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Fine Specificity of Neonatal Lymphocytes to an Abundant Malaria Blood-Stage Antigen: Epitope Mapping of *Plasmodium falciparum* MSP1₃₃¹

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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 MSP1₃₃ allele (*MAD20*) and two T cell epitopes for the second allele (*KI*); 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.

A number of studies have shown that cord blood mononuclear cells (CBMC)³ proliferate and produce cytokines in response to various environmental allergens and Ags usually associated with chronic infections in pregnant women. Notable infections that reproducibly stimulate an immune response in the fetus are HIV and CMV, and the parasitic infections *Trypanosoma cruzi*, toxoplasmosis, and malaria (1–6). This prenatal priming has generated considerable interest because of the impact it might have on the infant. Allergen exposure may predispose children to develop allergic diseases. Viral or parasitic Ags could either enhance or diminish the child's subsequent acquisition of immunity to these infections. Studies of this phenomenon have important implications for the development of pediatric vaccines, as attempts are made to manipulate vaccines or their adjuvants to better generate mature responses in human newborns and infants.

Despite this interest, CBMC to various Ags have been poorly characterized. It is known that CD4⁺ and/or CD8⁺ T cells are stimulated

(7), but whether these represent classical CD45RO⁺ memory T cells eliciting a recall response or nonspecific activation of naive T cells is not well-understood. Indeed, memory function of low numbers of CD45RO⁺ T cells in the fetal circulation remains undetermined. Recent characterization of CBMC to environmental allergens indicated that a significant component of T cell reactivity was not mediated by memory T cells, but rather via a default response of recent thymic emigrants, inducing a transient cellular immune response in the absence of conventional T cell memory (8, 9).

A defining property of fetal TCR is the shorter size of their CDR3 regions in comparison to CDR3s in adults (10). The CDR3 (11) is encoded by nucleotide sequences derived from the junctions of V, D, and J segments and flanking regions, forming part of the Ag-binding groove of the TCR; it is a key site of fine Ag recognition (12). Short fetal CDR3 regions may result in flat Ag-binding sites (13). This may explain the frequent presence of low-affinity polyreactive specificities in naive frequent thymic emigrants in the fetal immune system (14, 15). This low-affinity response is illustrated in T cell epitope mapping of OVA, a common food allergen, in cord compared with adult blood. CBMC demonstrate broadly reactive responses to multiple OVA peptides that are absent in adults (15), suggesting fetal lymphocytes' lack of fine antigenic specificity to certain Ags.

To examine further whether fetal lymphocytes can acquire fine specificity of immune response to a different Ag, we examined the recall responses to peptides corresponding to the malaria blood-stage Ag merozoite surface protein 1 (MSP1). In contrast to inhalant or food allergens, malaria offers a good paradigm to study fetal immune responses to exogenous Ags. Malaria infects erythrocytes, releasing large quantities of Ag intravascularly, which can cross the placenta to directly expose the fetus, either through soluble Ag (16, 17) or infected erythrocytes during pregnancy

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

(17–19). 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 MSP1 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 or *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 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 by 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 diagnosed 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 Institutional Review Boards 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, thick and thin films were stained with 4% Giemsa and examined microscopically for asexual *P. falciparum* under $\times 100$ oil immersion fields to determine parasitemia levels. DNA was extracted from 200 μ l of whole blood using individual spin blood kits (Qia-

gen). A total of 2.5 μ l of DNA was used for amplification of the multicopy 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 intervillous 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 amplicon "M15" are: 5'-CCATTTTGGAGAATCCGAAG-3' (aa 1329–1336) and 5'-TTCGTCTGTTTTGCTGGTG-3' (aa 1504–1510), while "M16" primers are 5'-AAGGTTTTAGCGAAATATAAGGATGA-3' (aa 1475–1484) and 5'-TTCTCTTTTCATCTAAATGTCTGAA-3' (aa 1661–1669). *K1* primers are as follows: amplicon "K15" 5'-TTGGAGAATCCGAAGAA GATT-3' (aa 1332–1338) and 5'-TTTCACCTGTGTTGTCGTGA-3' (aa 1489–1515) and "K16" 5'-TTCAATAGATACGGATATAAAATTT TGC-3' (aa 1556–1564) and 5'-ACATTCTTCTTTCATCTAAAT GTCT-3' (aa 1650–1658). The PCR was conducted in a 21- μ l reaction volume using 10.84 μ l of sterile water, 1 μ l of 2.5 mM dNTPs, 2 μ l of 10 \times buffer, 2 μ l of 25 mM MgCl₂, and 0.16 μ l of PE AmpliTaq Gold polymerase (5 U/ μ l), with 2 μ l of forward and reverse primers (2.5 μ M). 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 is 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 out on 1% agarose gel via electrophoresis in 1 \times 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.

