Fine Specificity of Neonatal Lymphocytes to an Abundant Malaria Blood-Stage Antigen: Epitope Mapping of Plasmodium falciparum MSP1

Indu Malhotra, Alex N. Wamachi, Peter L. Mungai, Elton Mzungu, Davy Koech, Eric Muchiri, Ann M. Moormann and Christopher L. King

J Immunol 2008; 180:3383-3390; doi: 10.4049/jimmunol.180.5.3383
http://www.jimmunol.org/content/180/5/3383

References
This article cites 48 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/180/5/3383.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Fine Specificity of Neonatal Lymphocytes to an Abundant Malaria Blood-Stage Antigen: Epitope Mapping of Plasmodium falciparum MSP1\textsubscript{33}\textsuperscript{1}

Indu Malhotra,\textsuperscript{2*} Alex N. Wamachi,\textsuperscript{†} Peter L. Mungai,* Elton Mzungu,‡ Davy Koech,\textsuperscript{†} Eric Muchiri,‡ Ann M. Moormann,* and Christopher L. King\textsuperscript{2*§}

Cord blood T cells have been reported to respond to a variety of exogenous Ags, including environmental allergens and various viruses and parasites, as demonstrated by enhanced proliferation and cytokine secretion. This finding is evidence that Ags in the maternal environment transplacentally prime and result in fetal development of memory T cells. Some studies suggest these neonatal T cell responses may arise by nonspecific activation of T cells that express TCRs with low binding affinity, thus lacking fine lymphocyte specificity. To address this question, we examined malaria Ag stimulation of human cord and adult blood mononuclear cells in samples from residents of a malaria endemic area in Kenya. We constructed overlapping 18-mer peptides derived from sequences contained in dimorphic alleles of the C-terminal 33-kDa fragment of Plasmodium falciparum merozoite protein 1. This study identified a dominant T cell epitope for one MSP1\textsubscript{33} allele (MAD20) and two T cell epitopes for the second allele (K1); these epitopes were nonoverlapping and allele specific. In a given donor, peptide-specific proliferation and IFN-γ secretion were highly concordant. However, IL-10 and IL-13 secretion were not correlated. Importantly, the fine specificity of lymphocyte proliferation and cytokine secretion in cord and adult blood mononuclear cells was similar. Cord blood cells obtained from malaria-infected pregnant women were 4-fold more likely to acquire a peptide-specific immune response. We conclude that the fetal malaria response functions in a fully adaptive manner and that this response may serve to help protect the infant from severe malaria during infancy. The Journal of Immunology, 2008, 180: 3383–3390.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1}This work was supported by Health and Human Services Grant AI064687 and by the Veterans Affairs Research Service.

\textsuperscript{2}Address correspondence and reprint requests to Drs. Indu Malhotra and Christopher L. King, Case Western Reserve University, Wolstein Research Center Room 4132, 2103 Cornell Road, Cleveland, OH 44106. E-mail addresses: jmj@case.edu and cxk21@case.edu

\textsuperscript{3}Abbreviations used in this paper: CBMC, cord blood mononuclear cell; MSP1, merozoite surface protein 1; ssu, small subunit; SI, stimulation index.

www.jimmunol.org
We and others have shown that 20–70% of CBMC collected from newborns of women living in malaria holoendemic areas of Africa have recall responses to MSP1 and/or other malaria blood-stage Ags (5, 6, 20). Recall responses to blood-stage Ags in CBMC occur more frequently in newborns of primi- and secundigravid women, who are at increased risk of parasite sequestration in the placenta during pregnancy, and therefore more likely to expose their fetus to soluble malaria Ags (17, 19). No recall responses to peptides corresponding to malaria pre-erythrocytic-stage Ags are noted with CBMC, which occur in very low concentrations and are primarily found in hepatocytes and not intravascularly (5, 6), suggesting a high level of specificity to Ags expressed during different stages of the malaria parasite life cycle. In contrast, recall responses to pre-erythrocytic-stage peptides occur in young children and adults (21, 22). This study examines the hypothesis that the fetus can acquire a similar fine specificity of the adaptive immune responses to MSP1 compared with adults.

