The Journal of Immunology
RESEARCH ARTICLE | JULY 15 2002

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J Immunol (2002) 169 (2): 944–951.
https://doi.org/10.4049/jimmunol.169.2.944

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[1]

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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 $MSP1_{33}$, which is shed from the merozoite surface, and $MSP1_{19}$, which is the only fragment carried into a new RBC. In this study, we have identified T cell epitopes on $MSP1_{33}$ of *Plasmodium yoelii* and have examined their function in immunity to blood stage malaria. Peptides 20 aa in length, spanning the length of $MSP1_{33}$ 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 were able to induce effector T cells capable of delaying growth of lethal *P. yoelii* YM following adoptive transfer into immunodeficient mice without inducing detectable Ab responses. Homologs of these epitopes could be candidates for inclusion in a subunit vaccine. ***The Journal of Immunology*****, 2002, 169: 944–951.**

Malaria, a parasitic infection caused by protozoan parasites of the *Plasmodium* genus, is an important cause of morbidity in many parts of the world. It is estimated that malaria kills 1–2 million people each year, mostly children under the age of 5 years and a significant number of pregnant women in sub-Saharan Africa (1). The emergence of drug resistance means that adequate treatment of malaria is becoming increasingly difficult and effective controls are urgently needed. The development of a malaria vaccine is one strategy that could prove the most cost-effective means of controlling both the transmission of infection and the impact of disease.

Merozoite surface protein 1 (MSP1)[3] is a high molecular mass (~185- to 205-kDa) glycoprotein expressed on the surface of merozoites (2–4). It is a potential vaccine candidate because it is directly exposed and interacts with the host milieu during RBC invasion. The MSP1 precursor protein, which has been shown to induce complete protection against *Plasmodium falciparum* in monkeys (5), is processed by proteases into a number of fragments. The primary processing at schizont rupture cleaves the precursor protein into major fragments of ~83 ($MSP1_{83}$), 30 ($MSP1_{30}$), 38 ($MSP1_{38}$), and 42 ($MSP1_{42}$) kDa. The fragments are found as a noncovalently associated complex held together on the free merozoite surface by the C-terminal membrane-bound 42-kDa fragment (2–4). At the time of merozoite invasion, $MSP1_{42}$ is cleaved into two products. The soluble 33-kDa fragment ($MSP1_{33}$), corresponding to the N-terminal region of $MSP1_{42,}$ is shed from the free merozoite surface (6). The membrane-bound 19-kDa C-terminal fragment ($MSP1_{19}$), 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 $MSP1_{19}$, 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 $MSP1_{19}$ was unable to induce any level of protection and adoptively transferred T cells specific for either $MSP1_{19}$ 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 $MSP1_{19}$. Since $MSP1_{42}$ has been shown capable of protecting both monkeys (11, 13) and mice (14) from homologous challenge, we decided to examine $MSP1_{33}$ (which is contained within $MSP1_{42}$) for the presence of protective T cell epitopes.

Materials and Methods

Animals and parasites

Six- to 8-wk-old female BALB/c (H-2^d), BALB/c *nu*/*nu* (nude), BALB/c SCID, and C57BL/6 (H-2^b) 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

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Received for publication January 22, 2002. Accepted for publication May 7, 2002.

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[1] This investigation received financial support from the United Nations Development Program/World Bank/World Health Organization special program for Research and Training in Tropical Diseases (to T.D.R.), the Cooperative Research Center for Vaccine Technology, and the Australian Center for International and Tropical Health and Nutrition.

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[3] Abbreviations used in this paper: MSP1, merozoite surface protein 1; pRBC, parasitized RBC.

0022-1767/02/$02.00

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$_{19}$

Twenty-six peptides corresponding to MSP1$_{33}$ (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 Cm 1–Cm 26 (Fig. 1). The purity of peptides was >85%, except peptide Cm 2, where crude peptide was used.

Recombinant MSP1$_{19}$ of *P. yoelii* was produced in *Saccharomyces cerevisae* 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 μM 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 [^{3}H]thymidine. Incorporation of radiolabel 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$_{19}$ in CFA. Some groups were vaccinated with the pool of dominant peptides at 20 μg of each peptides. 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 10^4 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, Narrabean, Australia).

Adoptive transfer study

A total of 10^7 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 10^4 *P. yoelii* YM pRBC.

