Lee, EAM; Flanagan, KL; Odhiambo, K; Reece, WHH; Potter, C; Bailey, R; Marsh, K; Pinder, M; Hill, AVS; Plebanski, M; (2001) Identification of frequently recognized dimorphic T-cell epitopes in Plasmodium falciparum merozoite surface protein-1 in West and East Africans: Lack of correlation of immune recognition and allelic prevalence. The American journal of tropical medicine and hygiene, 64 (3-4). pp. 194-203. ISSN 0002-9637 http://researchonline.lshtm.ac.uk/id/eprint/17098

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IDENTIFICATION OF FREQUENTLY RECOGNIZED DIMORPHIC T-CELL EPITOPES IN PLASMODIUM FALCIPARUM MEROZOITE SURFACE PROTEIN-1 IN WEST AND EAST AFRICANS: LACK OF CORRELATION OF IMMUNE RECOGNITION AND ALLELIC PREVALENCE

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Abstract. The merozoite surface protein-1 (MSP1) is the most studied malaria blood-stage vaccine candidate. Lymphokines such as interferon gamma (IFN-γ) and interleukin 4 (IL-4) may mediate blood-stage specific protection. Here we identify Plasmodium falciparum MSP1 T-cell epitopes capable of rapid induction of IFN-γ and/or IL-4 from peripheral blood mononuclear cells of East and West African donors. Both allelic forms of these novel MSP1 T-cell epitopes were stimulatory. An unusually high numbers of Gambian responders (> 80%) to these epitopes were observed, suggesting that MSP1 reactivity may have been underestimated previously in this population. Surprisingly, IFN-γ responses to allelic T-cell epitopes failed to correlate with differential antigenic exposure in The Gambia compared to Kenya. These results suggest an unexpected level of immunoregulation of IFN-γ response with variable allelic T-cell reactivity independent of the level of antigenic exposure. Further analysis of the mechanisms determining this response pattern may be required if vaccines are to overcome this allelic reactivity bias in malaria-exposed populations.

INTRODUCTION

The success of an effective malaria blood-stage vaccine may depend on its ability to induce both cellular and humoral immunity.1 A major blood-stage vaccine candidate is the merozoite surface protein-1 (MSP1) of Plasmodium falciparum.2 Antibodies to MSP1 can mediate murine, primate and human blood-stage immunity.3-8 The role of blood-stage immune responses to allelic T-cell epitopes failed to correlate with differential antigenic exposure in The Gambia compared to Kenya. These results suggest an unexpected level of immunoregulation of IFN-γ response with variable allelic T-cell reactivity independent of the level of antigenic exposure. Further analysis of the mechanisms determining this response pattern may be required if vaccines are to overcome this allelic reactivity bias in malaria-exposed populations.

MATERIALS AND METHODS

Study area and volunteers. Malaria-exposed adults were from the villages of Brefet, Jali, and Berending in The Gambia, West Africa and from the villages around Kilifi in Kenya, East Africa. These studies were approved by the Ethical Committee of The Gambian Medical Research Council and the Kenya Medical Research Institute. All adult subjects were aged between 18–60 years-old. Thick and thin blood films were prepared at the time of bleeding, stained with Giemsa, and examined by microscopy for the presence of circulating malaria parasites. All malaria-exposed donors were clinically healthy and parasite-negative by microscopy at the time of blood sampling.

High malaria transmission in The Gambia commences shortly after the start of the rainy season and occurs between August and November.26 Gambian samples were collected both before (March to April, 1998) and after the wet season (October to November, 1998). No statistical differences in Gambian IFN-γ reactivity patterns between samples collect-
ed during the wet and dry seasons were observed (data not shown). Malaria transmission in Kenya is highest during and following the long rains (March to May) with a second peak of transmission occurring after the short rains in October to December. Kenyan samples were taken after the wet season (July to August, 1999). Malaria-unexposed donors were used as negative controls for IFN-γ and IL-4 ELISPOT detection assays. These were clinically healthy European-Caucasian volunteers aged between 21–50 years from Oxford, UK, who had not traveled to a malaria-endemic country.