Plasmodium falciparum MAD20 is a 195-kDa GPI-anchored protein on the merozoite surface, representing the most abundant merozoite surface protein (23). It undergoes a series of proteolytic cleavages during merozoite invasion of erythrocytes (23). The final cleavage of the C-terminal 42-kDa portion of MSP1 releases a soluble 33-kDa fragment (24), while the 19-kDa fragment is retained on the merozoite surface and is carried into the erythrocyte during erythrocyte invasion (25). T cell responses are primarily directed to the 33-kDa fragment (MSP133) whereas Ab responses are primarily directed to the 19-kDa fragment (MSP119; Ref. 26). MSP133 is highly polymorphic and forms two distinct allelic families, referred to as the MAD20 allele (3D7 strain) or the Wellcome K1 allele (or FVO parasite strain). These alleles have 51% amino acid identity (27). Previous studies have examined PBMC recall responses from individuals living in a malaria endemic area to several peptides corresponding to predicted T cell epitopes for both MSP133 and MSP119 (28, 29). No published studies, however, have demonstrated a systematic mapping of T cell epitopes for MSP133 or whether these epitopes differ between the two dominant allelic families. Moreover, no studies have shown detailed T cell epitope mapping of an immunogenic molecule in cord blood. The current study examines 58 peptides that span both MAD20 and K1 alleles of MSP133, constructed as 18-mer peptides overlapping by nine amino acids, with respect to proliferation and cytokine responses from CBMC from newborns and PBMC from adults residing in holoendemic area for P. falciparum in Kenya.

Materials and Methods

Study population and sample collection

CBMC were prepared from umbilical cord blood using previously described protocols (5, 30) from 48 newborns of pregnant women who delivered at the Msambweni District Hospital (Coast Province, Kenya). Intervillous placental and maternal peripheral blood was collected to determine the presence of malaria at delivery. Impression smears from placental tissues were prepared as previously described (5, 19, 20). Peripheral venous blood was collected from three adult men who had been diagnostically treated with malaria and successfully treated within a month of blood collection. PBMC preparations used a previously described protocol (5, 30). CBMC representing negative controls were prepared from healthy North American newborns vaginally delivered at University Hospitals of Cleveland (n = 10; Cleveland, OH). Ethical approval was obtained from the Human Investigations Review Board of University Hospitals of Cleveland and the Kenya Medical Research Institute in Nairobi.

Determination of malaria infection status and genotyping malaria isolates

For blood smear diagnosis, thin and thick films were stained with 4% Giemsa and examined microscopically for asexual P. falciparum under ×100 oil immersion fields to determine parasitemia levels. DNA was extracted from 200 μl of whole blood using individual spin blood kits (Qiagen). A total of 2.5 μl of DNA was used for amplification of the multicytokine 18S (small subunit (ssu)) ribosomal RNA genes (ssu rRNA) of P. falciparum by real-time quantitative PCR as described previously. PCR was performed using a quantitative thermocycler (GeneAmp 5700 Sequence Detector; ABI Research). Each run included no template DNA (negative control) and serial log-fold dilutions of plasmid containing the ssu rRNA genes (GenBank AF145334) (positive control).