FIGURE 1. Amino acid sequences of synthetic peptides corresponding to MSP1$_{33}$ of *P. yoelii* YM. The solid line *above* Cm1 indicates sequences out of the N terminus of MSP1$_{33}$, and the line *under* Cm26 indicates sequences that overlap with the N terminus of MSP1$_{19}$.

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, TCR$\alpha\beta$, and TCR$\gamma\delta$ (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% paraformaldehyde. 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 CTLL-2 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$_{33}$

To define T cell epitopes on MSP1$_{33}$, 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 Cm3 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. 2*E*), 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$_{19}$ (10) and were then challenged with *P. yoelii* YM. Control groups were immunized with

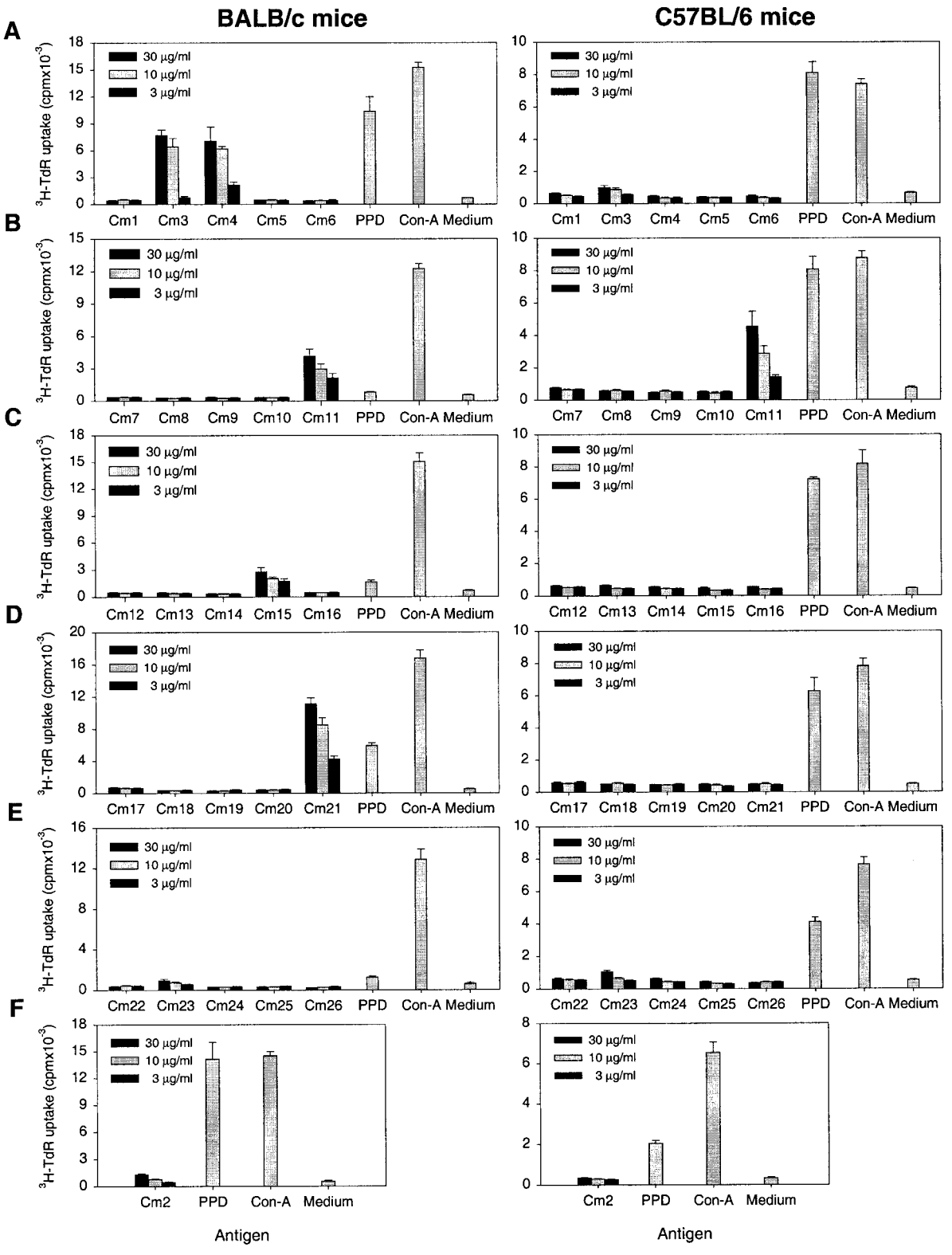

FIGURE 2. Identification of T cell epitopes on MSP1$_{33}$. Peptide-specific proliferative responses of T cells from draining lymph nodes of BALB/c and C57BL/6 mice immunized with the pool of peptides 1, 3–6 (*A*), 7–11 (*B*), 12–16 (*C*), 17–21 (*D*), 22–26 (*E*), and crude peptide Cm2 (*F*). Data show mean ± SE from three mice of one representative of two independent experiments. PPD, Purified protein derivative.

