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The *Plasmodium falciparum* Antigen MB2 Induces Interferon-γ and Interleukin-10 Responses in Adults in Malaria Endemic Areas of Western Kenya

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INTRODUCTION

Malaria is a leading cause of morbidity and mortality world-wide, accounting for about 250 million clinical cases and nearly one million deaths annually.[1] Malaria vaccines targeting pre-erythrocytic and blood stage antigens have had mixed success, with only a few vaccines showing efficacy in malaria endemic areas.[2,3,4] Identifying a suitable vaccine candidate capable of inducing stable and protective immunity in people with different genetic backgrounds and malaria transmission histories remains a challenge. This necessitates a continued exploration of additional vaccine candidates. One such candidate is the multi-domain sporozoite surface protein MB2, a 1610 amino acid long protein (molecular weight of 187 kDa) encoded by the MB2 gene. MB2 gene products are present in the sporozoite, asexual blood stages and gametocytes and have a distinct pattern of stage dependent subcellular localization and proteolytic processing at the various stages of the parasite life cycle.[5] The stage-dependent localization of MB2 on the other hand provides a possible target for a cellular immune attack during the sporozoite stage of the parasite.[6] The MB2 protein possesses tandemly repeated amino acid motifs and a highly conserved antigenic region[5] as is seen in other sporozoite surface antigens such as circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP).[6,7] This highly immunogenic

region may divert the immune response away from more important T cell epitopes. A recent study indicated that anti-MB2 antibodies are possessed by 83% of individuals living in an area of seasonal malaria transmission in Western Kenya.[6]

Interferon-gamma (IFN-γ) and interleukin-10 (IL-10) responses to *Plasmodium falciparum* antigens have been reported to correlate with protection from clinical malaria.[8] IFN-γ is a Th1, pro-inflammatory cytokine that is important in protection from infection in human,[9,10] and animal,[11,12] malaria models. Its responses to CSP correlated with protection in individuals immunized with RTS,S, a CSP-based malaria vaccine candidate.[13,14] In addition, IFN-γ responses to several pre-erythrocytic antigens including CSP.[15,16] TRAP,[15] and liver-stage antigen-1 (LSA-1),[10,17,18] have been associated with protection in individuals in different malaria endemic areas of Africa. IL-10 on the other hand is involved in regulation of the inflammatory response,[19,20] and together with other anti-inflammatory cytokines has been shown to be secreted following an initial IFN-γ-dominated response to *Plasmodium* infection in animal models.[21] In human, severe malarial anemia has been associated with low-levels of IL-10,[22] and a low IL-10 to tumor necrosis factor-α ratio.[23] LSA-1-specific IL-10 has been associated with protection from infection in children in Western Kenya.[24] Thus, both IFN-γ and IL-10-specific responses to *P. falciparum* pre-erythrocytic antigens may be important to protection from clinical malaria. Neither IFN-γ nor IL-10 responses to *P. falciparum* MB2 have been described to date in naturally exposed populations. In the present study, we assessed IFN-γ and IL-10 responses to MB2 9-mer peptides in adults in two populations in Kenya, one with stable and the other with unstable malaria transmission.

MATERIALS AND METHODS

Study area and population

The study was conducted in two areas of western Kenya, Kanyawegi and Kipsamoite. Kanyawegi is located in Kisumu District, a lowland area (elevation ˜1200 m) of stable and intense malaria transmission, with an entomological inoculation rate (EIR) estimated at >300 infectious bites per person/year during this period.[25] In contrast, Kipsamoite in Nandi District, an epidemic-prone highland area (elevation 1850-2100 m) with sporadic, unstable transmission patterns and an estimated EIR of <1 infectious bite/year.[26] This cross-sectional sample collection were conducted in August 2001 at a time of high, stable malaria incidence in the lowland area and during a peak of seasonal malaria incidence in the highland area.[27]