Peripheral or intervillosus blood that was positive by blood smear or PCR was then genotyped for the 33-kDa region of MSP1. PCR primers were chosen to distinguish between the MAD20 and K1 dimorphic variants, allowing for the detection of mixed or polyclonal infections to generate a product of ~500 bp. MAD20 primers for amplification “M15” are: 5′-CCATTGGGAGAATCCGAAG-3′ (aa 1329–1336) and 5′-TTGCTGTGGTTTCTGTTGTT-3′ (aa 1504–1510), while “M16” primers are 5′-AATTAGGATCATATCCGCAATACATGAGTA-3′ (aa 1550–1564) and 5′-TTCTCTCTTTCTACATGCTGAA-3′ (aa 1661–1669). K1 primers are as follows: amplion “K15” 5′-TTGGGAATACCTGGAGAAATTG-3′ (aa 1332–1338) and 5′-TTTACCTTGTTTACGTGTTGA-3′ (aa 1498–1515) and “K16” 5′-TCTAATAATACGATATATTTGCC-3′ (aa 1556–1564) and 5′-ACATTTCTCTCTTTCTACATGCTATGGTTGTA-3′ (aa 1650–1658). The PCR was conducted in a 21-μl reaction volume using 10.64 μl of sterile water, 1 μl of 2.5 mM dNTPs, 2 μl of 10× buffer, 2 μl of 25 mM MgCl2, and 0.16 μl of PE AmpliTaq Gold polymerase (5 U/ml), with 2 μl of forward and reverse primers (2.5 μM). The cycling conditions included initial denaturation at 94°C for 4 min, 45 cycles of denaturation for 1 min 45 s at 94°C, extension for 1 min at 63°C. This was followed by 1 min annealing at 72°C and a final extension for 10 min at 72°C. Three microliters of the PCR product was run on 1% agarose gel via electrophoresis in 1× Tris-acetate-EDTA buffer. Band visualization was used on CyberGold. Only samples with bands in both block 15 and block 16 for a particular allele are reported as positive.

Peptides, recombinant proteins, and mitogens

Peptides were synthesized by using F-moc biochemistry by Sigma-Genosys. Peptides were 70–80% pure and used without further purification. Thirty 18-mer peptides overlapping by nine amino acids were prepared; these peptides spanned MSP142 based on the P. falciparum MAD20 allele (GenBank sequence no. X03371.1, Fig. 1 designated at K26–53). Note two additional peptides were synthesized for the MAD20 allele because of the 20-aa insertion for this allele. Lyophilized peptides were reconstituted in 20% (w/v) DMSO and resuspended in PBS. A working concentration of 100 μg/ml in complete RPMI 1640 (10% human serum) was prepared as sterile solutions. Ten microliters of 10 μg/ml in sterile water, 1 μl of 2.5 mM dNTPs, 2 μl of 10× buffer, 2 μl of 25 mM MgCl2, and 0.16 μl of PE AmpliTaq Gold polymerase (5 U/ml), with 2 μl of forward and reverse primers (2.5 μM). The cycling conditions included initial denaturation at 94°C for 4 min, 45 cycles of denaturation for 1 min 45 s at 94°C, extension for 1 min at 63°C. This was followed by 1 min annealing at 72°C and a final extension for 10 min at 72°C. Three microliters of the PCR product was run on 1% agarose gel via electrophoresis in 1× Tris-acetate-EDTA buffer. Band visualization was used on CyberGold. Only samples with bands in both block 15 and block 16 for a particular allele are reported as positive.

Proliferation assay

A total of 4 × 105 CBMC or PBMC in 200 μl of culture medium was added to each well of a round-bottom 96-well plate (Costar) in triplicate. Cultures were incubated at 37°C in 5% CO2 for 5 days, labeled with 1μCi [3H] thymidine (DuPont NEN Research Products) for 14 h then harvested and radioactivity incorporation was determined using a Matrix 96 beta counter (Packard Instrument). The mean cpm of each set of triplicate wells was calculated and the simulation index (SI) was determined as the mean cpm of peptide-stimulated culture divided by the mean cpm of unstimulated cultures. A cutoff value of SI = 2 was considered positive based on proliferation of the same peptide preparations in CBMC preparations from 10 newborns born in Kenya, whose mothers had never been exposed to malaria in responses. Among these unexposed CBMC SI ranged from 0.7 to 1.9, mean = 0.97, SD = 0.36.
FIGURE 1. Alignment of sequences corresponding to the MAD20 and K1 alleles of MSP133. Amino acid residues in bold are identical between the two alleles. The beginning alanine (A) for the MAD20 allele is amino acid position 1349 in MSP1 and for the K1 allele, position 1350. Lines under sequences correspond to 18-mer peptides that stimulated the greatest frequency of lymphocyte proliferation and/or IFN-γ response shown in Figs. 2 and 3. Dominant T cell epitopes for the MAD20 allele is peptide number 42 and for the K1 allele numbers 30 and 49 (boxed regions).