PBS or MSP1$_{19}$. BALB/c mice that were immunized with PBS or individual peptides succumbed to infection (Fig. 3*A*) 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$_{19}$ 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. 3*B*). However, three of

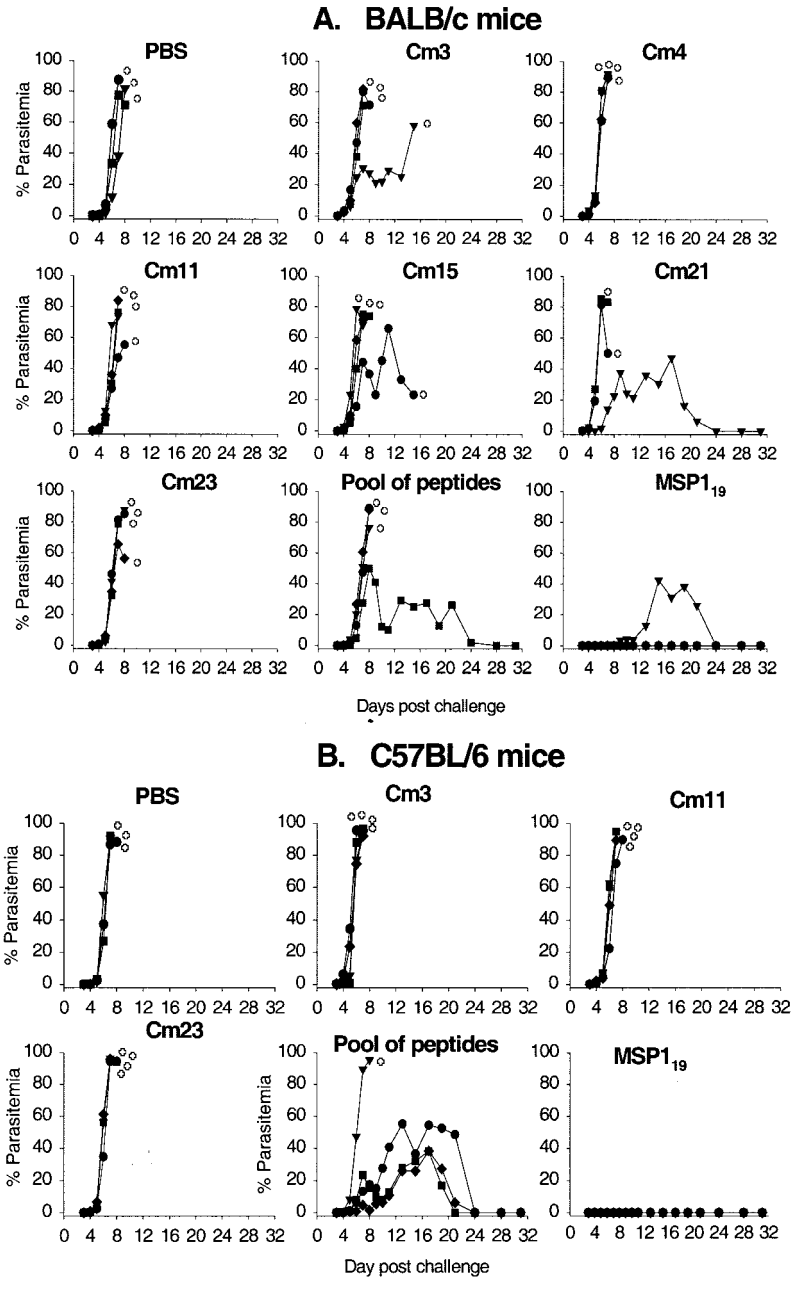

FIGURE 3. Parasitemia of BALB/c (*A*) or C57BL/6 (*B*) mice immunized with defined T cell epitopes. Groups of three to four mice were immunized with $MSP1_{33}$-defined T cell epitope peptides or the pool of peptides (Cm3, 4, 11, 15, 21, and 23 for BALB/c mice and Cm3, 11, and 23 for C57BL/6 mice). Mice immunized with PBS or $MSP1_{19}$ were used as controls. Mice were challenged with 1×10^4 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*[a]