**Blood collection and cell preparations.** After informed consent, 20 ml of venous blood was collected into heparin. Cells were separated by density gradient centrifugation on Lymphoprep® (Pharmacia, Oslo, Norway) at 800 g for 20 min. Peripheral blood mononuclear cells (PBMCs) were collected from the blood-ficoll interface and washed three times in RPMI-1640 medium (Sigma, Irvine, UK). PBMCs were resuspended at 4×10^6 per ml in RPMI-1640 medium supplemented with 5% heat inactivated human AB serum, 2 mM glutamine (Gibco, Paisley, UK), 100 μg per ml streptomycin (Gibco) and 100 units per ml penicillin (Gibco) (RN5) prior to use in ELISPOT assays.

**MSP1 peptides.** HLA-DRB1*1302 (DR1302) and HLA-DRB1*1301 (DR1301) alleles are identical except for a glycine to valine change at position 86 of the HLA-DRB1*1301 (DR1301) alleles may present different peptides based on their allelic type (MAD20 or WELLCOME). A total of 26 allelic variant peptides were synthesized (Table 1). This computer program utilizes pool sequencing data from HLA class-II proteins and scans the MSP1 amino acids negative controls for IFN-γ and IL-4 ELISPOT detection assays. These were clinically healthy European-Caucasian volunteers aged between 21–50 years from Oxford, UK, who had not traveled to a malaria-endemic country.

**Blood collection and cell preparations.** After informed consent, 20 ml of venous blood was collected into heparin. Cells were separated by density gradient centrifugation on Lymphoprep® (Pharmacia, Oslo, Norway) at 800 g for 20 min. Peripheral blood mononuclear cells (PBMCs) were collected from the blood-ficoll interface and washed three times in RPMI-1640 medium (Sigma, Irvine, UK). PBMCs were resuspended at 4×10^6 per ml in RPMI-1640 medium supplemented with 5% heat inactivated human AB serum, 2 mM glutamine (Gibco, Paisley, UK), 100 μg per ml streptomycin (Gibco) and 100 units per ml penicillin (Gibco) (RN5) prior to use in ELISPOT assays.

**MSP1 peptides.** HLA-DRB1*1302 (DR1302) and HLA-DRB1*1301 (DR1301) alleles are identical except for a glycine to valine change at position 86 of the HLA-DR8 chain which affects the preference for particular hydrophobic peptide residues. DR1302, but not DR1301, was shown to be protective against severe malaria in The Gambia. Hence, DR1302 and DR1301 alleles may present different peptides to CD4+ T cells. Thus, a “subtracted motif” which characterized peptides that could bind to the allelic motif of DR1302 but not to DR1301 was defined. By contrast in Kenya, HLA-DRB1*0101 (DR1) but not DR1302 or DR1301 was shown to be protective against severe malaria (Yates SN, unpublished data). Two selection criteria for choosing MSP1 peptides were employed: one based on the peptide-binding motifs of the two protective class II alleles, HLA-DRB1*0101 (DR1) and DR1302, the other based on this “subtracted motif.” Using a computer prediction algorithm which predicted potential T-cell epitopes that differed between two major allelic types of MSP1, MAD20 and WELLCOME type, a total of 26 allelic variant peptides were synthesized (Table 1). This computer program utilizes pool sequencing data from HLA class-II proteins and scans the MSP1 protein for candidate epitopes that fit the HLA class-II peptide motif. A probability of being processed and presented (PPP) algorithm score is assigned to each amino acid of the protein which determines the probability of a 15-mer peptide beginning with that amino acid to be naturally processed and presented. All peptides were purified by high-performance liquid chromatography (HPLC) and purity ranged from >80% to >95%. Peptides were reconstituted in sterile phosphate-buffered saline (PBS) and used at a pre-determined optimal culture concentration of 25 μg/ml. When tested in pools each peptide was used at 25 μg/ml. Table 1 shows the composition of the peptide pools. Peptides were grouped into non-overlapping pools of 2 or 3 peptides based on their allelic type (MAD20 or WELLCOME) and their peptide motif to either DR1 or DR1302.

**Control antigens.** The mitogen phytohemagglutinin (PHA) (Wellcome Pharmaceuticals, Stevenage, UK) and two recall antigens, tetanus toxoid (TT) (Evans Biomedical, Horsham, UK), and purified protein derivative (PPD) from Mycobacterium bovis (Statens Serum Institut, Copenhagen, Denmark) were used as positive controls antigens. PHA was used at 1 μg/ml and the two recall antigens were used in cultures at 10 μg/ml.