\[ n = 580 \text{ (e.g., 58 peptides in 10 CBMC samples). Thus, a mean + 3 SD was equal to 2.05 which we rounded to 2.0 as the cutoff.} \]

ELISPOT and ELISA for cytokine production

IFN-γ ELISPOT was performed as described (5). Briefly, ELISPOT plates (Millipore) were coated with capture Abs in sterile PBS overnight at 4°C and blocked with complete RPMI 1640 with 10% pooled human AB serum. Plates were then washed three times with sterile PBS. To measure the frequency of Ag-specific IFN-γ-secreting cells, 4 x 10^5 CBMC were added to each well in 200 μl of medium in triplicate and were incubated at 37°C in 5% CO₂ for 72 h. Fewer CBMC were added to each well (1 x 10^5) to measure mitogen-driven cytokine production. A positive response was scored when one of the following conditions was met: 1) an average of >4 IFN-γ-secreting cells in response to any one of the peptides or rMSP1, when no IFN-γ-secreting cells were present in negative control wells (medium alone); or 2) in cases where IFN-γ-secreting cells were observed in negative control wells, the number of spots generated by Ag-driven CBMC was at least 2-fold greater. Malaria Ag-driven, IFN-γ-secreting cells were also screened in CBMC from 10 healthy North American newborns or in PBMC from 10 malaria-naïve adults. None of the peptides or rMSP1 induced secretion of more than two spots if there were no spontaneous IFN-γ production (only observed in two normal CBMC from nonendemic area) and for the two with spontaneous IFN-γ secretion peptides induced never exceeded a 1.6-fold increase above background for any peptides.

Quantification of IL-2, IL-4, IFN-γ, and IL-13 by ELISA was performed on culture supernatants collected at 72 h. Results were expressed in picograms per milliliter by interpolation from standard curves based on recombinant lymphokines (31). Ab pairs for cytokine capture and detection (all biotinylated) were used as previously described (20). A positive response was scored when the following two criteria were fulfilled: 1) a net value for Ag-stimulated cells that was at least 2-fold greater than that of parallel cultures (only observed in two normal CBMC from nonendemic area) and for the two with spontaneous IFN-γ secretion peptides induced never exceeded a 1.6-fold increase above background for any peptides.

Statistics

The significance of differences between groups was evaluated using the Student t test, and relationships between variables were examined by simple linear regression. Comparisons of the proportions of responders in various groups of donors were evaluated by \( \chi^2 \) analysis using Fischer’s exact test.

FIGURE 2. The proportion of cord blood samples (\( n = 48 \)) with a lymphocyte proliferation SI of >2 (left panels). Right panels, Individual responders with a SI > 2 to peptides that induced a response in >30% of subjects examined.
Results

**Mapping T cell epitopes to MSP1**

To identify dominant epitopes, Fig. 2 shows the pattern of lymphocyte proliferation by CBMC \((n = 48)\) to 18-mer peptides spanning the sequence for both the MSP1\(_{33}\), MAD20 and K1 alleles. A positive response had a stimulation index of \(>2\). Peptides that stimulated a positive response in the greatest proportion of individuals tended to have higher SI (Fig. 2). Fig. 3 shows peptide-driven IFN-\(\gamma\) secretion by a subset of CBMC \((n = 23)\) measured by ELISPOT. Overall, peptides that induced lymphocyte proliferation also stimulated IFN-\(\gamma\). MAD20 peptides 41–43 stimulated the strongest lymphocytes responses in the greatest proportion of individuals indicating the presence of a dominant T cell epitope in this region of the molecule. Additionally, K1 peptides 29–31 and 48–50 stimulated lymphocytes responses in the greatest proportion of individuals indicating that these regions of the molecule also contain strong T cell epitopes. T cell epitopes were distinct and nonoverlapping between the two alleles (Figs. 1–3).

