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Ex Vivo Cytokine and Memory T Cell Responses to the 42-kDa Fragment of \textit{Plasmodium falciparum} Merozoite Surface Protein-1 in Vaccinated Volunteers\textsuperscript{1}

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A number of blood-stage malaria Ags are under development as vaccine candidates, but knowledge of the cellular responses to these vaccines in humans is limited. We evaluated the nature and specificity of cellular responses in healthy American volunteers vaccinated with a portion of the major merozoite surface protein-1 (MSP1) of \textit{Plasmodium falciparum}, MSP1\textsubscript{142}, formulated on Alhydrogel. Volunteers were vaccinated three times with 80 \textmu g of either MSP1\textsubscript{42}-FVO/Alhydrogel or MSP1\textsubscript{42}-3D7/Alhydrogel. Cells collected 2 wk after the third vaccination produced Th1 cytokines, including IFN-\gamma and IL-2 following Ag stimulation, and greater levels of the Th2 cytokines IL-5 and IL-13; the anti-inflammatory cytokine IL-10 and the molecule CD25 (IL-2R\alpha) were also detected. The volunteers were evaluated for the MSP1\textsubscript{42–FVO} or MSP1\textsubscript{42–3D7} specificity of their T cell responses. Comparison of their responses to homologous and heterologous Ags showed ex vivo IFN-\gamma and IL-5 levels that were significantly higher to homologous rather than to heterologous Ags. The epitopes involved in this stimulation were shown to be present in the dimorphic MSP1\textsubscript{133} portion of the larger MSP1\textsubscript{42–3D7} polypeptide, and indirect experiment suggests the same for the MSP1\textsubscript{42–FVO} polypeptide. This contrasts with B cell responses, which were primarily directed to the conserved MSP1\textsubscript{19} portion. Furthermore, we explored the maturation of memory T cells and found that 46% of vaccinees showed specific memory T cells defined as CD4\textsuperscript{+}CD45RO\textsuperscript{+}CD40L\textsuperscript{+} after long-term in vitro culture. The identification of human-specific CD4\textsuperscript{+} memory T cells provides the foundation for future studies of these cells both after vaccination and in field studies. The Journal of Immunology, 2008, 180:1451–1461.

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\textsuperscript{3}Abbreviations used in this paper: MSP1, merozoite surface protein-1; SFU, spot-forming unit; FoxP3, forkhead-box-P3 transcriptional repressor; Treg, regulatory T cell.


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(13). Although the basis for this Ab decay is not understood, at least two mechanisms have been proposed: 1) evasion and modulation of cells of the immune system by the parasite, and 2) normal regulatory mechanisms of the immune system. Wykes et al. (14) studied the generation of memory B cells and long-lived plasma cells after vaccination with MSP119 in a rodent model of malaria. They suggested that malaria infection triggers apoptosis of Ag-specific B cells following vaccination and speculated that repeated infection of humans could delete circulating Ag-specific memory B cells. In this context, Dorfman et al. (15) compared memory B cell ELISPOTs directed to MSP1 to those of tetanus toxoid in individuals living in Kenya and found fewer malaria-specific cells, suggesting reduced parasite specific memory B cells. Recently, Lee et al. (16) reported a putative mechanism by which memory T cells are turned off and T cell priming is suppressed in response to an allelic form of MSP1 common in a malaria endemic region. They found inhibition of T cell proliferation and IFN-γ production after stimulation with malaria peptides from the common allelic form of the protein but not the alternate one.

In contrast, Drakely et al. (17) have recently reported that, once acquired, Ab responses to MSP1, and other asexual stage Ags seem to persist for many years based on studies in multiple villages in Tanzania. Moreover, Struijk and Riley (18) have compiled a number of epidemiological studies that assert Ab and B cell memory responses to malaria appear relatively normal, although certain characteristics of Plasmodium infection might undermine the effectiveness of memory responses, including possible influence of regulatory T cells (Tregs) that suppress Th1 and Th2 responses by secretion of IL-10 and TGF-β. This subpopulation of Tregs has not been examined in malaria until recently (19, 20), and may be critical to adaptive responses to infection.

Most studies of MSP1 as a malaria vaccine candidate have focused on Ab production, whereas little is known about the induced cellular responses induced or about specific memory T cell generation. A phase I clinical trial of two allelic forms of MSP1 formulated independently on Alhydrogel or MSP1-3D7 formulated independently on Alhydrogel has allowed us to study T cell responses to this important vaccine candidate in malaria-naive adult volunteers. Participants received 80 µg of either MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel at 0, 1, and 6 mo (21). We investigated the ex vivo cytokine responses of PBMCs from the vaccinated volunteers to these two allelic forms of MSP1 and asked whether the epitopes responsible for these responses were localized to the dimorphic N-terminal MSP113 or the relatively conserved C-terminal MSP119. Furthermore, we sought to identify specific memory T cells directed to MSP142 using long-term in vitro culture of PBMC, focusing on CD4+ T cells expressing the CD40L and CD45RO markers. The optimization of these techniques and the results obtained provide important information for future MSP1 vaccine trials and set the groundwork for additional investigation of cellular responses to this major blood-stage Ag in field studies.

