Recombinant Attenuated *Toxoplasma gondii* Expressing the *Plasmodium yoelii* Circumsporozoite Protein Provides Highly Effective Priming for CD8\(^+\) T Cell-Dependent Protective Immunity Against Malaria

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Recombinant Attenuated *Toxoplasma gondii* Expressing the *Plasmodium yoelii* Circumsporozoite Protein Provides Highly Effective Priming for CD8$^+$ T Cell-Dependent Protective Immunity Against Malaria

Hughes Charest,* Martha Sedegah,†‡ George S. Yap,* Ricardo T. Gazzinelli,§ Patricia Caspar,* Stephen L. Hoffman,‡ and Alan Sher*†

The protozoan parasite *Toxoplasma gondii* elicits strong cell-mediated immunity against itself as well as nonspecific resistance against other pathogens and tumors. For this reason, we asked whether recombinant *Toxoplasma* could be utilized as an effective vaccine vehicle for inducing immunity against heterologous microbial infections. The circumsporozoite protein (PyCSP) of *Plasmodium yoelii* was engineered into a *T. gondii* temperature-sensitive strain (ts-4), a mutant that induces complete protection against virulent *Toxoplasma* challenge. When administered to mice in a single dose, a recombinant ts-4 (CSC3) that both secretes and expresses surface PyCSP induced strong anti-CSP Ab responses, with an isotype distribution pattern similar to that stimulated by the *T. gondii* carrier. When challenged with *P. yoelii* sporozoites during the first month after CSC3 vaccination, these animals displayed substantial levels of nonspecific resistance attributable entirely to the *T. gondii* carrier. Nevertheless, after the nonspecific protection had waned, high levels (up to 79%) of specific immunity against sporozoite challenge were achieved by boosting the animals with recombinant vaccinia virus expressing PyCSP. These CSC3-primed PyCSP-vaccinia-boosted mice displayed high frequencies of splenic PyCSP-specific IFN-γ-producing cells, as well as CD8$^+$ T cell-dependent cytolytic activity. In vivo depletion of CD8$^+$ lymphocytes at the time of challenge completely ablated protective immunity in the *T. gondii*-primed/vaccinia-boosted animals, while neutralization of IFN-γ or IL-12 caused a partial but significant reduction in resistance. Together these findings establish the efficacy of recombinant attenuated *Toxoplasma* as a vaccine vehicle for priming CD8$^+$-dependent cell-mediated immunity. *The Journal of Immunology*, 2000, 165: 2084–2092.

Live vaccine vehicles offer a powerful approach for inducing protective immunity against pathogenic microorganisms. These genetically engineered infectious agents provide a method for delivering heterologous vaccine Ags in a form that mimics that utilized by the carrier, exploiting its particular presentation capabilities. A variety of different attenuated viruses, bacteria and protozoan, have been utilized successfully as vaccine delivery systems in several experimental models, and a number of these constructs have been tested in clinical trials (1).

*Toxoplasma gondii* is an apicomplexan protozoan that normally produces an asymptomatic infection in its vertebrate intermediate hosts. The parasite has been shown to elicit a potent cell-mediated immune response that efficiently controls its own growth and can lead to nonspecific resistance to unrelated pathogens and tumors (2–5). Because of its unusual immunogenicity, we have considered the use of this protozoan as a live vaccine carrier. Genetic transformation of *T. gondii* has been achieved using tachyzoites of the RH strain that replicate rapidly in tissue culture (6–10). However, since the RH strain is highly virulent and produces lethal infections in mice (11, 12), it is unsuitable for use as a vaccine carrier in humans. The temperature-sensitive ts-4 mutant strain of RH, in contrast, is avirulent in the experimental murine model. While the injection of only one wild-type RH tachyzoite results in death within 12 days, mice inoculated with up to 10$^7$ ts-4 tachyzoites survive and do not show acute disease symptoms (13, 14). Importantly, mice inoculated with ts-4 tachyzoites develop complete protection against lethal challenge with RH (14–16). No other vaccination method described in the literature provides such solid immunity against virulent *T. gondii* infection.

Previous studies have indicated that the protection against virulent challenge conferred by ts-4 vaccination involves a Th1-type cell-mediated immune mechanism. Thus, lymphocytes from immunized animals produce high levels of IFN-γ and IL-2, but undetectable amounts of IL-4 or IL-5 after restimulation in vitro. Furthermore, protective immunity is abolished by depletion of CD8$^+$ cells or administration of anti-IFN-γ at the time of challenge (15, 16). Based on the above properties, one would predict that ts-4 as a vaccine carrier should promote strong Th1 cytokine and CD8$^+$ T cell responses against the heterologous Ag it expresses.