**Ex-vivo ELISPOT assays.** Ninety-six well polyvinylidene difluoride (PVDF) backed plates (MAIP S45, Millipore, Molsheim, France) were coated with 15 μg/ml of monoclonal anti-human IFN-γ (1-D1K), or IL-4 (IL-4-I), or IL-10 (9-D7) antibodies (all from Mabtech, Nacka, Sweden) for 3 hours at room temperature (RT) or overnight at 4°C. The plates were then washed 5 times with sterile PBS pH 7.4, (Sigma) and then blocked for 1 hour at RT with RPMI-1640 supplemented with 10% heat inactivated human AB serum (RN10). PBMCs were plated at 100 μl (4×10^5 cells) per well. MSP1 peptides, peptide pools, or antigens were added to each well. The plates were incubated at 37°C in 5% CO2/95% air (18 hr for IFN-γ detection, 24 hr for IL-4 and IL-10 detection). Plates were then processed by washing 4 times in PBS with 0.05% Tween-20 (Sigma) and 2 times with PBS. One microgram per milliliter of the second biotinylated monoclonal antibody of either IFN-γ (7-B6-1-Biotin), IL-4 (IL-4-II-Biotin) or IL-10 (12G8-Biotin) (all from Mabtech) was added and incubated for 2 hours at RT. The plates were washed as described above and incubated for 1 hour at RT with 1 μg/ml strepavidin alkaline phosphatase (ALP). These were washed again and developed with the ALP conjugate substrate kit (Biorad, Hercules, CA). Individual cytokine-producing cells within each well leave a “footprint” or spot which is visualized with the aid of a dissecting microscope (magnification × 2). Data is presented as spot-forming-units (SFU) per million PBMC.

A response was considered positive if the probability of a cell forming a spot was higher in the test well compared to the negative-control well (P < 0.05). The significance test was a chi-squared comparison of the odds ratios of numbers of lymphokine-positive cells in the test well and the control well. This method of determining a significant positive ELISPOT response is as powerful as non-parametric or parametric comparisons of the means or medians of several replicate wells. The null hypothesis tested by our significance test is: the probability of a cell forming a spot in the control well is the same as the probability of a cell forming a spot in the well with peptide. The null hypothesis is rejected at the 95% confidence limit if there are at least 5 spots in the test well than in the control well. This significant cut-off value above background spots higher than zero increased based on a binomial distribution assuming 4 × 10^5 cells per well. Similar significant values were obtained if a Poisson model was used.

**Molecular detection of MSP1 allelic strain distribution.** DNA samples of Gambian and Kenyan (Yates SN, unpublished data) children with clinical malaria were collected as part of an earlier case-control study of malaria. Part of the P. falciparum MSP1 gene (283 base pairs [bp]) corresponding to the allelic variant epitope pair, M3/M4 was amplified by polymerase chain reaction (PCR) with biotinylated primers (5’ to 3’): TACAATAAACAATTACAAGAAGC and TTAAATATTCTTAACTCAAGTGG. Polymerase chain reaction was carried out in 16 mM (NH4)2SO4, 67 mM tris
Peripheral blood mononuclear cells from adult Gambians were stimulated separately with either MSP1 allelic variant peptide (MAD20 or WELLCOME) in a 16 hr ex-vivo IFN-γ release assay. Positive IFN-γ enzyme-linked immunospots (ELISPOTs) detect a precursor frequency of > 1 per 1×10^5 responding cells (P < 0.05). The MSP1 protein is divided into 17 blocks based on amino-acid homology between dimorphic types. Conserved [■], semi-conserved (shaded [■]) and polymorphic [■] blocks are shown. Untested MSP1 allelic variant peptides are represented by an asterisk.

HCl (pH 8.8), 0.01% Tween 20, 0.5 mM MgCl₂, and 0.5 mM spermidine, with deoxynucleotide triphosphates (0.2 mM) and a 0.5 mM concentration of each primer, for an initial denaturation step of 94°C for 1,080 sec, followed by 32 cycles each of 10 sec at 94°C, 30 sec at 48°C, and 60 sec at 72°C. To obtain single-stranded PCR products, 5 μl of PCR product was denatured in equal volume of 1.6% sodium hydroxide solution for 5 min at room temperature. The denatured samples were then transferred to a pre-washed strepavidin coated microtitration plates (Wallac Oy, Turku, Finland).