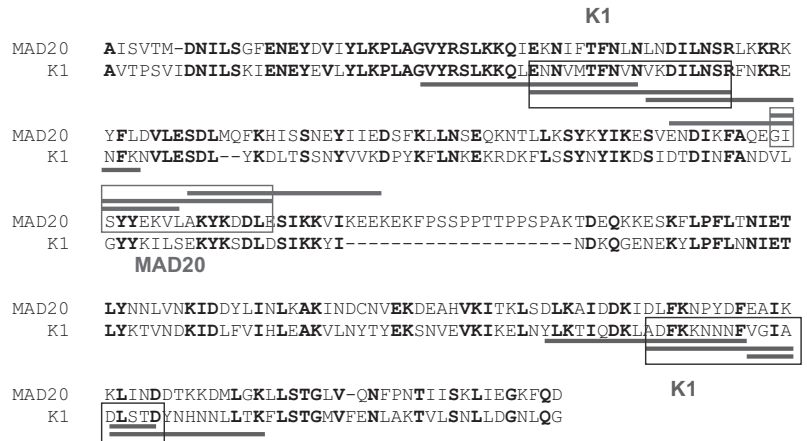
Peptides, recombinant proteins, and mitogens

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 MSP1₃₃ based on the *P. falciparum* *MAD20* allele (GenBank sequence no. Z35327.1; Fig. 1 designated as M28–57). Similarly, 28 18-mer peptides overlapping by nine amino acids were prepared corresponding to the *P. falciparum* the Wellcome or *K1* 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 μ g/ml in complete RPMI 1640 (10% human AB serum) was prepared from stock (1 mg/ml) solutions before addition to cultures. rMSP1₄₂ corresponding to both *MAD20* and *K1* alleles was provided by Drs. C. Long, S. Singh, and D. Narum (Malaria Vaccine Development Unit (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD)). PMA plus ionomycin (Calbiochem) and PHA (Sigma-Aldrich) were used in parallel cultures for positive mitogen controls. The concentration of endotoxin in preparations used for evaluation of lymphocyte responses was < 0.5 ng/ml, which is 5- to 50-fold less than that required for stimulation of cytokine production by human lymphocytes. Only preparations that responded to the mitogen are reported. Media alone (negative control), 10 μ g/ml *MAD20* and *K1* peptides, 5 μ g/ml MSP1₄₂, and PMA (50 pg/ml) plus ionomycin (1 μ g/ml) or PHA (1 μ g/ml) were added to wells after optimal concentrations were determined in pilot experiments in CBMC and PBMC for lymphocyte proliferation and ELISPOT experiments. Of note, higher concentrations of MSP1₄₂ (e.g., 10 and 20 μ g/ml) did not induce a greater frequency of MSP1₄₂-specific responses in pilot studies or prior investigations (5, 20), which is why the 5 μ g/ml was used in the current experiments.

Proliferation assay

A total of 4×10^5 CBMC or PBMC in 200 μ l 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 1 μ Ci [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 stimulation 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 of > 2.0 was considered positive based on proliferation of the same peptide preparations in CBMC preparations from 10 newborns born in Ohio, 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).



$n = 580$ (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, 4×10^5 CBMC were added to each well in 200 μ l of medium in triplicate and were incubated at 37°C in 5% CO₂ for 72 h. Fewer CBMC were added to each well (1×10^4) 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 rMSP1₄₂, 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-naive adults. None of the peptides or rMSP1₄₂

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 , IL-10, 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 containing medium alone and 2) responses to three or more MSP1₃₃ peptides. If cytokine production was not detectable in the negative control cultures, then >40 pg/ml cytokine was considered to be a positive response.

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 χ^2 analysis using Fischer's exact test.