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In the present study, we have assessed the utility of T. gondii as a vaccine delivery system by generating a series of ts-4 stable recombinant lines expressing the circumsporozoite protein of Plasmodium yoelii (PyCSP) and testing the ability of these transgenic lines to induce PyCSP-specific immune responses as well as to confer protection against malaria. The circumsporozoite protein (CSP), the major surface Ag of the infective sporozoite stage, was chosen as a model Ag for use in this vaccine delivery system for the following reasons. First, vaccination against CSP has been studied in a number of experimental animal models for immunization against preerythrocytic forms of the parasite, and the protein is currently a major malaria vaccine candidate. In the case of P. yoelii and several other Plasmodium species, the molecule has been extensively characterized, and the location of the B cell, CD4+ and CD8+ T cell epitopes mapped (reviewed in Ref. 17). Moreover, the protective immunity induced by CSP against sporozoites involves similar cell-mediated components as those implicated in the protection induced by ts-4 against virulent T. gondii strains. Thus, in many rodent malaria model systems analyzed, the resistance to challenge resulting from vaccination with CSP appears to be dependent on the induction of a cell-mediated immune mechanism involving T cells and IFN-γ (18–20). A number of different live carriers (e.g., vaccinia, Salmonella, pseudorabies, Leishmania, adenovirus, influenza) expressing CSP have been tested for their protective activity (21–26). Several of these CSP recombinants have been shown to be effective and have proven particularly useful for priming or boosting immunity induced by a heterologous delivery system.

As shown in this study, ts-4 constructs expressing CSP induce specific immune responses against this malaria protein that resemble those triggered by the T. gondii carrier against its own Ags. Since immunization with the nontransgenic ts-4 carrier itself unexpectedly conferred transient but high levels of nonspecific protection against a stringent sporozoite challenge, the direct efficacy of ts-4 recombinants as primary vaccines for induction of CSP-specific immunity could not be evaluated. Nevertheless, CSP recombinant ts-4 vaccination was highly effective at priming for Ag-specific CD8+ T cell responses and protective immunity following boosting with a recombinant vaccinia expressing the CSP. Together, these results demonstrate the potential of recombinant T. gondii as a live vaccine vehicle for induction of protective cell-mediated immunity against heterologous pathogens.

Materials and Methods
Toxoplasma strains and cell cultures
The temperature-sensitive mutant ts-4 strain of Toxoplasma (14) and stably transfected derivative lines were maintained by serial passages in human foreskin fibroblast (HFF) monolayers cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (HyClone, Logan, UT) and antibiotics. All cultures were incubated in a 5% CO2 environment and at 37°C, a permissive temperature for the ts-4 mutant strain.

The ts-4 HXGPR (hypoxanthine-xanthine-guanine-phosphoribosyltransferase) knockout strain (ts-4H) used in this study was produced by targeted homologous recombination using the RH knockout construct pHXGPRtg11ΔSalI (gift from Dr. David Roos, University of Pennsylvania, Philadelphia, PA) and selection with 6-thioxanthine, as described (10). The HXGPR K/O genotype was confirmed by PCR and Southern blot analyses (not shown).

Recombinant DNA constructs and procedures
The plasmid pNTPSec (27) (kindly provided by Dr. Keith Joiner, Yale University, New Haven, CT) containing untranslated regions and the signal peptide sequence of the T. gondii nucleoside triphosphate hydrolase 3 gene (NTPase3) was used to express PyCSP fusion proteins. The HXGPR mini-gene selectable marker, suitable for mycophenolic acid selection (MPA) in cell culture, was subcloned from the ts-4 knockout strain (10), was donated from the vector development facility (Frederick, MD) as a 1.96-kb XhoI/BamHI fragment and inserted into pNTPSec upstream of the NTPase3 gene sequences, in the same transcriptional orientation to produce pNTPSecH.

Fragments of the P. yoelii 17XL circumsporozoite gene (28) were amplified by PCR from plasmid CS2053 (gift from T. McCutchan, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) using sequence-specific oligonucleotides engineered with restriction sites suitable for subcloning into pNTPSecH. The oligonucleotide CSAVRI (AGGCTAGGGTTCAACA GATTACTTGGCCGAT) was used in 5′ for in-frame fusions with the NTPase3 signal peptide sequence. The restriction site AvrII (shown in bold letters above) from this oligonucleotide introduced an extra glycine residue between the NTPase3 signal peptide and the CSP sequence. In 3′, oligonucleotides CSBGII (TAGATCTCATTTAAGACCCCTCTCT TGTGAGTTG), CSRIBGII (TAGATCTCATATCAATATCCTCACTGTA), and CSSTOBGII (TAGATCTTAATAGAAGTACTAATATAC) were used to amplify CSN1, CSNA, and CS3C inserts, respectively (the restriction site BglII is shown in bold). The truncated PyCSP open reading frame amplified fragments were inserted into pNTPSecH using the AvrI I and BglII restriction sites.