The molecular detection of M3/M4 allelic types was by allele-specific hybridization of Europium (Eu) labeled M3 (MAD20) or Samarium (Sm) labeled M4 specific probe to single-stranded PCR products immobilized on pre-washed strepavidin-coated microtitration plates (Wallac Oy). To obtain single-stranded PCR products, 5 μl of PCR product was denatured by an equal volume of 1.6% sodium hydroxide solution for 5 min at room temperature and added to 100 μl of DELFIA assay buffer (Wallac Oy), containing 2 ng Eu-labeled M3 (MAD20) specific probe (5’ to 3’), Eu-GAAGATATAGATAA and 5 μg Sm-labeled M4 (WELLCOME) specific probe (5’ to 3’), Sm-GATAAGATAAATGAA per well. Plates were incubated for 1hr at room temperature on a plate shaker and washed 6 times in Wallac wash solution to remove excess unbound probes. 200 μl of Wallac enhancement solution was added per well to activate the fluorescence labeled probes and the plates were shaken for a further 45 min at room temperature. Fluorescent signals from simultaneously hybridized Eu- and Sm-labeled specific probes were then measured and quantified using a time-resolved 1234 Wallac Fluorometer. The number of samples that hybridized to Eu (MAD20) or Sm (WELLCOME) probes was expressed as a percentage of the total number of Gambian (234 total) and Kenyan (212 total) samples. Similar results were obtained typing the same Gambian donors by standard dot-blot hybridization with allele-specific oligonucleotide probes (data not shown).

RESULTS

Peripheral blood mononuclear cells from 22 malaria-exposed Gambian adults were tested in IFN-γ ELISPOT responses to MSP1 peptides (Figure 1). Table 1 lists the peptides, selected on the basis of sequence differences between MAD20 and WELLCOME types and predicted binding to protective African HLA class II alleles HLA-DRB1*1302 (DR1302) and HLA-DRB1*0101 (DR1). The majority (18 of 22) of individuals gave an IFN-γ response to at least one MSP1 peptide. A minimum of four individuals responded to each of these MSP1 peptides. This unexpectedly high level of reactivity was unlikely to be due to stimulation of naive
The Gambia have shown a stable parasite distribution over time with MAD20 at 95% and WELLCOME at 5%. Heterogeneity was observed in the prevalence of IFN-γ responses: 28.4% to MAD20 allele (95%) in The Gambia. Molecular typing of IFN-γ T-cell responses in Gambian donors were found distributed throughout the MSP1 protein, with no immunodominant regions (Figure 1). However, the MSP1 dimorphic peptide with allelic type designated MAD20 and WELLCOME. Seroepidemiological studies in The Gambia have shown a stable parasite distribution over time with MAD20 at 95% and WELLCOME at 5%. However, the MSP1 dimorphic epitopes identified here were recognized overall to a similar degree in Gambian donors; MAD20 responses: 36.8% ± 2.6 standard error (SE), WELLCOME responses: 28.4% ± 4.6 SE. This reactivity pattern was surprising given the marked excess of the MAD20 allele (95%) in The Gambia. Molecular typing of MSP1 allelic strain distribution using the sequence-encoding variant peptides M3 and M4 in block 3 of the gene in > 200 Gambians with clinical malaria confirmed the high prevalence of MAD20 (MAD20: 88%, WELLCOME: 12%). Heterogeneity was observed in the prevalence of IFN-γ responses to MAD20 or WELLCOME for different MSP1 allelic T-cell epitope pairs. Thus, in the M5/M6 region, IFN-γ responses to the abundant MAD20 parasite form were prevalent (MAD20: 65% versus WELLCOME: 35%), whereas the adjacent allelic pair M3/M4 showed equal reactivity to both forms. Surprisingly, the allelic pair M7/M8 showed higher prevalence of responses to the rare WELLCOME strain represented by M8 (M7: 25% versus M8: 75%). All epitopes selected on the basis of peptide motifs to DR1 (e.g., M3/M4), DR1302 (e.g., M5/M6) or the “subtracted motif” (e.g., M7/M8) could bind to varying degrees to DR1, DR7, DR1301, DR1302, and DR1501 in peptide binding assays (data not shown). Thus, these unusual allelic reactivity patterns were unlikely to be due to lack of binding to HLA-DR.