Fig. 4 shows the distribution of individual CBMC samples that responded to one or more peptides for both lymphocyte proliferation and IFN-\(\gamma\) production by ELISPOT. A cord blood sample was considered to have positive recall responses to MSP1\(_{33}\) if there was significant lymphocyte proliferation and/or ELISPOT response to more than or equal to three peptides. Based on this definition, 25 of 48 (52\%) CBMC responded to MSP1\(_{33}\) based on proliferation and 14 of 23 (61\%) of CBMC responded to MSP1\(_{33}\) as measured by IFN-\(\gamma\) ELISPOT. Of the 14 subjects with significant IFN-\(\gamma\) responses to more than or equal to three peptides, 11 also had a significant response as measured by lymphocyte proliferation whereas only 2 of 9 non-IFN-\(\gamma\) responders had significant responses by lymphocyte proliferation. Thus, there was significant correlation between lymphocyte proliferation and ELISPOT results \((\chi^2, p = 0.01)\). CBMC responses could be allele specific. Seven CBMC samples responded only to peptides corresponding to the dominant MAD20 T cell epitopes and three CBMC only to peptides corresponding to the dominant K1 T cell epitope.

PBMC samples from three Kenyan adults known to be recently infected with *P. falciparum* demonstrated the same dominant T cell epitope recognition patterns as CBMC. For the MAD20 allele,
MSP133 peptides stimulate lymphocyte proliferation in a greater proportion of CBMC samples compared with rMSP142.

We have previously observed that peptides corresponding to previously described T cell epitopes from malaria blood-stage Ags stimulate lymphocyte responses in a greater proportion of CBMC samples compared with a recombinant malaria blood-stage protein tested in the same sample (5, 19, 20). These studies, however, did not directly compare peptides derived from sequences contained within the recombinant protein. To examine this relationship further, a subset of CBMC were also stimulated with rMSP142 corresponding to the MAD20 allele. MSP142 includes the full sequence of MSP133. As shown in Table I, a significantly greater proportion of CBMC samples responded to multiple peptides than to rMSP142 (see Fig. 4). Positive lymphocyte proliferation responses to rMSP142 were 2.1 and 2.2 and frequency of IFN-γ-secreting cells to rMSP142 was 85, 147, and 3119 spots per 4 × 10^5 PBMC. By contrast, all three adult PBMC generated significant lymphocyte proliferation and IFN-γ release to both peptides and rMSP142 (data not shown). Similar results were observed with the K1 allele, although the overall number of responders was lower (data not shown). This suggests a possible defect in Ag processing and/or presentation by CBMC.

Six of 11 malaria-positive women were genotyped as to whether they were infected with MAD20 and K1 alleles for MSP133. All six had the MAD20 allele; no K1 alleles were detected. To further evaluate the frequency of parasites with the two alleles in the same population, we examined an additional 31 peripheral venous or intervillosus placental blood samples from women collected at delivery in the year preceding the current study. At least 42 genotypes were identified (because of mixed infections) with 35 (83%) corresponding to the MAD20 allele and the remaining 7 (17%) to the K1 allele.

Primi- and secundigravid women have increased frequency of malaria infection and MSP133 peptide-specific lymphocyte proliferation and IFN-γ release by CBMC compared with multigravid women

Lymphocyte priming in the fetus is likely to occur by transplacental transfer of malaria Ags or infected erythrocytes during gestation, and not by nonspecific activation of lymphocytes. Therefore,

![FIGURE 5](image-url)
Table II.  Relationship of maternal malaria infection at delivery and parity with MSP133 peptide-specific proliferation and IFN-γ production by CBMC

<table>
<thead>
<tr>
<th>Parity</th>
<th>Maternal Malaria (%)</th>
<th>Positive by Proliferation (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Positive by IFN-γ ELISPOT (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primigravid</td>
<td>38 14 (37)</td>
<td>23 (61)</td>
<td>13/19 (68)</td>
</tr>
<tr>
<td>Multigravid</td>
<td>10 1 (10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 (30)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/6 (17)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Criteria for a positive response is given in the legends of Figs. 2 and 3 and Table I.

<sup>b</sup> Value of p < 0.01 by χ² analysis.

