6-15-2009

Kinetics and Phenotype of Vaccine-Induced CD8+ T-Cell Responses to Toxoplasma gondii

Kimberly A. Jordan
University of Pennsylvania

Emma H. Wilson
University of California, Riverside

Elia D. Tait
University of Pennsylvania

Barbara A. Fox
Dartmouth College

David S. Roos
University of Pennsylvania

See next page for additional authors

INFECTION AND IMMUNITY, Sept. 2009, p. 3894–3901 Vol. 77, No. 9
0019-9567/09/$08.00+0 doi:10.1128/IAI.00024-09

Kinetics and Phenotype of Vaccine-Induced CD8$^+$ T-Cell Responses to *Toxoplasma gondii*$^\nabla$

Kimberly A. Jordan,[1] Emma H. Wilson,[2] Elia D. Tait,[1] Barbara A. Fox,[3] David S. Roos,[4] David J. Bzik,[3] Florence Dzierszinski,[5] and Christopher A. Hunter[1]*

Department of Pathobiology, University of Pennsylvania, 380 South University Ave., Philadelphia, Pennsylvania 19104[1]; Division of Biomedical Sciences, University of California, Riverside, 900 University Ave., Riverside, California 92521[2]; Department of Microbiology and Immunology, Dartmouth Medical School, 1 Medical Center Drive, Lebanon, New Hampshire 03756[3]; Department of Biology, 304B Carolyn Lynch Laboratories, University of Pennsylvania, 433 South University Ave., Philadelphia, Pennsylvania 19104[4]; and Institute of Parasitology, McGill University, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Québec H9X 3V9, Canada[5]

Received 7 January 2009/Returned for modification 25 February 2009/Accepted 4 June 2009

Multiple studies have established that the ability of CD8$^+$ T cells to act as cytolytic effectors and produce gamma interferon is important in mediating resistance to the intracellular parasite *Toxoplasma gondii*. To better understand the generation of the antigen-specific CD8$^+$ T-cell responses induced by *T. gondii*, mice were immunized with replication-deficient parasites that express the model antigen ovalbumin (OVA). Class I tetramers specific for SIINFEKL were used to track the OVA-specific endogenous CD8$^+$ T cells. The peak CD8$^+$ T-cell response was found at day 10 postimmunization, after which the frequency and numbers of antigen-specific cells declined. Unexpectedly, replication-deficient parasites were found to induce antigen-specific cells with faster kinetics than replicating parasites. The generation of optimal numbers of antigen-specific CD8$^+$ effector T cells was found to require CD4$^+$ T-cell help. At 7 days following immunization, antigen-specific cells were found to be CD62L^{low}, KLRG1$^+$, and CD127^{low}, and they maintained this phenotype for more than 70 days. Antigen-specific CD8$^+$ effector T cells in immunized mice exhibited potent perforin-dependent OVA-specific cytolytic activity in vivo. Perforin-dependent cytolysis appeared to be the major cytolytic mechanism; however, a perforin-independent pathway that was not mediated via Fas-FasL was also detected. This study provides further insight into vaccine-induced cytotoxic T-lymphocyte responses that correlate with protective immunity to *T. gondii* and identifies a critical role for CD4$^+$ T cells in the generation of protective CD8$^+$ T-cell responses.

The intracellular parasite *Toxoplasma gondii* infects a wide range of mammalian hosts and cell types and is an important opportunistic pathogen in humans (25). Early following infection, the proinflammatory cytokine interleukin-12 (IL-12) is produced by antigen-presenting cells, including macrophages and dendritic cells, which induce NK cell production of gamma interferon (IFN-γ). This environment allows for the development of a Th1-polarized CD4$^+$ and CD8$^+$ adaptive T-cell response dominated by IFN-γ, the maintenance of which is critical for resistance to chronic toxoplasmosis (17). An additional function of CD8$^+$ T cells in mediating resistance to intracellular pathogens is their capacity to be cytolytic, and perforin-mediated cytolytic activity contributes to the control of chronic toxoplasmosis (12). Consistent with this model, immunocompromised patients with suppressed T-cell numbers or functionality can experience reactivation of latent *T. gondii* (25). Furthermore, multiple studies using murine models have established the critical role of CD4$^+$ and CD8$^+$ T cells in protective immunity to *T. gondii* during primary and secondary challenges (16, 17, 34).