The study was carried out as part of a larger study that focused on responses to other previously described antigens,[28,29] in which 228 individuals aged 18-85 years (114 from the area of stable transmission and 114 from the area of unstable transmission) were randomly selected to participate in the study. Sample size for the study was determined by the ability to detect a difference of >50% in responses to different *P. falciparum* antigens with >80% power. Individuals who had sufficient cell numbers for the assays of the present study were included in this study. 129 individuals had testing by the enzyme-linked immunosorbent spot (ELISPOT) (64 from stable transmission area, 65 from unstable

transmission area), 89 individuals had testing by enzyme-linked immunosorbent assay (ELISA) (40 from stable transmission area, 49 from unstable transmission area) and 41 individuals had testing by both ELISA and ELISPOT (22 from stable transmission area, 19 from unstable transmission area). *Plasmodium* infection was determined by microscopy as previously described.[17] Blood smears were obtained and stained at the time of sample collection, but read after the completion of collection as all individuals were asymptomatic. Individuals who developed symptoms of fever any time after the collection were asked to return to the health center, where an immediate blood smear was done and treatment for malaria was provided to all individuals with a blood smear positive for *Plasmodium* species.

This study was approved by the Ethical Review Committee of the Kenya Medical Research Institute and Institutional Review Board for Human studies at University Hospitals of Cleveland, Case Western Reserve University. The work was performed while the principal investigator Prof. Chandy C. John (CCJ) was at Case Western Reserve University. Written informed consent was obtained from all participants prior to sample collection.

Antigens and mitogens for cytokine testing

To test for the cytokine responses to MB2 peptide pool, peptide sequences from the MB2 antigen that were predicted by algorithm to be T cell epitopes for human leukocyte antigen (HLA) class I alleles common in both populations were used. The sequence for MB2,[30] was entered into the http://www-bimas.cit.nih.gov/molbio/hla_bind/ and the two sequences with the highest predicted binding for HLA supertypes A24 and B7, which are common and seen at similar frequencies in both sites,[31] were used. The MB2 peptides were SVSSINTNL (aa 191-199; Sigma, St. Louis, MO, USA) and KPKKKYYEV (aa 119-127; Invitrogen, Carlsbad, CA, USA). The peptides were synthesized and purified by high performance liquid chromatography to >90% purity and used at a concentration of 10 μg/mL. The mitogen phytohemagglutinin (PHA) at 1 μg/mL was used as a positive control.

Peripheral blood mononuclear cell (PBMC) isolation, cytokine testing, and HLA typing

Venipuncture blood (10-20 mL) was drawn into sodium-heparin vacutainers (BD BioScience, San Jose, CA, USA). Samples were transported from the field to the laboratory for processing within 6 h of collection. PBMCs were isolated by Ficoll-Hypaque (Amersham Biosciences, Upsala, Sweden) density gradient centrifugation and resuspended in complete RPMI 1640 (Gibco Invitrogen Paisley, Scotland, UK), supplemented with 10% heat inactivated human AB serum (Sigma, H4522, St. Louis, MO, USA), 10 μg/mL gentamicin (Amresco, E737, Solon, OH, USA), 10 mM HEPES (Sigma, H3375, St. Louis, MO, USA) and 10 mM L-glutamine (Gibco, Invitrogen Paisley, Scotland, UK).

Individuals with cell recoveries of $>15 \times 10^6$ cells had both ELISPOT and ELISA testing carried out, individuals with $8\text{-}15 \times 10^6$ cells had only ELISPOT testing carried out and individuals with $<8 \times 10^6$ cells had only ELISA testing performed.

ELISPOT testing was carried out as previously described.[28] Briefly, sterile 96-well Millipore MAIP ELISPOT microtiter plates (Millipore, Corp., Billerica, MA, USA) were pre-coated at 4°C overnight with 5 μg/mL of human anti-IFN-γ monoclonal antibody (Endogen M-700A, Rockford, IL, USA). After washing with sterile phosphate buffered saline (PBS) (Sigma, P2194, St. Louis, MO, USA) and blocking with 10% heat-inactivated fetal bovine serum (Sigma, F7524, St. Louis, MO, USA) in PBS, PBMCs were plated at a final concentration of 5×10^5 cells/mL in duplicate wells along with either 10 μg/mL MB2 antigen, or 1 μg/mL PHA (as positive control), or PBS alone (as negative control). The cells were incubated at 37°C in 5% CO2 for 5 days (˜120 h). Plates were washed and a biotinylated anti-IFN-γ monoclonal antibody (Endogen M-701B, Rockford, IL, USA) was applied (0.75 μg/mL) for 1.5 h at 37°C. This treatment was followed by washing, incubation with a 1:2000 dilution of streptavidin-conjugated horseradish peroxidase (DAKO, P0397, Dako, Denmark) for 2 h at room temperature, washing and color development by addition of 1% 3-amino-9-ethyl-carbazole (Sigma, A6926, St. Louis, MO, USA) in 0.1 M acetate buffer catalyzed by 0.015% hydrogen peroxide. The reaction was stopped after 10-20 min by washing with deionized water. Plates were dried in the dark at room temperature. The number of spot-forming units (SFU) per well was counted by using C.T.L. ImmunoSpot Scanning and Imaging Software Version 5.0 (Cellular Technology Ltd., Shaker Heights, OH, USA). Results were expressed as numbers of SFU per 10^6 PBMC. A sample was considered positive if the proportion of SFUs in the stimulated well was significantly greater than that of the unstimulated wells, i.e., PBS alone ($P < 0.05$).[17,32] Responses to the malaria peptides were not detected for PBMC from 18 malaria-non-exposed healthy adults (data not shown).

