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References

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Unique T Cell Effector Functions Elicited by Plasmodium falciparum Epitopes in Malaria-Exposed Africans Tested by Three T Cell Assays

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Natural immunity to malaria is characterized by low level CD4 T cell reactivity detected by either lymphoproliferation or IFN-γ secretion. Here we show a doubling in the detection rate of responders to the carboxyl terminus of circumsporozoite protein (CS) of Plasmodium falciparum by employing three T cell assays simultaneously: rapid IFN-γ secretion (ex vivo ELISPOT), IFN-γ secretion after reactivation of memory T cells and expansion in vitro (cultured ELISPOT), and lymphoproliferation. Remarkably, for no individual peptide did a positive response for one T cell effector function correlate with any other. Thus these CS epitopes elicited unique T cell response patterns in malaria-exposed donors. Novel or important epitope responses may therefore be missed if only one T cell assay is employed. A borderline correlation was found between anti-CS Ab levels and proliferative responses, but no correlation was found with ex vivo or cultured IFN-γ responses. This suggested that the proliferating population, but not the IFN-γ-secreting cells, contained cells that provide help for Ab production. The data suggest that natural immunity to malaria is a complex function of T cell subgroups with different effector functions and has important implications for future studies of natural T cell immunity. The Journal of Immunology, 2001, 167: 4729–4737.
it is not known whether anti-CS Ab levels correlate with IFN-γ responses by T cells.

It has been shown that T cells may selectively perform different effector functions, such as lymphokine secretion (45, 46) or cytotoxicity (47), and may also differ in their susceptibility to apoptotic signals (48, 49) and their rapidity of response (50). Indeed, use of the ELISPOT assay has allowed the identification of T cells that can secrete IFN-γ within 6 h of Ag stimulation (51). The rapidity of this response makes it a prime candidate for a protective role during the short period (5–10 days) during which the malaria parasite is found inside liver cells. Moreover, rapid IFN-γ ELISPOT responses to CS have been correlated with protection in the mouse model of malaria (52). This highly sensitive assay is also particularly useful, since it determines the precise number of IFN-γ-producing cells circulating in peripheral blood.

To address whether such rapid T cell effector functions focus on the same T cell epitopes as those identified by proliferative assays, we performed three T cell assays simultaneously in 48 malaria-exposed Gambian adults. Thus, proliferation assays, rapid IFN-γ ELISPOT on freshly isolated PBMC (ex vivo ELISPOT), and IFN-γ ELISPOT assays after restimulation and expansion in vitro (cultured ELISPOT) were performed using eight peptides spanning residues 328–397 of the carboxyl terminus of CS (see Fig. 1). This region contains the previously described dominant T cell epitope regions (20). Serum Ab levels to recombinant CS were assessed in parallel for each donor by ELISA to look for a correlation with rapid or cultured IFN-γ release or lymphoproliferation. Rapid IL-4 ELISPOT assays were also performed on selected donors, and malaria blood films were performed on all donors.

By assaying three T cell effector functions simultaneously we obtained a higher number of CS responders than found in previous studies employing proliferation assays alone. Surprisingly, the pattern of reactivity for each donor varied according to the effector function assessed, exposing different immunodominant regions in the carboxyl terminus of CS and providing a broader picture of T cell reactivity to CS for each donor. There was no correlation between positive responses in any of the three T cell assays, suggesting that each assay detects a distinct functional T cell subset. These findings have important implications in our understanding of protective immunity to malaria and other disease processes in which T cell responses play a protective role. Furthermore, these results should influence the design of future field studies of natural T cell immunity and vaccine efficacy.

Materials and Methods

Study site and volunteers

PBMCs were collected from 50 healthy adult male volunteers (donors D1–D50) from Dampna Kunda, Upper River Division, The Gambia, between February and March 1997. This represents the middle of the dry season when malaria transmission rates are low. The malaria season begins 5 mo later after the rains commence, with high malaria transmission rates occurring between August and November. Forty-eight of the PBMC samples were used for the study of the three T cell assays (donors D1–D48), and two (D49–D50) were tested for responses to the Th2R/Th3R CS epitopes. Twenty malaria-naïve adult donors were recruited from the laboratory in Oxford to assess naïve T cell responses to the malaria epitopes.

Peptides

Thirty-seven 15-mer peptides (overlapping by five residues) extending from the NANP repeat region to the carboxyl terminus of CS (aa 273–412) from strains NF54 and 7G8 were synthesized commercially. All peptides were tested in the IFN-γ ELISPOT assay in five randomly selected Gambian adults (Table I). Eight peptides spanning residues 328–397 (C1–C8) were chosen for the study (Fig. 1), consisting of a combination of NF54-derived (peptides C1–C4, C7, and C8) and 7G8-derived (C4–C8) sequences. These two strains vary by six nucleotide substitutions across residues 328–397, all of which lead to amino acid changes (53). Peptides C4, C7, and C8 are identical for NF54 and 7G8. The relative frequencies of NF54 and 7G8 in the Basse region of The Gambia in 1997 were as follows: 7G8 Th2R epitope, 0.12; NF54 Th2R epitope, 0.07; 7G8 Th3R epitope, 0.05; and NF54 Th3R epitope, 0.26 (54). Peptides corresponding to the immunodominant Th2R (aa 326–347) and Th3R (aa 361–380) CD4+ T cell epitope regions of CS clone NF54 were similarly synthesized commercially (Fig. 1). Purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark) was used as a positive control.