Understanding how the adaptive immune response to *T. gondii* is generated will be helpful for the development of an effective vaccine. Numerous *T. gondii* protein or DNA vaccines have been tested in mice and have provided various levels of protection (31, 37). Further, a vaccine against ovine toxoplasmosis is commercially available, but the strain is not suited for human use because, although it is attenuated, it can still cause disease in vaccinated animals (5). Perhaps the most effective protection has been provided by infection with an attenuated temperature-sensitive strain of parasite, called ts-4 (17, 34); however, this strain possesses the ability to replicate and cause disease in immunodeficient as well as immunocompetent hosts (30). One possible solution to this safety issue is to genetically modify parasites so that they are unable to replicate in vivo and would be incapable of reverting to a virulent phenotype. One such clone, cps1-1, was generated by knocking out a regulatory enzyme of the de novo pyrimidine biosynthesis pathway, carbamoyl phosphate synthetase II (13). This mutation renders the parasites unable to replicate in the absence of exogenous uracil, and the cps1-1 strain is nonvirulent in mice. Thus, mice that lack IFN-γ survive infection with this organism. Further, immunization protects wild-type (WT) mice following challenge with the virulent RH strain (13). At the time that these studies were initiated, little was known about the nature of the adaptive immune response that these parasites induced.

The studies presented here examine the kinetics, phenotype, and function of endogenous antigen-specific CD8$^+$ effector T cells generated in response to the cps1-1 parasite. Several reports have demonstrated the utility of using model antigens

* Corresponding author. Mailing address: Room 313, Hill Pavilion, 380 South University Avenue, Philadelphia, PA 19104-4539. Phone: (215) 573-7772. Fax: (215) 746-2295. E-mail: chunter@vet.upenn.edu.
$^\nabla$ Published ahead of print on 15 June 2009.

to study antigen-specific responses to *T. gondii* (24, 29); in addition, previous work demonstrated that secreted but not cytosolic antigens induce an effective adaptive immune response (24, 29). Consequently, to allow the tracking of an endogenous $CD8^+$ T-cell response, the cps1-1 parasites were engineered to express a secreted form of the model antigen ovalbumin (CPS-OVA). The studies presented here show that the protection induced by immunization with CPS-OVA required T cells at the time of challenge. The peak of $CD8^+$ T-cell expansion to CPS-OVA was seen at day 10 in both the site of infection and secondary lymphoid tissue. Unexpectedly, the replication-deficient parasites induced an OVA-specific response which was comparable in magnitude to that observed using a replicating parasite, Pru-OVA, but which actually developed more rapidly. Optimal generation of the antigen-specific cells in response to CPS-OVA required the presence of $CD4^+$ T cells at the time of immunization. Nonetheless, the $CD8^+$ T cells that were generated in the absence of $CD4^+$ T-cell help were able to produce IFN-γ and were cytolytic. CD4 help also was critical for the upregulation of the killer-like lectin receptor G1 (KLRG1), a marker associated with an effector phenotype (20, 35). Cytolytic activity was mediated mainly via a perforin-dependent pathway, while Fas-FasL interactions were not required. This work provides some of the first data regarding the phenotype and function of antigen-specific cells induced by a nonreplicating strain of *T. gondii*. The findings presented here suggest that an effective vaccine targeting cell-mediated immunity should engage both $CD4^+$ and $CD8^+$ T-cell subsets.