For IFN-γ and IL-10 measurements by ELISA, isolated PBMCs were plated at a final concentration of 1×10^6 cells/mL in 96-well U-bottomed microtiter plates (Microtest; BD Biosciences, San Jose, CA, USA). Cell culture supernatants were harvested after 5 days and tested for the presence of IFN-γ and IL-10 by ELISA as previously described.[33] Optical densities were measured at 405 nm using an OpsysMR™ Microplate Reader (Dynex Technologies, Chantilly, VA, USA) and analyzed using Revelation QuickLink Software, Version 4.04 (Dynex Technologies, Chantilly, VA, USA). Values of unstimulated culture supernatants were subtracted from those of peptide and PHA-stimulated culture supernatants. The cut-off value of a positive IFN-γ or IL-10 ELISA response was defined as greater than the mean plus two standard deviations of the values from 18 North American control subjects. Cut-off values for the MB2 peptide pool were 67 pg/mL for IFN-γ and 31 pg/mL for IL-10.

HLA typing and supertype classification was performed using the PCR-SSOP method as previously described.[31]

Statistical analysis

Differences in proportions were compared by the Chi-square or Fishers exact test. Spearman's rank test was used to assess the correlation between the IL-10 and IFN-γ levels between sites and assays. Differences in cytokine levels were compared by the Mann-Whitney rank sum test. Stata 10.0 (Stata Corporation, College Station, TX, USA) was used for all statistical analysis. *P* values <0.05 were considered significant for all tests.

RESULTS

IFN-γ and IL-10 responses to MB2 as measured by ELISA

IFN-γ responses to MB2 were detected by ELISA in 27.5% of individuals in the stable malaria transmission area compared to 18.8% of individuals in the unstable malaria transmission area ($P = 0.33$) [Table 1]. Levels of MB2-specific IFN-γ were not different between the areas of stable (geometric mean = 113.9 pg/mL) and unstable-transmission (63.9 pg/mL) ($P = 0.26$) [Table 1].

IL-10 responses were observed in 25.0% of individuals in the stable transmission area compared to 22.5% of individuals in the unstable malaria transmission area ($P = 0.78$) [Table 1]. No significant difference was observed in the levels of MB2-specific IL-10 between the stable (geometric mean = 65.3 pg/mL) and the unstable transmission areas (39.6 pg/mL; $P = 0.55$) [Table 1].

No association was observed between levels of IFN-γ and the IL-10 within the individuals from stable transmission area ($r = 0.007$; $P = 0.97$) or the unstable transmission area ($r = 0.27$; $P = 0.06$).

Sixteen of 40 individuals tested by ELISA for IFN-γ or IL-10 in the stable transmission area had a positive blood smear for *P. falciparum* at the time of blood collection. None of the 49 individuals tested by ELISA for IFN-γ or IL-10 had a positive blood smear for *P. falciparum*. There was no relationship between blood smear results and IFN-γ or IL-10 responses or levels in the stable transmission area (all *P* values for differences in IFN-γ or IL-10 responses or levels between smear positive and smear negative individuals >0.30).

