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The Induction and Persistence of T Cell IFN-γ Responses after Vaccination or Natural Exposure Is Suppressed by *Plasmodium falciparum*[1]

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Abstract

Epidemiological observations suggest that T cell immunity may be suppressed in malaria-endemic areas. In vitro studies, animal models, and limited data in humans link immunosuppression with malaria, malnutrition, and other parasitic infections. However, there are no data to determine whether malaria-induced immunosuppression is significant in the long-term, or relative data comparing it with other factors in malaria-endemic areas, so as to measure the impact of malaria, other parasitic disease, nutritional status, age. and location on the acquisition and longevity of IFN-γ responses in children in Kenya. We studied these factors in two cohorts of 1- to 6-year-old children in a malaria-endemic area. T cell responses were induced by vaccination in one cohort, and acquired as a result of natural exposure in a second cohort. Serial ELISPOT assays conducted over a 1-year period measured the induction and kinetics of IFN-γ production in response to the malaria Ag thrombospondin-related adhesion protein. Induced responses in both cohorts and the longevity of response in the vaccinated cohort were fitted to potential explanatory variables. Parasitemia was prospectively associated with reduced IFN-γ-producing T cells in both cohorts (by 15-25%), and both parasitemia and episodes of febrile malaria were associated with 19 and 31% greater attrition of T cell responses, respectively. Malaria may reduce the efficacy vaccinations such as bacillus Calmette-Guérin and investigational T cell-inducing vaccines, and may delay the acquisition of immunity following natural exposure to malaria and other pathogens.

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Some epidemiological observations suggest that cellular responses may be suppressed in malaria-endemic areas (1, 2), but a number of environmental factors might be responsible. Malaria-infected erythrocytes modulate dendritic cell function in vitro, inhibiting Th cell induction (3, 4) and stimulating T regulatory cells (5), but the evidence that associates malaria with immunosuppression in vivo is limited. Although routine childhood Ab-inducing vaccinations appear to be effective in malaria-endemic areas, and are mostly unaffected by chemoprophylaxis (6), there may be a transient suppression of cellular responses during acute infection in Thai adults (7, 8). Acute infection has also been associated with a loss of T cell inhibition of EBV-infected cells (9), and there is a transient suppression of vaccine-induced Ab responses after inpatient treatment for malaria (10). However, the long-term in vivo impact of malaria-induced immunosuppression in the populations at risk has not been determined.

Furthermore, other factors might also suppress cellular responses in malaria-endemic areas. Severely malnourished children have a suppressed cellular response to mycobacterial Ags (11). Mild malnutrition is common in malaria-endemic areas and might also lead to depressed cellular immunity. Concurrent helminth infections suppress T cell responses by inducing a TH2 cytokine profile (12). T cell responses to tetanus toxoid (13, 14) and bacillus Calmette-Guérin (BCG)[3] (15) may be inhibited by helminth infection.

We measured T cell responses by IFN-γ production in two cohorts of children in rural Kenya, for whom data on malaria infection, helminth infection, nutritional status, age, and village was available. We used both ex vivo and cultured ELISPOT assays. Responses detected by cultured ELISPOTs are sustained for at least 6 mo after vaccination of naive subjects, but responses detected by ex vivo ELISPOT begin to fall soon after induction (16).

The vaccination regimen induces T cell responses by sequential immunization with the attenuated fowlpox strain, FP9, and modified virus Ankara, to deliver a multiple epitope (ME) string (17) coupled to the pre-erythrocytic malaria Ag thrombospondin-related adhesion protein (TRAP) (18).

Vaccine efficacy, safety, and immunogenicity are described elsewhere (19). The study presented here examines whether malaria, other parasitic infections, or malnutrition influence natural or vaccine-induced acquisition of T cell responses. The impact of these exposures is studied on three different data sets.

The first data set comprises T cell responses measured in a cross-sectional bleed of children 1 wk after the last of three vectored ME-TRAP vaccinations (the second cross-sectional bleed). These responses were adjusted by T cell responses at the first cross-sectional bleed, taken before vaccination. The second data set comprises naturally acquired T cell responses measured in rabies (i.e., control) vaccine recipients at the end of the malaria season (the third cross-sectional bleed). This analysis was adjusted by T cell responses at the cross-

[3]Abbreviations used in this paper:

BCG bacillus Calmette-Guérin

ME multiple epitope

TRAP thrombospondin-related adhesion protein

MUAC mid upper arm circumference

CI confidence interval

sectional bleed before the start of the malaria season (the first cross-sectional bleed). The third data set comprises T cell responses measured in ME-TRAP vaccinees 9 mo after the last vaccination (the fourth cross-sectional bleed). Analysis was adjusted by responses immediately after vaccination (the second cross-sectional bleed). Thus, the analysis in each data set is adjusted by T cell responses at a prior cross-sectional bleed. Parasitemia is considered as a potential factor, identified on the cross-sectional bleed analyzed (referred to as “concurrent” parasitemia), and also on the prior bleed (referred to as “previous”).

Materials and Methods

Study design

The study was conducted in the context of a randomized, controlled, and double-blind vaccine trial. Ethical approval was obtained from the Kenyan Medical Research Institute National Ethics Committee, the Central Oxford Research Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee. Five milliliters of blood was taken by venipuncture for immunology and safety; cross-sectional assessments of malaria parasitemia were conducted prevaccination, at screening, 1 wk after the third vaccination, then at 3 mo and at 9 mo later. Children were screened in February 2005, immunized between March 2005 and May 2005, and followed up until February 2006.