MATERIALS AND METHODS

Mice and infections. C57BL/6, C57BL/6-*Prf1*^{tm1Sdz}/J (22), and B6.MRL-*Fas*^{lpr}/J mice (1) were purchased from Jackson Laboratory (Bar Harbor, ME). B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice were purchased from Taconic. For depletion of $CD4^+$ or $CD8^+$ T cells, mice were injected twice during the week prior to immunization with 0.5 mg anti-CD4 (GK1.5), anti-CD8 (2.43), or control antibody (rat immunoglobulin G2b). Mice were maintained under specific-pathogen-free conditions, and all animal work was done in accordance with the Institutional Animal Care and Use Guidelines of the University of Pennsylvania. For all experiments using CPS-OVA and Pru-OVA, mice were injected intraperitoneally with 10^5 parasites. For challenge experiments, mice were given 10^3 RH parental or RH-OVA (RH expressing secreted OVA) tachyzoites (29). Tachyzoites were grown in human foreskin fibroblast monolayers in Dulbecco modified Eagle medium containing 1% (CPS and RH parasites) or 10% (Pru parasites) fetal calf serum and 1% penicillin-streptomycin. CPS parasites were cultured in medium containing 0.2 mM uracil, and OVA-transgenic parasites were maintained in medium containing 20 μM chloramphenicol.

In vitro T-cell responses. Spleen cells, lymph node cells, and peritoneal exudate cells (PECs) were harvested and dissociated into single-cell suspension in complete RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum, 10 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 25 mM HEPES, 0.1 mM nonessential amino acids, and 50 μM 2-mercaptoethanol. Spleen and lymph node cells (5×10^5/well) and PECs (10^5/well) were plated out in 96-well round-bottom plates (Costar, Carlsbad, CA) and cultured to assess cytokine production. Cells were restimulated with anti-CD3/CD28 (1 μg/ml each), soluble *Toxoplasma* antigen (25 μg/ml), 500 μg/ml OVA (Worthington Biochemical Corporation, Lakewood, NJ), or 1 μg/ml SIINFEKL peptide (CHI Scientific, Maynard, MA). Supernatants were removed after 40 h and assayed for the production of IFN-γ by enzyme-linked immunosorbent assay.

Flow cytometry. For identification of OVA-specific T-cell responses, single-cell suspensions were washed in fluorescence-activated cell sorter buffer (phosphate-buffered saline, 2 mM EDTA, and 2% bovine serum albumin) and incubated for 15 to 20 min with Fc block (fluorescence-activated cell sorter buffer containing 1 μg/ml 2.4G2 from BD Pharmingen and 1 μg/ml rat and mouse immunoglobulin G from Caltag/Invitrogen). Cells were stained with OTI-SIINFEKL tetramer conjugated to phycoerythrin (PE) or allophycocyanin (APC) (from Beckman Coulter or a generous gift from the Wherry lab at Wistar Institute) for 25 min at room temperature, washed once, and then stained for other surface markers for 15 min at 4°C. The following monoclonal antibodies were used: CD8 (conjugated to fluorescein isothiocyanate, peridinin chlorophyll protein, or APC), CD45.1-PE, CD127-biotin, CD122-biotin, and CD62L-APC (BD Biosciences); tumor necrosis factor alpha-APC, IFN-γ–APC, IFN-γ–PE, KLRG1-APC, and streptavidin-APC (eBioscience, San Diego, CA); and anti-human granzyme B-APC (Caltag, Carlsbad, CA).

For intracellular cytokine analysis, splenocytes or PECs were incubated (with cell and antigen concentrations as noted above) for 5.5 h total, with the addition of 10 μg/ml brefeldin A (Sigma) for the final 4 h. Cells were first stained for surface markers, followed by fixation overnight with 2% paraformaldehyde (Electron Microscopy Sciences). Cells were permeabilized with 0.1% saponin and then stained for intracellular cytokines for 1 h at 4°C. Flow cytometry samples were collected on a FACSCalibur or FACSCanto machine (BD) and analyzed with FlowJo software (Tree Star Inc. Ashland, OR).