All peptides were used at a final concentration of 25 µg/ml in the ELISPOT assay, following preliminary titrations with PPD and the CS-derived Th2R and Th3R CD4+ epitopes in selected donors (data not shown). Peak ELISPOT responses occurred at between 25 and 50 µg/ml; thus, a concentration of 25 µg/ml was chosen for the study. There was no change in the PPD response following the addition of peptides C1–C8 to the PPD wells in IFN-γ ELISPOT assays, thus demonstrating that the peptides were nontoxic (data not shown). Four peptide pools, consisting of two nonoverlapping peptides per pool, were prepared in which to culture cells for a later ELISPOT pilot study of 37 15 mer CS peptides from strains NF54 and 7G8 of circumsporozoite protein for 5 Gambian donors.

| Peptide | 1st Mer | 1st Mer | NF54 or 7G8 | Donor |
|---------|---------|---------|-------------|-------|
| 1       | 212     | Both    | Both        | T1    |
| 2       | 279     | Both    | Both        | T2    |
| 3       | 263     | Both    | Both        | T3    |
| 4       | 298     | Both    | Both        | T4    |
| 5       | 293     | Both    | Both        | NA    |
| 6       | 298     | Both    | Both        | NA    |
| 7       | 303     | Both    | NF54        | NA    |
| 8       | 306     | Both    | 7G8         | NA    |
| 9       | 313     | Both    | NF54        | NA    |
| 10      | 318     | Both    | NF54        | NA    |
| 11      | 323     | Both    | 7G8         | NA    |
| 12      | 328     | Both    | 7G8         | NA    |
| 13      | 333     | Both    | NF54        | NA    |
| 14      | 338     | Both    | 7G8         | NA    |
| 15      | 343     | Both    | C1          | C1    |
| 16      | 346     | Both    | C1          | C1    |
| 17      | 353     | Both    | C1          | C1    |
| 18      | 358     | Both    | C1          | C1    |
| 19      | 363     | Both    | 7G8         | NA    |
| 20      | 368     | Both    | 7G8         | NA    |
| 21      | 373     | Both    | 7G8         | NA    |
| 22      | 375     | Both    | C2          | C2    |
| 23      | 376     | Both    | C2          | C2    |
| 24      | 388     | Both    | C2          | C2    |
| 25      | 395     | Both    | C2          | C2    |
| 26      | 396     | Both    | C2          | C2    |
| PPD     | NA      | NA      | NA          | NA    |

"The peptides marked with an asterisk (C1–C8) were selected for the main study. NA, not applicable.

Blood collection and sample processing

Twenty milliliters of venous blood were collected into heparinized 25-ml universal containers. Thick and thin blood films for malaria were prepared using a standard Giemsa stain for each donor at the time of sampling. Heparinized blood was centrifuged, and 3 ml plasma was collected and frozen. PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway), collected from the interface, washed three times in RPMI, and resuspended in complete medium RM10 (RPMI medium supplemented with 10% heat-inactivated human AB serum, 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin).

CD4+ and CD8+ T cell depletions were performed using anti-human CD4 and CD8 magnetic beads, respectively (Dynal, Bromborough, U.K.) according to the manufacturer’s instructions. The protocol reliably depleted...
The ELISPOT assay was conducted using 96-well MAIP S4510 0.45-μm plates coated with a hydrophobic high protein binding Immunomol P membrane (Millipore, Watford, U.K.). ELISPOT kits supplied by Mabtech (Mabtech, Nacka, Sweden) were used for all ELISPOT assays. Each kit consists of an mAb for coating the plates, a biotinylated mAb for capture after cell culture, and streptavidin alkaline phosphatase (ALP), which was used as the third Ab before color development with the ALP substrate kit. The plates were then washed (4× PBS-0.05% Tween 20, 2× PBS), and incubated for an additional 1 h at room temperature with 1 μg/ml streptavidin-ALP (MabTech). They were then washed again as described above and developed with ALP conjugate substrate kit (Bio-Rad, Hercules, CA) as follows. For each 96-well ELISPOT plate 4.8 ml water (Sigma) was combined with 50 μl each of color reagents A then B, followed by 200 μl ALP color development buffer. Fifty microliters of this solution were added per well, and the reaction was stopped after 10–20 min (according to the color intensity of spots) by flicking off and running under tap water.

Each IFN-γ-producing cell leaves a single spot or “footprint” in the ELISPOT well. Wells were scored visually, using a dissection microscope, for the number of cells producing IFN-γ according to the number of purple spots or spot-forming units (SFUs) per well. Three observers scored every positive well to ensure our confidence in this subjective scoring system. The results were expressed as SFUs per 10^6 PBMC added. ELISPOT assays were conducted in single wells (depletion studies were performed in duplicate), and the number of responder cells in the test well was compared (4× 10^5) because the frequency of specific cells rises during 14 days of culture. Preliminary experiments confirmed that the same precursor frequencies were detected using 1 × 10^5 or 4 × 10^5 cells/well, although at high precursor frequencies 1 × 10^5 cells/well improved the accuracy of scoring.

**Proliferation assay**

PBMC were resuspended in MEM10 at 5 × 10^5/ml and plated in triplicate in 96-well round-bottom tissue culture plates at 200 μl/well (1 × 10^3 cells/well) in the presence of peptides C1–C8, with PPD as a positive control. RPMI was used as the background response. Plates were incubated for 120 h at 37°C in 5% CO₂, at which time 1 μCi [³H]thymidine was added to each well. Thymidine incorporation was measured by liquid scintillation in a 1205 Beta-plate beta counter, and expressed as a stimulation index (SI) of counts per minute according to the formula: mean test cpm/mean background cpm. An SI value of >2 was taken as the cutoff value for a positive response in this study.