Materials and Methods

PBMC isolation

PBMC were isolated from whole blood (BD Vacutainer Cell Preparation Tubes) from 17 volunteers that participated in a phase I trial in malaria-naive U.S. adult volunteers (21). Nine volunteers received three vaccinations of MSP142-3D7/Alhydrogel and eight received three vaccinations with MSP142-FVO/Alhydrogel and were used for this study. Because PBMC were not collected on day 0 before vaccination, all methods used in this study were optimized to allow the use of unstimulated cells from vaccinees or PBMC from healthy nonvaccinated volunteers as controls, which were provided by DTM Research (Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD). The clinical protocol and related documents were reviewed and approved by the Heartland Institutional Review Board (trial site Institutional Review Board), the PATH Human Subjects Protection Committee, and the National Institute of Allergy and Infectious Diseases Institutional Review Board (Protocol NCT00340431; www.clinicaltrials.gov). All subjects gave informed consent for samples collected for this study. PBMC were frozen in heat-inactivated FBS (Invitrogen Life Technologies) containing 5% DMSO (Sigma-Aldrich) in liquid nitrogen until analysis.

PBMC thawing and stimulation

Cells were thawed in incomplete medium (RPMI 1640; Invitrogen Life Technologies) containing 50 U/ml DNase (Benzonase nuclease; Novagen), then washed, resuspended in complete medium (RPMI 1640 containing 10% FBS; Invitrogen Life Technologies), and incubated at 37°C and 5% CO2 in air for 24 h without any stimulation. Cells were then resuspended at 2 × 10^6 cells/ml and stimulated with 10 µg/ml clinical grade MSP142 produced in Escherichia coli (21), in other recombinant proteins described below, or in mitogens PMA and ionomycin (Sigma-Aldrich) at concentrations of 10 and 500 ng/ml, respectively. Mitogen stimulation was used as positive control to assess the functional potential of each sample. Viability for each sample was tested and only samples with >90% viable cells were used.

Recombinant proteins

The MSP142-FVO and the MSP142-3D7 proteins used in these studies were produced in E. coli under cGMP conditions and were the same lots used in the MSP142 clinical trial. For the polypeptides representing portions of the MSP142, E. coli BL21 (DE3) transformed with MSP142-3D7 plasmid and Saccharomyces cerevisiae clones expressing MSP119-3D7 or MSP119-FVO were fermented and purified essentially as described by Shimpi et al. (22). E. coli MSP119-FVO was produced in the same manner, but because of its low solubility in an aqueous buffer, it was not suitable for use in cellular assays and was not further investigated. MSP142-3D7 and MSP119-FVO proteins were supplied in PBS, while the MSP119-3D7 was in PBS plus 0.2% polysorbate 80. Purified recombinant proteins were fully characterized by reverse-phase HPLC, size exclusion-multiaight light scatter HPLC, N-terminal sequencing, electron-spray ionization mass spectrometry, SDS-PAGE (reduced and nonreduced), and immunoblot essentially as previously described (22). The observed results were similar to those expected for each recombinant protein. The endotoxin concentrations were <26 endotoxin units/ml for all of the recombinant proteins by chromogenic Limulus amebocyte lysate assays (LAL Associates of Cape Cod). Recombinant proteins were stored at −80°C before use.

ELISPOT assays

Ex vivo ELISPOT assays were conducted to enumerate Ag-specific PBMC producing IFN-γ, IL-5, or IL-10. MultiScreen 96-well plates (Millipore) were coated overnight at 4°C separately with either 15 µg/ml anti-human IFN-γ (clone 1-D1K), anti-human IL-5 (clone TRFK5), or anti-human IL-10 (clone 9D7) Abs; all Abs used for ELISPOT were obtained from Mabtech. PBMC previously stimulated with MSP142, MSP133, or MSP119 Ags for 24 h were transferred to coated plates and incubated for an additional 24 h to detect IFN-γ, and 48 h to detect IL-5 or IL-10. The assay plates were washed, and 1 µg/ml biotinylated anti-human IFN-γ (clone 7-B6-1), anti-human IL-5 (clone TRFK5), or anti-human IL-10 (clone 9D7) Abs were added, and plates were incubated with biotinylated Abs for 2 h at room temperature and washed, streptavidin-alkaline phosphatase (Mabtech) was added, and plates were incubated for 1 h at 37°C. After washing, 50 µl of substrate NBT/BCIP (Pierce) were added to the plates to visualize the spots. Plates were scanned and counted by Cellular Technology.

Measurement of cytokines in supernatants

Cytokines were quantified in culture supernatants of PBMC collected 72 h after cell stimulation with MSP142 proteins using SearchLight proteome arrays (Pierce). Briefly, samples were diluted 1/50 or 1/1000, 1 h before incubation on the array plates that were prespotted with capture Abs specific for each protein biomarker. Plates were decanted, and washed three times before adding a mixture of biotinylated detection Abs to each well. After incubating with detection Abs for 30 min, plates were washed three times and incubated for 30 min with streptavidin-HRP. Plates were again washed before adding SuperSignal Femto Chemiluminescent substrate. Plates were immediately analyzed using the SearchLight imaging system, which is a cooled charge-coupled device camera, and data was analyzed using ArrayVision software (Pierce). The luminescent signal produced is proportional to the amount of each protein present in the original
standard or sample and concentrations in test samples were estimated from a standard curve.

