Identification of T Cell Epitopes on the 33-kDa Fragment of *Plasmodium yoelii* Merozoite Surface Protein 1 and Their Antibody-Independent Protective Role in Immunity to Blood Stage Malaria

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Identification of T Cell Epitopes on the 33-kDa Fragment of Plasmodium yoelii Merozoite Surface Protein 1 and Their Antibody-Independent Protective Role in Immunity to Blood Stage Malaria

Jiraprapa Wipasa,* Chakrit Hirunpetcharatt,† Yuvadee Mahakunkijcharoen,‡ Huji Xu,* Salenna Elliott,* and Michael F. Good2*

Merozoite surface protein 1 (MSP1) of malaria parasites undergoes proteolytic processing at least twice before invasion into a new RBC. The 42-kDa fragment, a product of primary processing, is cleaved by proteolytic enzymes giving rise to MSP133, which is shed from the merozoite surface, and MSP119, which is the only fragment carried into a new RBC. In this study, we have identified T cell epitopes on MSP119 of Plasmodium yoelii and have examined their function in immunity to blood stage malaria. Peptides 20 aa in length, spanning the length of MSP133 and overlapping each other by 10 aa, were analyzed for their ability to induce T cell proliferation in immunized BALB/c and C57BL/6 mice. Multiple epitopes were recognized by these two strains of mice. Effector functions of the dominant epitopes were then investigated. Peptides Cm15 and Cm21 were of particular interest as they could induce complete protection against Plasmodium falciparum in monkeys (5), is processed by proteases into a number of fragments. The 42-kDa fragment, a product of primary processing, is cleaved by proteolytic enzymes giving rise to MSP133, which is shed from the merozoite surface, and MSP119, which is the only fragment carried into a new RBC. The soluble 33-kDa fragment (MSP133), corresponding to the N-terminal region of MSP142, is cleaved into two products. The soluble 33-kDa fragment (MSP133), corresponding to the N-terminal region of MSP142, is shed from the free merozoite surface (6). The membrane-bound 19-kDa C-terminal fragment (MSP119), which contains two epidermal growth factor-like domains (7), is the only fragment carried with an invading merozoite into the new RBC (8).

Early studies suggested that T cells specific for native MSP1 could protect mice independent of Ab (9). However, when MSP119, which can induce high levels of protection from homologous challenge in mice (10) and monkeys (11), was assessed for its ability to stimulate protective T cells, results were uniformly negative (12). Vaccination with T cell epitopes from MSP119 was unable to induce any level of protection and adoptively transferred T cells specific for either MSP119 or defined T cell epitopes did not render the recipients resistant to infection. It thus appeared that if T cell epitopes capable of protecting mice in the absence of Ab existed on MSP1, they must occur on fragments other than MSP119. Since MSP142 has been shown capable of protecting both monkeys (11, 13) and mice (14) from homologous challenge, we decided to examine MSP133 (which is contained within MSP142) for the presence of protective T cell epitopes.

Materials and Methods

Animals and parasites

Six- to 8-wk-old female BALB/c (H-2d), BALB/c nu/nu (nude), BALB/c SCID, and C57BL/6 (H-2b) mice were used. Animals were purchased from Animal Resources Center (Willetton, Western Australia, Australia) and were housed in the animal house under pathogen-free conditions. Nude and
SCID mice were housed in filter top cages and were handled in laminar flow cabinets.

*P. yoelii* YM strain was used. The parasite was maintained by i.p. injection with 10^6 parasitized RBCs (pRBC) every 5 days.

**Synthetic peptides and recombinant MSP1**

Twenty-six peptides corresponding to MSP1,aa (1394–1657) (15) were produced at either the Queensland Institute of Medical Research (Queensland, Australia) or Mimotopes (Clayton, Victoria, Australia). Peptides were 20 aa in length, overlapping each other by 10 aa, and were termed Cm1–Cm 26 (Fig. 1). The purity of peptides was >85%, except peptide Cm 2, where crude peptide was used.

Recombinant MSP1 of *P. yoelii* was produced in *Saccharomyces cerevisiae* as described previously (16).