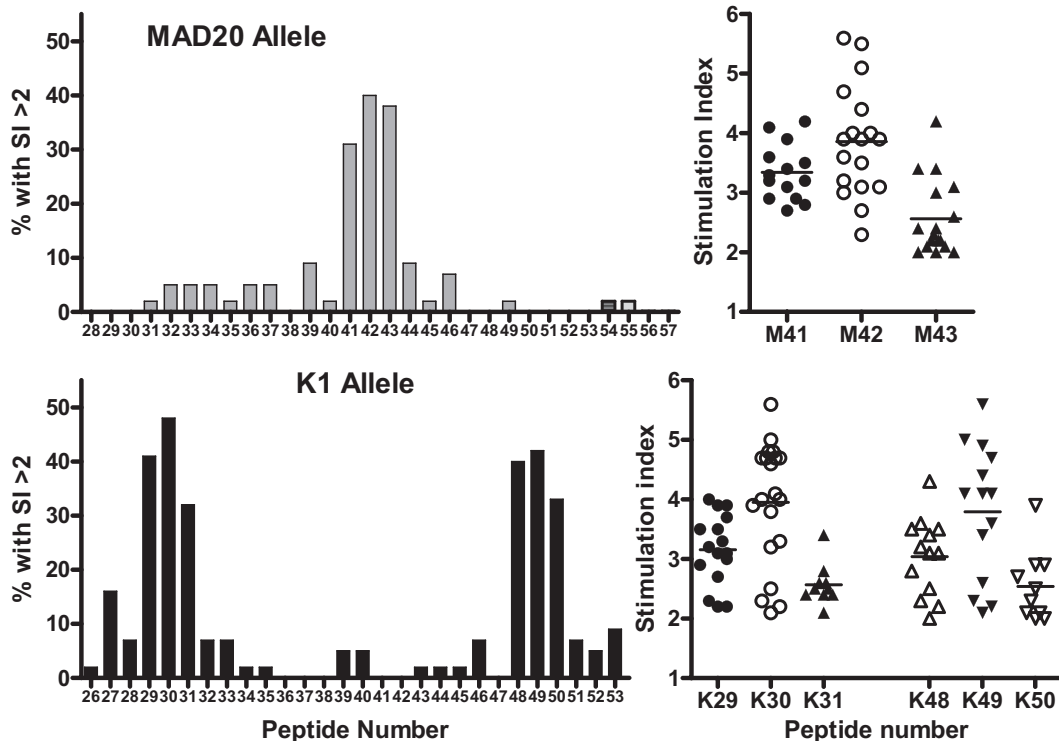


FIGURE 2. The proportion of cord blood samples ($n = 48$) with a lymphocyte proliferation SI of >2 (left panels). Right panels, Individual responders with a SI > 2 to peptides that induced a response in >30% of subjects examined.