**Long-term culture for memory cell enrichment**

Due to limited PBMC numbers, only 14 samples were available for this experiment, seven PBMC samples from each group vaccinated with MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel were cultured for a total of 10 days. Long-term culture of PBMC was used to investigate the generation of memory T cells, following a procedure previously described (23). In that previous experiment, PBMC from seven normal healthy non-vaccinated volunteers were included. Briefly, PBMC were stimulated with 10 µg of MSP142-FVO or MSP142-3D7, independently, for 72 h. Following Ag removal, cells were maintained in culture until the day 10, and fresh culture medium was added on days 7 and 9. On day 10, PBMC were pelleted and adjusted to 1 × 10⁶ cells in 500 µl of culture medium per sample and stimulated for 12 h with the same Ags.

**MSP142-specific IgE measurement**

Serum samples from volunteers vaccinated with MSP142-FVO/Alhydrogel (n = 3 volunteers providing samples) and MSP142-3D7/Alhydrogel (n = 5 volunteers providing samples), each produced the highest levels of specific IgE Abs against homologous Ag (400 or more ELISA units on day 194) (21), were selected for IgE evaluation against homologous and heterologous Ags using suspension array technology (24). Serum samples from days 0 and 194 were diluted 1/50 in 1% BSA/PBS and incubated for 1 h with microspheres coupled with MSP142-FVO or MSP142-3D7 in Multiscree plates (Millipore). Goat anti-human IgE (Kirkegaard & Perry Laboratories) and secondary PE-labeled donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) were added to develop the reaction. The mean fluorescence intensity corresponding to MSP142-specific IgE was measured by Luminex X-MAP (Millipore) with the software Bioplex (Bio-Rad).

**Abs and flow cytometry**

All Abs and flow cytometry reagents were purchased from BD Biosciences Immunocytometry unless otherwise indicated. Reagents were used according to the manufacturer’s instructions. Abs against CD154 (anti-CD40L-PE-Cy5) were added in the last 12 h, essentially as previously described (25-26). Monoclonal Abs anti-CD28 and anti-CD94 were added to enhance stimulation, and Golgi Stop (monensin-based reagent) was used to block the release of cytokines and facilitate intracellular detection of cytokines. For phenotypic analysis, PBMC were stained simultaneously with the following panels of Abs: Panel 1 (for memory cell identification) CD3-Pacific blue, CD4-PE-alkaline phosphatase, CD8-PE-Cy7, CD45RA-PE-Cy5, CD45RO-PE-Cy5, CD45RA-PE-Cy7, and Panel 2 (for proliferation and viability) CD3-Pacific blue, CD4-alkaline phosphatase, CD5-PE-Cy5, 10 mM CFSE was added in day 5 of the 10-day culture (Invitrogen Life Technologies), 7-aminomatinycin D, CD25-PE Cy7, CD45RO-alkaline phosphatase. Positive controls included PBMC stimulated with mitogen PMA/Ionomycin. The fluorescence minus one procedure described by Baumgarth and Roederer (27) was used for negative control and gating. All but one of the reagents of each stain panel are present to discriminate between background and specific staining. Labeled cells were fixed with 1% paraformaldehyde, and data acquisition of at least 40,000 events was performed using a Cyan Flow Cytometer (DakoCytomation).

**Data analysis**

Flow cytometric data was analyzed using FlowJo version 8.3 (Tree Star). A paired t test was used for comparisons of cytokine production and IgE determination. ANOVA and Dunnett’s multiple comparison tests were performed when more than two variables were compared with a control.

**Results**

**Ex vivo Th1 and Th2 cytokine responses to MSP1 vaccine Ags**

PBMC were obtained from volunteers immunized with 80 µg of either MSP142-FVO or MSP142-3D7, each formulated on Alhydrogel. To examine the cytokine profiles produced in response to Ag stimulation, PBMC obtained 2 wk after the third vaccination were incubated with 10 µg/ml immunizing Ag. This Ag concentration was optimized so that background levels of cytokines were obtained when 10 nonvaccinated volunteers were tested. Spot-forming units (SU) obtained per 10⁶ cells were 0–10 for IFN-γ, IL-5, and IL-10, indicating that the SU frequency after Ag stimulation was specific for the Ag tested. When PBMC from vaccin-ated individuals was tested by ELISPOT, the number of both IFN-γ and IL-5-producing cells was significantly higher than the number found in unstimulated controls (p < 0.01, by ANOVA and Dunnett’s multiple comparison test). Fig. 1A shows that after stimulation with the immunizing Ag, the average number of SUF in PBMC of volunteers immunized with MSP142-FVO increases from 60 to 600 SUF/10⁵ cells in the case of IFN-γ and from 12 to 305 SUF/10⁵ cells in the case of IL-5 (Fig. 1B); similar increases over the control are seen with PBMC from volunteers immunized with the 3D7 allelic form of MSP142 and stimulated with the same protein (Fig. 1, C and D). The increase in SUF was greater in the PBMC from volunteers immunized with the FVO than with the 3D7 allele for IFN-γ, suggesting that the FVO might be more immunogenic (p = 0.0007 by unpaired t test).