Discussion

This first comprehensive epitope mapping of MSP1<sub>33</sub>-driven responses identifies dominant T cell epitopes that could be useful guides in the development of blood-stage malaria vaccines. The C-terminal 42-kDa fragment of MSP1 that includes MSP1<sub>33</sub> is a leading malaria vaccine candidate. Although the conserved C-terminal 19-kDa fragment contains B cell epitopes recognized by Abs which inhibit merozoite invasion of erythrocytes, the inclusion of the polymorphic MSP1<sub>33</sub> with strong T cell epitopes is necessary because it markedly enhances MSP1 immunogenicity and vaccine efficacy in nonhuman primates (26, 27, 32). The observation that the K1 allele of MSP1<sub>33</sub> has two distinct T cell epitopes that differ from a single dominant T cell epitope for the MAD20 allele (Fig. 1) suggests that a vaccine against P. falciparum may require rMSP1<sub>42</sub> for both alleles to obtain optimal immunogenicity. This study also provides the basis for finer mapping of the dominant T cell epitopes such that these epitopes could be incorporated into a synthetic MSP1<sub>1</sub>-based vaccine.

The magnitude of proliferation and IFN-γ responses to the three dominant peptides among positive responders was generally low and never reached a stimulation index of >6 (Fig. 2) and mean number of peptide-induced IFN-γ cells of ~60 cells per 4 × 10<sup>5</sup> CBMC (Fig. 3). The relatively low response in cord blood likely represent recent priming and/or strong immune modulation such as by T regulatory cells (33, 34).

The dominant T cell epitopes identified in this study both confirm and contradict past studies. Prior studies selected T cell epitopes of MSP1<sub>33</sub> in humans based on computer algorithms (28, 29), but did not systematically map T cell epitopes as performed in this study. Udhayakumar et al. (28) tested nine peptides, ranging from 14 to 20 aa for MSP1<sub>33</sub> for the MAD20 allele. One peptide that stimulated the greatest proportion of adult responders corresponded exactly to the dominant MAD20 peptide observed in our study (GISYYEKVLAKY KDDL). Only one other study examined peptides corresponding to MSP1<sub>33</sub> in humans (29). This study selected three peptides corresponding to MAD20 and three of the K1 allele of MSP1<sub>33</sub> using a different computer algorithm than the study by Udhayakumar et al. (28) Overall, they observed a lower frequency of lymphocyte responses to their peptides compared with our study, even though the malaria endemicity was similar between study populations. None of the peptides corresponded to dominant T cell epitopes. They did, however, show clear allele-specific differences in some donors, as observed in our study. Overall, our findings are consistent with earlier studies, but they emphasize the importance of a systematic mapping of T cell epitopes because algorithms used previously did not consistently identify the dominant T cell epitopes.

The presence of CBMC responses to MSP1<sub>33</sub> peptides was greater in primigravid and multigravid women, consistent with exposure to malaria in utero. Parity is a useful surrogate for malaria infection because primigravid women are more susceptible to malaria during pregnancy compared with multigravid women (19). Table II shows that primigravid women in the study were ~4-fold more likely to be infected at delivery, and ~3-fold more likely to show a positive lymphocyte proliferation and/or IFN-γ response to MSP1<sub>33</sub> peptides compared with multigravid women. Additionally, the portion of CBMC samples that show positive proliferative responses to more than or equal to three peptides was compared between malaria-infected women and those without detectable malaria at the time of delivery. CBMC from 9 of 15 (60%) malaria-infected women had significant proliferative responses compared with significant proliferative responses in 14 CBMC born to 33 (42%) women uninfected at delivery (p = 0.2, by χ² analysis).

Even though MAD20 was the dominant circulating genotype of MSP1<sub>33</sub> in the community and in the maternal infections screened in this study, the proportion of CBMCs and PBMCs samples responding to peptides from one or both variants was similar. A prior study also found a similar discordance between the frequency of responses to allele-specific peptides and the epidemiological distribution of different MSP1 alleles (29). The reasons for this discordance are unclear, however, there are several plausible explanations. First, it is possible that pregnant women in the current study could have been infected with parasites expressing the K1 allele earlier during pregnancy sufficient to prime the fetus and to generate T cell memory. Second, the K1 allele has two dominant T cell epitopes, whereas MAD20 has just one and is thus more likely to be HLA class II restricted. Third, this allelic dimorphism may have evolved independent of parasite strain frequency-dependent selection, such as by allelic altered peptide ligands to interfere with T cell priming (36).