In vivo CTL assay. Cytolytic activity was assessed using the in vivo cytotoxic T-lymphocyte (CTL) assay, modified slightly from previous protocols (3). Briefly, spleen and lymph node cells from CD45.1 mice were pooled and pulsed with 1 μg/ml OTI peptide (CHI Scientific) for 1 h at 37°C. Cells were washed extensively in phosphate-buffered saline, labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (Molecular Probes/Invitrogen) at a concentration of 5 μM (pulsed with OTI peptide) or 0.1 μM (unpulsed), and then counted and resuspended at a 1:1 ratio. A total of 6×10^6 cells were then transferred intravenously to anesthetized recipient mice. Mice were sacrificed 4 h later, and spleens were analyzed for specific lysis of the peptide-pulsed population by gating on $CD45.1^+$ donor cells. Specific lysis was calculated as described previously (3).

Detection of parasite DNA. Tissue samples were taken from spleen, liver, and peritoneal exudate at various time points following immunization, and DNA was extracted using the High Pure PCR template preparation kit (Roche). For measurement of parasite burden, the 35-fold repetitive *T. gondii* B1 gene was amplified by real-time PCR with SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in an AB7500 fast real-time PCR machine (Applied Biosystems) using published conditions (36).

Statistics. Absolute numbers and frequencies of tetramer-positive cells were statistically analyzed using a Student *t* test. Where appropriate, data are shown as means ± standard deviations.

RESULTS

T cells are required for CPS-OVA-induced protective immunity. Earlier work demonstrated that cps1-1 parasites could provide protective immunity to challenge with the virulent RH strain of *T. gondii* (13). However, these studies did not provide an analysis of the adaptive immune response to immunization or indicate which cell types were important for protection. In order to define which T-cell subsets were required for CPS-induced protective immunity, mice were injected intraperitoneally with a single dose of 10^5 CPS parasites that expressed the model antigen OVA and then challenged 30 days later. Similar to what was seen previously with the parental (non-OVA-expressing) strain, unimmunized mice succumbed to RH-OVA infection by day 12, while mice immunized with CPS-OVA survived this challenge (Fig. 1). One week prior to RH-OVA challenge, immunized mice were depleted of either $CD4^+$ or $CD8^+$ T cells. As shown in Fig. 1, depletion of $CD8^+$ T cells resulted in increased susceptibility to RH-OVA challenge. In contrast, while there was some variation between experiments, $CD4^+$ depletion had only a modest effect on the survival of challenged mice. Thus, in this model of immunization with a single dose of 10^5 CPS-OVA parasites, $CD8^+$ T cells appeared to be the most important subset required for protection following a lethal RH-OVA challenge. It is also worth noting that CPS-OVA immunization was also able to provide resistance to challenge with the parental RH strain (Fig. 1), indicating that CPS-OVA can induce a protective polyclonal response to *T. gondii*.

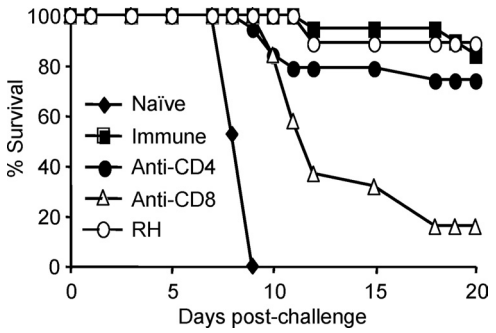

FIG. 1. Immunization with CPS-OVA provides resistance to rechallenge. Mice immunized intraperitoneally with 10^5 CPS-OVA parasites or naïve control B6 mice were challenged with 1,000 RH-OVA parasites at 30 days following immunization, and survival was assessed. Mice were depleted of $CD4^+$ or $CD8^+$ T cells 1 week prior to RH-OVA challenge. Mice immunized with CPS-OVA and challenged with the RH parental strain were also protected. Combined results from four experiments with four or five mice per group, except for RH challenge group ($n = 9$ from two similar experiments), are shown.