When ex vivo cytokine analysis of supernatant fluids was performed on the same set of cells, significantly more IL-2, IL-5, and IL-13 (paired t test, p < 0.05) was secreted by cells stimulated for 72 h with the homologous FVO or 3D7 allelic forms of MSP142 for each group of vaccinees as compared with unstimulated cells (Fig. 2, A and B). However, no statistically significant differences in levels of IFN-γ were measured between Ag-stimulated and unstimulated PBMC (Fig. 2, A and B). This finding was true whether the supernatant fluids were tested at 24, 48, or 72 h or in vitro culture. The differences in the results for IFN-γ and the other cytokines might be explained by the different methodologies involved in these two techniques and the higher sensitivity of ELISPOT analysis.

Taking the response to homologous Ag in all volunteers together, the ratio of IL-5 to IFN-γ SUF (6.9 ± 1.7), and the ratio of IL-5 to IFN-γ cytokine levels in supernatant fluids (6.07 ± 1.3) both reflect a predominately Th2-skewed cytokine profile. T cells (CD3⁺CD4⁺) were shown to be the source for both IFN-γ and IL-5 production.
IL-5 by intracellular cytokine staining experiments as determined after 72 h of stimulation with the homologous Ag, whereas CD3+CD8+ cells did not produce these cytokines (data not shown). Stimulation with homologous vaccine Ags also induced the secretion of the inflammatory cytokine TNF-α (p < 0.05 by paired t test) in culture supernatants (data not shown). In addition, our results suggest stronger responses in volunteers vaccinated with MSP142-FVO than those vaccinated with MSP142-3D7, which is statistically greater for IFN-γ-producing cells and IL-13 in supernatants (p < 0.05 by unpaired t test).

Finally, we measured MSP142-specific IgE Abs in serum samples from a subset of volunteer samples on days 0 and 194 using suspension array technology with MSP142-coupled beads incubated with sera and anti-IgE Abs, which were quantified using a Luminex system. In a subset of serum samples with IgG anti-MSP142, tested with five selected samples that previously were shown to produce >200 IL-5 SFU/10^6 cells. Also, six available samples from the group that received MSP142-FVO, with variable responsiveness, were stimulated with MSP142-FVO and MSP142-FVO. For both groups of vaccinees, the homologous MSP142 polypeptides did not induce significant activation of PBMC (p > 0.05, determined by ANOVA and Dunnett’s multiple comparison test) as compared with activation in unstimulated control cultures. *p < 0.05.

4.3 and 210, respectively, *p < 0.01, as determined by paired t test), also indicative of a Th2-type response (Fig. 2C).

Allelic specificity induced by vaccination

PBMC from the two groups of volunteers vaccinated with MSP142FVO/Alhydrogel or MSP142-3D7/Alhydrogel were stimulated independently with the homologous and heterologous Ags to examine the allelic specificity of cellular responses and to assess whether there was cross-reactivity in the ex vivo cytokine responses. Fig. 1 illustrates IFN-γ and IL-5 ELISPOT data from cells from the eight volunteers immunized with MSP142-FVO/Alhydrogel and nine volunteers immunized with MSP142-3D7/Alhydrogel. Although there were significant increases in both IFN-γ- and IL-5-producing cells after stimulation with the homologous Ag, when the heterologous Ag was used, the differences in responses compared with background levels with medium alone were not statistically significant (p > 0.05, determined by Dunnett’s multiple comparison test and considered not significant). This result suggests that the responses to T cell epitopes measured in this experiment are allele-specific. The region of MSP142 that induced the observed cellular stimulation was localized by measuring cytokine responses to MSP142-3D7 and its fragments MSP119 and MSP133, tested with five selected samples that previously were shown to produce >200 IL-5 SFU/10^6 cells. Also, six available samples from the group that received MSP142-FVO, with variable responsiveness, were stimulated with MSP142-FVO and MSP142-FVO. For both groups of vaccinees, the homologous MSP142 polypeptides did not induce significant activation of PBMC (p > 0.05, determined by ANOVA and Dunnett’s multiple comparison test) as compared with activation in unstimulated cells (Fig. 3). In contrast, MSP133-3D7-stimulated cytokine responses

**FIGURE 2.** Ex vivo cytokine production by PBMC from volunteers vaccinated with MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel and IgE measurement of high responders. Cytokine production of PBMC from volunteers vaccinated with 80 µg of MSP142-FVO/Alhydrogel (n = 8) (A) or MSP142-3D7/Alhydrogel (n = 9) (B). PBMCs were obtained 2 wk after three vaccinations. Cytokine levels were assessed at 72 h after in vitro restimulation with specific Ag and are expressed in picogram/milliliter. Data are mean cytokine production by unstimulated PBMC (□) and PBMC after stimulation with the homologous MSP142 protein (●), and error bars represent SE. *p < 0.05 statistically significant differences between stimulated PBMC and control determined by paired t test. C. Ag-specific IgE measurement in serum obtained on day 0 and 2 wk after three vaccinations (day 194) in samples in which anti MSP142-IgG were higher than 400 ELISA units (n = 8). *p < 0.01 for significant difference between MFI in day 0 and day 194 samples.