Participants

The participating children were aged 1-6 years old (inclusive), healthy, and resident in the Junju sublocation, Kilifi District. After a series of public meetings and individual discussions, parents were invited to bring their children to a screening visit. Recruiting continued until the target sample size was reached.

Children were screened by history, examination, and blood tests (full blood count, creatinine, alanine transaminase). Subjects with clinically significant illness were excluded. HIV prevalence among children is low in Kilifi, at <2% (20), and screening did not include HIV serology. Clinically evident immunosuppression was an exclusion criteria, but no children were excluded on that basis. The mid upper arm circumference (MUAC) was measured to determine nutritional status. The median MUAC was 15 cm among the children recruited (95% range 13-17 cm). A cutoff of 13 cm determines severe malnutrition (21), and the median MUAC is 16 cm in nonaffluent populations in the age-group studied (22). Eligible children were invited to attend vaccination in the order in which they were screened.

Location

The study was conducted in the Junju sublocation in the Kilifi District, on the Kenyan coast. Junju contains a group of five closely related villages within the Chonyi area of the Kilifi District. Kilifi is malaria endemic, with all-year-round transmission and two high transmission seasons (23), with a transmission intensity of 22-53 infective bites per year (24).

Vaccinations

Recombinant viral vectored vaccinations were used. The Ag insert was TRAP, joined to a ME string from six *Plasmodium falciparum* pre-erythrocytic Ags (17). The ME string contains 14 pre-erythrocytic MHC class I epitopes from six *P falciparum* pre-erythrocytic Ags, three class II epitopes, two pre-erythrocytic B cell epitopes, and pb9 (a *Plasmodium berghei* T cell epitope that allows preclinical potency and stability testing). The vectors were an attenuated fowlpox virus, FP9, and modified vaccinia virus Ankara. The trial vaccination regimen was two sequential FP9 ME-TRAP vaccinations (5×10^7 PFU) followed by

modified virus Ankara ME-TRAP vaccination (1.5×10^8 PFU), given intradermally. The control was rabies vaccine (WISTAR strain; Aventis Pasteur), also given intradermally. Vaccinations were spaced 4 wk apart (acceptable range 3-5 wk). Curative antimalarial treatment was given 1 wk after the final vaccination, using 7 days of directly observed dihydroartemisinin monotherapy (2 mg/kg on the first day, followed by 1 mg/kg for 6 days).

Follow-up

Children were monitored for episodes of malaria, defined as an axillary temperature >37.5 degrees centigrade, with a *P. falciparum* parasitemia >2500 parasites per microliter. Children were visited every week by field workers. When the temperature was >37.5 degrees, a blood film was made and a rapid near-patient test (Optimal, which detects parasite lactate dehydrogenase) for malaria was conducted. Treatment for episodes of malaria was with the Government of Kenya recommended first line treatment, artemether-lumefantrine.

Four cross-sectional bleeds were conducted to measure T cell responses and to examine blood films on all children. Results were not available for several weeks, during which monitoring of febrile illnesses continued. Asymptomatic parasitemia was therefore not treated unless the child developed a fever. Samples of urine and stool were collected during screening, and examined by direct, wet microscopy for OVA, cysts, and parasites.

ELISPOTs

PBMC were separated at screening, 1 wk after the last vaccination, then at 3 mo and at 9 mo. PBMCs incubations were in RPMI 1640 medium (Sigma-Aldrich) with 10% human AB serum. ELISPOTs used Millipore MAIP S45 plates and MabTech Abs according to manufacturer's instructions. A total of 4×10^5/well of freshly isolated PBMC were incubated in 100 μl with 10 μg/ml peptides for 18-20 h before developing. Individual 8-17 residue epitopes were pooled for the ME string. Twenty residue peptides overlapping by 10 residues were used for TRAP. TRAP peptides were pooled according to region. 20 μg/ml PHA (Sigma-Aldrich) was used as positive control, and PBMC cultured in medium alone as negative control. Spot-forming cell numbers were counted by ELISPOT plate reader (Autoimmun Diagnostika, version 3.0).

For cultured ELISPOTs, 1×10^6 PBMC were incubated in 0.5 ml of 10 μg/ml/peptide of pooled TRAP and ME peptides in a 24-well plate. On days 3 and 7, 250 μl of culture supernatant was replaced with 250 μl of culture medium containing 20 IU/ml rIL 2. On day 9, the cells were washed three times and left overnight before the standard ELISPOT assay.

Analysis

The origin of data sets used in analysis is shown in Fig. 1. ELISPOT wells were assayed in duplicate, taking the mean and subtracting the negative control well result. Assays were discounted if the positive control was <50 spots, or the negative control >20 spots for ex vivo ELISPOTs, or 40 spots for cultured ELISPOTs. Results are presented per million incubated PBMC. Geometric means and 95% confidence intervals (CI) are reported, substituting 1 spot/well above background for blank wells (the lower limit of detection).

To examine the impact of various exposures on T cell responses, multiple linear regression analysis was conducted, using log-transformed spot numbers for three peptide pools per individual. Significance testing was adjusted with peptide pool as a factor to account for the multiple measures (one for each pool) per individual. Independent variables for nutritional status, gastrointestinal helminth infection, urinary schistosomiasis, eosinophilia (as a categorical variable with three levels), malaria parasitemia (concurrent and previous), and

episodes of febrile malaria (binary variables, present, or absent) were examined for their impact on T cell response. Analysis was adjusted by village and age (as a categorical variable). Because longitudinal data was available, T cell responses in each data set were adjusted by previous T ELISPOT responses (rather than being analyzed as a cross-section in isolation). Log-likelihood testing was used to derive a single p value for categorical variables with more than two levels.