**ELISA to measure Ab to NAPN repeats of CS**

The Ab response to the tetrapeptide (NANP) repeat of CS was measured using a specific ELISA with plate-adsorbed R32LR Ag. A standard reference serum of known concentration was provided by Walter Reed Army Institute of Research (Washington, DC) (54) and was used on every plate as a positive control. Microwells were coated with 50 μl R32LR at 0.1 mg/ml in PBS-0.2% boiled casein and were incubated overnight at room temperature. After washing, wells were blocked with 0.5% boiled casein. The Ab was then washed in PBS for 1 h at room temperature. Wells were aspirated, and 50 μl of the appropriate dilution was added per well. Assays were conducted in triplicate with blank wells and standard reference positive and negative sera on each plate. After 2-h incubation at room temperature plates were washed four times with PBS-1% Tween, and 50 μl anti-human conjugated peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. Plates were incubated for 1 h at RT, washed four times with PBS-1% Tween, and 100 μl ABTS substrate solution was added per well. After a further 1-h incubation at room temperature, 10 μl 20% SDS (Sigma) was added to stop the reaction, and absorbance was read at 414 nm. Titters were expressed in comparison with the standard reference serum.

**Statistical methods**

A simulation approach was used to test whether the pattern of response to each peptide was correlated over the ex vivo ELISPOT, cultured ELISPOT, and proliferative assays. For each donor we summarized the correlation between a pair of assays by counting the number of times the same result was found for the corresponding peptides. This is a maximum of eight when the individual shows the same response to each peptide in each assay. The average value was calculated over all donors for each pair of assay comparisons. The distribution of this average index under the null hypothesis of no correlation was calculated using binomial distributions with n/48, and the probability of a success estimated from the average number of positive responses in each assay. χ² tests were used to assess associations among Ab level (categorized as low (<4 μg/ml), medium (4–10 μg/ml), high (>10 μg/ml)), blood smears (positive or negative), and assay responses (residents to at least one peptide or no response). In addition, proliferative SI values were compared directly with Ab levels (micrograms per milliliter) to assess the association.

**Results**

**Lack of correlation between responses to CS determined by simultaneous assayment of three different T cell effector functions**

Forty-eight donors were tested for responses to a panel of eight overlapping peptides, C1–C8, spanning residues 328–397 of the carboxyl terminus of CS (Fig. 1) in ex vivo ELISPOT, cultured ELISPOT, and lymphoproliferative assays. PPD was used as a positive control Ag, as it is frequently positive in this population of blood donors. Eighty-four percent (32 of 38) of the 36 donors for whom there were sufficient cells to perform all three assays responded to one or more CS peptide (Table II), and all but two donors gave a positive response to PPD in ex vivo ELISPOT or proliferation. Twenty malaria-naïve adult donors recruited in Oxford were tested for ex vivo IFN-γ ELISPOT responses to peptides
C1–C8, and no positive responses were found, whereas all naive donors responded to PPD (data not shown). Five malaria-naive donors were tested in cultured ELISPOT, and four donors were tested in lymphoproliferative assays, and again no positive responses were observed to the eight CS peptides (data not shown).

More than half the donors who gave a positive response did so to more than one peptide. The maximum number of peptides to which an individual responded was seven in the ex vivo ELISPOT assay (donor D46), four in the cultured ELISPOT assay (donor D32), and five by lymphoproliferation (donor D45; Table II). For each T cell effector function those donors that gave multiple responses were different (Table II). It was possible that a response to two overlapping peptides in a particular donor represented a single response to a shared T cell site; for example, D42 responded in the ex vivo ELISPOT to the overlapping peptides C7 (aa 378–392) and C8 (aa 383–397). However, responses were also observed to each peptide individually over the three assays; for example, D31 and D32 responded to C7, but not C8, and D30 and D40 responded to C8, but not C7, suggesting that each peptide must also represent a T cell epitope in its own right.

We found that 40% of donors (19 of 48) responded to one or more peptide in the ex vivo ELISPOT assay, 55% (22 of 40) in the cultured ELISPOT assay, and 40% (17 of 42) by proliferation. Eleven (23%) of the donors tested in the ex vivo ELISPOT assay responded to one peptide only, and eight donors (17%) responded to more than one peptide. Eight (20%) of the donors tested by cultured ELISPOT assay responded to one peptide only, and 14 (35%) responded to more than one peptide. Nine donors (21%) had a proliferative response to one peptide, and 8 (19%) donors had

Table II. Summary of ex vivo ELISPOT, cultured ELISPOT, proliferative responses, and anti-CS Ab levels (anti-R32LR) for 48 Gambian donors (D1 to D48)*

*Filled in squares represent a positive response, and are shaded according to the magnitude of the response (see key). Donors are ordered according to response. Ab levels are expressed in micrograms per milliliter; *, Malaria blood film positive. ELIS, ELISPOT; Prol, proliferative responses.
multiple responses by lymphoproliferation. Thus, cultured ELISPOT assay produced double the number of multiple epitope responders compared with the other two assays. The cultured ELISPOT responses were of a much higher magnitude (mean ± SE, 224 ± 30.6/10⁶ PBMC) than the ex vivo responses (39 ± 5.3/10⁶ PBMC), possibly reflecting their 2wk expansion period in vitro (Table III). The lymphoproliferative responses were generally low (mean SI, 4.6 ± 0.7). Low level T cell responses are characteristic of malaria-induced natural immunity both to pre-erythrocytic stage and blood stage Ags (13, 20, 43, 55).