	T Cell Lines Specific for					
	Cm3	Cm4	Cm11	Cm15	Cm21	OVA
Cell surface phenotyping						
% $CD3^+$ cells	99.7	98.1	99.9	99.4	99.9	99.3
% $CD4^+$ cells	99.5	95.1	99.8	99.3	99.5	98.9
% $CD8^+$ cells	1.9	0.4	0.6	0.7	1.7	2.7
% $B220^+$ cells	0.4	0.4	0.6	0.5	0.7	1.0
% TCR$\alpha\beta^+$ cells	99.7	98.9	92.6	98.8	98.1	98.8
% TCR$\gamma\delta^+$ cells	0.5	0.5	0.6	1.1	0.4	0.6
Cytokine production						
IFN-γ (U/ml)	<1	<1	129.7	58.6	126.7	4035
IL-2 (U/ml)	<1	<1	584.0	<1	56.4	210
IL-4 (U/ml)	<1	<1	<1	<1	<1	<1

[a] 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.

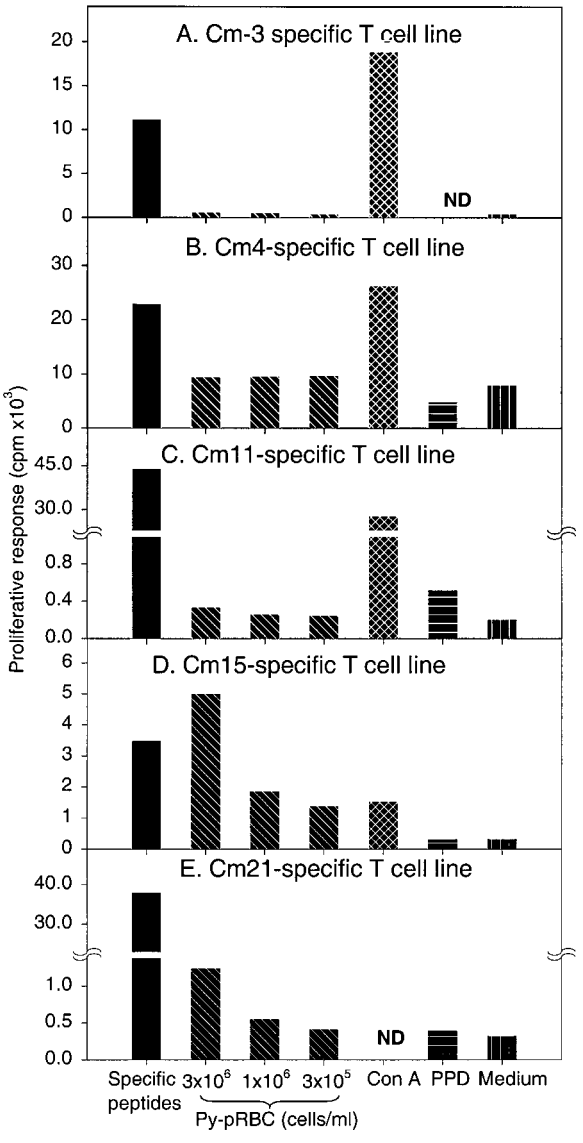

FIGURE 4. Proliferative responses of $MSP1_{33}$ peptide-specific T cell lines against different Ags. Resting T cells were stimulated with specific peptides (10 μg/ml), *P. yoelii* YM pRBC (3×10^6, 1×10^6, or 3×10^5 cells/ml), Con A (10 μg/ml), purified protein derivative (10 μg/ml), or medium alone for 4 days. Data show mean of quadruplicate wells. ND, Not done; PPD, purified protein derivative.

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 $MSP1_{19}$ 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 cells 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 $TCR\alpha\beta^+$ 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-, Cm 4-, and Cm11-specific T cells died within 11 days, similarly to mice transfused with a T cell-line specific to an irrelevant Ag, OVA (Fig. 5). However, nude mice that were administered Cm15- or Cm21-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 $\pm$ 2.7% to 19.2 $\pm$ 10.6% for Cm15-specific T cell recipients ($p < 0.01$) and from 87.8 $\pm$ 10.6% to 24.6 $\pm$ 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).