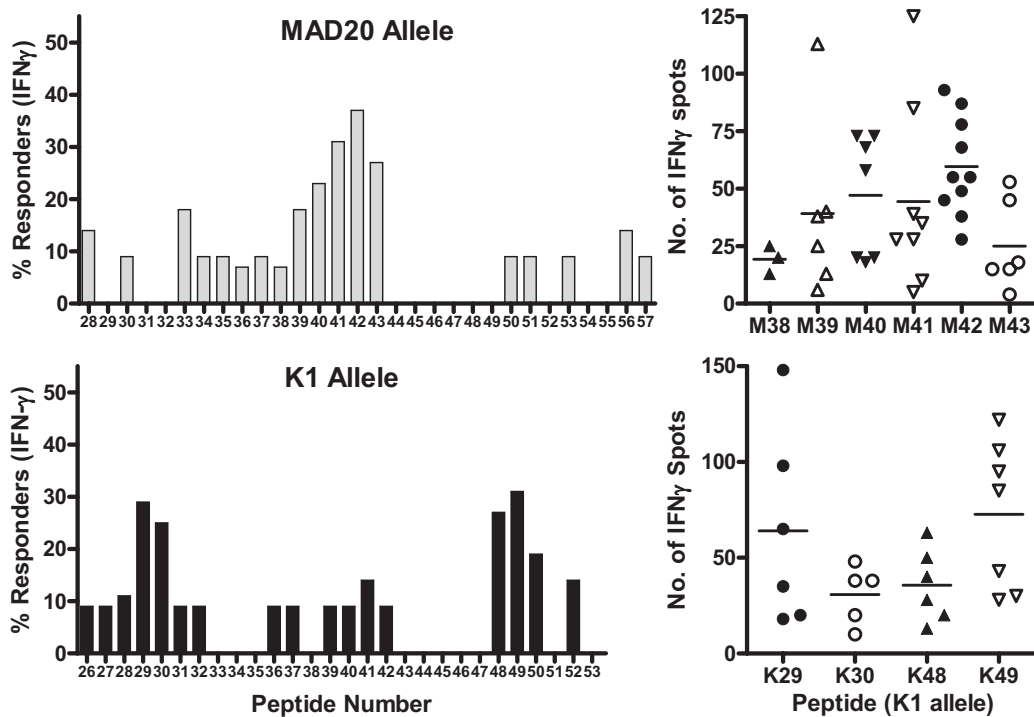


FIGURE 3. The proportion of cord blood samples ($n = 23$) with lymphocytes secreting IFN- γ as measured by ELISPOT in response to each peptide (left panels). Right panels, The mean number of IFN- γ -secreting cells minus the mean number of cells in the cultures with medium alone among positive responders for peptides showing the highest frequency of responses. Variation was generally <20% among replicates.

Results

Mapping T cell epitopes to MSP1₃₃

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 MSP1₃₃ 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- γ secretion by a subset of CBMC ($n = 23$) measured by ELISPOT. Overall, peptides that induced lymphocyte proliferation also stimulated IFN- γ . 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- γ production by ELISPOT. A cord blood sample was considered to have positive recall responses to MSP1₃₃ 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 MSP1₃₃ based on proliferation and 14 of 23 (61%) of CBMC responded to MSP1₃₃ as measured by IFN- γ ELISPOT. Of the 14 subjects with significant IFN- γ 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- γ responders had significant responses by lymphocyte proliferation. Thus, there was significant correlation between lymphocyte proliferation and ELISPOT results (χ^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 epitopes.

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,

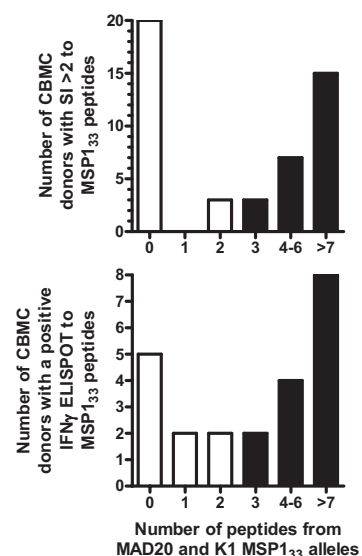


FIGURE 4. Distribution of CBMC samples responses to MSP1₃₃ peptides measured by lymphocyte proliferation ($n = 48$, upper panel) and IFN- γ ELISPOT ($n = 23$, lower panel). A positive response is defined in *Materials and Methods*. ■, CBMC samples with a positive response (to more than or equal to three peptides). □, Samples classified as nonresponders (less than or equal to two peptides). A cord blood sample was considered to have an overall positive response to MSP1₃₃ if three or more peptides stimulated a positive response (■). Peptides of both MAD20 and K1 alleles were included.