PCR amplifications and subcloning steps were performed using standard procedures and protocols. All PCR-amplified DNA fragments were first inserted into the pCRII vector (Invitrogen,墙壁bad, CA) and partially sequenced to confirm fusion open reading frames before subcloning in expression vectors. Plasmid DNA preparations were used for electroporations were purified using anion-exchange resin columns (Qiagen, Chatsworth, CA), following the manufacturer’s protocols. DNA sequencing was performed at the National Cancer Institute-Frederick Cancer Research and Development Center/Science Applications International Corp. LCMS sequencing facility (Frederick, MD) using ABI automated sequencers and dye terminator cycle sequencing kits, according to the manufacturer’s protocols.

Transfections and characterization of stable T. gondii transfectants
Electroporation of tachyzoites was performed as described (8). After transfection, tachyzoites were allowed to infect HFF in drug-free culture medium for 18 h to permit the phenotypic expression of the HXGPR mini-gene selectable marker, at which time MPA was then added at a final concentration of 25 μg/ml in combination with xanthine (50 μg/ml), as described (10). Polyclonal transfected MPA-resistant tachyzoite cultures were passaged three times (1/10 to 1/50 dilutions) in the presence of the drug before cloning in 96-well plates containing HFF, in drug-free medium. Clonal lines were selected from wells containing a single plaque.

MPA-resistant cultures after electroporation and clonal lines were analyzed for PyCSP fusion expression by Western blot analyses. SDS-PAGE and immunoblot analyses were performed using a X-Cell II mini-Cell system and precast gels (NOVEX, San Diego, CA) and Protran nitrocellulose transfer membranes (Schleicher & Schuell, Keene, NH), following manufacturer’s recommended protocols. The purified anti-PyCSP mAb NYS1 (29) was a gift from Dr. Yupin Charoenvit (Naval Medical Research Institute, Rockville, MD). HRP-conjugated secondary Abs were detected using the chemiluminescent ECL system substrate (Amersham Pharmacia Biotech, Piscataway, NJ), following the manufacturer’s protocols.

Immunofluorescence microscopy and flow cytometric analyses of recombinant tachyzoites
For immunofluorescence, nonconfluent HFF monolayers cultured on gelatin-coated LAB-TEK glass chamber slides (Nalge Nunc International, Naperville, IL) were infected for 30 min with tachyzoites at a multiplicity of infection of ~5:1. Free extracellular tachyzoites were then removed by washing, and the infected HFF were further incubated for 6 h at 37°C in medium with 4% parafomaldehyde, 0.1% glutaraldehyde, 15 min at 4°C and permeabilization (acetone/methanol 1:1, 1 min at room temperature). Cells were sequentially stained with NYS1 (5 μg/ml) and a goat anti-mouse Ig (H+L) FITC conjugated (human adsorbed; Southern Biotechnology Associates, Birmingham, AL) for 30-min periods at 4°C in PBS supplemented with 1% FBS. Slides were mounted in ProLong anti-fade (Molecular Probes, Eugene, OR). For flow cytometric analyses,
tachyzoites at a concentration of 2 × 10⁶ cells/ml in PBS supplemented with 1% FBS were incubated at 4°C sequentially with biotinylated NYS1 Ab (1 μg/ml for 30 min), followed by streptavidin-R-PE conjugate (60 μg/ml; Caltag Laboratories, Burlingame, CA) for 30 min. After washing, parasites were fixed in 4% paraformaldehyde for 15 min at 4°C. Fluorescence was assayed using a FACSScan flow cytometer (Becton Dickinson, San Jose, CA) and the data analyzed using CellQuest software.

Recombinant vaccinia constructs

Recombinant vaccinia virus expressing PyCSP (NYVAC-K1L-PyCSP or vP1258) and control parental virus (NYVAC-K1L or vP993) particles (30) were kindly provided by John A. Tine (Virogenetics, Troy, NY).

Laboratory animals, immunizations, and challenges

For all experiments, female BALB/c mice (>8 wk old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in filter-top cages at a National Institute of Allergy and Infectious Diseases American Association for the Accreditation of Laboratory Animal Care-approved facility. Recombinant vaccinia (1 × 10⁶ to 5 × 10⁶ PFU/mouse) and T. gondii ts-4 immunization doses were prepared in PBS, pH 7.4, and injected i.p. P. yoelii sporozoites from the nonlethal strain 17XNL used for challenges were obtained by hand dissection of infected mosquito salivary glands, resuspended in medium 199 containing 5% normal BALB/c serum, and injected through the tail vein at 100 parasites/mouse. For all challenges, a group of five naive BALB/c mice was injected last with the same parasite preparation to control for sporozoite infectivity. Blood smears from challenged mice were prepared at day 7, 10, and 15 postchallenge and evaluated for blood-stage malaria. Protection was defined as the absence of infected cells in the blood smears of vaccinated animals as compared with control animals failing to develop blood-stage malaria were excluded.