It was possible that T-cell responses to the MAD20 epitope variant were underestimated by assessing only IFN-γ, a characteristic Th1 lymphokine. Therefore, the allelic pairs of M3/M4, M5/M6, and M7/M8 were used to assess in parallel both early IFN-γ and IL-4 production in a further 14 malaria-exposed adults in The Gambia (Figure 2 and Table 2). In general, fewer donors produced IL-4 (6 of 14 individuals) to these MSP1 epitopes compared to IFN-γ (12 of 14 individuals). Malaria-naive donors (n = 10) tested in similar IL-4 ELISPOT assays did not react to these allelic peptide pairs (data not shown). Individual donors could respond to different MSP1 epitopes either by IL-4 or IFN-γ. Thus, T cell release of IL-4 did not preclude the ability of a donor to respond by IFN-γ (Figure 2). Indeed, 58% (7 of 12 positive) of total IL-4 responses also had IFN-γ responses to the same epitopes. Analysis of IFN-γ and IL-4 reactivity in all Gambian donors showed an average higher IFN-γ (49%) reactivity to MSP1 allelic variant epitopes compared to IL-4 (14%) (t-test, P < 2 × 10−5) (Table 2).
The analysis of the limited number of IL-4-specific responses detected suggested that they may reflect the MAD20 prevalence of parasites (Table 2). This contrasted with IFN-γ reactivity. Thus, IFN-γ and IL-4 production to allelic-variant epitopes may be independently regulated. However, total T-cell responsiveness (IFN-γ and IL-4) still failed to reflect the higher MAD20 prevalence for allelic pairs M3/M4 and M7/M8.
TABLE 2
Summary of IFN-γ and IL-4 positive responders to MSP1 allelic variant peptide pairs (M3/M4, M5/M6, and M7/M8) in malaria-exposed donors in The Gambia

<table>
<thead>
<tr>
<th>Peptide Pair</th>
<th>IFN-γ Reactivity</th>
<th>IL-4 Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3/M4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide M3</td>
<td>20/45 (44%)*</td>
<td>3/14 (21%)*</td>
</tr>
<tr>
<td>Peptide M4</td>
<td>24/45 (53%)*</td>
<td>2/14 (14%)*</td>
</tr>
<tr>
<td>Specific (MAD20 versus WELLCOME)†</td>
<td>17% versus 31%</td>
<td>50% versus 25%</td>
</tr>
<tr>
<td>Both variants‡</td>
<td>52%</td>
<td>25%</td>
</tr>
<tr>
<td>M5/M6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide M5</td>
<td>24/40 (60%)*</td>
<td>2/14 (14%)*</td>
</tr>
<tr>
<td>Peptide M6</td>
<td>19/40 (48%)*</td>
<td>0/14 (0%)*</td>
</tr>
<tr>
<td>Specific (MAD20 versus WELLCOME)†</td>
<td>30% versus 11%</td>
<td>100% versus 0%</td>
</tr>
<tr>
<td>Both variants‡</td>
<td>59%</td>
<td>0%</td>
</tr>
<tr>
<td>M7/M8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide M7</td>
<td>16/44 (36%)*</td>
<td>3/14 (21%)*</td>
</tr>
<tr>
<td>Peptide M8</td>
<td>23/44 (52%)*</td>
<td>2/14 (14%)*</td>
</tr>
<tr>
<td>Specific (MAD20 versus WELLCOME)†</td>
<td>9% versus 39%</td>
<td>33% versus 0%</td>
</tr>
<tr>
<td>Both variants‡</td>
<td>52%</td>
<td>67%</td>
</tr>
</tbody>
</table>

* Percentages shown indicate the percentage of positive responders in each group.
† Percentage of specific responses induced by either the MAD20 or the WELLCOME variant from the total number of positive responders.
‡ Percentage of responses induced to both the MAD20 and the WELLCOME variant from the total number of positive responders.

It was interesting to speculate whether the high MSP1 reactivity and pattern of distribution of allelic variant specific immune responses were specific to The Gambia. A different pattern of reactivity might be expected in Kenya, since MSP1 allele frequencies in East Africa are different; equal frequencies of MAD20 and WELLCOME allelic types have been observed in Sudan.25

MSP1 peptides (M19 to M42) were tested in pools of peptides (Table 1) in early IFN-γ ELISPOT assays in 58 Kenyan malaria-exposed adults. Only limited IFN-γ reactivity was observed in this population (Kenyan: MAD20 responses: 6.0% ± 2.5 SE and WELLCOME responses: 8.6% ± 2.8 SE) compared to Gambian reactivity to these epitopes (M19 to M42) (Gambian: MAD20 responses: 36.8% ± 1.7 SE and WELLCOME responses: 24.6% ± 2.5 SE). Parasite typing of MSP1 strain distribution in > 200 Kenyans with clinical malaria showed a significant (chi-square, *P* < 5×10⁻⁸) difference in distribution of MAD20 and WELL-
Summary of dry season IFN-γ reactivity to MSP1 allelic variant peptide pairs (M3/M4, M5/M6 and M7/M8) in malaria-exposed donors in The Gambia and Kenya