**Lymphoproliferation assay and generation of T cell lines**

Mice were immunized in hind footpads with Ags (30 µg of peptide) emulsified in CFA (Sigma-Aldrich, St. Louis, MO). Nine to 10 days later, inguinal and popliteal lymph nodes were removed and single-cell suspensions were prepared. Cells were washed with Eagle’s MEM and were cultured in a volume of 200 µl in MEM supplemented with 50 µg/ml of 2-ME and 2% heat-inactivated normal mouse serum at 2 × 10^6 cells/ml in flat-bottom 96-well plates. Cells were cultured with different concentrations of Ag (final concentration of 30, 10, and 3 µg/ml) for 72 h and then were pulse labeled with 0.25 µCi of [3H]thymidine. Incorporation of radio-label was estimated 18–24 h later by beta emission spectroscopy. Δcpm were determined by subtraction of cpm in the absence of Ag from cpm in the presence of Ag.

T cell lines specific to dominant epitopes or OVA were generated as described previously (17).

**Immunization and challenge infection**

Mice were immunized with PBS or Ags using a vaccination protocol described previously (10). Briefly, mice were immunized s.c. with PBS, 20 µg of peptides, or MSP1 in CFA. Some groups were vaccinated with the pool of dominant peptides at 20 µg of each peptide. The mice were then boosted four times with the same dose of Ag, s.c. in IFA (Sigma-Aldrich) at 21 days, i.p. in IFA at 42 and 56 days, and finally i.p. in PBS at 63 days.

Ten days after the last immunization, the mice were challenged i.v. with 10^6 live *P. yoelii* YM pRBC. Parasitemia was monitored after infection by microscopic examination of smears from tail blood stained with Diff-Quick stain (Lab Aids, Narrabeen, Australia).

**Adoptive transfer study**

A total of 10^6 viable resting T cells was purified by centrifugation over Ficoll-Paque, washed twice, and injected i.v. into nude or SCID mice. The mice were then challenged i.v. 4–24 h later with 10^6 *P. yoelii* YM pRBC.

**Priming mice with T cell epitopes**

Mice were primed s.c. with 20 µg of peptides emulsified in CFA. Two weeks later, the mice were immunized by infection and cured as described previously (12). Sera were collected to assess Ab responses.

**Cell surface phenotype characterization**

Single-cell suspensions of T cell lines were stained with PE- or FITC-conjugated mAbs specific for mouse CD4, CD3, CD19, NK1.1 cells, TCRβ, and TCRγδ (Caltag Laboratories, Burlingame, CA). Cells were incubated for 30 min at 4°C, washed twice with washing buffer (0.1% BSA/0.1% sodium azide/PBS), and resuspended in 250 µl of 1% parafomaldehyde. The percentage of positive cells was measured by a FACS (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences).

**ELISA**

Serum Ab levels against crude parasite Ags were analyzed by ELISA as described previously (12).

**Western blot**

Crude parasite Ag was separated on SDS-polyacrylamide gels (4% stacking and 12% separating gel; Bio-Rad, Hercules, CA) at 100 V for 1 h. Gels were electrophoretically blotted onto nitrocellulose paper, from which strips were cut and blocked overnight with 1 ml of PBS containing 5% skim milk. Strips were incubated with 1 ml of 1/100 mouse sera in 0.05% skim milk/PBS at room temperature for 2 h. After washing three times with 0.05% Tween 20/PBS, strips were incubated with 1 ml of 1/3000 goat anti-mouse Ig HRP conjugate (Silenus Labs, Melbourne, Australia) for 1 h at room temperature. Strips were washed three times, incubated with substrate (4-chloro-1-naphtol; Sigma-Aldrich) for 20 min, and washed three times with water.

**Bioassay for IFN-γ, IL-2, and IL-4**

Culture supernatants from T cell lines were collected 24, 48, and 72 h after stimulation. IFN-γ, IL-2, and IL-4 activity were determined as described previously (18). IFN-γ activity was determined by measuring inhibition of WEHI-279 cell proliferation. IL-2 and IL-4 activities were determined using the cytokine-dependent cell lines CTL-L2 and CT-4S, respectively. The concentrations were calculated from cytokine standards in the assays.