Recent human vaccine studies with rMSP1<sub>42</sub> have shown good immunogenicity and safety, but failed to demonstrate protection in phase 2 trials (Refs. 37 and 38 and E. Angov, et al. Abstract no. 12, 2007 American Society for Tropical Medicine and Hygiene Meeting). This encouraging, however, that a better vaccine formulation is required. Based on findings from the current study, the vaccine may be modified, for example, to include repeats of the different variants of the dominant T cell epitopes of MSP1<sub>33</sub> that could enhance protective Ab responses, increase Ab-independent protection to MSP1<sub>33</sub> as has been observed with murine malaria (39), and improve strain-transcending immunity. It is possible to immunize with separate MSP1<sub>33</sub> and MSP1<sub>49</sub> fragments that would provide the necessary conditions...
T cell help and also produce Abs targeting MSP1\textsubscript{13} that could enhance protective anti-MSP1\textsubscript{19} Ab responses (40).

Circulating neonatal T cells are fundamentally different from those of naive adult T cells that could potentially affect the response to exogenous Ags. Not surprisingly, fetal T cells have many characteristics of recent thymic emigrants including 1) high concentrations of TCR excision circles which are episomal DNA by-products of a TCR \(\alpha\)-chain rearrangement that are not replicating but are diluted during cell division; 2) a high proportion of cells in cycle; and 3) increased propensity to undergo apoptosis (41). Together, this indicates high cell turnover (42), which is thought to expand TCR diversity, establish the T cell repertoire, and populate peripheral lymphatic tissues (41, 43). As a consequence, many naïve T cells may have low-affinity TCRs and reduced threshold for T cell activation and expansion without production of conventional memory T cells (8, 9). Therefore, the presence and pattern of cytokine responses to Ags in cord blood might have little relevance to development of immune responses to the same Ags later in childhood. The current study challenges this proposition by showing CBMC demonstrated a distinct pattern of lymphocyte responses to MSP1\textsubscript{13} peptides, providing clearly definable dominant T cell epitopes. Most peptides, however, failed to stimulate any response by CBMC, disproving nonspecific activation of lymphocytes. Moreover, the dominant T cell epitopes were distinct for the two different allelic forms of PfMSP1\textsubscript{13}, indicating a clear specificity of cord blood T cell responses. These dominant T cell epitopes were identical with those identified in PBMC from adults known to be previously infected or exposed to \textit{P. falciparum} from the same geographical area. Thus, the fetus is capable of generating a repertoire of peptide-specific T cells similar to adult responses.

How the fetus is exposed to Ags may account for the difference in CBMC responses vs environmental allergens. Inhalant or food Ags/allergens are likely to have much lower systemic levels compared with malaria and only certain Ags may cross the placenta. By contrast, malaria-infected erythrocytes can pass transplacentally, exposing the fetus to a wide variety of molecules including those that activate innate immune responses. A notable molecule is GPI which anchors MSP1 and other membrane-associated molecules to the plasma membrane of intact parasites (44) and is a potent TLR agonist (45). Because APCs in the fetus show diminished responses to TLR ligands and deliver less costimulatory signals compared with adult APC, altered T cell responses in the fetus may occur (46). However, if the fetus is exposed to molecules that stimulate innate immunity, lymphocytes may acquire functional characteristics similar to those of adult lymphocytes. Supporting this explanation is detection of functionally normal cord blood Ag-specific CD8\textsuperscript{T} T cells in response to congenital infections with CMV and \textit{T. cruzi} (1, 2) and Ag-specific neonatal CD4\textsuperscript{+} T cell to intravascular hemelinthes and mycobacterial Ags (30, 47). Thus, an immune response in the fetus is more likely to resemble that of an adult if the fetus is exposed to sufficient quantities of Ag in the context of stimulation of innate immunity, thereby overriding functional defects in neonatal APCs.