**FIGURE 3.** Localization of T cell epitopes to MSP142. The mean frequency of IL-5 producing PBMC was enumerated by ELISPOT assay of samples from volunteers vaccinated with MSP142-3D7/Alhydrogel (n = 5 samples) (A) or MSP142-FVO/Alhydrogel (n = 6) (B) after stimulation with the homologous recombinant MSP142, MSP133 (only MSP142,3D7 polypeptide), or MSP119 polypeptides or medium alone (control). Mean of triplicate values from each volunteer sample are represented (●), and mean of the group is also shown (□). Significant differences were determined among vaccinees T cell responses by ANOVA analysis and Dunnett’s multiple comparison tests. Only MSP133 and MSP142 induced responses significantly different from unstimulated control cultures. *p < 0.05.
were comparable to responses induced with MSP1<sub>42</sub>-3D7 when tested with PBMC of volunteers vaccinated with MSP1<sub>42</sub>-3D7/Alhydrogel (p < 0.01, Dunnett’s multiple comparison test), as seen in Fig. 3; for technical reasons it was not possible to do the corresponding experiment with the MSP1<sub>13</sub>-FVO. The represented values are obtained from a subset of vaccinees with sufficient PBMC available for study and are not a comparison of responsiveness between the two groups.

These results show directly that nearly all the stimulation with the intact 3D7 Ag can be accounted for by the N-terminal 33-kDa portion of the MSP1<sub>13</sub>-3D7 polypeptide; indirect evidence supports a similar conclusion for the FVO allelic form of the protein.

**IL-10 production and CD25 (IL-2Rα)**

The cytokines IL-10, TGF-β, IL-2, and other molecules such as CTLA-4 or CD25 (IL-2Rα) have been implicated in negative regulation of immune reactions to maintain immune homeostasis and self-tolerance (28). We investigated the production of IL-10 in culture supernatants of stimulated PBMC in eight volunteers vaccinated with MSP1<sub>42</sub>-FVO/Alhydrogel and eight vaccinated with MSP1<sub>42</sub>-3D7/Alhydrogel, and in both cases we found IL-10 production significantly higher than the controls (p < 0.05, by paired t test) (Fig. 4, A and B). By using ELISPOT, five samples from volunteers vaccinated with MSP1<sub>42</sub>-3D7 showed also to have more IL-10 SFU than found in samples from the controls (p < 0.05, by paired t test) (data not shown). To examine whether cells from individual volunteers showed any relationship between production of IFN-γ and IL-10, we tested the correlation of the production of these two cytokines after stimulation with the homologous Ag and found significant positive correlation (p < 0.0001, R² = 0.79) (Fig. 4C).

The presence of IL-2Rα in supernatants was investigated in both groups of vaccinees, receiving MSP1<sub>42</sub>-FVO/Alhydrogel and MSP1<sub>42</sub>-3D7/Alhydrogel (n = 8 volunteers in each group), and we found greater levels of soluble IL-2Rα in PBMC stimulated with the homologous Ag than in unstimulated PBMC (p < 0.01, by paired t test), as shown in Fig. 5, A and B. Both the cytokine IL-2 and the soluble IL-2Rα detected in supernatants at day 3 correlated with lymphocyte proliferation measured in the last part of the 10-day culture, measured from day 5 through day 10 (p = 0.0081, R² = 0.49 and p = 0.0175, R² = 0.39, respectively) (Fig. 5, C and D). Low correlation values could be accounted for by the difference in timing for these measurements (one value on day 3 and the other on day 10). Measurements of IL-2 and soluble IL-2Rα showed a positive correlation (p = 0.01, R² = 0.41) (Fig. 5E). When we compared expression of IL-2Rα (CD25) on lymphocyte surfaces measured at day 10 of culture with proliferation, determined as dilution of CFSE added on day 5, a significant inverse correlation was found (p = 0.0001, R² = 0.8) (Fig. 5F); greater expression of CD25 was associated with less proliferation.