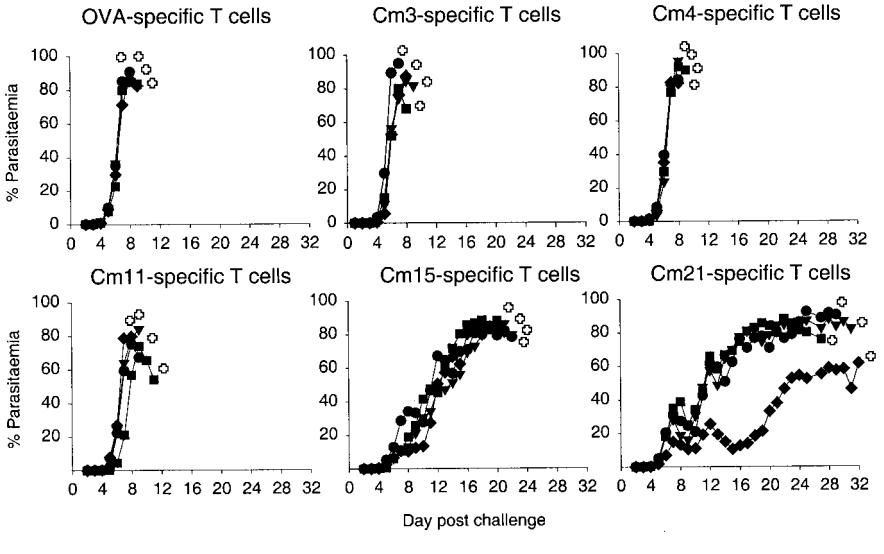

FIGURE 5. Parasitemia following challenge infection of T cell-transfused nude mice. A total of 10^7 T cells specific for $MSP1_{33}$-dominant epitopes or OVA was transferred into nude mice. The mice were challenged with 10^4 *P. yoelii* YM pRBC. Data show percent parasitemia of each individual mouse of one representative of two independent experiments. Cross symbols indicate the days on which mice died.