<table>
<thead>
<tr>
<th>Peptide</th>
<th>M3/M4</th>
<th>M5/M6</th>
<th>M7/M8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide M3</td>
<td>10/20 (50%)*</td>
<td>10/19 (53%)*</td>
<td>4/19 (21%)*</td>
</tr>
<tr>
<td>Peptide M4</td>
<td>10/20 (50%)*</td>
<td>6/19 (48%)*</td>
<td>11/19 (57%)*</td>
</tr>
<tr>
<td>Specific (MAD20 versus WELLCOME)†</td>
<td>9% versus 18%</td>
<td>10% versus 0%</td>
<td>0% versus 64%</td>
</tr>
<tr>
<td>Both variants‡</td>
<td>73%</td>
<td>60%</td>
<td>36%</td>
</tr>
<tr>
<td>Peptide M5</td>
<td>3/30 (10%)*</td>
<td>1/31 (3%)*</td>
<td>1/31 (3%)*</td>
</tr>
<tr>
<td>Peptide M6</td>
<td>2/30 (7%)*</td>
<td>3/31 (10%)*</td>
<td>4/31 (13%)*</td>
</tr>
<tr>
<td>Specific (MAD20 versus WELLCOME)†</td>
<td>33% versus 0%</td>
<td>0% versus 67%</td>
<td>0% versus 75%</td>
</tr>
<tr>
<td>Both variants‡</td>
<td>67%</td>
<td>33%</td>
<td>25%</td>
</tr>
</tbody>
</table>

* Percentages shown indicate the percentage of positive responders in each group.
† Percentage of specific responses induced by either the MAD20 or the WELLCOME variant from the total number of positive responders.
‡ Percentage of responses induced to both the MAD20 and the WELLCOME variant from the total number of positive responders.

COME in Kenya compared to The Gambia. The general pattern of T-cell reactivity in Kenyan donors (Table 3, Figure 3) reflected the local parasite strain distribution (MAD20: 68%, WELLCOME: 32%).

T-cell responses to MSP1 in East Africa may be Th2 mediated with concomitant down-regulation of IFN-γ responses. To address this possibility, the peptide pools were tested for early IFN-γ and IL-4 secretion in parallel in another seven exposed Kenyan donors. Figure 3A shows that few IL-4 responses were observed. In another six Kenyan donors tested for early Th1 (IFN-γ) and Th2 (IL-4 and IL-10) production assays to M3, M4, M5, M6, M7, and M8, no increase in Th2 responses over Th1 responses by assessing either lymphokine was observed in Kenya compared to The Gambia (Figure 3B). Therefore, the lower MSP1 reactivity in Kenya assessed by IFN-γ release was unlikely to be due to cross-regulation by Th2 responses to these epitopes.

In The Gambia, the allelic IFN-γ T-cell responses to the three variant pairs either followed (M5/M6) or failed to reflect (M3/M4, M7/M8) parasite prevalence. Whether differences in MSP1 parasite distribution could affect T-cell allelic reactivity patterns were characterized in thirty Kenyan malaria-exposed individuals (Table 3). In general, there was a lower IFN-γ responder rate to these allelic pairs in Kenya than in The Gambia (paired t-test, P < 1 × 10^-3) (Table 3). Moreover, in Kenya, allelic pair M3/M4 gave slightly higher reactivity to MAD20 (M3:60% versus M4:40%). In contrast, both allelic pairs of M5/M6 and M7/M8 gave higher reactivity to the WELLCOME allelic type (M5:25% versus M6: 75%, M7:20% versus M8:80%). Thus, similar to data in The Gambia, there was a heterogeneity in the prevalence of MAD20 or WELLCOME responses in the three allelic variant epitopes, with only some epitope-specific responses following East African parasite prevalence.