The coefficients derived from regression models were transformed to percentages by the formula $10^{(\text{coefficient})}/10 \times 100\%$. This expresses the increase or decrease in T cell responses as a percentage of the responses seen without that factor. The baseline factor, with which others are compared, is given as 100% in the results of all regression models (see Tables II-IV). Where data was not available from both time points used in the data set, children were excluded from the analysis.

Results

Study profile

Numbers lost to follow-up are shown in Fig. 2, and the numbers contributing to each analysis are marked in Figs. 3 and 4.

Kinetics of responses

Vaccination with sequential FP9 virus vaccinations (twice) FFM then modified Vaccinia Ankara virus (once), all encoding Ag construct ME-TRAP induced both ex vivo ELISPOT measured responses (from a geometric mean of 9 spots/million, CI 7-11 to 55/million, CI 45-67, $p < 0.0005$) and cultured ELISPOT measured responses (from a geometric mean of 55 spots/million, CI 44-68 to 240/million, CI 200-290, $p < 0.0005$) (Fig. 3, data set 1).

There was also a significant rise in IFN-γ-producing T cells during the malaria season among control vaccinees. Ex vivo responses rose from 10 spots/million (95% CI 8-12) before the malaria season (January 2005), to 23 spots/million (95% CI 18-28) in the malaria season (August 2005), $p = 0.0005$. Resting memory (cultured) responses also rose significantly during the malaria season, from 53/million (CI 42-66) in January 2005, to 97/million (CI 79-120) in August 2005, $p = 0.0001$ (Fig. 3, data set 2).

Responses that had been induced by vaccination then fell over the following 9 mo. Ex vivo responses fell from a geometric mean of 55 spots/million (CI 43-66) in early May 2005 (1 wk after vaccination) to 19 spots/million (CI 16-24) in January 2006 (9 mo after vaccination), $p < 0.00005$. However, resting memory responses were preserved, from 234 spots/million in May 2005 (CI 194-281) to 213 (CI 169-269) in January 2006, $p = 0.51$. The attrition of responses during the 9 mo after vaccination is shown in Fig. 3, data set 3.

Multiple regression models to determine factors associated with variation of responses

The responses at the second time point for each of the three data sets are modeled as outcomes, using the earlier T cell responses and blood film results at both time points as explanatory variables. The frequencies of other covariates in the population are given in Table I, and the results of multiple regression models to identify explanatory variables for T cell responses are shown in Table II-IV.

Covariates

The pool of TRAP peptides that was tested (three pools covering the full length of the molecule), the child's village and prior immunity were all significant factors in each data set. Neither vaccine-induced nor naturally acquired responses examined in data sets 1 and 2

varied by age. Nine months after vaccination, ex vivo responses in data set 3 were significantly better sustained among older children (2-7 year olds) compared with 1- to 2-year-old children, but cultured responses did not vary.

In data set 1, the ex vivo ELISPOT results measured before vaccination predicted the ex vivo responses after vaccination ($p = 0.012$). In data set 3, cultured responses 9 mo after vaccination were predicted by cultured responses 1 wk after vaccination ($p < 0.0005$), and ex vivo responses 9 mo after vaccination were predicted by ex vivo responses 1 wk after vaccination ($p = 0.009$). However, in data set 2, neither cultured nor ex vivo responses during the dry season predicted naturally acquired responses seen during the rainy season.

Nutrition and helminth infection

ELISPOT results did not vary by nutritional status (as determined by MUAC in January 2005). Parasitic infection was determined by stool and urine microscopy, and peripheral blood eosinophil counts (conducted in January 2005). There were no associations between T cell responses and microscopy results for urinary schistosomiasis or gastrointestinal helminth infections, but eosinophilia was strongly associated with a greater attrition of ex vivo ELISPOT responses, examined in data set 3 ($p = 0.006$ by log-likelihood ratio testing, Table IV). Responses in moderate and highly eosinophilic subjects were 69 or 79% of the magnitude of responses without eosinophilia (Table IV). However, cultured responses did not vary by eosinophilia, and eosinophilia did not alter vaccine induction of responses (Table II) or naturally acquired responses (Table III), examined by data sets 1 and 2, respectively.

Parasitemia and malaria infection

Asymptomatic malaria parasitemia as vaccination began was associated with a reduction in postvaccination T cell responses (i.e., data set 1) to 85% of the magnitude of nonparasitemic children ($p = 0.09$) by ex vivo assays and 79% ($p = 0.043$) by cultured ELISPOT assays (Table II). Naturally acquired responses (data set 2) were also suppressed by previous parasitemia, to 75% ($p < 0.0005$) by ex vivo assays and 75% ($p = 0.032$) by cultured assays (Table III). However, parasitemia at the time blood was drawn for ELISPOT assays did not reduce T cell responses for either vaccine-induced or naturally acquired responses.

Ex vivo responses to vaccination with FFM ME-TRAP were less well-sustained among children previously parasitemic (at 81%, $p = 0.002$), with concurrent parasitemia (69%, $p < 0.0005$) and with episodes of febrile malaria before the ELISPOT assays (79% $p = 0.014$). Cultured responses did not vary by asymptomatic parasitemia, but were reduced to 72% by episodes of febrile malaria ($p = 0.04$).

Fig. 4 shows the frequencies of T cell responses by previous exposure to malaria parasitemia, displayed for each data set studied. The change in ELISPOT results compared with the prior studies used to adjust results is shown for each data set (i.e., for the first data set, the change in response from pre- to postvaccination is shown. For the second data set, the change in response from pre- to post-malaria season, and for the third data set, the change in response from 1 wk to 9 mo postvaccination is shown.