**Statistics**

Student’s t test for unpaired observations was used to determine differences between groups.

**Results**

**Identification of T cell epitopes on MSP1**

To define T cell epitopes on MSP1, BALB/c mice or C57BL/6 were immunized in the footpads with pools of purified peptides grouped as Cm1 and 3–6, Cm7–11, Cm12–16, Cm17–21, and Cm22–26. Crude peptide Cm2 was used alone. Ten days after immunization, draining lymph nodes were removed and tested for their proliferative response in vitro to an individual peptide at three different concentrations. Proliferative responses were assessed to be significant at a stimulation index of 3 or above. BALB/c mice recognized peptides Cm3, 4, 11, 15, and 21 (Fig. 2), whereas C57BL/6 mice recognized peptide Cm11. Peptides Cm13 and Cm23 induced low-level proliferative responses in C57BL/6 mice (stimulation index, <3) on two occasions (Fig. 2, A and E) and peptide Cm23 induced low-level responses in BALB/c mice (Fig. 2F), so these peptides were selected along with the dominant epitopes for further study.

**Vaccination of immunocompetent mice with dominant epitopes**

To determine whether the dominant T cell epitopes could induce effector T cells capable of mediating protection against *P. yoelii* infection, we used two approaches. First, normal BALB/c and C57BL/6 mice were immunized with the peptides following the standard vaccination protocol for MSP1 (10) and were then challenged with *P. yoelii* YM. Control groups were immunized with
PBS or MSP1,9. BALB/c mice that were immunized with PBS or individual peptides succumbed to infection (Fig. 3A) with the exception of one mouse that was immunized with Cm21 which developed parasitemia, but gradually recovered. There was also one mouse immunized with the pool of peptides (Cm3, 4, 11, 15, 21, and 23) that developed parasitemia but recovered. All mice that were vaccinated with MSP1,9 survived infection with one mouse developing patent parasitemia. C57BL/6 mice that were vaccinated with individual peptides developed parasitemia and died at the same rate as the PBS control group (Fig. 3B). However, three of
FIGURE 3. Parasitemia of BALB/c (A) or C57BL/6 (B) mice immunized with defined T cell epitopes. Groups of three to four mice were immunized with MSP1α-defined T cell epitope peptides or the pool of peptides (Cm3, 4, 11, 15, 21, and 23 for BALB/c mice and Cm3, 11, and 23 for C57BL/6 mice). Mice immunized with PBS or MSP19 were used as controls. Mice were challenged with $1 \times 10^5$ live P. yoelii YM pRBC. Data show percent parasitemia of individual mice. Cross symbols indicate the days on which mice died.

Table I. **Characteristics of T cell lines specific for dominant epitopes**

<table>
<thead>
<tr>
<th>T Cell Lines Specific for</th>
<th>Cm3</th>
<th>Cm4</th>
<th>Cm11</th>
<th>Cm15</th>
<th>Cm21</th>
<th>OVA</th>
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<tbody>
<tr>
<td>Cell surface phenotyping</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% CD3+ cells</td>
<td>99.7</td>
<td>98.1</td>
<td>99.9</td>
<td>99.4</td>
<td>99.9</td>
<td>99.3</td>
</tr>
<tr>
<td>% CD4+ cells</td>
<td>99.5</td>
<td>95.1</td>
<td>99.8</td>
<td>99.3</td>
<td>99.5</td>
<td>98.9</td>
</tr>
<tr>
<td>% CD8+ cells</td>
<td>1.9</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>% B220 cells</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>% TCRαβ cells</td>
<td>99.7</td>
<td>98.9</td>
<td>92.6</td>
<td>98.8</td>
<td>98.1</td>
<td>98.8</td>
</tr>
<tr>
<td>% TCRγδ cells</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>1.1</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Cytokine production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ (U/ml)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>129.7</td>
<td>58.6</td>
<td>126.7</td>
<td>4035</td>
</tr>
<tr>
<td>IL-2 (U/ml)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>584.0</td>
<td>&lt;1</td>
<td>56.4</td>
<td>210</td>
</tr>
<tr>
<td>IL-4 (U/ml)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* T cells were analyzed for cell surface phenotype and cell culture supernatants at 24 h after Ag-specific stimulation were examined for cytokine production. The concentration of IFN-γ and IL-2 was determined from a standard curve.
four mice that received the pool of peptides (Cm3, 11, and 23) developed parasitemia but were then able to control parasite growth. Positive control mice that were immunized with MSP119 did not develop detectable parasitemia. One representative mouse from each group was sacrificed on the day of challenge to assess proliferation of spleen cells induced by specific peptides used for immunization. In all cases, spleen cells proliferated in response to specific peptides (data not shown).