Day 7 splenic lymphocytes were obtained by hand dissection of infected mosquito salivary glands, a group of five naive BALB/c mice was injected last with the same parasite preparation to control for sporozoite infectivity. Blood smears from challenged mice were prepared at day 7, 10, and 15 postchallenge and evaluated for blood-stage malaria. Protection was defined as the absence of infected cells in the blood smears of vaccinated animals as compared with control animals failing to develop blood-stage malaria were excluded.

In some experiments, vaccinated animals were injected at the time of challenge with mAb to deplete specific T cell subsets or cytokines. The mAb employed were directed against CD4 (GK1.5), CD8 (2.43), IFN-γ, and Gal-3. For all depletion experiments, each mouse was injected i.p. with 1 mg of mAb (or 0.5 mg for α-CD8) on days −2, −1, 0 (3 h prechallenge), and +2. For cytokine depletions, an additional dose of 1 mg of mAb was given at day 0, 6 h postchallenge. The various hybridoma cell lines and methods for mAb purification have been described (31–33) previously. The effectiveness of CD4 and CD8 depletion was confirmed to be greater than 95% by FACS analyses performed on blood samples obtained on the day of challenge. The significance of the effects of the depletions on protective immunity was analyzed using an uncorrected χ² test.

Results

Generation of stably transfected T. gondii lines expressing PyCSP

Several different strategies were tested to generate T. gondii stable transfectants expressing PyCSP. The chloramphenicol acetyltransferase gene and chloramphenicol system (6) was inefficient in our hands to select for stably transfected cell lines of T. gondii ts-4. We therefore generated a HXGPRT knockout strain of ts-4 (ts-4H) to allow the use of the more stringent mycophenolic acid (MPA) selection. Fusions with the major surface protein gene SAG1, in various configurations, generated either undetectable, or short truncated protein products expressed in the cytoplasm of recombinant parasites. However, full-length rPyCSP expression and secretion were obtained when fusion constructs with the NTPase3 gene sequences were designed to directly target the dense granule pathway of secretion in Toxoplasma. The three CSP/NTPase3 constructs generated for this study are depicted in Fig. 1. The first 64 amino-terminal residues of PyCSP carrying its signal peptide as

Assays for PyCSP-specific IFN-γ production

The frequency of PyCSP CTL epitope-specific IFN-γ-producing CD8⁺ T cells was determined using an ELISPOT assay performed on freshly isolated unstimulated spleen cells from the same animals used for CTL assays. This procedure has been described in detail previously (30). Briefly, spleen cells were cultured for 24–28 h in nitrocellulose plates precoated with rat anti-mouse IFN-γ mAb in the presence of either unpulsed P815 cells or P815 cells pulsed with 1 μM of the PyCSP synthetic peptide SYYVPSAEQL. After washing, the presence of IFN-γ-specific spots was revealed using a second biotinylated anti-mouse IFN-γ mAb, followed by incubation with streptavidin-peroxidase and development using a DAB Reagent Set (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The results were expressed as the number of IFN-γ-producing cells per 10⁶ spleen cells. As an additional readout of IFN-γ response, supernatants were collected on the third day from the same restimulated cultures used for CTL assays and levels of IFN-γ measured using a two-site ELISA, as described previously (36).

Antimalarial immunity induced by recombinant Toxoplasma

Schematic representation of the PyCSP structure and of DNA constructs designed to express the recombinant fusion protein in T. gondii. The structure of PyCSP, as well as the description of T cell CD4 and 3 oligonucleotides used for the PCR amplification were designed to insert in-frame stop codons that translated into proteins truncated by 62 and 29 aa in carboxyl terminus, respectively, compared with CSC3, P1, P2, and P3 arrows map the location and orientation of primers used to confirm the integration of recombinant plasmids in stably transfected ts-4 cell lines (Fig. 2). I and II refer to regions conserved among circumsporozoite gene sequences from Plasmodium strains and isolates.