Combined multiple regression analysis for sustained ex vivo responses

In data sets 1 and 2, only one factor (malaria parasitemia) significantly altered responses after adjusting for peptide pool, previous ELISPOT results and village. However, several additional factors were predictors of the attrition of ex vivo responses after vaccination (Table IV, data set 3), and so were entered into a further multiple regression analysis, to examine whether any were confounding factors. The effect of eosinophilia was still highly

significant ($p = 0.0005$, with reductions in response to 63 and 79%, respectively, for moderate and high eosinophilia). Concurrent parasitemia (74%, $p = 0.001$), previous parasitemia (85%, $p = 0.048$), and episodes of febrile malaria (81%, $p = 0.033$) were also independently associated with reduced ex vivo ELISPOT results.

Protection against episodes of malaria

Vaccination with FFM ME-TRAP was not protective, and neither ex vivo nor cultured ELISPOT results predicted protection within FFM ME-TRAP vaccinees (19). The naturally acquired T cell responses were not protective, either for ex vivo (incident rate ratio = 1.17 for each 10-fold rise in T cell numbers, 95% CI 0.7-2) or cultured ELISPOT (incident rate ratio = 0.84, 95% CI 0.5-1.3, $p = 0.48$).

Relationship of ex vivo to cultured responses

Although cultured and ex vivo responses did not correlate with each other among unvaccinated children ($r = -0.017$, $p = 0.71$), there was a significant association between cultured and ex vivo responses 1 wk after vaccination, in May 2005 ($r = 0.18$, $p < 0.00005$ unadjusted, coefficient = 0.14, $p = 0.006$ when adjusted for peptide pool and village).

Discussion

Parasitemia immediately before vaccination suppressed the acquisition of T cell responses, as measured by IFN-γ production, in data set 1 (where responses were acquired from vaccination) and in data set 2 (where responses were acquired as a result of natural exposure in the unvaccinated group). Parasitemia immediately after vaccination did not suppress responses. The data sets are demonstrated in Fig. 3, and the effect of parasitemia in Fig. 4. This might reflect an effect of parasitemia per se at the time point immediately before vaccination, or simply that more highly exposed children are both parasitemic and immunosuppressed. However, concurrent parasitemia would be equally strongly linked to overall exposure, but did not influence T cell responses. Furthermore, malaria episodes during the time of monitoring were not associated with lower T cell responses in data sets 1 and 2.

What are the limitations of this study? The link between immunosuppression and parasitemia is made by association. In vitro studies suggest malaria is immunosuppressive (25). However, the association could be the result of T cell responses protecting against infection. But naturally acquired ex vivo responses to TRAP were not associated with protection previously (26), and in this study neither cultured or ex vivo detected responses were protective. Furthermore, the temporal nature of the association is between parasitemia at a previous cross-sectional bleed to vaccination and reduced T cell responses at a later time point. It therefore seems likely that the direction of causation is from the prior event (malaria infection) to the latter outcome (immunosuppression).

Multiple comparisons have been made. Three data sets are analyzed for the impact of nine different variables. One would expect to see some p values <0.05 by chance alone. However, because previous parasitemia was a significant factor on four of six occasions, this suggests the result is unlikely to be due to chance alone, and two of these p values were <0.0005. One should be more cautious in interpreting the single significant association seen for eosinophilia in the third data set.

The study measures responses to one Ag (TRAP), albeit using all potential epitopes (using 57 peptides in three pools), and only used IFN-γ responses. The ELISPOT assay allows reproducible, high throughput measurement of T cell responses (27), but does not allow detailed phenotyping of the cellular response. Although vaccination can produce very

diverse cytokine responses (28), IFN-γ responses are strongly predictive of at least one other cytokine response (IL-2) after vaccination with the viral vectors used in our study (29, 30). Furthermore, although we did not conduct magnetic bead depletion studies, previous work shows that natural infection (26) and vaccination (31) induces primarily CD4 responses. Acute malaria induces a monocytosis (32), but the ELISPOT studies were of well, afebrile children, and the monocytosis associated with malaria appears to last only 3-7 days (32). Therefore, despite these limitations, the study allows reasonable conclusions to be drawn from the relative magnitude of cellular responses in the children studied.

Previous studies have identified a temporary cellular immunosuppression in febrile malaria (7, 8), but our data suggest a more long-lasting immunosuppression associated with asymptomatic parasitemia. By what mechanism might early parasitemia but not parasitemia at the time of the assay for cellular responses influence T cell induction? Children received priming vaccinations 2 wk after the earlier blood films, and boosting vaccinations 1 wk before the concurrent blood films. In this vaccinated cohort, parasitemia appeared to inhibit T cell priming, but not the subsequent boosting of responses. In the cohort studied for natural exposure, malaria transmission was high throughout the interval between blood film examinations. Because a single exposure to sporozoites in naive volunteers does not appear sufficient to generate T cell responses (16), it is likely that the T cell responses acquired in this cohort were as a result of multiple exposures. Although this natural exposure is not a heterologous prime boost, it nevertheless seems that parasitemia concurrent with early exposure was associated with suppressed responses, but that parasitemia was concurrent with later exposure. Thus, for vaccination and natural exposure, it appears that parasitemia has an influence on initial priming, but not subsequent recall and/or boosting. The boosting of cultured and ex vivo responses was equally affected.