Protection against P. yoelii YM infection by adoptively transferred T cell lines

The second approach used to establish the effector function of peptide-specific T cells was to adoptively transfer T cell lines specific for defined epitopes to naive nude mice. This approach was followed because we (19, 20) and others (21) have shown that T cells can adoptively transfer protection to malaria and because it is possible that the vaccination protocol (above) was unable to induce sufficient numbers of T cells. Furthermore, the phenotypes of vaccine-induced T cells is likely to be heterogeneous. The experiments were performed with T cell lines derived from BALB/c mice generated to peptides Cm3, Cm4, Cm11, Cm15, and Cm21.

T cell lines specific for dominant epitopes were generated by repeated cycles of stimulation and rest in vitro. They were CD3+, CD4+, and TCRαβ+ and produced IFN-γ and IL-2 following Ag-specific stimulation (Table I). All T cell lines proliferated following stimulation with the immunogen (Fig. 4). Cm15- and Cm21-, but not Cm3-, Cm4-, and Cm11-, specific T cell lines responded to whole parasite Ag. Nude mice administered peptide-specific T cell lines were challenged with P. yoelii YM and parasitemia was monitored. Nude mice that received Cm3-, Cm4-, and Cm11-specific T cell lines demonstrated significantly suppressed parasite growth, indicating that these epitopes were able to induce functional T cells. In these mice, survival was prolonged from 8.5 to 21.5 days for mice that received Cm15-specific T cells (p < 0.01) and from 8.5 to 29.8 days for mice that received Cm21-specific T cells (p < 0.01). In addition, parasite density at day 8 was reduced from 87.8 ± 2.7% to 19.2 ± 10.6% for Cm15-specific T cell recipients (p < 0.01) and from 87.8 ± 10.6% to 24.6 ± 11.2% for Cm21-specific T cell recipients (p < 0.01). Sera taken from recipient mice after challenge did not show detectable Ab to crude parasite Ags as determined by ELISA and Western blot (Fig. 6).
To confirm that Cm21-specific T cells were able to control parasite growth in the absence of Abs, SCID mice were transfused with $10^7$ Cm21- or OVA-specific T cells and were then challenged with $10^3$ P. yoelii YM pRBC. Data show mean ± SE of parasitemia from five mice. Cross symbols indicate the days on which mice died.

SCID mice was prolonged from 8.6 to 12.6 days ($p < 0.02$) and parasite density at day 7 was reduced from 59.94 ± 7.51% to 10.56 ± 4.96% ($p < 0.01$).

Discussion

Although significant efforts have been invested in malaria research, a malaria vaccine is still not available for common use. Immunity to malaria is commonly species, stage, strain, and variant specific (22–24). Thus, an ideal malaria vaccine should comprise multiple epitopes that would cover various stages of the complex life cycle and induce cross-protection against many strains. Most studies investigating the C-terminal region of MSP1 as a vaccine candidate have focused on developing Abs that will neutralize or prevent invasion of merozoites. However, acquired protective immunity to blood stage malaria involves both Ab-mediated and cell-mediated immunity (25).