**Generation of memory cells**

The hallmark of vaccines is the generation of specific memory cells and their maintenance over time. To address the question whether these MSP1<sub>42</sub> vaccines induced the generation of specific memory T cells, we followed a procedure established for enriching memory T cells developed by Keating et al. (23). Fourteen PBMC samples (seven samples from the group that received MSP1<sub>42</sub>-FVO/Alhydrogel and seven samples from the group that received MSP1<sub>42</sub>-3D7/Alhydrogel), were enriched for memory cells by a 10-day culture period consisting of 3 days of Ag stimulation, 7 days of resting, and restimulation again on day 10 with the same Ag. After the second in vitro stimulation with homologous MSP1<sub>42</sub> Ag, cells were analyzed by flow cytometry using two different panels of Abs (a panel for proliferation and viability and a panel for memory cell identification). Fig. 6A shows that two populations of cells (R1 and R2) can be identified by forward and side light scatter in 10-day cultures. CFSE staining showed that the larger size of the second population is the result of proliferation (Fig. 6A, R2). As identified by progressive dilution of CFSE, >80% of cells proliferated (Fig. 6B, R2) vs only 40% (or less) of cells proliferated (Fig. 6B, R1). In Fig. 6C, R2 shows predominately live cells when stained with 7-aminoactinomycin D with >95% viable cells as compared with 55% or more dead cells (Fig. 6C, R1), or with >90% of CD3<sup>+</sup> cells (R2, data not shown). Hence, we defined R2 as our population of interest for subsequent analysis, and cultured PBMC were gated on this population. Fig. 7A shows R2 population gated and plotted for expression of CD4 and CD40L. When the memory differentiation marker CD45RO<sup>+</sup>
was examined (Fig. 7B), vaccinated volunteers expressed CD45RO+ at a significantly higher frequency than nonvaccinated volunteers \((p < 0.05)\) by unpaired \(t\) test. An arbitrary threshold of positivity for specific responses to MSP1 42, defined by a CD4\(^+\)CD45RO\(^+\)CD40L\(^+\) phenotype, equal to the mean of coexpression values for CD4, CD45RO, and CD40L plus two SDs, was determined in samples from seven healthy malaria-naive volunteers used as a negative control group. Fig. 7C shows 6 of 13 individuals with positive specific memory T cells against MSP1 42 based on this threshold. In addition we observed that specific memory T cells against MSP1 42 ranged from 0.4 to 2.04% in the vaccinated population. The lymph node homing receptors CCR7 and CD62 ligand were also used to identify central memory T cells (29); four samples were CCR7+ and two samples were CD62

FIGURE 6. Flow cytometric analysis for quantification of memory T cells. **A**, Cell populations detected in PBMC cultured for 10 days with homologous MSP1 42 are designated R1 and R2. Population R2 has a higher forward scatter dispersion, which indicates larger size. **B**, CFSE staining indicates that the R2 population (red) shows more proliferation compared with the R1 population (blue) (81.6 and 42.7%, respectively). **C**, The viability of the two populations determined by 7-aminoactinomycin D (7AAD) staining shows that the majority of the R2 population (blue) cells were alive (4.4% dead cells or 95.6% viable), whereas R1 (red) stained with 7-aminoactinomycin D indicating few viable cells (44.8%). Data are from one representative sample of 14 analyzed.
ligand-positive (data not shown). These results suggest the presence of a specific memory T cell population in some vaccinees.

Ex vivo IFN-γ production correlates with specific memory T cell formation

Direct demonstration of memory T cells involves laborious techniques, thus we sought to correlate the frequency of specific memory T cells (CD4+CD154+ (CD40L) identifies MSP142-specific T cells. Only vaccinated volunteers had detectable Ag-specific T cells. Unpaired t test p < 0.01). B, The frequency of double positive CD4+CD45RO+ population is also different from nonvaccinated volunteers, with 2-fold increase in MSP142/Alhydrogel-vaccinated volunteers. Data from representative samples in A and B of a nonvaccinated volunteer (left) and a vaccinated volunteer (right). Comparable results were obtained in two different experiments (n = 15). C, Expression of CD4+CD154+CD45RO+ as a percentage of R2 in samples from nonvaccinated (controls) and vaccinated volunteers; positivity (dashed horizontal line) was designated as the mean of the control volunteers plus two SDs.

Discussion

For malarial Ags of pre-erythrocytic stages, particularly the circumsporozoite protein, investigations of the cellular response elicited by these Ags suggest that CD8+ T cell-mediated immunity plays a crucial role against parasite Ags found in the infected liver cell (30, 31). In general, most investigations of Plasmodium blood-stage Ags have focused on humoral immunity, with the understanding that T cell priming is an important determinant of B cell differentiation, Ab class switching, and memory maintenance. However, there is little information about cellular response induced by vaccination with Plasmodium blood-stage Ags in humans. We have evaluated the cellular immune response directed toward two MSP142 protein subunit vaccines in a phase I clinical study.
trial with cells collected from volunteers 2 wk after the third and final vaccination. Using ex vivo ELISPOTs and cytokine measurements in supernatants of PBMC from vaccinated malaria-naive volunteers, we demonstrated that MSP1_{42} elicits T CD4-specific cellular responses after Ag-specific activation of PBMC.

An important issue in the development of subunit vaccines for blood-stage malaria is the use of antigenically variant proteins and the degree of immune coverage that can be achieved by the inclusion of a limited number of allelic variants. We addressed this question by studying the allelic specificity of immune responses induced by the two different forms of the dimorphic MSP1_{42} protein. PBMC from two groups of volunteers, vaccinated with either MSP1_{42}-FVO or MSP1_{42}-3D7 formulated independently on Alhydrogel, were stimulated with the homologous or heterologous immunogens to assess whether there was cross-reactivity in T cell responses. Both the level of cytokines in supernatants and the ELISPOT frequency demonstrated that significant stimulation occurred with the homologous Ag, whereas there was no response above the background levels when PBMCs were stimulated with the heterologous MSP1_{42} protein. This result contrasts markedly with the humoral response to the same Ag MSP1_{42} that is predominantly directed to MSP1_{19} and showed essentially complete cross-reactivity between dimorphic forms in these same volunteers (21).