Immunization with a replication-deficient parasite induces a robust endogenous $CD8^+$ T-cell response and upregulation of activation markers. Because the parasite used in these experiments expressed the model antigen OVA, a robust endogenous $CD8^+$ T-cell response that could be tracked using an OVA-specific class I tetramer was induced (Fig. 2A [day 7 data are shown]). These experiments revealed that OVA-specific cells could be readily detected in the spleen as early as 5 days following immunization (Fig. 2B). Analysis of the kinetics indicated that the peak of this response occurred at 10 days postinfection, after which the response declined, though low frequencies of OVA-specific cells were maintained (Fig. 2B, top panel). A similar pattern of expansion and contraction of OVA-specific $CD8^+$ T cells was also seen in the PECs, the site of infection (Fig. 2B, bottom panel). In both the spleen cells and PECs, the total number of OVA-specific $CD8^+$ T cells followed a similar pattern of expansion and contraction (data not shown). Splenocytes and PECs were stimulated directly ex vivo with OTI peptide for 5 h, revealing antigen-specific IFN-γ production following intracellular cytokine staining (Fig. 2C, day 7). Further, antigen-specific IFN-γ production was detected in the supernatant following a 40-hour restimulation of splenocytes with OTI peptide, whole OVA protein, or soluble *Toxoplasma* antigens (Fig. 2D).

To examine the expression of phenotypic markers associated with activation and effector capacity, cells were stained directly ex vivo from the spleen and peritoneal exudate for tetramer binding as well as phenotypic markers, including CD62L,