A positive response to each peptide in the ex vivo ELISPOT assay was not significantly correlated with the corresponding peptide response in proliferation (p = 0.57). This result is in agreement with the lack of correlation between proliferative responses and IFN-γ release seen in response to the two blood stage malaria Ags P/155/ring-infected erythrocyte surface Ag (RESA) (56) and merozoite surface protein 1 (P/MSP1) (44) in naturally exposed Gambian adults. We also failed to find a correlation between the ex vivo and cultured ELISPOT responses (p = 0.65). One might predict that proliferation and cultured ELISPOT assays would detect the same T cells, since both assays measure cells restimulated in culture. However, in our study the pattern of peptide responses was not significantly correlated over the two assays (p = 0.12). Thus, for no individual peptide did a positive response for one of the three T cell effector functions correlate significantly with a positive response to another. Indeed, with the exception of two donors (D26 and D27), the reactivity pattern for each responder was unique to the eight CS peptides over the three T cell assays (Table II).

The PBMC were stimulated differently for each of the three T cell assays, which may contribute to their lack of correlation, for example by preferentially promoting the activation of a different T cell subset. Thus, the cultured ELISPOT cells were cultured for 14 days in MEM-10 medium in a 48-well tissue culture plate, and IL-2 was added on two occasions. The proliferating cells were also cultured in MEM-10, but for only 5 days, and no IL-2 was added. The ELISPOT assays using the ex vivo cells and cultured cells were performed in the same way, except that fewer cultured cells (1 × 10⁶) were added per well because after culture the reactive cells became too numerous to score visually.

Different immunodominant epitopes are the targets of diverse T cell effector functions

Lymphoproliferative assays in naturally exposed donors have identified three immunodominant T cell epitope domains located outside the repetitive region of CS, called the Th2R (aa 326–347), Th3R (aa 361–380) (20, 57), and CS.T3 (aa 378–398) regions (58). All these domains are covered by the peptides assayed in this study (Fig. 1). Up to 25% of naturally exposed adults respond to the Th2R region, and 43% respond to the Th3R region of CS (20). Sinigaglia and colleagues (59) found that 56% of naturally exposed adults from the Ivory Coast respond to region CS.T3, and a surprisingly high proportion (one-third) of malaria-naive subjects also had a proliferative response to CS.T3. However, Good and colleagues (20) found no proliferative response to an equivalent peptide (peptide 27, aa 380–399) in 35 Gambian adult volunteers. Similarly, Hoffmann and colleagues (36) found no proliferative response to CS residues 381–400, and only one responder to residues 376–395, in a study of 28 Kenyan adults.

The most frequent positive response in ex vivo ELISPOT in this study was to CS peptide C6 (aa 368–382; 19% donors), which overlaps the Th3R region (aa 361–380), whereas in the cultured ELISPOT assay it was to peptide C2 (aa 333–347; 27% donors), which is contained within the Th2R region (aa 326–347). In assays of T cell proliferation, peptide C6 (Th2R; 19% of donors) was recognized most frequently, with C2 (Th2R) next in frequency (17% of donors). Peptide C8 (aa 383–397) was the second most commonly recognized epitope in ex vivo ELISPOT assays (15% of donors) in our study. This peptide corresponds to the CS.T3 (aa 378–398) region that is recognized in association with at least seven different class II HLA DR types (59), although the minimal epitope corresponding to C8 is recognized by only three of these seven DR types.

Each assay therefore gave a different hierarchy of epitope responses, although individual epitopes were recognized at frequencies similar to those in previous studies. Importantly, no proliferative responses were seen to peptide C4 (aa 348–362) in our study despite ex vivo (three donors) and cultured (two donors) IFN-γ ELISPOT responses (Table II). Thus, an entire epitope region would have been missed if only lymphoproliferative assays had been performed. Since peptide C4 lies in a conserved region of CS, it is a potentially useful vaccine candidate epitope that could provide cross-variant protection. CD4⁺ and CD8⁺ cell depletion studies confirmed that overnight ex vivo IFN-γ-ELISPOT responses to epitopes C1, C2, C3, and C6 were CD4⁺ mediated (Fig. 2).

Correlates with Ab responses to CS in naturally exposed adults

Several studies of natural immunity to CS found no correlation between proliferation and anti-CS Ab levels (13, 20, 40); however, Hoffmann and colleagues (36) did observe a correlation. To explore

Table III. Magnitude of ex vivo and cultured ELISPOT responses (SFU/million PBMC) and lymphoproliferative responses (SI) for 14 of the donors recruited to the study*