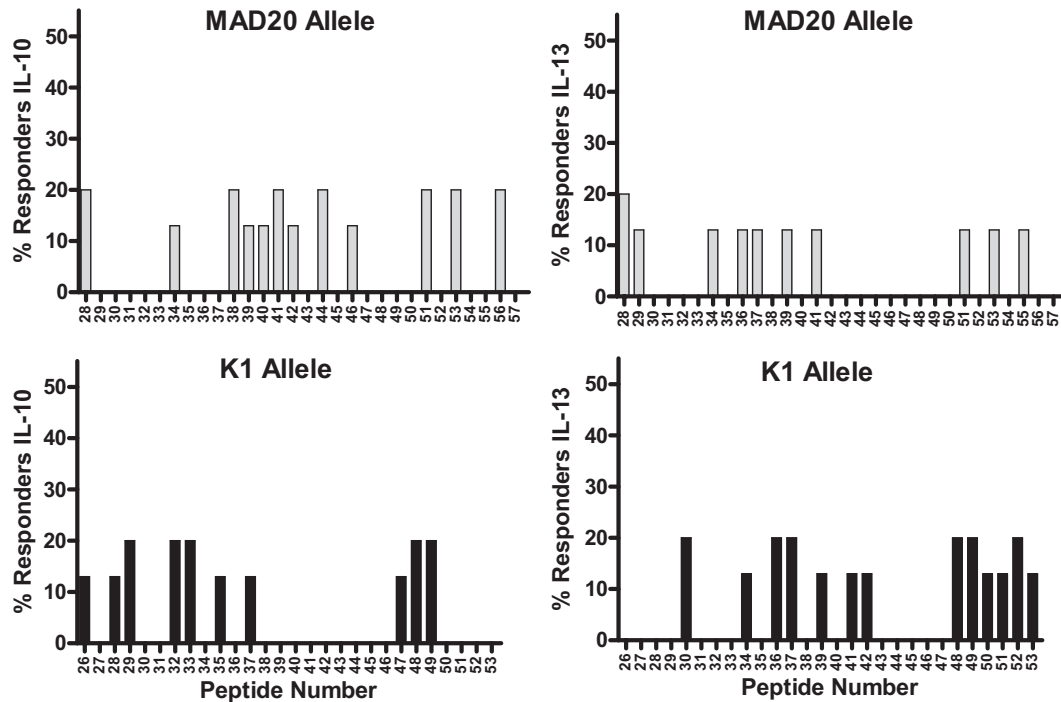


FIGURE 5. The proportion of cord blood samples ($n = 48$) with lymphocytes secreting IL-10 and IL-13 as measured by ELISA in response to each peptide corresponding to the *K1* allele of MSP1₃₃ (upper panels) and *MAD20* allele (lower panels).

peptides 41 and 42 induced SI >2 (2.3–4.8) in all three adults, peptide 43 SI >2 (2.9, 3.7) in two adults. None of the other *MAD20* peptides stimulated a significant proliferation response. For the *K1* allele, only peptides 29, 30, 48, and 49 stimulated SI >2 (2.1–5.3) in all three adults. *K1* peptides 27, 31, and 50 induced SI >2 in one or two adult PBMC. All other *K1* peptides failed to induce a SI >2. A similar pattern of responses for adult PBMCs compared with CBMCs was observed for IFN- γ secretion by ELISPOT (data not shown).

To evaluate other cytokine production, their release was measured in supernatants from the same cultures used for lymphocyte proliferation at 96 h, before the addition of [³H]thymidine. Little appreciable peptide-induced IL-2 or IL-4 was detected in cultures. IFN- γ production measured by ELISA paralleled that observed for the ELISPOT assay, however, with a lower sensitivity (data not shown). By contrast, net peptide-induced IL-10 (150–1268 pg/ml) and IL-13 (130–1220 pg/ml) was detected from 12 to 20% of CBMCs, often to peptides that did not induce lymphocyte proliferation or IFN- γ production (Fig. 5).

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 gener-

ated 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 intervillous 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,

Table I. Frequency of MSP1₃₃ peptides and rMSP1₄₂-driven lymphocyte proliferation by CBMC corresponding to the *MAD20* allele

	<i>n</i>	Positive Response to rMSP1 ₄₂ (%)	Positive Response to MSP1 ₃₃ (% Responders)
Proliferation ^a	23	2 (9)	10 (43) ^b
IFN- γ production ^c	23	3 (13)	11 (48) ^d

^a The presence of a positive response was defined for lymphocyte proliferation as a SI more than two, to more than or equal to three, peptides.

^b Value of $p = 0.021$ by χ^2 compared to rMSP1₄₂.

^c IFN- γ detected by ELISA in culture supernatants. A positive response >40 pg/ml or >2-fold above medium alone, to more than or equal to three peptides.

^d Value of $p = 0.023$ by χ^2 compared to rMSP1₄₂.

Table II. Relationship of maternal malaria infection at delivery and parity with MSP1₃₃ peptide-specific proliferation and IFN- γ production by CBMC

Parity	n	Maternal Malaria (%)	Positive by Proliferation (%) ^a	Positive by IFN- γ ELISPOT (%) ^a
Primi/secundigravida	38	14 (37)	23 (61)	13/19 (68)
Multigravida	10	1 (10) ^b	3 (30) ^b	1/6 (17) ^b

^a Criteria for a positive response is given in the legends of Figs. 2 and 3 and Table I.