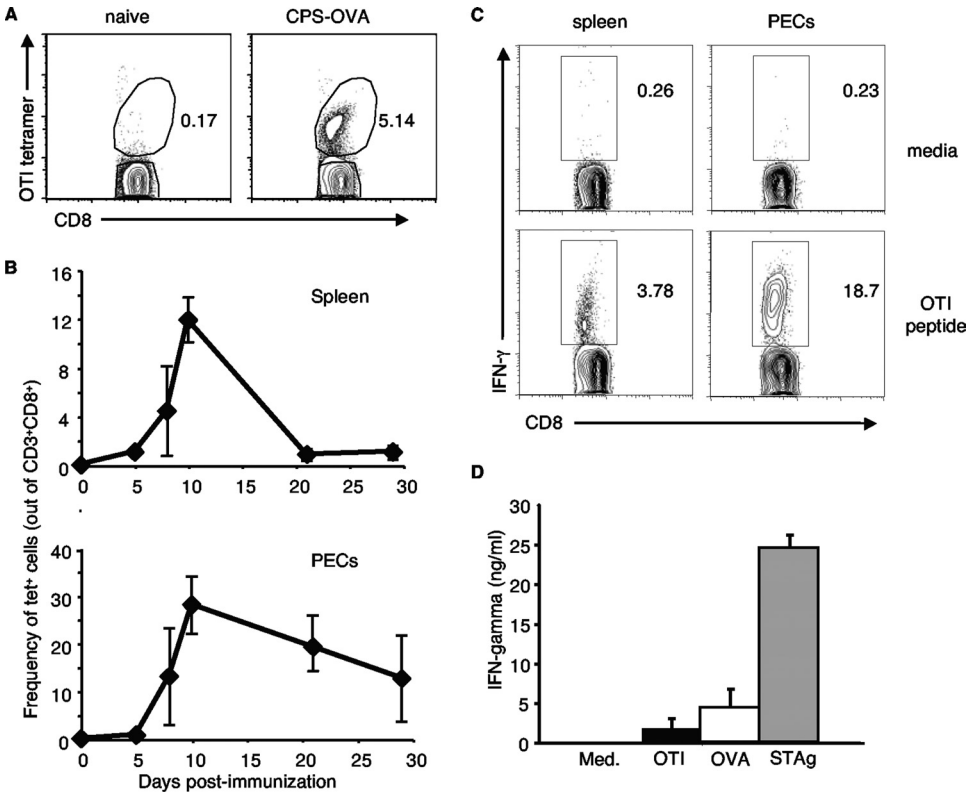

FIG. 2. Endogenous OVA-specific $CD8^+$ T-cell responses can be detected following immunization with CPS-OVA. (A) Tetramer-positive cells following immunization with CPS-OVA on day 7 (spleen). Numbers indicate the frequency of live $CD3^+$ $CD8^+$ cells that are tetramer positive. (B) Kinetics of antigen-specific response in spleen cells and PECs, showing frequency (out of $CD3^+$ $CD8^+$ gated cells) of OVA-specific $CD8^+$ T cells over time following immunization. Data points represent mean values ± standard deviations. (C) Spleen cells and PECs restimulated ex vivo for 5 h with OTI peptide and stained for intracellular IFN-γ, gated on $CD8^+$ lymphocytes. (D) IFN-γ production by splenocytes was determined by enzyme-linked immunosorbent assay following a 40-h incubation with OTI peptide, OVA protein, or soluble *Toxoplasma* antigen (STAg) (average + standard deviation). Representative data from one of three similar experiments with five mice per group are shown.

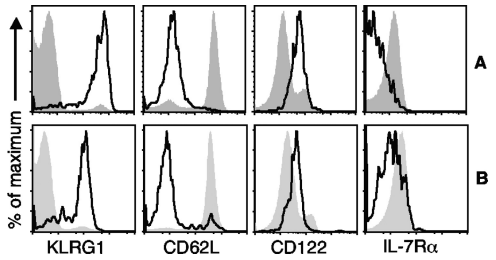

FIG. 3. Endogenous OVA-specific $CD8^+$ T cells have an effector phenotype following immunization with CPS-OVA. Histograms show $CD3^+$ $CD8^+$ tetramer-negative splenocytes (shaded gray histograms) versus OTI tetramer-positive cells (black open histograms) at 7 (A) or 75 (B) days following immunization. Representative results from one mouse per time point are shown; each time point has been repeated at least twice with three mice per group.

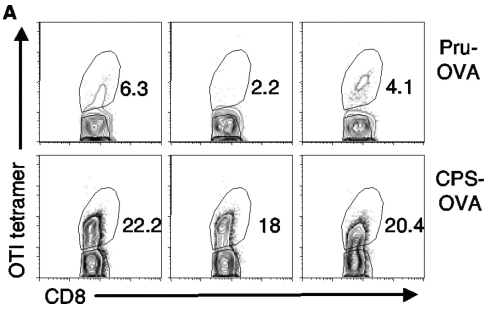

FIG. 4. CPS-OVA induces more potent $CD8^+$ responses than Pru-OVA at 8 days following infection. Tetramer responses in the PECs at 8 days following infection, gated on $CD3^+$ $CD8^+$ lymphocytes, are shown. Numbers indicate the frequency of tetramer-positive cells for Pru-OVA and CPS-OVA from three individual mice per group.

KLRG1, CD122, and CD127. These particular markers were chosen because they have been used to discriminate between naïve, effector, and memory subpopulations in a variety of viral and bacterial systems (21). However, antigen-specific responses to *T. gondii* in particular have not been extensively characterized. We found that at day 7, the majority of tetramer-positive cells in the spleen (Fig. 3) as well as the PECs (data not shown) had upregulated KLRG1 and had also downregulated CD62L, thus exhibiting an effector phenotype similar to what has been noted in other infection models. Here, OVA-specific cells are compared to the tetramer-negative $CD8^+$ T-cell population, which contains activated cells but at a very low frequency compared with the immunodominant tetramer-positive population. Antigen-specific cells also showed higher expression of the IL-2/IL-15 receptor β (IL-2/IL-15Rβ) (CD122) than tetramer-negative cells (Fig. 3). Consistent with recent publications (21), at 7 days following immunization expression of IL-7Rα was decreased on tetramer-positive cells compared to $CD3^+$ $CD8^+$ tetramer-negative cells, a population that would include primarily naïve cells but also a small population of activated cells (Fig. 3).