| Ab   | Ex-vivo ELISPOT | Cultured ELISPOT | Proliferation |
|------|----------------|-----------------|--------------|
|      | Bg  | C1   | C2   | C3   | C4   | C5   | C6   | C7   | C8   | PPD |
| 2.6  | 12  | 12   |      |      |      |      |      |      |      |     |
| 4.8  | 0   | 0    |      |      |      |      |      |      |      |     |
| 16.8 | 12  | 12   | 23   | 155  |      |      |      |      |      |     |
| 3.1  | 12  |      | 30   | 355  |      |      |      |      |      |     |
| 4.0  | 2   | 15   |      |      | 315  |      |      |      |      |     |
| 2.4  | 10  | 25   | 23   | 235  |      |      |      |      |      |     |
| 14.1 | 10  | 25   | 43   | 20   | 25   | 22   | 90   |      |      |     |
| 8.3  | 2   | 23   | 13   | 15   | 48   | 40   |      |      |      |     |
| 2.0  | 25  | 88   | 43   | 80   |      | 43   | 50   |      |      |     |
| 17.4 | 7   | 25   |      |      |      | 30   |      |      |      |     |
| 14.4 | 3   |      |      |      |      |      | 130  |      |      |     |
| 0.8  | 0   | 13   | 30   | 55   | 15   | 20   | 58   | 50   |      |     |
| 0.8  | 2   | 15   | 23   |      | 18   | 75   |      |      |      |     |
| 10.1 | 7   | 41   | 36   | 55   | 258  |      |      |      |      |     |
| 180  | 180  | 150   | 170  |      |      |      |      |      |      |     |
| 2224 |      |      |      |      | 9    |      |      |      |      |     |
| 46   |      |      |      |      |      | 96   |      |      |      |     |
| 73   |      |      |      |      |      |      | 15   | 22   | 5    | 5    |
| 1406 |      |      |      |      |      |      |      |      | 200  |      |
| 1355 |      |      |      |      |      |      |      | 2   | 4    | 39   |
| 69   |      |      |      |      |      |      |      |      |      | 41   |
| 500  |      |      |      |      |      |      |      |      |      | 117  |
| 60   |      |      |      |      |      |      |      |      |      | 842  |
| 65   |      |      |      |      |      |      |      |      |      | 85   |
| 133  |      |      |      |      |      |      |      |      |      |      |
| 700  |      |      |      |      |      |      |      |      |      |      |
| 44   |      |      |      |      |      |      |      |      |      |      |
| 171  |      |      |      |      |      |      |      | 4   | 6    | 3    |
| 455  |      |      |      |      |      |      |      | 60  |      |      |
| 69   |      |      |      |      |      |      |      |      |      | 10   |
| 173  |      |      |      |      |      |      |      |      |      | 35   |
| 4    |      |      |      |      |      |      |      |      |      |      |
| 5    |      |      |      |      |      |      |      |      |      |      |
| 15   |      |      |      |      |      |      |      |      |      |      |
| 95   |      |      |      |      |      |      |      |      |      |      |

*The background values (Bg) to media alone are shown in italics in the left hand column for the three assays. ELISPOT background values are expressed as spot-forming cells per million PBMC added to the well, and proliferative background values as average cpm. ELISPOT values are expressed as specific cells above the background response (SFU/million). Proliferative values are expressed according to the formula mean test cpm/mean background cpm for triplicate samples.
with Ab levels, no correlation with ex vivo or cultured IFN-γ secretion. Thus, although proliferation correlated rather weakly and positive lymphoproliferation no longer reached significance when only those donors with high Ab levels were found. Anti-CS Ab levels were thus assessed in parallel with IL-4 and IFN-γ ELISPOT assays to the immunodominant epitopes Th2R (aa 326–347) and Th3R (aa 361–380) of the NF54 strain of CS (see Fig. 1) for 19 of the 48 donors recruited to this study. An additional two donors were also tested for IL-4 and IFN-γ responses to these two epitopes (D49 and D50) (Table IV). Two donors had a positive IL-4 ELISPOT response to the Th2R epitope, one of whom gave a positive IFN-γ response. The three donors who produced IL-4 in response to the Th3R region also produced IFN-γ to the same epitope. The frequency of IFN-γ responders (5 of 21; 24%) was comparable to the IL-4 response rate (4 of 21, 19%). No correlation was apparent between Ab levels and IL-4 ELISPOT responses in these donors, although the number of IL-4 responders was small (n = 4).

**Parasitaemia does not correlate with immunological parameters**

All donors had thick and thin malaria blood films performed at the time of blood collection, and 14 donors (29%) were found to be parasitic with asexual forms of *P. falciparum*. Four of the blood film-positive donors had low anti-CS Ab levels (<4 µg/ml), six had moderate levels (4–10 µg/ml), and four donors had high Ab levels (>10 µg/ml), with no correlation between smear positivity and Ab level (by χ² test, p = 0.5). Similarly, there was no association between blood smear positivity and any of the three T cell assays employed in this study (ex vivo ELISPOT, p = 0.8; cultured ELISPOT, p = 0.9; proliferation, p = 0.7).

**Discussion**

Previous field studies of natural T cell immunity to malaria have generally focused on lymphoproliferative responses. In a seminal study of T cell proliferative responses in Gambian adults using peptides spanning the length of the CS protein, Good and colleagues (20) found that 60% of individuals respond to at least one peptide. We found a similar level of proliferative responses to equivalent peptides used in Good’s study, but were able to detect additional responders (84% of donors tested) to a much smaller region of CS by assessing three T cell effector functions simultaneously. Thus, the number of individuals capable of responding to a particular Ag can be greatly underestimated unless multiple parameters of T cell activation are measured. Indeed, we showed that an entire epitope region would have been missed if only proliferative assays were performed.

The proliferative assay, ex vivo, and cultured ELISPOT assays gave differing reactivity patterns for all donors, and no correlation was found among any responses to the three assays. Thus, different regions of CS emerged as immunodominant according to the assay employed. The reactivity pattern seen for each donor was unique.

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** Effect of depletion of cells expressing CD4 and CD8. Overnight ex vivo ELISPOT assays were performed before and after depletion of cells expressing the markers CD4 (CD4⁺/⁻) and CD8 (CD8⁺/⁻). Responses present in the undepleted PBMC (Total) and in the CD8-depleted wells (CD8⁺/⁻), but absent in the CD8⁺/⁻ wells. This suggested that rapid IFN-γ secretion to CS peptides C1, C2, C3, and C6 were CD4 T cell mediated. Assays were performed in duplicate; responses from three donors (D35 to peptide C6, D38 to peptide C1, and D46 to peptides C2 and C3) are shown. Bg, Background values.