However, the more rapid attrition of T cell responses associated with parasitemia, episodes of febrile malaria, and eosinophilia was seen only with ex vivo responses. Cultured responses were not affected (data set 3). Furthermore, the variation in attrition of response seen according to village was more pronounced for ex vivo than for cultured responses. These data suggest that the effect of malaria on T cell induction is seen on ex vivo and cultured ELISPOT, but that once induced, cultured responses are relatively durable. Ex vivo responses are susceptible to attrition from a number of environmental factors, of which malaria infection had the greatest impact in our study. This is consistent with previous comparisons of cultured and ex vivo ELISPOTs. Cultured ELISPOT responses appear to identify a more durable T cell response than the ex vivo assay in naive volunteers (16), and did not correlate with ex vivo responses in semi-immune adults in a single cross-sectional survey (33).

The priming of T cells responses depends on dendritic cells, whereas subsequent memory appears to be independent of dendritic cells (34), but is dependent on cytokines such as IL-7 and IL-15 (35, 36). Previous parasitemia may act on dendritic cells to inhibit T cell priming, but intercurrent febrile malaria, parasitemia, and the TH2 responses associated with eosinophilia may impair the cytokine milieu required to support the longevity of responses. T cell responses are depleted but for the inflammatory cytokine response during malaria infection in mice (37). Our data suggest that the cytokine milieu acts differently on T cells seen to respond on ex vivo assays (i.e., with an immediate effector phenotype) than on cultured ELISPOTs (i.e., with a “resting memory” phenotype).

The 15-30% reductions in T cell responses in parasitemic children are likely to be an underestimate of the overall immunosuppression caused by malaria in endemic areas. Parasitemia below the level of blood film detection is frequent in endemic areas (38), and some children may have cleared their parasitemia soon after the film was taken. It is likely

that the majority of children in the study (film negative and positive) were exposed to malaria, but those with positive blood films had a greater suppression of cellular responses.

Neither gastrointestinal helminth infection nor urinary schistosomiasis was associated with altered cellular immunity. However, only single specimens of stool and urine were examined, and children with lower parasite burdens might have been identified by repeated specimens or concentration techniques. The only common cause of eosinophilia among children in Africa is helminth infection, and eosinophilia may be a more discriminatory marker for altered immunity, because it is more closely associated with the TH2 cytokine responses than the presence or absence of parasites (39). Because eosinophilia was associated with greater attrition of cellular responses, it is likely that helminths do play a role in the attrition of responses.

What are the implications of these data? Malaria parasitemia and helminth infection should be considered in assessing the immunogenicity and efficacy of experimental and routine (i.e., BCG) T cell-inducing vaccinations. The efficacy of BCG is less in malaria-endemic areas (40), and more immunogenic vaccines are in development (41). T cell-inducing vaccines are also in development for HIV (42) and malaria (43). Estimates of immunogenicity and efficacy should be sought in populations chosen for different malaria and helminth endemicities. Age appears to be a less important consideration over the 1- to 6-year range, and mild and moderate malnutrition does not appear to reduce immunogenicity. The clinical development plans of experimental vaccines, intermittent presumptive treatment of malaria, and observational studies of immunity to malaria should consider the immunosuppressive effect of malaria in their designs.

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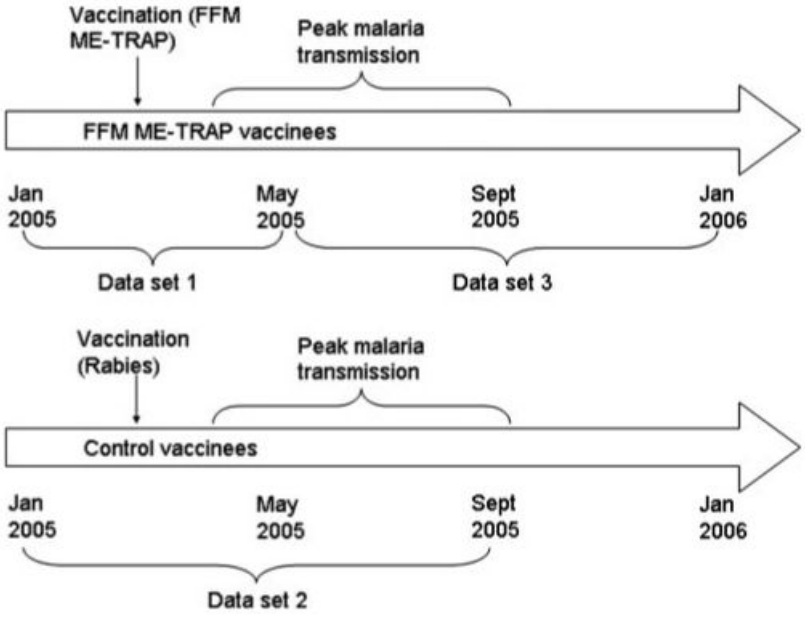

FIGURE 1.

The cross-sectional bleeds used for each data set and their relationships to malaria transmission and vaccination are shown. Each data set uses two cross-sectional bleeds, modeling the impact of T cell responses at the earlier time point, parasitemia at both time points, and malaria episodes during the intervening time on T cell responses seen at the later time point.