Similar broad reactivity (responses to both allelic forms) patterns to the allelic pairs M3/M4, M5/6, and M7/8 in The Gambia and Kenya were observed (Table 3). However, a discordance between allelic specific responses and MSP1 parasite strain distribution was observed. Thus, only variant specific responses to M5/M6 reflected the epidemiological distribution of MSP1 alleles in both West and East Africa with a prevalence of M5 (MAD20) responses in The Gambia and higher M6 (WELLCOME) responses in Kenya. In contrast, allelic pair M3/M4 gave higher specific responses to M4 (WELLCOME) in The Gambia and a higher response to M3 (MAD20) in Kenya, in contrast to the expected epidemiological data. Surprisingly, the allelic pair M7/M8 had higher specific responses to the WELLCOME allele in both The Gambia and Kenya, independent of geographical differences in MSP1 allelic distribution. Thus, in this analysis of MSP1 allelic responses in two different populations in distinct geographical areas, IFN-γ effector T-cell function to certain allelic T-cell epitopes failed to reflect MSP1 epidemiological distribution.

**DISCUSSION**

T-cell production of IFN-γ has been implicated in protective immunity to blood-stage malaria.11,16,17 IFN-γ can activate non-lymphocytic effector cells such as monocytes34,35 to control parasite growth through phagocytosis,36 generate reactive oxygen intermediates37 or L-arginine-derived nitric oxide.38,39 However, IFN-γ does not control parasite growth by direct elimination of intra-erythrocytic parasites.40-42

In the present study, frequently recognized T-cell epitopes of MSP1 distributed throughout the protein were characterized in thirty Kenyan malaria-exposed individuals (Table 3). In general, there was a lower IFN-γ responder rate to these allelic pairs in Kenya than in The Gambia (paired t-test, P < 1 × 10^-3) (Table 3). Moreover, in Kenya, allelic pair M3/M4 gave slightly higher reactivity to MAD20 (M3:60% versus M4:40%). In contrast, both allelic pairs of M5/M6 and M7/M8 gave higher reactivity to the WELLCOME allelic type (M5:25% versus M6: 75%, M7:20% versus M8:80%). Thus, similar to data in The Gambia, there was a heterogeneity in the prevalence of MAD20 or WELLCOME responses in the three allelic variant epitopes, with only some epitope-specific responses following East African parasite prevalence.

Similar broad reactivity (responses to both allelic forms) patterns to the allelic pairs M3/M4, M5/6, and M7/8 in The Gambia and Kenya were observed (Table 3). However, a discordance between allelic specific responses and MSP1 parasite strain distribution was observed. Thus, only variant specific responses to M5/M6 reflected the epidemiological distribution of MSP1 alleles in both West and East Africa with a prevalence of M5 (MAD20) responses in The Gambia and higher M6 (WELLCOME) responses in Kenya. In contrast, allelic pair M3/M4 gave higher specific responses to M4 (WELLCOME) in The Gambia and a higher response to M3 (MAD20) in Kenya, in contrast to the expected epidemiological data. Surprisingly, the allelic pair M7/M8 had higher specific responses to the WELLCOME allele in both The Gambia and Kenya, independent of geographical differences in MSP1 allelic distribution. Thus, in this analysis of MSP1 allelic responses in two different populations in distinct geographical areas, IFN-γ effector T-cell function to certain allelic T-cell epitopes failed to reflect MSP1 epidemiological distribution.
protein (20%) and thrombospandin-related anonymous protein (TRAP) (30%) tested in the same population (Flanagan KL and others, unpublished data). It remains to be determined whether this represents a general difference in response rate between blood-stage and pre-erythrocytic antigens. Since cells from individuals not exposed to malaria did not produce significant amounts of IFN-γ, it can be inferred that malaria exposure was required to observe this reactivity.

The extensive IFN-γ reactivity to these epitopes in The Gambia supports the view that MSP1 is a major blood-stage antigenic target which may play a significant role in IFN-γ mediated protection in vivo. In murine studies, IFN-γ secreting T cell clones have been shown to be protective against malaria by a nitric-oxide dependent mechanism which may be dependent on macrophages and neutrophils. Alternatively, T-cell secretion of IFN-γ may help in the induction of protective cytophilic MSP1 IgG blood-stage specific antibodies and may assist in antibody-dependent cellular inhibitory mediated mechanisms. In recent prospective case-control studies of African children, IFN-γ responses to merozoite antigens were associated with resistance to malaria reinfection.