To assess the phenotype of the memory cell population, later time points were also examined. Parasite DNA was not detected in the PECs or spleen at 3 weeks following CPS infection, and it was difficult to consistently detect parasite DNA even at 10 days following immunization (Fig. 3B and data not shown); thus, we decided to investigate the phenotype of antigen-specific cells at 30 days after CPS-OVA immunization. At this time point, and as late as 75 days following immunization, the tetramer-positive cells maintained their $KLRG1^{hi}$ $CD62L^{lo}$ $IL\text{-}7R\alpha^{hi}$ effector-like phenotype (data not shown). Overall, these experiments demonstrated that antigen-specific cells at the site of infection as well as in secondary lymphoid tissues resembled an effector population up to 75 days following immunization.

As data emerged from these studies, it became apparent that the kinetics of the $CD8^+$ T-cell response to CPS-OVA differ from what has been reported for replication-sufficient strains of *T. gondii*. While the antigen-specific response induced by CPS-OVA peaks at approximately 10 days following immunization in both the spleen and PECs (Fig. 2), work from other groups has demonstrated that $CD8^+$ T-cell responses induced by replicating parasites are not detectable until 2 weeks following peroral infection (24, 26). To directly compare the development of antigen-specific responses, mice were given the same dose of 10^5 CPS-OVA or Pru-OVA tachyzoites intraperitoneally, and tetramer responses were assessed at two time points. In this comparison, CPS-OVA induced significantly higher $CD8^+$ T-cell responses in the spleen as well as the PECs at 8 days following infection (Fig. 4; Table 1). However, by day 14 following infection, the pattern had reversed such that Pru-OVA responses were now significantly higher in the spleen with a trend toward higher responses in the PECs, while CPS-OVA $CD8^+$ T-cell responses had started to contract (Table 1). CPS-OVA and Pru-OVA are derived from different strains of *T. gondii* and have been previously shown to induce disparate responses (15). However, work from our laboratories (E. D. Tait et al., unpublished data) has compared replicating and nonreplicating type 1 strains and demonstrated that CPS-OVA did induce higher frequencies of antigen-specific $CD8^+$ T cells than its replication-sufficient parental strain, RH-OVA. These data must be interpreted with care, but the same construct to drive production of OVA was used in all strains, and these parasites appear to express similar levels of OVA. Both CPS-OVA and Pru-OVA generated antigen-specific splenocytes with a similar effector-like phenotype at these time points, expressing low levels of CD62L and CD127 and high levels of CD44, KLRG1, and CD122 (data not shown).

OVA-specific antigen-specific $CD8^+$ T cells are cytotoxic. In addition to production of cytokines, another important function of antigen-specific $CD8^+$ T cells is the lysis of infected target cells. Indeed, at 1 week following immunization, many tetramer-positive splenocytes had upregulated expression of

TABLE 1. Frequencies of tetramer-positive cells

Antigen	Frequency of tetramer-positive cells[a]			
	Day 8		Day 14	
	Spleen cells[b]	PECs[b]	Spleen cells[b]	PECs
Pru-OVA	0.81 ± 0.37	4.2 ± 1.22	6.76 ± 1.32	17.47 ± 2.85
CPS-OVA	6.69 ± 1.45	20.2 ± 1.2	1.55 ± 0.25	6.70 ± 0.37

[a] Frequencies are out of $CD3^+$ $CD8^+$ lymphocytes. The data are means ± standard deviations for three mice per group from one representative experiment.

[b] $P < 0.02$ (Pru-OVA versus CPS-OVA).