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**Table IV. Anti-CS Ab levels assessed in parallel with IFN-γ and IL-4 ex vivo ELISPOT responses for 21 donors**

|   | D1 | D3 | D4 | D6 | D7 | D8 | D10 | D12 | D15 | D17 | D19 | D26 | D30 | D31 | D34 | D41 | D47 | D49 | D50 |
|---|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Anti-CS Ab |    |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Th2R | IFN-γ |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PSDKHIKELCNKLSLSTESW | IL-4 | 4.3 | 3.3 | 5.6 | 4.1 | 24 | 13 | 5.7 | 17 | 17 | 2.1 | 17 | 2.1 | 2.7 | 4.8 | 0.9 | 0.5 | 4.3 | 8.3 | 0.8 |
| Th3R | IFN-γ |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| IKPSANKPKDELYANDIE | IL-4 | ND | ND | ND | ND | ND |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

*IFN-γ (black squares) and IL-4 (hatched squares) ex vivo ELISPOT responses to the immunodominant Th2R and Th3R epitope regions of CS strain NF54 were assessed in parallel to anti-CS Ab levels (in micrograms per milliliter) for 21 donors. Filled squares represent a positive response by ELISPOT.*
in all but two donors, who gave identical reactivity patterns to the eight CS peptides over the three assays. Similar observations of unique T cell specificities according to the peptide Ag determinant have been made for lepromatous leprosy. Different T cell clones derived from leprosy patients recognize distinct fragments from Mycobacterium leprae Ags (61), and M. leprae Ags can differentially induce Th1- or Th2-type T cell responses, suppressor T cells, and/or lymphoproliferation in different donors (62). Studies of the hen egg-white lysosome model of autoimmunity show heterogeneity of T cell responses to different immunodominant determinants of hen egg-white lysosome (63). Milich and colleagues (64) showed for hepatitis B responses that Th cells with the same specificity can develop into different Th subsets depending on the structural form of the immunogen and local cytokine milieu.

The reasons for such unique specificities in our study is not known. It may reflect the different culture conditions used for the three assays, which, in turn, favored the expansion of different T cell subsets. The medium for the ex vivo ELISPOT (RN10) was different from the cultured ELISPOT or proliferative medium (MEM10), and IL-2 was added to the cultured ELISPOT cells only. Moreover, the cultured ELISPOT cells had a 14-day period of growth in culture, and the proliferating cells had 5 days. Circumsporozoite protein is capable of activating other T cell types, such as γδ cells (65–67) and NK cells (68), but αβ T cell responses are generally considered to predominate. We have not attempted to determine the roles of these different cells in the responses observed in this study, but it is feasible that they contributed to the differential reactivity patterns elicited by the three T cell assays. Depletion studies did confirm that four of our CS epitopes elicited CD4+ T cell ex vivo IFN-γ ELISPOT responses. Ex vivo and cultured IFN-γ ELISPOT responses using the same peptides were also shown to be CD4+, and not CD8+, T cell-mediated in volunteers immunized with the RTS.S candidate malaria vaccine (69). Whether these results apply to natural malaria exposure is not known. We did not investigate the phenotype of T cells mediating proliferative responses to these peptides, but since discrete CD8+ T cell epitope regions exist within the carboxyl terminus of CS (24), one possibility is that proliferating CD8+ T cells caused the lack of correlation with the ELISPOT assays. However, since CD8+ T cells normally have limited proliferative capacity in the absence of IL-2, supplied either by CD4+ T cells or exogenously, this is unlikely.

All adults living in this region of The Gambia are repeatedly exposed to malaria throughout their life and have developed a state of partial immunity by adulthood. Despite this, 46% of donors had low anti-CS Ab levels (<4 μg/ml). We found that proliferative responses correlated with anti-CS Ab levels, a result consistent with the findings of Hoffman and colleagues (36), although other studies report no such correlation (13, 20, 40). It is possible that the proliferating T cell population contains CD4+ Th cells that provide help for Ab production and cause the correlation with Ab production. Indeed, the authors interpret the correlation between IL-4 production to peptides from the blood stage Ag Pf 155/RESA and elevated serum Ab levels in a study of adult Gambians as being due to help from IL-4-producing Th cells (56). We found no such correlation in our study between IL-4 production by ex vivo ELISPOT and anti-CS Ab levels. The correlation between proliferation and Ab levels in our study was no longer significant if only those donors with high Ab levels (>10 μg/ml) were considered, and thus this result merits reassessment in a larger study population. Neither the ex vivo or cultured IFN-γ ELISPOT responses correlated with Ab levels.

Thirty-five percent (17 of 48) of naturally exposed donors in this study gave a T cell response with a concurrent low Ab response (<4 μg/ml) suggesting that T cell functions other than help in Ab production play a role in natural immunity to malaria. A further 10% (5 of 48) of the donors in this study had low Ab levels and no peptide response in any of the three assays. Such T cell nonresponsiveness is characteristic of natural immunity to malaria Ags in both parasitemic and healthy aparasitemic individuals (20, 70–72). A number of factors might contribute to such nonresponsiveness, including intervening disease, genetic diversity of the donors (73), antigenic polymorphism (74), or T cell tolerance induction in the neonate (75).

The ELISPOT assay has the advantage over the proliferation assay of being less time consuming (overnight vs 5 days) and not requiring the use of radioactivity, which is not always available in the tropics. It is a robust, simple, and sensitive technique and can be used to screen for multiple epitope responses at one time. Indeed, we have used the ELISPOT assay to map new T cell epitope responses from a panel of 62 peptides in adult Gambians (55). The effect of continuous natural exposure on ex vivo ELISPOT responses is not known. However, we have recent evidence that ex vivo IFN-γ ELISPOT responses change over a 1-yr time period in naturally exposed donors (K. L. Flanagan, manuscript in preparation) and therefore may not provide long-lasting protection.