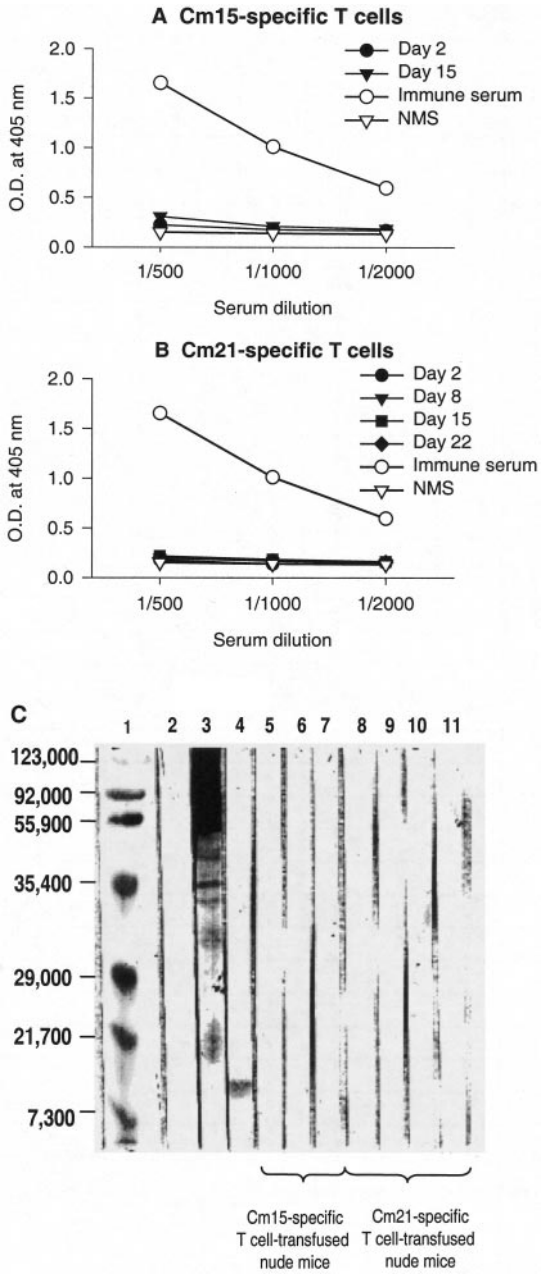

FIGURE 6. Ab responses against whole parasite Ag of T cell-transfused nude mice as determined by ELISA (*A* and *B*) or Western blot (*C*). *A* and *B*, The level of Abs on days 2 and 15 after challenge for Cm15-specific T cell recipients (closed symbols) and days 2, 8, 15, and 22 after challenge for Cm21-specific T cell recipients (closed symbols). Open symbols show immune serum and normal mouse serum (NMS) controls, respectively. *C*, Ab response to crude parasite Ags in Cm15- and Cm21-specific T cell-transfused nude mice; *Lane 1*, Molecular mass marker; *lane 2*, normal mouse serum; *lane 3*, anti-whole parasite Abs; *lane 4*, anti-$MSP1_{19}$ Abs; *lanes 5–7*, Cm15-specific T cell-transfused nude mice prechallenge and days 8 and 22 after challenge, respectively; *lanes 8–11*, Cm21-specific T cell-transfused nude mice prechallenge and days 2, 15, and 22 after challenge, respectively.

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 *P. yoelii* YM. Mice that received OVA-specific T cells could not control parasite growth and all mice died within 10 days (Fig. 7). In contrast, the survival of Cm21-specific T cell-transfused

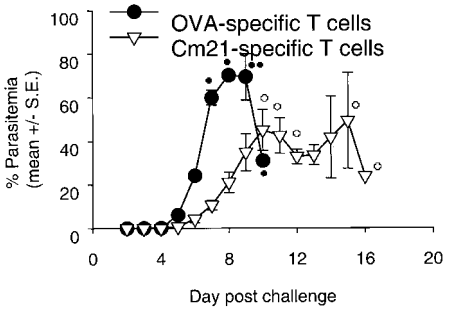

FIGURE 7. Parasitemia following challenge infection of T cell-transfused SCID mice. A total of 10^7 T cells specific for Cm21 or OVA was transferred into SCID mice. The mice were challenged with 10^4 *P. yoelii* YM pRBC. Data show mean ± SE of parasitemia from five mice. Cross symbols indicate the days 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).

$MSP1_{42}$, which gives rise to $MSP1_{33}$ and $MSP1_{19}$, has been shown to induce protective immunity in mice (14) and monkeys (11, 13). The degree of protection induced by $MSP1_{42}$ 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 $MSP1_{42}$ can inhibit parasite growth in vitro (13, 26). Preincubation of anti-$MSP1_{42}$ sera with $MSP1_{19}$ results in loss of the binding of anti-$MSP1_{42}$ sera to parasite MSP1 or $MSP1_{42}$. Furthermore, anti-$MSP1_{19}$-depleted sera failed to inhibit parasite growth in vitro, suggesting that inhibitory epitopes of $MSP1_{42}$ are localized on $MSP1_{19}$ (27). Sera from monkeys immunized with recombinant $MSP1_{42}$ recognize the 19-kDa fragment on Western blot (13). These data suggest that B cell epitopes of $MSP1_{42}$ are localized on $MSP1_{19}$. Studies of natural immune responses to $MSP1_{42}$ in humans have shown that B cell epitopes on $MSP1_{19}$ are recognized by immune sera from *P. falciparum*-exposed individuals, whereas the T cell proliferative responses are predominantly induced by the dimorphic regions of $MSP1_{33}$ (28). Similar observations by Egan et al. (29) have shown that T cell epitopes on $MSP1_{33}$ are commonly recognized by mononuclear cells from malaria-exposed individuals.

Although T cell responses against $MSP1_{33}$ have been demonstrated (28, 29), their roles in immunity to blood stage malaria remained unclear. In this report, T cell epitopes on *P. yoelii* $MSP1_{33}$ were identified and their roles in immunity to malaria were then studied. Using overlapping peptides, we found that $MSP1_{33}$ contains multiple T cell epitopes. C57BL/6 mice recognized fewer epitopes than BALB/c mice, suggesting that responses to $MSP1_{33}$ 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 $MSP1_{33}$-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-$MSP1_{33}$ mAb recognizing $MSP1_{42}$ 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 $MSP1_{33}$ are able to induce effector T cells capable of controlling *P. yoelii* growth in an Ab-independent manner. Natural T cell responses to $MSP1_{33}$ are directed toward the conserved regions on *P. falciparum* $MSP1_{42}$ (28, 29). This along with data presented here suggests a strong case for further investigation of T cell epitopes of $MSP1_{33}$ 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.

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