Functional defects of neonatal APCs do persist as suggested by the observation in the current study that CBMC are 3-fold more likely to respond to peptides compared with rMSP1\textsubscript{42}. By contrast, adult PBMC responded equivalently to peptides and rMSP1\textsubscript{42}. Processing of MSP1\textsubscript{42} may be particularly difficult because the C-terminal region is cysteine-rich with two epidermal growth factor-like motifs, each with multiple disulfide bonds (26). We have previously observed a greater proportion of individuals responding to malaria peptides compared with rAg (5, 20); however, we have never directly compared responses to the same rAg and peptides as was done here. This suggests that detection of recall responses to specific Ags in cord blood may be made more sensitive by using peptides. An alternative explanation may account for the difference in response to rMSP1\textsubscript{42} and peptides. The fetus may have been preferentially exposed to MSP1\textsubscript{13} compared with the full-length MSP1\textsubscript{42} because secondary processing of MSP1\textsubscript{42} leads to release of MSP1\textsubscript{13} in serum (24). These fragments may be preferentially transported transplacentally, whereas the MSP1\textsubscript{13} fragment would more likely result from the less frequent fetal exposure to the merozoite or infected erythrocytes (19).

Peptide-induced IL-10 and IL-13 production by CBMC failed to correlate with that observed for lymphocyte proliferation and IFN-\(\gamma\) release. The proportion of individuals producing these cytokines was also >2-fold lower than that observed for lymphocyte proliferation and/or IFN-\(\gamma\) release. There are several possible explanations for this disparity. First, certain peptides recognize low-affinity receptors on naive, recent thymic emigrants and this low receptor engagement stimulates the release of certain cytokines rather than lymphocyte proliferation. Nonspecific binding to naive T cells is unlikely because addition of these same peptides to CBMC obtained from North American newborns failed to stimulate any detectable cytokine response. Second, partial engagement of low-affinity TCRs on previously activated T cells could generate a different signal leading to cytokine production rather than lymphocyte proliferation (48). This is in contrast to peptides with higher affinity binding to TCR which stimulates lymphocyte proliferation and IFN-\(\gamma\) production (48). Third, certain peptides of MSP1\textsubscript{13} stimulate T cells to develop into Th1-type phenotypes whereas other MSP1\textsubscript{13} peptides induce differentiation of T cells into alternative phenotypes that secrete other cytokines, but weakly proliferate and release little IFN-\(\gamma\). Ultimately, we can better differentiate these pathways by looking at the T cell phenotype using various cell surface markers, e.g., CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}, and CD45 isotypes and by examining cytokine production using flow cytometry and proliferation by CSFE labeling.

In conclusion, these results support a model in which human neonates can mount a spectrum of immune responses to exogenous Ags, ranging from nonspecific activation to a fully mature immune response. This flexibility of responsiveness may have arisen to protect the fetus from harmful effects due to excessive inflammatory responses and yet enable the fetus to develop adaptive immune responses against potentially life-threatening pathogens. The adaptive responses to malaria Ags in the fetus may play functional roles in generating both immunoregulatory and immunoprotective states against severe disease during infancy and early childhood.

Acknowledgments

We appreciate the help of Dr. Dawood Mwaura—Medical Superintendent, Elizabeth Chomba—Clinical Officer, and nurses Victoria Saidi, Hashora Mwanguku, Zaituni Mwakilelo, Fatuma Ngare, Ruth Notina, and Florence Wambua in helping with recruitment of women to the study, collection of cord blood samples, and care of the women and their newborns. We are grateful for the help of Charles NgaNga and Alex Osore who undertook all the parasitological examinations and Grace Methenge and Christine Lucas for data entry and management. Dr. Michelle Spring helped with peptide design and development of the methods for detection of MSP1\textsubscript{13} alleles. Kevin Steiner participated in conception of the study. Dr. Arlene Dent provided helpful comments on the manuscript. Finally, we appreciate the willingness of the women residing in the Msambweni location to participate in the study.

Disclosures

The authors have no financial conflict of interest.