IFN-γ response to MB2 as measured by ELISPOT

The proportion of individuals with a positive ELISPOT response was 10.9% in the stable malaria transmission area and 10.8% in the unstable malaria transmission area ($P = 0.98$) [Table 2]. The magnitude of responses also did not differ between stable (geometric mean = 11.7 SFU) and unstable transmission areas (23.6 SFU; $P = 0.16$) [Table 2]. 34 of 64 individuals in the stable transmission area tested by ELISPOT for IFN-γ had a positive blood smear for *P. falciparum* at the time of blood collection. One of the 65 individuals tested by ELISPOT for IFN-γ had a positive blood smear for *P. falciparum*. There was no relationship between blood smear results and IFN-γ ELISPOT responses or levels in the stable transmission area (all *P* values for differences in IFN-γ ELISPOT responses or levels between smear negative and smear positive >0.30). Comparisons were not carried out for the unstable transmission area as only one individual had a positive blood smear in the unstable transmission area.

Forty-one individuals had samples tested by both ELISA and ELISPOT. Of the 22 individuals tested in the stable-transmission area, five were positive by ELISA and one was positive by ELISPOT, but only one was positive by both methods. In the unstable-transmission area, of the 19 individuals tested, one was positive by ELISA and four were positive by ELISPOT; however, none were positive by both methods. Furthermore, there was

no relationship between the numbers of IFN-γ-secreting cells determined by ELISPOT and IFN-γ cytokine levels measured by ELISA in the stable transmission area (r = –0.05; P = 0.83) or unstable transmission area (r = –0.11; P = 0.67).

HLA class I restriction of IFN-γ and IL-10 responses to MB2

There were no significant differences in the frequencies of IFN-γ responses to MB2 as measured by ELISPOT or ELISA in individuals with the HLA class I supertypes (HLA-A24 and/or B7) to which the 9-mer MB-2 peptides were predicted to bind as compared to those without these HLA class I supertypes [Table 3]. Similarly, IL-10 responses did not differ according to presence HLA-A24 or B7 [Table 3]. Numbers of IFN-γ secreting cells and levels of IFN-γ and IL-10 produced in response to MB2 also did not differ significantly in those with versus without HLA A24 or B7 supertype [Table 3].

DISCUSSION

This study demonstrates that the *P. falciparum* antigen MB2 induces IFN-γ and IL-10 responses in adults in areas of stable and unstable malaria transmission. The MB2 antigen is a novel multi-domain sporozoite surface protein of *P. falciparum*. Its antigenic region is highly conserved,[5] and it is localized in a stage-dependent manner at sporozoite, liver, blood and gametocyte stages;[6] thus, it is a potential target for both antibody- and cell-mediated immune responses at multiple stages.[5,6] IFN-γ and IL-10 responses to other pre-erythrocytic antigens such as CSP and TRAP have been associated with protection from clinical malaria.[15,17] The induction of these responses by MB2 suggests that MB2 may be a potential vaccine candidate antigen, though further studies are required to assess whether these responses to MB2 are also associated with protection from clinical malaria. Our study was not powered to detect small differences in frequency of response between the two sites; so, we can conclude that MB2 induces cytokine responses in areas of stable and unstable transmission and that there are not large differences in immune responses (>50% difference) between the areas.

Previous studies in areas of stable malaria transmission have shown that IFN-γ and IL-10 responses are associated with protection from malaria infection and disease. In Kenya, Gambia, Papua New Guinea, Gabon, IFN-γ responses to CSP,[15] LSA-1,[10,17,18] and merozoite surface protein-1,[34] and IL-10 responses to LSA-1,[24] correlated with protection from clinical malaria with *P. falciparum*. In areas of unstable transmission, IFN-γ and IL-10 responses to CSP,[17] and LSA-1,[9,17,29] have also correlated with protection from infection or disease.

There was no difference in response to MB2 antigen between individuals who had or did not have the HLA-A24 and/or B7 alleles, suggesting that the IFN-γ and IL-10 responses to the MB2 antigen is not HLA restricted. HLA is important to the host in the defense against malaria infection,[35] and an HLA restricted immunity to T-cell epitopes has been suggested to reduce immunogenicity and efficacy of a subunit vaccine.[36] HLA class I restriction has been shown to be important in cellular immune response to *P. falciparum* antigens in some studies,[36,37] but not others.[29]