In summary, we found that the eight CS peptides elicited unique specificities in malaria-exposed Gambians when tested by three T cell assays simultaneously. This suggested that the three assays detect different T cell subsets, and that the currently accepted classification of memory T cells is incomplete. The precise role of each T cell subset in protection against malaria, and indeed other infectious diseases, remains to be determined, with the exciting prospect that understanding these important issues will facilitate development of a new generation of immunoregulatory vaccines.

References

1. Doolan, D. L., and S. L. Hoffman. 1997. Multi-gene vaccination against malaria: a multistage, multi-immune response approach. Parasitol. Today 13:171.
2. Kwiatkowski, D., and K. Marsh. 1997. Development of a malaria vaccine. Lancet 350:1696.
3. Miller, L. H., and S. L. Hoffman. 1997. Research towards vaccines against malaria. Nat. Med. 4:520.
4. Nussenzweig, R. S., J. P. Vanderberg, H. Most, and C. Orton. 1969. Protective immunity induced by the injection of x-irradiated sporozoites of Plasmodium berghei. Nature 216:160.
5. Clyde, D. F., H. Most, V. McCarthy, and J. P. Vanderberg. 1973. Immunization of man against sporozoite-induced falciparum malaria. Am. J. Med. Sci. 266:169.
6. Nussenzweig, V., and R. S. Nussenzweig. 1985. Circumsporozoite proteins of malaria parasites. Cell 42:401.
7. Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos, and V. Nussenzweig. 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of Plasmodium falciparum sporozoites. Cell 70:1021.
8. Frevert, U., P. Sinnis, C. Cerami, W. Schreiber, B. Takacs, and V. Nussenzweig. 1993. Malaria circumsporozoite protein binds to heparin sulfate proteoglycans associated with the surface membrane of hepatocytes. J. Exp. Med. 177:1287.
9. Potocnjak, P., N. Yoshida, R. Nussenzweig, and V. Nussenzweig. 1980. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malignant infection. J. Exp. Med. 151:1504.
10. Egan, J. E., J. L. Weber, W. R. Ballou, M. R. Hollingdale, W. R. Majarian, D. M. Gordon, W. L. Maloy, S. L. Hoffman, R. A. Wirtz, I. Schneide, et al. 1987. Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. Science 236:453.
11. Hoffman, S. L., R. Wistar, W. R. Ballou, M. R. Hollingdale, R. A. Wirtz, I. Schneider, H. A. Marwoto, and W. T. Hockmeyer. 1986. Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of Plasmodium falciparum. N. Engl. J. Med. 315:601.
12. Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig. 1987. γ-Interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. Nature 330:664.
13. Riley, E. M., S. J. Allen, S. Bennett, P. J. Thomas, A. O’Donnell, S. W. Lindsay, M. E. Good, and B. M. Greenwood. 1990. Recognition of dominant T cell-stimulating epitopes from the circumsporozoite protein of Plasmodium falciparum and relationship to malaria morbidity in Gambian children. Trans. R. Soc. Trop. Med. Hyg. 84:648.
14. Zavala, F., J. P. Tam, A. H. Cochran, I. Quakyi, R. S. Nussenzweig, and V. Nussenzweig. 1995. Rationale for development of a synthetic vaccine against Plasmodium falciparum malaria. Science 228:1436.
15. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Pombo, I. A. Quakyi, E. M. Riley, A. Menon, W. J. Britton, J. R. Pink, et al. 1987. Inhibitory activity of interferons and interleukin 1 on the development of Plasmodium falciparum in human hepatocyte cultures. J. Immunol. 139: 1492.

16. de Groot, A. S., A. H. Johnson, W. L. Maloy, I. A. Quakyi, E. M. Riley, A. Menon, S. M. Banks, J. A. Berzofsky, and M. F. Good. 1989. Human T cell recognition of polymorphic epitopes from malaria circumsporozoite protein. J. Immunol. 142:4000.

17. Verheegen, Y., R. A. Houghten, I. H. Frazer, and M. F. Good. 1990. Major population differences in T cell response to a malaria sporozoite vaccine candidate. In Immunol. 1990: 2-945.

18. Aebischer, M., M. Aido, H. C. Whittle, and A. V. S. Hill. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of Plasmodium falciparum in West Africa. J. Immunol. 158:2849.

19. Ballou, W. R., J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

20. Good, M. F., D. Pombo, I. A. Quakyi, J. C. Beier, J. D. Chulay, J. A. Sherwood, W. R. Ballou, R. A. Houghten, A. Menon, D. W. Alling, A. J. Berzofsky, and L. H. Miller. 1988. Human T cell recognition of the circumsporozoite protein of Plasmodium falciparum: immunodominant T-cell domains map to the polymorphic regions of the molecule. Proc. Natl. Acad. Sci. USA 85:1899.

21. Moreno, A., P. Clavijo, P. Edelman, J. Davis, M. Sztein, F. Sinigaglia, and J. L. Chong. 1990. Differential ability of T cell subsets to undergo activation-induced cell death. Proc. Natl. Acad. Sci. USA 87:3360.

22. Aebischer, M., M. Aido, H. C. Whittle, and A. V. S. Hill. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of Plasmodium falciparum in West Africa. J. Immunol. 158:2849.

23. Ballou, W. R., J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

24. Good, M. F., D. Pombo, I. A. Quakyi, J. C. Beier, J. D. Chulay, J. A. Sherwood, W. R. Ballou, R. A. Houghten, A. Menon, D. W. Alling, A. J. Berzofsky, and L. H. Miller. 1988. Human T cell recognition of the circumsporozoite protein of Plasmodium falciparum: immunodominant T-cell domains map to the polymorphic regions of the molecule. Proc. Natl. Acad. Sci. USA 85:1899.