FIGURE 1. Schematic representation of the PyCSP structure and of DNA constructs designed to express the recombinant fusion protein in T. gondii. The structure of PyCSP, as well as the description of T cell CD4 helper and CD8 epitope (H-2Kb) on the molecule are as published previously (17, 28, 37). Truncated PyCSP gene DNA fragments amplified by PCR were inserted between NTPase3 regulatory sequences (5’ and 3’ UTR), in frame with the NTPase3 signal peptide (SS), for expression and targeting through the dense granule pathway of secretion (27). In CSN1 and CSN3, the 3’ oligonucleotides used for the PCR amplification were designed to insert in-frame stop codons that translated into proteins truncated by 62 and 29 aa in carboxyl terminus, respectively, compared with CSC3, P1, P2, and P3 arrows map the location and orientation of primers used to confirm the integration of recombinant plasmids in stably transfected ts-4 cell lines (Fig. 2). I and II refer to regions conserved among circumsporozoite gene sequences from Plasmodium strains and isolates.
well as the CD4 epitope Py.1 were not incorporated in the fusions. Expression and secretion were designed to be controlled by the NTPase3 regulatory sequences (1.65 kb in 5′ and 1.2 kb in 3′). The CSC3 plasmid construct contains the complete carboxy-terminal part of PyCSP, including the conserved regions I and II, the repeat portion (including the B cell epitope) comprised of 25 repeated QGPGAP sequence in the T. gondii 17xL strain (unpublished observation), the H-2d-restricted CD8 T cell epitope, and the terminal hydrophobic domain (THD). In CSNA, the last 29 aa (which includes the THD) were not included in the fusion. In the third construct (CSN1), region II, the THD, as well as the sequences encoding the CD4 epitope AS44 (37) were omitted.

For each selected T. gondii ts-4 transgenic clonal cell line, PCR analyses using primers annealing on the 5′ and 3′ flanking sequences of the NTPase3 open reading frame (P1 and P3, respectively; refer to Fig. 1 for the location of primers) amplified the genomic NTPase3 gene (~2-kb fragment), as well as the inserted sequences of the NTPase3 open reading frame (P1 and P3, respectively), the H-2d-restricted CD8 T cell epitope, and the terminal hydrophobic domain (THD). In CSNA, the last 29 aa (which includes the THD) were not included in the fusion. In the third construct (CSN1), region II, the THD, as well as the sequences encoding the CD4 epitope AS44 (37) were omitted.

Expression of PyCSP by recombinant ts-4 cell lines

rPyCSP-truncated proteins were readily detected from T. gondii ts-4-infected HFF lysates by immunoblot analyses using the mAb NYS1, which recognizes the reduced repetitive QGPGAP sequence (38). The reduced protein products migrated at ~80 kDa on 6% SDS-PAGE (Fig. 3A), a result consistent with the reported molecular mass for the P. yoelii 17x(NL) CSP recognized by NYS1 (from 56 to 84 kDa) (29).

Localization of PyCSP in recombinant ts-4 tachyzoite-infected HFF by immunofluorescence showed that the expressed malaria protein product accumulates mainly within the parasitophorous vacuolar space, suggesting trafficking and secretion through the dense granule pathway. A representative fluorescence micrograph of a HFF infected with CSNA is shown in Fig. 3B. The same staining pattern was observed when HFF infected with CSN1 or CSC3 were analyzed. Flow cytometric analyses of extracellular tachyzoites (Fig. 3C) revealed that, in the case of CSC3, PyCSP is also surface expressed in addition to being secreted and/or shed. A residual but significant surface staining was also observed for both CSN1 (not shown) and CSC3 parasites was estimated to be ~1 log.

Single dose immunization with ts-4 tachyzoites expressing PyCSP leads to both specific immune responses as well as nonspecific protection against T. gondii sporozoites

We first compared the ability of the CSN1, CSNA, and CSC3 stably transfected ts-4 lines to elicit PyCSP-specific responses following a single dose i.p. immunization of BALB/c mice. Animals were inoculated with 10⁷ CSC1, CSNA, or CSC3 tachyzoites, and serum PyCSP as well as Toxoplasma-specific total Ig Ab titers were evaluated 22 days later. Significant PyCSP-specific titers were detected by ELISA in mice inoculated with each of the three recombinant lines (Fig. 4). These titers were highly reproducible within each animal group. The CSC3 and CSNA lines, which express the PyCSP AS44 cryptic CD4 epitope, induced significantly higher ELISA and IFAT titers (see inset, Fig. 4) than CSN1, which lack this sequence. All strains, including the H1 control HXGPRT-complemented line, elicited similar T. gondii-specific titers, as judged by serum reactivity against a Toxoplasma soluble extract (STAg) in ELISA (Fig. 4, inset), demonstrating that expression of PyCSP did not modify the response to the ts-4 carrier in vivo.
Since ts-4 immunization has previously been shown to result in strong Th1 cytokine responses (16), we asked whether a comparable Th1 bias would be reflected in the isotypes of the anti-sporozoite Abs elicited by CSC3 vaccination. Interestingly, CSC3 immunization induced high levels of PyCSP-specific IgG3 and IgG2a with little IgG1 and IgG2b Ab (Fig. 5A). This isotypic profile was similar to that of the T. gondii-specific Ab elicited by CSC3, as measured by ELISA against soluble Toxoplasma Ags (Fig. 5B).