The frequencies and levels of IFN-γ responses to MB2 by ELISA and ELISPOT were comparable to those reported for other pre-erythrocytic and blood-stage antigens.[17,28,29] Similar to analysis of IFN-γ responses to TRAP and LSA-1 in the same study populations,[17] no relationship was observed between the numbers of IFN-γ-secreting cells (as measured by ELISPOT) and the IFN-γ cytokine production (as measured by ELISA) in response to MB2 peptides in individuals in the present study. These findings suggest that a few IFN-γ-secreting cells might be responsible for the production of large amounts of IFN-γ. The IFN-γ ELISPOT assay was found to be slightly more sensitive for detection of immune responses to *P. falciparum* antigens in one study,[38] but not in another.[17] In the only study to date comparing IFN-γ responses to *P. falciparum* antigens by both assays to protection from clinical malaria, responses by ELISA, but not ELISPOT were found to correlate with protection.[17] However, in vaccine studies of the CSP-based RTS, S vaccine, IFN-γ responses to CSP by ELISPOT were correlated with vaccine-associated protection from clinical malaria.[39] At present, it is not clear which assay provides the most relevant information for field studies of malaria and further assessment with both assays is needed.

CONCLUSIONS

This study establishes that MB2 induces IFN-γ and IL-10 responses in adults in areas of stable and unstable transmission and that these responses are not HLA restricted. Since IFN-γ and IL-10 responses to other *P. falciparum* antigens have been associated with protection from clinical malaria, the present study sets the stage for future studies, in which the correlation between IFN-γ and IL-10 responses to MB2 and protection from clinical malaria is assessed.

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Footnotes

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Conflict of Interest: None declared.

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Figures and Tables

Table 1

ELISA IFN-γ and and IL-10 responses to MB2 in adults in areas of stable and unstable transmission

Cytokine	Stable transmission area	Unstable transmission area	*P*
IFN-γ			
No. positive/total (%)	11/40 (27.5)	9/48 (18.8)	0.33*
Geometric mean (range), pg/mL	113.9 (0-2896.4)	63.9 (0-1064.2)	0.26†
IL-10			
No. positive/total (%)	10/40 (25.0)	11/49 (22.5)	0.78
Geometric mean (range), pg/mL	65.3 (0-353.4)	39.6 (0-506.9)	0.55

*χ^2 test comparing frequencies of positive responses in areas of stable and unstable transmission; $P<0.05$ significant. †Wilcoxon rank-sum (Mann-Whitney) test; $P<0.05$ significant. IFN-γ: Interferon-gamma; IL: Interleukin

Table 2

ELISPOT IFN-γ response to MB2 in adults in areas of stable and unstable transmission

	Stable transmission area	Unstable transmission area	*P*
No. positive/total tested (%)	7/64 (10.9)	7/65 (10.8)	0.98*
Geometric mean SFU (range)	11.7 (0-98.0)	23.6 (0-195.0)	0.16†

*χ^2 test; $P<0.05$ significant. †Wilcoxon rank-sum (Mann-Whitney) test; $P<0.05$ significant. ELISPOT: Enzyme-linked immunosorbent spot; IFN-γ: Interferon-gamma; SFU: Spot-forming units

Table 3

Cytokine response in individuals with/without HLA A24 and/or B7 in areas of stable and unstable malaria transmission

Assay	Stable transmission area			Unstable transmissio	
	Supertype +ve	Supertype –ve	*P*	Supertype +ve	Supertyp
IFN-γ ELISPOT					
No. positive/total (%)*	1/24 (4.2)	7/36 (19.4)	0.13	3/26 (11.5)	5/36 (1
Geometric mean SFU (range)†	13.2 (0-54.0)	12.1 (0-98.0)	0.22	24.7 (0-142.5)	23.0 (0-1
IFN-γ ELISA					
No. positive/total (%)*	4/13 (30.8)	5/21 (23.8)	0.70	2/17 (11.8)	4/27 (1
Geometric mean pg/ml (range)†	194.6 (0-2896.4)	84.5 (0-1497.5)	0.77	40.8 (0-248.1)	61.6 (0-5
IL-10 ELISA					
No. positive/total (%)*	2/13 (15.4)	5/21 (23.8)	0.68	3/17 (17.7)	6/27 (2
Geometric mean pg/ml (range)†	42.7 (0-114.2)	67.9 (0-193.6)	0.96	48.6 (0-279.6)	32.2 (0-5

*Fisher's test; $P<0.05$ significant. †Wilcoxon rank-sum test; $P<0.05$ significant. HLA: Human leu ELISA: Enzyme-linked immunosorbent assay; ELISPOT: Enzyme-linked immunosorbent spot; SFU: Spot-f