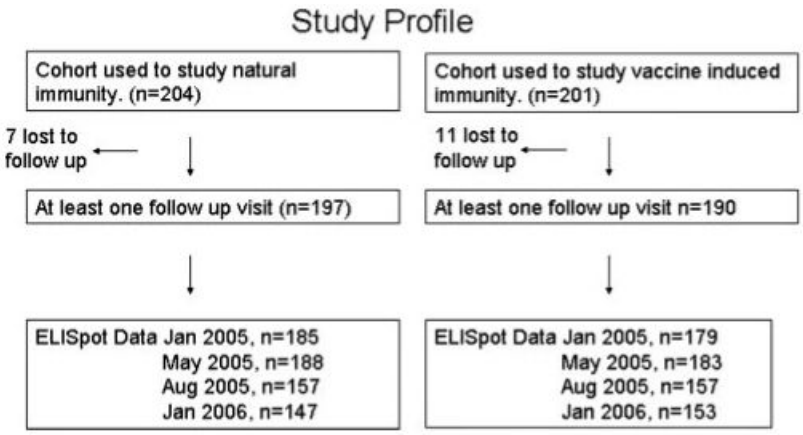

FIGURE 2.

The study profile for the 405 subjects recruited is shown. ELISPOT data was missing from the four time points because of assay failure in 51, 8, 45, and 7 instances, and because of nonattendance in 0, 33, 57, and 102 instances, respectively. The high number of nonattendances at the last time point appeared to be related to seasonal traveling patterns.

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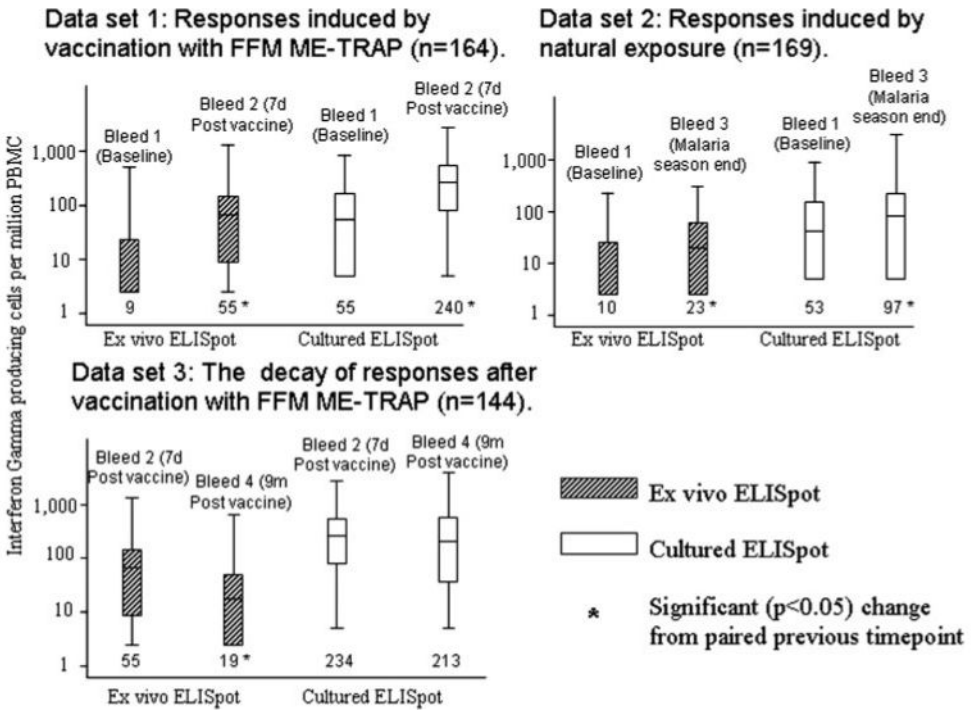

FIGURE 3.

T cell responses to vaccination identified by both ex vivo and cultured ELISPOT are displayed over time for each data set analyzed. Median, 25th and 75th quartile, 5th and 95% quartile and outlying results are given by box and whisker plots. Vaccination with FFM ME-TRAP induced ex vivo ELISPOT and cultured responses ($p < 0.0005$, $p < 0.0005$, data set 1). There was also a significant rise in T cell responses during the malaria season among control vaccinees (data set 2, $p < 0.0005$, $p = 0.001$ for ex vivo and cultured responses, respectively). Vaccine-induced ex vivo response fell over the next 9 mo (data set 3, $p < 0.0005$), but cultured responses were sustained ($p = 0.51$). The geometric mean responses are shown below each box and whisker plot.

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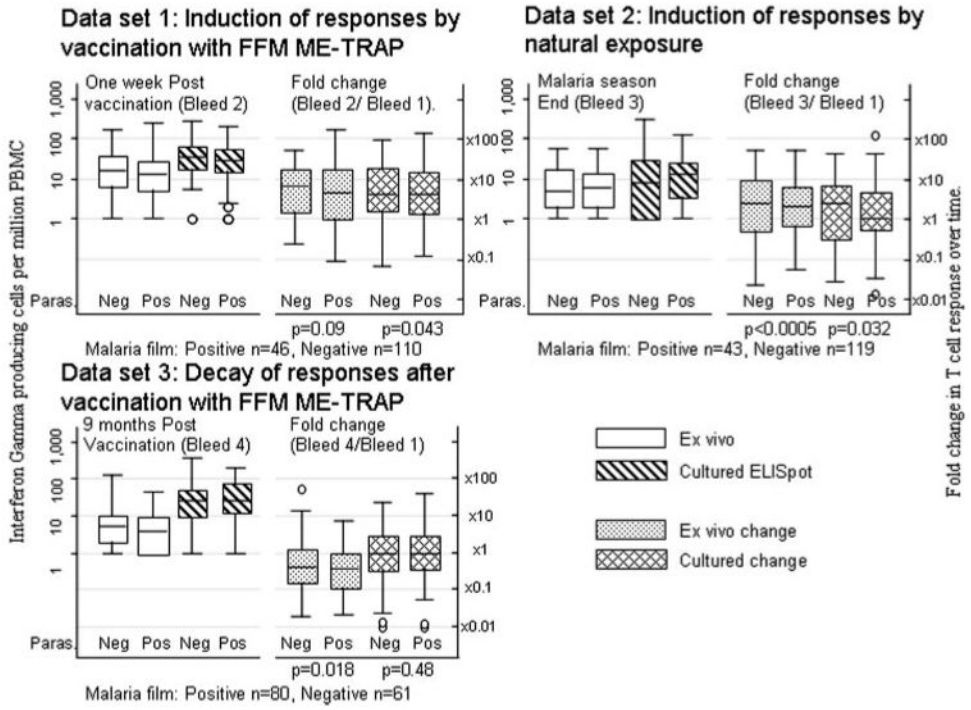

FIGURE 4.