MSP142, which gives rise to MSP133 and MSP119, has been shown to induce protective immunity in mice (14) and monkeys (11, 13). The degree of protection induced by MSP142 correlates with the level of specific Abs (11), and passive transfer of immune sera from immunized mice confers partial protection to the recipient animals (14). It has been shown that immune sera from animals immunized with MSP142 can inhibit parasite growth in vitro (13, 26). Preincubation of anti-MSP142 sera with MSP119 results in loss of the binding of anti-MSP142 sera to parasite MSP1 or MSP142. Furthermore, anti-MSP119-depleted sera failed to inhibit parasite growth in vitro, suggesting that inhibitory epitopes of MSP142 are localized on MSP119 (27). Sera from monkeys immunized with recombinant MSP142 recognize the 19-kDa fragment on Western blot (13). These data suggest that B cell epitopes of MSP142 are localized on MSP119. Further studies of natural immune responses to MSP142 in humans have shown that B cell epitopes on MSP119 are recognized by immune sera from P. falciparum-exposed individuals, whereas the T cell proliferative responses are predominantly induced by the dimorphic regions of MSP133 (28). Similar observations by Egan et al. (29) have shown that T cell epitopes on MSP133 are commonly recognized by mononuclear cells from malaria-exposed individuals.

Although T cell responses against MSP133 have been demonstrated (28, 29), their roles in immunity to blood stage malaria remained unclear. In this report, T cell epitopes on P. yoelii MSP133 were identified and their roles in immunity to malaria were then studied. Using overlapping peptides, we found that MSP133 contains multiple T cell epitopes. C57BL/6 mice recognized fewer epitopes than BALB/c mice, suggesting that responses to MSP133 are MHC class II restricted. Cm3 and Cm11, which...
were recognized by both strains of mice, and Cm4, which was only recognized by BALB/c mice, did not induce protection following immunization with individual peptides. One mouse that received Cm21 was able to clear parasitemia following challenge infection, suggesting that Cm21 may be able to induce effector T cells capable of protection. To confirm the effector role of T cells induced by MSP113-dominant epitopes, T cell lines specific to individual peptides were generated and were then transferred into nude mice. Cm15 and Cm21 were of particular interest, as they were able to confer partial protection against lethal P. yoelii YM to recipient mice. It is probably relevant that these T cell lines were the only ones to respond to whole parasite Ag in vitro. No malaria-specific Abs were detected in these mice, suggesting that Cm15- and Cm21-specific T cells are able to control parasite growth independently of Abs. It is unlikely that T cell-transfused nude mice developed their own Abs at levels below detection, since adoptive transfer of T cells into SCID mice gave similar results. Since C57BL/6 mice vaccinated with the pool of peptides were better protected than mice immunized with individual peptides, adoptive transfer of pooled T cell lines may induce more effective protective immunity by ensuring that mice have higher numbers of effector T cells at the time of challenge. However, the mechanism of this enhanced immunity warrants further study. Cm15- and Cm 21-specific T cell lines used in the adoptive transfer study were of the Th1 type, producing IFN-γ following Ag-specific stimulation in vitro. We noted that Cm11-specific T cells also produced IFN-γ (as much as Cm21 cells and more than Cm15 cells) but did not induce protection. Cm15-and Cm21-specific T cells proliferated in response to whole parasite Ags whereas Cm11 specific T cells did not. IFN-γ and TNF are thought to be important in immunity as are downstream molecules (30). However, the most important correlate in our study was with the ability to recognize whole parasites. Cm11 may be a “cryptic” epitope (31) and although it may induce a IFN-γ response following peptide stimulation, that is inconsequential if the cells cannot be activated by parasites.

It is worth noting that passive transfer of an anti-MSP113 mAb recognizing MSP1142 in merozoite extracts and the intact MSP1 precursor did not affect the course of parasitemia following infection with P. yoelii YM (32). This further suggests that the protection we have observed is Ab independent.

It is generally considered that immunity to P. yoelii is primarily Ab-mediated (25). Here, we demonstrate that T cell epitopes on MSP113 are able to induce effector T cells capable of controlling P. yoelii growth in an Ab-independent manner. Natural T cell responses to MSP113 are directed toward the conserved regions on P. falciparum MSP1142 (28, 29). This along with data presented here suggests a strong case for further investigation of T cell epitopes of MSP113 as potential candidates for inclusion in a subunit vaccine. As cellular immune responses are crucial in mediating protection against many infectious diseases (33), incorporating epitopes that would induce effector T cells may enhance the efficacy of a vaccine against malaria.

References