We identified the region of MSP1_{42} that induced T cell stimulation by assessing cytokine responses to the MSP1_{19} and MSP1_{133} fragments of 3D7 allelic form in addition to the full-length protein (MSP1_{42}). For either vaccine group, the homologous MSP1_{19} regions did not induce significant activation of T cells as compared with unstimulated cells. In contrast, MSP1_{33} produced strong T cell responses when tested with PBMC from volunteers that received the MSP1_{42}-3D7/Alhydrogel (p < 0.01, ANOVA and Dunnett’s multiple comparison test) and these were comparable to responses with the complete polypeptide. The fact that MSP1_{19} did not induce cytokine production is in agreement with previous findings indicating that the compact disulfide-rich structure of MSP1_{19} interferes with Ag processing for presentation to T cells (32, 33). A study in mice immunized with overlapping peptides of the homologous Plasmodium yoelii MSP1_{42} suggested that MSP1_{133} contains several T cell epitopes of which two (Cm15 and Cm21) confer partial protection against lethal P. yoelii (34). In addition, previous field studies have identified T cell epitopes located primarily in the MSP1_{42} region (35, 36), and our study directly demonstrates that the MSP1_{133} portion of MSP1_{42} is responsible for virtually all the stimulation of T cells in MSP1_{42}-vaccinated human volunteers. Moreover, the dimorphism of MSP1_{133}, with 47% homology between allelic forms FVO and 3D7, explains the lack of cross-reactivity we found at the cellular level.

Interestingly, we did not find a correlation between cellular responses and serum Ab levels (21), which may be due to T and B cells recognizing completely different epitopes of MSP1_{42} or because weak responses limit our ability to see possible correlations. However, our finding is in agreement with a field study in which no correlation was found between cellular responses, judged by IFN-γ production and proliferation, and Ab levels against MSP1_{42} (32).

To further investigate the nature of the T cell responses, cytokines reflecting a Th1 profile (IFN-γ, IL-2) and a Th2 profile (IL-5, IL-13) were measured. The results obtained by ELISPOT showed a significantly greater number of IFN-γ spots in stimulated PBMC as compared with unstimulated cells, whereas amounts of IFN-γ detected by ELISA in supernatants were not significantly different from the control. The discordance of these results may be explained by a greater sensitivity of ELISPOT when compared with ELISA. As IFN-γ is produced, it is consumed by activated cells, and measurement in supernatants at a single time point by ELISA may not accurately reflect the true levels induced by a given stimulus. With ELISPOT, the high-affinity Abs coating the ELISPOT plate compete with the uptake by activated cells and even small amounts of the secreted cytokine are immobilized by these specific Abs that facilitate detection (37). Compared with Th1 cytokines, the Th2 cytokines IL-5 and IL-13 were produced in a much greater quantity when measured both by ELISPOT frequency and by secreted cytokine levels, indicating predominantly a Th2-biased response. Additional experiments confirmed significantly increased anti-MSP1_{42} IgE in several samples comparing titers at day 0 and 2 wk after the third vaccination. The Th2 bias and the increase in the levels of Ag-specific and total IgE Abs found in this study are concordant with the properties of Alhydrogel- or aluminum salt-adjuvanted vaccines (38, 39). Additional experiments with selected samples using intracellular cytokine staining indicated those cells identified as CD3^+ CD4^+ are the main source for both IFN-γ and IL-5, whereas CD8^+ T cells did not produce these cytokines (data not shown).

When we compared the response to the homologous MSP1_{42} between both groups of vaccinees, IFN-γ and IL-13, but not IL-5, were significantly higher in PBMC from volunteers vaccinated with MSP1_{42}-FVO. Preclinical animal studies assessing the immunogenicity of MSP1_{42}-FVO and MSP1_{42}-3D7 by Ab production in BALB/c mice and Aotus nancymai monkeys indicated that the MSP1_{42}-FVO was more immunogenic than the 3D7 allelic form (L.B. Martin and C.A. Long, unpublished data). However, in the human clinical trial from which the current PBMC were obtained, no statistically significant differences could be detected in the IgG Ab titers elicited by the FVO or the 3D7 allelic forms of MSP1_{42} (21). In future studies overlapping peptides of MSP1_{42}-FVO and MSP1_{42}-3D7 will be used to identify epitopes, which are more immunogenic.

IL-10 was measured in supernatants and IL-10-producing cells were enumerated by ELISPOT in the group that received MSP1_{42}-3D7. With these data, we established that IL-10 was produced by Ag-specific stimulated cells and was detected in culture supernatants at significantly greater levels than unstimulated controls. IL-10 is a cytokine initially described as a product of Th2 T cells, also as a product of Treg and more recently, as a product of Th1 T cells (40). Known functions of IL-10 include inhibiting IL-12 production in macrophages (41), which interferes with Th1 differentiation (42), and limiting Th2 responses (43). Our interest in ex vivo IL-10 measurements upon vaccination is based on the reported ratio of IFN-γ to IL-10 as an indicator of vaccine success in a mouse model for Leishmania (44) and in influenza vaccine in humans (45).