To assess whether single dose CSC3 immunization results in protection against preerythrocytic stages of malaria, mice were challenged i.v. with 100 sporozoites and monitored for blood-stage parasites for a 2-wk period. Combined data obtained from three independent experiments are presented in Table I. Unexpectedly, the non-PyCSP-transfected ts-4 H1 line that was used as a negative control conferred up to 70% protection in mice challenged 27 days postimmunization. This level of resistance was comparable with that displayed by the mice vaccinated with CSC3. To attempt to circumvent the problem of the nonspecific resistance to sporozoites induced by the ts-4 carrier, we conducted a series of single dose vaccination experiments in which the challenge was delayed and determined that by day 38 all of the protection induced by the control line H1 had waned. However, when CSC3-immunized animals were challenged at the same day 38 time point, less than 10% specific protection was observed (Table I).

Since the nonspecific production of IFN-γ (e.g., as induced in vivo by rIL-12) can lead to transient protection against the preerythrocytic stages of the malaria parasite (39, 40), we followed the expression of this cytokine in both H1- and CSC3-vaccinated mice. When we compared IFN-γ mRNA expression in spleen as well as in liver at day 27 and day 40 postimmunization by RT-PCR, we failed to detect any consistent differences in expression of this cytokine that would explain the presence or absence of nonspecific protection at the two time points (data not shown). In addition, we did not reveal any serological cross-reactivity by immunofluorescence between Toxoplasma-induced Ab in ts-4 H1-immunized mouse sera and P. yoelii sporozoite or liver-stage parasites.

When we attempted to boost the specific immunity induced by ts-4 CSC3 by administering a second higher dose (10^6) of recombinant parasites 40 days after the 10^4 priming dose, no significant specific or nonspecific protection was observed (Table I), nor were PyCSP titers significantly increased in the CSC3-primed boosted mice (data not shown).

*Ts-4/PyCSP provides highly effective priming for the induction of protective immunity against sporozoite challenge following boosting with recombinant vaccinia*

It was possible that the inability to induce antisporeozoite immunity by repeated vaccination with ts-4 CSC3 was due to the induction of a strong resistance to the Toxoplasma carrier, resulting in rapid clearance of the boosting dose of recombinant parasites. Therefore, we next attempted to boost the primary CSC3 vaccination with PyCSP produced by a different carrier. A recombinant vaccinia (vP1258) expressing PyCSP, which has been successfully utilized for boosting immunity induced by DNA vaccination, was employed for this purpose. As shown in Table II, mice given up to 5 × 10^7 PFU of vP1258, without priming, failed to display protection when challenged with 100 sporozoites 15 days later, consistent with previous findings (30). Similarly, mice primed with a single dose of control H1 ts-4

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**Table I. Recombinant ts-4 immunization induces nonspecific protection against sporozoite challenge**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Challenge</th>
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<tbody>
<tr>
<td>ts-4 strain</td>
<td>Dose (no.)</td>
</tr>
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<td>H1</td>
<td>2</td>
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<tr>
<td>CSC3</td>
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**Table II. Protection against sporozoite challenge induced by heterologous recombinant ts-4/vaccinia prime/boost**

<table>
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<tbody>
<tr>
<td>ts-4 strain</td>
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* Injected 50 days after ts-4 immunization.
* vP993, Parental vaccinia.
* vP1258, PyCSP recombinant vaccinia.
* Data pooled from two separate experiments with similar results.
tachyzoites and then boosted with either vP1258 or with the control parental vaccinia (vP993) were not protected against the same challenge, as were mice given a single dose of ts-4 CSC3 65 days before. In direct contrast, up to 79% of mice primed with CSC3 and boosted with vP1258 were protected from the development of blood-stage malaria following sporozoite challenge (see also Fig. 8). Thus, effective vaccination requires a combination of heterologous recombinant carriers both expressing PyCSP.

A number of different immunological parameters were examined to attempt to determine the basis of the high level of specific protection conferred by CSC3/vP1258 vaccination. When serum IFAT titers were compared at the time of sporozoite challenge, CSC3/vP1258-immunized mice showed significantly higher responses than animals vaccinated with any of the other combinations of recombinant ts-4 and vaccinia (Table II). Examination of Ab isotypes revealed that CSC3/vP1258-vaccinated mice displayed higher levels of IgG1 than mice exposed to CSC3 alone for the same time period, but did not differ significantly in any of the other isotypes (data not shown).