IFN-γ-producing T cells are compared by malaria parasitemia at the cross-sectional bleed before immunological studies (as paired box and whisker plots). The first two box and whisker pairs in each data set show the absolute values seen at the cross-sectional bleed that was analyzed as the outcome data in each data set. The second two box and whisker pairs show the fold change in T cell numbers when the outcome data is divided by T cell numbers seen at a previous cross-sectional bleed. Hence, x1 indicates no change in T cell responses over time. Values of p for each comparison (malaria parasites positive vs malaria parasites negative) are shown under each pair.

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Table I

The frequencies of covariates among study subjects[a]

Variable	Factor	Data Set 1 (%)	Data Set 2 (%)	Data Set 3 (%)
Village	Gongoni	34 (18)	31 (18)	35 (20)
	Junju	47 (25)	39 (23)	40 (23)
	Kolewa	51 (27)	50 (30)	44 (26)
	Mapawa	37 (20)	31 (18)	35 (20)
	Mwembe ts.	14 (7)	15 (9)	14 (8)
		n = 183	*n* = 166	*n* = 168
MUAC	1st tertile	44 (24)	45 (27)	38 (22)
	2nd tertile	54 (29)	62 (37)	53 (31)
	3rd tertile	85 (46)	59 (35)	77 (45)
		n = 183	*n* = 166	*n* = 168
GI worm	Infected	34 (23)	41 (29)	32 (24)
	Uninfected	111 (77)	96 (71)	103 (76)
		n = 145	*n* = 137	*n* = 135
Schist.	Infected	12 (8)	7 (5)	10 (7)
	Uninfected	127 (92)	117 (95)	119 (93)
		n = 139	*n* = 124	*n* = 129
Eosinophils	Norm	88 (48)	79 (47)	81 (48)
	Mod.	53 (28)	48 (28)	54 (32)
	High	25 (13)	33 (19)	27 (16)
		n = 166	*n* = 160	*n* = 162
Febrile mal.	≥1 episode	24 (13)	30 (18)	38 (23)
	No episodes	159 (87)	142 (82)	130 (77)
		n = 183	*n* = 166	*n* = 168
Prev. film	Positive	123 (70)	79 (50)	72 (43)
	Negative	52 (30)	79 (50)	96 (57)
		n = 175	*n* = 158	*n* = 168
Current film	Positive	78 (43)	38 (23)	47 (28)
	Negative	102 (57)	123 (77)	117 (72)
		n = 180	*n* = 161	*n* = 164

[a]Abbreviations: Age cat., age category; M. Tsungu, Mwembe Tsungu; GI worm, microscopy of stool positive for helminth infection; Schist, microscopy of urine positive for schistosomiasis; Mod., eosinophil count above 0.5×10^6/ml; High, above 1×10^6/ml. Prev film, blood film for malaria parasites at the cross-sectional bleed before the immunological studies analysed. Current film, blood film for malaria parasites at the cross-sectional bleed for immunological studies analyzed. Febrile Mal, number of febrile malaria episodes during surveillance in the 3 mo before immunological studies.

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Table II

Results of multiple regression model to examine factors influencing the acquisition of T cell responses after vaccination (data set 1)[a]

	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Peptides				
N-terminal	100		100	
Middle	60 (48-75)	<0.0005	50 (38-64)	<0.0005
C-terminal	91 (74-112)	0.4	74 (56-56)	0.016
Age cat.				
1-2 years	100		100	
2-5 years	109 (87-138)	0.41	79 (6-97)	<0.076
5-7 years	125 (97-165)	0.086	97 (7-128)	0.79
Prev. ex vivo	141 (108-189)	0.012	75 (56-102)	0.09
Prev. cult.	109 (89-134)	0.362	117 (91-147)	0.22
Village				
Gongoni	100		100	
Junju	91 (69-120)	0.51	93 (69-127)	0.66
Kolewa	72 (54-93)	0.013	69 (51-95)	0.024
Mapawa	72 (53-95)	0.019	47 (34-67)	0.0005
M. tsungu	104 (72-154)	0.8	138 (91-213)	0.123
MUAC				
1st tertile	100		100	
2nd tertile	109 (71-141)	0.45	83 (63-109)	0.2
3rd tertile	112 (89-141)	0.32	95 (74-123)	0.73
GI worm	117 (57-151)	0.136	81 (61-107)	0.13
Schist.	151 (103-218)	0.03	85 (56-125)	0.42
Eosin.				
Normal	100		100	
Mod.	134 (99-181)	0.06	114 (83-162)	0.38
High	112 (85-147)	0.39	12 (89-162)	0.23
Prev. Film	85 (69-102)	0.09	79 (63-99)	0.043

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	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Current film	95 (79-112)	0.57	99 (79-117)	0.7
Febrile mal	102 (77-134)	0.86	99 (72-134)	0.97

[a]The average effect associated with each factor is given as a percentage relative to individuals without that factor for each variable (for instance, responses to the middle region of the TRAP molecule are, on average, 60% of the responses seen to the N-terminal portion). Where more than two options exist for a given factor, a log-likelihood ratio test is given for the overall significance of that factor in the model. Log-likelihood testing (for ex vivo then cultured ELISPOTs, respectively) gave $p < 0.0005$ and $p < 0.0005$ for the effect of peptide, $p = 0.2$ and 0.1 for age, $p = 0.01$ and $p < 0.0005$ for village, $p = 0.59$ and $p = 0.38$ for MUAC, and $p = 0.12$ and $p = 0.48$ for eosinophilia.

