbers of IL-2–producing T cells—to generate an immediate and potent NK response as well as long-term memory—may be a better strategy. Although further study is required to determine whether T cell IL-2 production might be a reliable indicator of vaccine-induced immunity, IL-2 production is the most consistently observed function of RTS,S-induced CS-specific CD4+ T cells (26–28) and may underlie correlations between the induction of multifunctional (IFN-γ+TNF-α+IL-2+CD154+) CD4+ T cells and protection in murine vaccination models of leishmaniasis (36) and tuberculosis (37) and in human malaria vaccine trials (38, 39). The data from this study reveal a small number of easily testable hypotheses that might explain why IL-2 is emerging as a correlate of protection against these diverse infections.

Acknowledgments
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Disclosures
A.L., P.M., J.C., and J.V. are employed by GlaxoSmithKline-Biologicals and have received honoraria from the company for assistance with this study; Teun Bousema for statistical advice; and Robbert van der Most for comments on the manuscript.

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3. Ballou, W. R. 2009. Development and a better strategy. Although further study is required to determine whether T cell IL-2 production might be a reliable indicator of vaccine-induced immunity, IL-2 production is the most consistently observed function of RTS,S-induced CS-specific CD4+ T cells (26–28) and may underlie correlations between the induction of multifunctional (IFN-γ+TNF-α+IL-2+CD154+) CD4+ T cells and protection in murine vaccination models of leishmaniasis (36) and tuberculosis (37) and in human malaria vaccine trials (38, 39). The data from this study reveal a small number of easily testable hypotheses that might explain why IL-2 is emerging as a correlate of protection against these diverse infections.

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