When CSP-specific cellular responses were compared in the different groups of animals, a striking correlation with protective immunity was observed. As shown in Fig. 6A, cultures from CSC3/

FIGURE 6. CTL activity induced by single dose H1 or CSC3 or by heterologous prime-boost regimens. The results shown represent percent specific lysis of P815 target cells (H-2d) pulsed with the H-2Kd-restricted 9-mer PyCSP peptide 280–288 minus the percent lysis of unpulsed targets by restimulated effector cells pooled from three mice/group. A, Titration of CTL activity of spleen cells harvested from mice; 65 days postimmunization with CSC3, 15 days postimmunization with a single dose vP1258, 15 days postboost with either vP993 or vP1258 after priming with H1 or CSC3. B, Abrogation of CTL activity by in vitro depletion of CD8+ T cells. The effectors employed were spleen cells from mice primed with CSC3 and boosted with vP1258 as in A. Before the assay at an E:T ratio of 80:1, cells were depleted of T cell subsets by Ab-mediated complement lysis, as described previously (39).
vP1258-vaccinated mice restimulated with APC pulsed with a peptide spanning the defined CD8+ T cell epitope of PyCSP displayed strong CTL activity (up to 95%) even at low E:T ratios. This specific cytolytic activity was totally abrogated by depletion of CD8+, but not by CD4+ T cells from the effectors (Fig. 6B). In contrast, cultures from single dose CSC3, vP1258, or control recombinant carrier-immunized mice displayed only weak activity that disappeared upon titration. Moreover, CSC3/vP1258-vaccinated mice showed significantly higher frequencies of splenic IFN-γ-producing cells, as assessed by ELISPOT assay (Fig. 7A), and produced more of the cytokine in culture (Fig. 7B) than equivalent splenocyte populations from mice vaccinated with all other single carriers or combinations of carriers.

To assess whether the observed CD8 effector function is related to protective immunity, mice vaccinated with CSC3 and boosted with vP1258 were treated with either anti-CD8 and/or anti-CD4 at the time of sporozoite challenge. As shown in Fig. 8, depletion of CD8+ T cells resulted in a complete ablation of protection, while depletion of CD4+ T cells caused a partial but significant (p < 0.01, uncorrected \( \chi^2 \) test) loss in immunity. Furthermore, in vivo neutralization of either IFN-γ or IL-12 resulted in a partial loss in protection (p < 0.01 and p < 0.05, respectively), arguing that these cytokines also participate in the effector mechanism of CSC3/vP1258 vaccination.

Discussion

In this study, we demonstrate that nonpathogenic strains of T. gondii can be effectively used as recombinant carriers for heterologous immunization. Although the feasibility of expressing CSP in Toxoplasma has been previously documented, the Plasmodium knowlesi gene utilized in that study was transfected into the highly virulent RH strain, and the protective efficacy of the construct was not assessed (41). The use of the ts-4 strain of T. gondii as a carrier was an important element in the design of the recombinant vaccine constructs or combinations of carriers.

That the PyCSP expressed by our vaccine constructs is immunogenic in vivo was established in single dose immunization experiments using 104 recombinant tachyzoites. Mice immunized with recombinant ts-4 CSC3 or CSNA displayed higher PyCSP-specific ELISA and IFAT titers than animals inoculated with the CSN1 strain, while the humoral response directed to Toxoplasma Ags was similar in the different experimental groups. This finding confirms that the cryptic CD4 helper epitope AS44, present in CSC3 and CSNA but not in CSN1, contributes to the Ab response and can function efficiently in the absence of the dominant amino-terminal epitope Py1 (Fig. 1). No appreciable difference was observed in the titers elicited by CSC3 vs CSNA, suggesting that incorporation into T. gondii surface membrane is not critical for effective Ag presentation. Importantly, the IFAT titers induced by single dose CSC3 immunization were comparable with those induced by protective vaccination with irradiated P. yoelii sporozoites (38). The above findings confirmed that PyCSP is expressed in vivo in immunogenic form by recombinant ts-4 and can generate an efficient humoral response in the presence of antigenic competition from the Toxoplasma carrier.

Both humoral and cell-mediated immune mechanisms have been implicated in the protection against preerythrocytic stages of
malaria induced by CSP vaccination. In many of the experimental systems examined, cellular mechanisms appear to play a dominant role with CD4 and/or CD8 cells mediating protection (19, 20). T cell-derived IFN-γ has been shown to be a major effector of sporozoite immunity, and this protective cytokine response can be exogenously stimulated by IL-12. The above observations argue that protection induced by CSP vaccination depends on the induction of a Th1-type cellular immune mechanism similar to that triggered by ts-4 vaccination against T. gondii. Attempts to evaluate the profile of Th1/Th2 cytokines produced specifically in response to PyCSP in mice exposed to a single dose of CSC3 were unsuccessful in part due to the high background of IFN-γ induced by the ts-4 carrier against itself. Nevertheless, the isotypic profile of the PyCSP-specific Abs induced in these mice was consistent with a Th1 response pattern, in that IgG2a and IgG3 Ab predominated while IgG1 levels were low.