25. Aidoo, M., A. Lalvani, C. E. M. Allsopp, M. Plebanski, S. J. Meisner, P. Krausa, M. Brown, M. Morris-Jones, F. Sinigaglia, and J. R. Pink. 1995. Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine in West Africans.

26. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

27. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

28. Aebischer, M., M. Aido, H. C. Whittle, and A. V. S. Hill. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of Plasmodium falciparum in West Africa. J. Immunol. 158:2849.

29. Vanderberg, J., S. Chew, and M. J. Stewart. 1990. Immunity to sporozoite-induced immune reactivity in rodent models. J. Immunol. 142:1645.

30. Aebischer, M., M. Aido, H. C. Whittle, and A. V. S. Hill. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of Plasmodium falciparum in West Africa. J. Immunol. 158:2849.

31. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

32. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, J. D. Chulay, B. M. Greenwood, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein.

33. Requez, E., F. Vittori, L. Kabilan, S. Bennett, B. Takacs, H. Schonfeld, A. A. Holder, and B. M. Greenwood. 1992. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of Plasmodium falciparum are associated with reduced malaria morbidity. Parasite Immunol. 14:221.

34. Chen, D. H., R. E. Tigelaar, and F. I. Weinbaum. 1977. Immunity to sporozoite-infected immune reactivity in rodent models. J. Immunol. 142:1645.
62. Mutis, T. E. M. Kraakman, Y. E. Cornelisse, J. B. Haanen, H. Spits, R. R. de Vries, and T. H. Ottenhoff. 1993. Analysis of cytokine production by Mycobacterium-reactive T cells: failure to explain Mycobacterium leprae-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. J. Immunol. 150:4641.

63. Moudgil, K. D., J. Wang, V. P. Yeung, and E. E. Sercarz. 1998. Heterogeneity of the T cell response to immunodominant determinants within hen egg white lysozyme of individual syngeneic hybrid F1 mice: implications for autoimmunity and infection. J. Immunol. 161:6046.

64. Milich, D. R., F. Schodel, J. L. Hughes, J. E. Jones, and D. L. Peterson. 1997. The hepatitis B virus core particle and e antigens elicit different Th cell subsets: antigen structure can affect Th cell phenotype. J. Virol. 71:2192.

65. Tsuji, M., P. Mombaerts, L. Lefrancois, R. S. Nussenzweig, F. Zavala, and S. Tonegawa. 1994. β2 T cells contribute to immunity against the liver stages of malaria in αβ T-cell-deficient mice. Proc. Natl. Acad. Sci. USA 91:345.

66. Langhorne, J., P. Mombaerts, and S. Tonegawa. 1995. β2 and γδ T cells in the immune response to the erythrocytic stages of malaria in mice. Int. Immunol. 7:1005.

67. Rzepczyk, C. M., K. Anderson, S. Stamatiou, E. Townsend, A. Allworth, J. McCormack, and M. Whitby. 1997. γδ T cells: their immunobiology and role in malaria infections. Int. J. Parasitol. 27:191.

68. Doolan, D. L., and S. L. Hoffman. 1999. IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8+ T cells in the Plasmodium yoelii model. J. Immunol. 163:884.

69. Lalvani, A., P. Moris, G. Voss, A. A. Pathan, K. E. Kester, R. Brookes, E. Lee, M. Koutsoukos, M. Plebanski, M. Delchambre, et al. 1999. Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SA2, a recombinant Plasmodium falciparum malaria vaccine. J. Infect. Dis. 180:1656.

70. Riley, E. M., S. Jepsen, G. Andersson, L. N. Otoo, and B. M. Greenwood. 1988. Cell-mediated immune responses to Plasmodium falciparum antigens in adult Gambians. Clin. Exp. Immunol. 71:377.

71. Troye-Blomberg, M., E. M. Riley, H. Perlmann, G. Andersson, R. W. Snow, S. J. Allen, R. A. Houghten, O. Olerup, B. M. Greenwood, and P. Perlmann. 1989. T- and B-cell responses of Plasmodium falciparum malaria-immune individuals to synthetic peptides corresponding to epitopes in the conserved repeat regions of the P. falciparum antigen Pf155/RESA. J. Immunol. 125:1002.

72. Hvid, L., T. G. Theander, P. H. Jakobsen, Y. A. Abu-Zeid, N. H. Abdulhadi, B. G. Saeed, S. Jepsen, R. A. L. Bayoumi, K. Bendtzen, and J. B. Jensen. 1990. Cell-mediated immune responses to soluble Plasmodium falciparum antigens in residents from an area of unstable malaria transmission. APMIS 98:594.

73. Hill, A. V. S., C. E. M. Allsopp, D. Kwaitkowski, N. M. Anstey, I. P. Tvumas, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, and B. M. Greenwood. 1991. Common West African HLA antigens are associated with protection from severe malaria. Nature 352:595.

74. de la Cruz, V. F., W. L. Maloy, L. H. Miller, M. F. Good, and T. F. McCutchan. 1989. The immunologic significance of variation within malaria circumsporozoite protein sequences. J. Immunol. 142:5568.

75. Pombo, D., W. L. Maloy, J. A. Bezrosky, and M. F. Good. 1988. Neonatal exposure to immunogenic peptides: differential susceptibility to tolerance induction of helper T cells and B cells reactive to malarial circumsporozoite peptide epitopes. J. Immunol. 140:5594.