T-cell reactivity in the P. falciparum pre-erythrocytic antigen, the circumsporozoite (CS) protein is focused on two immunodominant epitope regions at its highly polymorphic carboxyl terminus. No previous study has characterized immunodominance in the MSP1 protein. Such efforts may help in the identification of potential polymorphic regions under selection immune pressures. Recently, such polymorphic epitopes in the CS protein have been shown to mutually inhibit both T-cell priming and effector responses. Such regions could be excluded from future blood-stage vaccines. Here, MSP1 epitopes were distributed along the entire protein with no immunodominant regions. This suggests that multiple fragments of the MSP1 protein, both shed before and during RBC invasion can be processed and presented to induce effector T cells.

It was surprising to observe overall lower levels of MSP1 reactivity in Kenya (27%, 8 of 31 positive IFN-γ responders) than The Gambia (>80%), given the higher malaria transmission rate in the former. It could be argued that immunosuppression due to malaria parasitemia may be responsible. However, this was unlikely since (i) all Kenyan adults were negative for malaria parasites and (ii) statistical analysis of Gambian IFN-γ reactivity patterns showed no difference between samples collected during the wet and dry seasons (data not shown).

It could be that low levels of reactivity to MSP1 simply reflect low rates of IFN-γ responsiveness in East Africa compared to West Africa. However, T cell reactivity to the \textit{P. falciparum} pre-erythrocytic antigens, CS protein and TRAP showed a similar response rate (30% in both locations) and a doubling of IFN-γ responses in Kenya (60%) compared to The Gambia (30%), respectively (Flanagan KL and Plebanski M, unpublished data). The same reagents and peptide batches were utilized when testing donors from The Gambia or Kenya, or control malaria-unexposed donors in Oxford. Since the testing of IFN-γ responses to CS protein, TRAP and MSP1 responses was performed using the same assay and on the same donors both in The Gambia and Kenya, the lower reactivity to MSP1 in Kenya cannot be due to a general suppression of IFNγ responses or assay differences. Interestingly, the general pattern of IFN-γ allelic T-cell reactivity to MSP1 in 58 Kenyan adults was consistent with MSP1 parasite strain distribution in East Africa. By contrast, MSP1 peptide specific IFN-γ responses in The Gambia did not show the expected excess of MAD20 over WELLCOME reactivity. Therefore, MSP1 immune reactivity in The Gambia may be regulated independently of antigenic exposure.

Malaria-exposed donors could respond to different MSP1 epitopes either by IFN-γ or IL-4 release. Thus, IL-4 T-cell production did not prevent IFN-γ release to other epitopes and 58% of positive IL-4 responses also had IFN-γ responses to the same epitopes, suggesting a Th1/Th2 profile. Independent IFN-γ or IL-4 production to different MSP1 epitopes was also observed in Kenya where responses were either Th1 or Th2 specific. Interestingly, there was a separation of Th2 responses into either IL-4 or IL-10 producing epitopes. Thus, MSP1 epitopes can induce independent T-cell production of Th1 or Th2 lymphokines. This independent immune regulation of IFN-γ and IL-4 responses was also reflected in the distribution of allele-specific T-cell responses to dimorphic epitopes. IL-4 responses in Kenyan donors, although found less frequently than IFN-γ in The Gambia, did follow the expected MAD20 prevalence.

It is not clear whether dimorphism in the MSP1 protein enables the parasite to evade variant-specific acquired immune responses. If this was the case, then such dimorphic epitopes might indeed have levels of immune responses that are not reflective of MSP1 parasite strain prevalence. Characterization of allele specific IFN-γ responses to allele-specific MSP1 T-cell epitopes suggests that mechanisms other than parasite strain frequency-dependent immune selection may influence reactivity patterns. We speculate that immune evasion focused on IFN-γ responses to allelic-altered peptide ligands of MSP1 may underlie the evolution of MSP1 dimorphism.

In conclusion, frequently recognized T-cell epitopes have been identified distributed throughout the \textit{P. falciparum} MSP1 with no immunodominant region. IFN-γ and IL-4 production were independently regulated and IFN-γ allelic T-cell reactivity patterns were discordant with epidemiological parasite allelic prevalence for specific MSP1 epitopes. These results suggest that MSP1 dimorphism may have evolved to evade Th1 T-cell responses. Further understanding of the immunological-selection pressure(s) that maintain MSP1 dimorphism will be required before deployment of candidate MSP1 vaccines in the field.

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Reactivity to Allelic P. falciparum T-Cell Epitopes