An unexpected finding of the present study was the high level of protection against sporozoite challenge induced by the ts-4 carrier itself. It is important to note that since the calculated ID₅₀ for P. yoelii challenge is often <2 sporozoites (47), the 100 sporozoite challenge dose used in our experiments is highly stringent. Since Ab reacting with sporozoite or liver-stage forms were not detected in ts-4-vaccinated mice, this high level of protection is unlikely to be due to the induction of cross-reactive immune responses. Although Toxoplasma has been shown to confer nonspecific protection against viral, bacterial, protozoal, or helminth infections, the parasite strains used in these previous studies all produce chronic infections (2–4). The results presented in this study provide the first demonstration that a nonspecific strain of Toxoplasma can induce protection against an antigenically distinct pathogen.

This highly effective nonspecific resistance was maintained for ~30 days, but then faded abruptly (Table I), perhaps due to the clearance of the attenuated parasite at this period. The avirulent phenotype of ts-4 is known to be dependent on the induction of both IL-12 and IFN-γ (36, 48, 49). Moreover, ts-4 vaccination induces strong T cell-derived IFN-γ responses (16). Since exogenous administration of IL-12 results in high levels of IFN-γ-dependent resistance against sporozoite challenge (39), one hypothesis is that the nonspecific immunity induced by ts-4 involves a similar mechanism. Indeed, it has been recently reported that immunization with heat-killed Toxoplasma tachyzoites protects mice against lethal P. yoelii malaria, and that this resistance is also associated with the induction of high levels of IFN-γ (50). We are currently attempting to test the involvement of this mechanism in our model by depleting IL-12 or IFN-γ at the time of challenge infection.

Consistent with the results of studies with other recombinant vaccine vehicles, attempts to boost specific protection by repeated CSC3 immunization once nonspecific protection had faded were unsuccessful. For this reason, we adopted a prime-boost approach using sequential immunization with heterologous T. gondii/vaccinia carriers for PyCSP. This strategy has proven effective in enhancing both T cell activity and protective immunity against preerythrocytic stages of malaria. Thus, under conditions in which vaccination with PyCSP vaccinia alone fails to result in significant protection against sporozoite challenge, the same recombinant efficiently boosts priming by CSP expressed by yeast, influenza, or naked DNA immunization against malaria (22, 26, 30, 51). Such boosting has been shown to lead to enhanced CSP-specific CD8 CTL activity as well as increased frequencies of CD8⁺ IFN-γ-secreting cells. Consistent with these previous findings, we observed that a regimen employing CSC3 priming and heterologous vaccinia boosting led to strong Ag-specific CD8⁺ T cell CTL- and IFN-γ-producing activity as well as high levels of CD8⁺ T cell-dependent protection against a stringent sporozoite challenge. Since little or no cellular response was detected in mice vaccinated with each of the carriers alone (Fig. 6), the effect of the prime boost in this model appears to result from a true synergy rather than the additive effects of both immunizations. These findings suggest that the recombinant ts-4 carrier, while failing to promote PyCSP-specific protection, nevertheless is highly efficient at eliciting CD8⁺ memory cells that can be induced to become effectors by heterologous boosting. The above hypothesis is consistent with the known ability of ts-4 to induce strong CD8-dependent immunity against T. gondii itself (16, 44). Alternatively, CSC3 may stimulate CD4⁺ Th1 lymphocytes that in turn provide help for the CD8⁺ T cell-dependent protective response induced by the vaccinia boost. Indeed, the protection conferred by the heterologous CSC3/vP1258 immunization was found to be partially abrogated by in vivo depletion of CD4⁺ T cells at the time of challenge, and one might expect an even greater effect if the treatment is instead initiated before boosting.

In conclusion, the results of this study establish the feasibility of using attenuated T. gondii as delivery system for priming protective immune responses against heterologous pathogens. An important issue concerns whether a live carrier that itself is potentially pathogenic could ever be made safe for use in humans. This objection, which can be leveled against many live Ag delivery vehicles currently under investigation, may be of lesser importance in the case of T. gondii, which normally gives asymptomatic infections in immunocompetent individuals. Indeed, avirulent strains of Toxoplasma have already been tested for immunizing livestock to prevent congenital toxoplasmosis (52). Moreover, because of the ease of genetically engineering stable parasite lines, it should be possible to rationally design attenuated strains of Toxoplasma, which are both efficient for heterologous immunization and non-pathogenic even in immunocompromised hosts. Thus, T. gondii should now be added to the list of vaccine carriers that are being considered for potential clinical use.

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