^b Value of $p < 0.01$ by χ^2 analysis.

fetal priming should be restricted to women infected with malaria during pregnancy. The presence of malaria in women at delivery was assessed by examining maternal peripheral and intervillous placental blood for the presence of malaria parasites. Because mothers may have been infected with malaria earlier during gestation, but not at the time of delivery, parity was also used a surrogate for prenatal exposure to malaria. Primi/secundigravida women are more susceptible to malaria during pregnancy compared with multigravida women (19). Table II shows that primi/secundigravida women in the study were ~4-fold more likely to be malaria infected at delivery, and ~3-fold more likely to show a positive lymphocyte proliferation and/or IFN- γ response to MSP1₃₃ peptides compared with multigravida 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 χ^2 analysis).

Discussion

This first comprehensive epitope mapping of MSP1₃₃-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 MSP1₃₃ 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 MSP1₃₃ 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 MSP1₃₃ 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 rMSP1₄₂ 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-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 4×10^5 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 MSP1₃₃ 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 MSP1₃₃ 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 (GISYYEKVLAKYKDDLE). Only one other study examined peptides corresponding to MSP1₃₃ in humans (29). This study selected three peptides corresponding to *MAD20* and three of the *K1* allele of MSP1₃₃ 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 identified in our study. This suggests that their peptides, although immunogenic, may not represent 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 MSP1₃₃ peptides was greater in primigravida and secundigravida compared with multigravida women, consistent with exposure to malaria in utero. Parity is a useful surrogate for malaria infection because primi- and secundigravida women are at significantly higher risk of malaria during pregnancy (35) and Table II and thus more likely to have fetal exposure to malaria Ags. This observation is consistent with the notion that recall responses to MSP1₃₃ peptides are specific and directed to fetal memory T cells previously exposed to malaria Ags, rather than due to nonspecific activation of naive T cells. Although there was trend toward greater CBMC responses to MSP1 among malaria-infected women compared with uninfected women at delivery, this difference was not significant. This is not surprising because some malaria infections may have been acquired shortly before or at delivery and therefore would not have sufficient time to prime the fetus.

Even though *MAD20* was the dominant circulating genotype of MSP1₃₃ 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 rMSP1₄₂ 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), indicating 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 MSP1₃₃ that could enhance protective Ab responses, increase Ab-independent protection to MSP1₃₃ as has been observed with murine malaria (39), and improve strain-transcending immunity. It is possible to immunize with separate MSP1₃₃ and MSP1₁₉ fragments that would provide the necessary

T cell help and also produce Abs targeting MSP1₃₃ that could enhance protective anti-MSP1₁₉ 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 α -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 naive 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 MSP1₃₃ peptides, showing 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 PfMSP1₃₃ 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 *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⁺ T cells in response to congenital infections with CMV and *T. cruzi* (1, 2) and Ag-specific neonatal CD4⁺ T cell to intravascular helminthes 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 rMSP1₄₂. By contrast, adult PBMC responded equivalently to peptides and rMSP1₄₂. Processing of MSP1₄₂ 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 rMSP1₄₂ and peptides. The fetus may have been preferentially exposed to MSP1₃₃ compared with the full-length MSP1₄₂ because secondary processing of MSP1₄₂ leads to release of MSP1₃₃ in serum (24). These fragments may be preferentially transported transplacentally, whereas the MSP1₄₂ 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- γ release. The proportion of individuals producing these cytokines was also >2-fold lower than that observed for lymphocyte proliferation and/or IFN- γ 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 stimulate lymphocyte proliferation and IFN- γ production (48). Third, certain peptides of MSP1₃₃ stimulate T cells to develop into Th1-type phenotypes whereas other MSP1₃₃ peptides induce differentiation of T cells into alternative phenotypes that secrete other cytokines, but weakly proliferate and release little IFN- γ . Ultimately, we can better differentiate these pathways by looking at the T cell phenotype using various cell surface markers, e.g., CD3⁺, CD4⁺, CD8⁺, and CD45 isoforms and by examining cytokine production using flow cytometry and proliferation by CFSE 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.

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Disclosures

The authors have no financial conflict of interest.

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