In our studies when IFN-γ and IL-10 were compared in individual samples, the production of IFN-γ and IL-10 were positively correlated, suggesting that the IL-10 may be synthesized to down-regulate the response to IFN-γ; additional studies will be required to investigate this possibility.

The molecule IL-2Rα (CD25) was also studied both in its soluble form in supernatants (sCD25) and on the surface of T cells by flow cytometry. The ex vivo measurement of soluble CD25 in supernatants of Ag-stimulated cells was significantly greater than the measurement of soluble CD25 from unstimulated cells in both MSP1_{42}-FVO/Alhydrogel and MSP1_{42}-3D7/Alhydrogel vaccinated groups. The cytokine IL-2 and its receptor IL-2Rα-chain are synthesized as part of the T cell proliferation process and as a consequence of the inflammatory response to Ag stimulation. Although measured at different times, IL-2 and IL-2Rα in supernatants (measured at 72 h) correlated with proliferation (measured at
10 days). Surface expression of IL-2Rα measured after 10 days of ex vivo culture was inversely correlated with proliferation. These findings are in agreement with the homeostatic role of IL-2 and its receptor IL-2Rα, which when produced upon stimulation form complexes and are released into medium to shape a population of cells that controls proliferation (46, 47). The expression of IL-2Rα on the surface is regulated directly by the transcription factor Foxp3, which has been recently described as a pivotal determinant of Tregs (48). These data suggest that this 10-day culture of PBMC could better define the dynamics of the memory and Treg populations.

There are no similar malaria blood-stage vaccine studies evaluating both Th1 and Th2 cytokines to which we can compare our results. The detection of IFN-γ-producing PBMC has been used previously to assess cellular responses to pre-erythrocytic stage malaria Ags. We obtained mean values of ELISPOTs to IFN-γ ranging from 40 to 60 SFU/10⁶ PBMC (six times less than values obtained for IL-5), which is comparable to other studies in which a recombinant fragment of the circumsporozoite protein was formulated with the adjuvants AS02A or Montanide ISA 720 (49). In previous studies, the use of a portion of the circumsporozoite protein with the hepatitis B surface Ag, formulated with AS02A (RTS,S/AS02A), showed greater IFN-γ production from PBMC that ranged from 100 to 500 SFU/10⁶ cells (50). Likewise, the recombinant poxvirus FP9 and modified vaccinia virus Ankara used as a prime and boost vaccine, respectively, for the thrombospondin-related adhesion protein and circumsporozoite protein vaccines also showed that ex vivo IFN-γ production predicted effector memory T cells for thrombospondin-related adhesion protein and circumsporozoite protein vaccines also showed that ex vivo IFN-γ production predicted effector memory response 3 mo after vaccination of malaria-exposed volunteers (57). We did not find correlation of specific memory T cells with members of the cytokine receptor γ-chain family, IL-2, IL-7, IL-15, or IL-4 (data not shown). These cytokines are known to control memory T cell development (58), and previous reports indicate that IL-4 and IL-4R are crucial to the development of memory T cells against sporozoite epitopes (59).

The cytokine profile associated with recombinant malaria protein vaccination is highly dependent on the adjuvant or carrier used in the formulation of the Ag. Our data suggest MSP1₄₂ adjuvanted on Alhydrogel elicits a weak Th1 response and a stronger Th2 response known to down-regulate the expression of IFN-γ (60). We also documented the production of IL-10 following vaccination and this might suppress Th1 or Th2 responses as well. Our findings that the Th1 responses correlate with specific memory T cells in a blood-stage vaccine, together with previously reported Th1 correlation with effector memory responses in pre-erythrocytic malaria vaccines (57), suggest that adjuvants that induce greater Th1 bias should be explored. A phase I clinical trial is in progress using these same Ags formulated with the addition of CpG oligodeoxynucleotide to the Alhydrogel formulation, which should promote a Th1 phenotype. Comparison of T cell results from the two trials will determine whether this case is indeed the result.

In conclusion, our study shows that MSP1₄₂/3D7/Alhydrogel and MSP1₄₂/FVO/Alhydrogel induce Th2-biased cytokine responses directed mainly to MSP1₃₃. Although the humoral response in vaccinated naive volunteers was directed predominantly to MSP1₁₀ and is highly cross-reactive (21), the cellular response was allele-specific and directed to the MSP1₃₃ region. If T cell responses to both allelic types are important, then vaccination with only one of the dimorphic forms will not elicit CD4+ T cell responses to both. Moreover, these vaccines induced specific memory T cells against MSP1₁₂₂, but the frequency of such cells was low and immunogenicity must be improved. The results provide an experimental foundation for future studies of cellular responses to malaria Ags both in subsequent clinical vaccine trials and in field studies to elucidate the mechanisms behind the development of immune responses to blood-stage malaria parasites.

Acknowledgments

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2510 –2516.
22: e12.
2: e12.
305: 6022 –6030.
154: 6022–6030.
1418 –1427.
17: 326 –332.
27: 257–297.
98: 10817–10822.
1113–1117.
48: 1047–1053.
269: 527–535.
46: 3024–3031.
26: 717–727.
24: 224 –228.
69: 2245–2251.
69: 2245–2251.
154: 944 –951.
2510 –2516.
289: 961–968.