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Table III

Results of multiple regression model to examine factors influencing the acquisition of T cell responses after natural exposure (data set 2)[a]

	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Peptides				
N-terminal	100		100	
Middle	75 (63-91)	0.005	74 (57-98)	0.038
C-terminal	125 (85-123)	0.74	89 (67-115)	0.357
Age cat.				
1-2 years	100		100	
2-5 years	95 (79-119)	0.73	98 (74-128)	0.88
5-7 years	79 (61-101)	0.064	93 (67-128)	0.63
Prev. ex vivo	75 (56-102)	0.09	117 (85-158)	0.34
Prev. cult.	117 (91-147)	0.22	125 (97-162)	0.079
Village				
Gongoni	100		100	
Junju	75 (6-93)	0.01	98 (74-131)	0.89
Kolewa	151 (123-19)	<0.0005	43 (32-58)	<0.0005
Mapawa	141 (109-181)	0.007	37 (26-51)	<0.0005
M. tsungu	112 (83-151)	0.43	77 (50-120)	0.255
MUAC				
1st tertile	100		100	
2nd tertile	109 (93-128)	0.27	77 (58-99)	0.046
3rd tertile	97 (83-114)	0.71	93 (7-121)	0.59
GI worm	98 (92-117)	0.86	83 (64-107)	0.16
Schist.	109 (75-154)	0.62	107 (64-173)	0.78
Eosinophils				
Normal	100		100	
Mod.	107 (85-134)	0.53	89 (66-123)	0.478
High	108 (83-125)	0.8	81 (61-107)	0.14
Prev. Film	75 (64-87)	<0.0005	75 (57-97)	0.032

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	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Current film	95 (79-114)	0.62	91 (72-117)	0.465
Febrile mal	102 (83-125)	0.828	91 (7-12)	0.51

[a]The average response associated with each factor is given as a percentage relative to the comparison for each variable. Where more than two options exist for a given factor, a log-likelihood ratio test is given for the factors addition to the model. Log-likelihood testing (for ex vivo then cultured ELISPOTs, respectively) gave $p < 0.0028$ and $p < 0.11$ for the effect of peptide, $p = 0.09$ and 0.88 for age, $p = <0.0005$ and $p < 0.0005$ for village, $p = 0.27$ and $p = 0.11$ for MUAC, and $p = 0.81$ and $p = 0.32$ for eosinophilia.

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Table IV

Results of multiple regression model to examine factors influencing the attrition of T cell responses after vaccination (data set 3)[a]

	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Peptides				
N-terminal	100		100	
Middle	66 (53-81)	<0.0005	69 (48-66)	0.028
C-terminal	21 (69-104)	0.134	69 (48-64)	0.027
Age cat.				
1-2 years	100		100	
2-5 years	128 (91-144)	0.198	169 (74-151)	0.775
5-7 years	97 (74-123)	0.776	165 (108-251)	0.02
Prev. ex vivo	138 (114-165)	<0.0005	154 (39-169)	0.155
Prev. cult.	93 (81-108)	0.384	141 (109-181)	0.009
Village				
Gongoni	100		100	
Junju	103 (14-129)	0.78	151 (102-223)	0.039
Kolewa	154 (123-198)	<0.0005	89 (57-134)	0.59
Mapawa	199 (157-263)	<0.0005	69 (44-102)	0.075
M. tsungu	144 (104-199)	0.025	74 (39-134)	0.3
MUAC				
1st tertile	100		100	
2nd tertile	112 (89-141)	0.31	114 (79-169)	0.45
3rd tertile	101 (82-125)	0.89	93 (66-128)	0.67
GI worm	95 (77-117)	0.66	113 (77-165)	0.51
Schist.	120 (89-165)	0.228	77 (42-144)	0.43
Eosinophils				
Normal	100		100	
Mod.	69 (53-87)	0.002	75 (50-120)	0.26
High	79 (63-99)	0.043	70 (48-112)	0.15
Prev. Film	81 (69-95)	0.018	111 (85-147)	0.48

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	Ex Vivo ELISPOTs		Cultured ELISPOTs	
Factor	**Proportional Difference %**	***p* Value**	**Proportional Difference %**	***p* Value**
Current film	69 (58-83)	<0.0005	112 (91-165)	0.2
Febrile mal	79 (66-95)	0.014	72 (52-98)	0.04

[a]The average response associated with each factor is given as a percentage relative to the comparison for each variable. Where more than two options exist for a given factor, a log-likelihood ratio test is given for the factors addition to the model. Log-likelihood testing (for ex vivo then cultured ELISPOTs, respectively) gave $p < 0.003$ and $p < 0.038$ for the effect of peptide, $p = 0.12$ and 0.019 for age, $p < 0.0005$ and $p = 0.0006$ for village, $p = 0.5$ and $p = 0.44$ for MUAC, and $p = 0.006$ and $p = 0.337$ for eosinophilia.