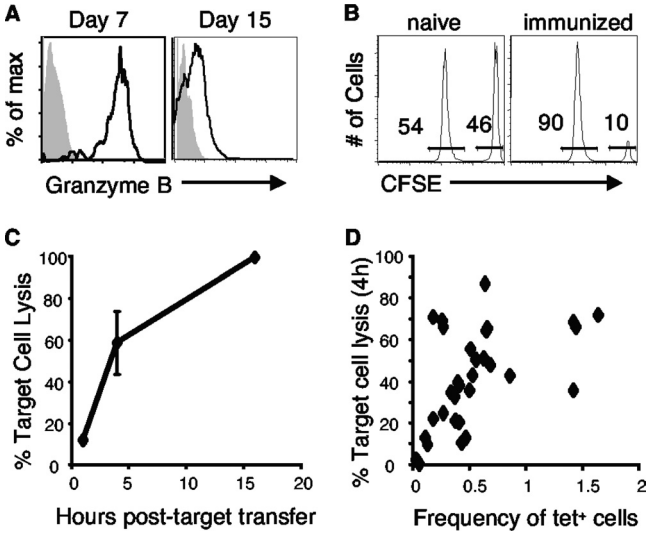

FIG. 5. Immunization induces cytolytic $CD8^+$ T cells. (A) Tetramer-positive splenocytes (black line) express high levels of granzyme B (89.8 ± 0.7 on day 7 and 40.9 ± 3.7 on day 15; $n = 3$ per group) ex vivo compared to tetramer-negative $CD3^+$ $CD8^+$ cells (shaded histogram). (B) In vivo CTL results for naïve and day 7 immunized mice, gated on $CD45.1^+$ donor cells in the spleen (unpulsed cells, $CFSE^{low}$; OTI peptide-pulsed cells, $CFSE^{high}$). Mice were analyzed at 4 h posttransfer of target cells. (C) Maximum cytolytic activity was seen at 16 h following target cell transfer. (D) The frequency of tetramer-positive splenocytes (out of live $CD3^+$ $CD8^+$ cells) was plotted against the percent target cell lysis at 4 h after target cell transfer. For panels C and D, results are combined from multiple comparable experiments).

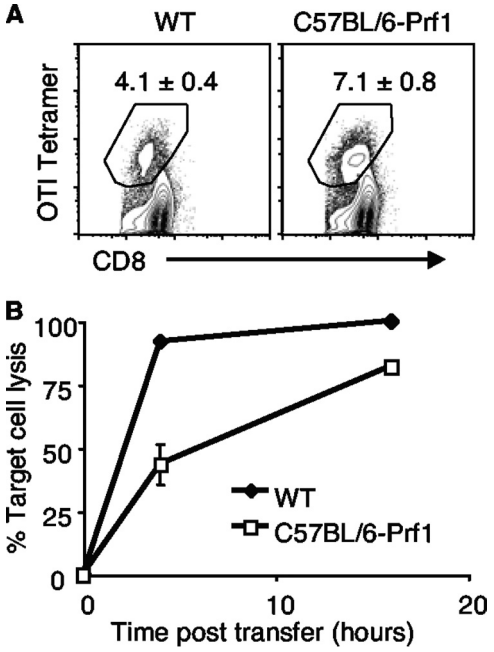

FIG. 6. Cytotoxic activity is decreased in the absence of perforin. (A) Tetramer staining from WT and perforin KO splenocytes at 1 week after immunization; numbers represent average tetramer-positive frequency ± standard deviation for three mice per group. (B) Cytolytic activity is decreased in perforin KO mice at 4 or 16 h following target cell transfer ($P < 0.001$ at both time points, Student's t test). Representative data from one experiment with three mice per group are shown; similar results were seen in three separate experiments.

granzyme B, consistent with possible cytolytic activity (Fig. 5A, day 7). While a proportion of the total $CD8^+$ tetramer-negative population had also upregulated granzyme B (5.5% ± 1.6%), this frequency was much lower than that for the tetramer-positive population (89.8% ± 0.7%). Two weeks following immunization, a lower proportion (40.9% ± 3.7%) of antigen-specific cells showed increased expression of this cytolytic effector molecule directly ex vivo (Fig. 5A, day 15). To directly assess the cytolytic ability of these cells, an in vivo CTL assay was modified based on earlier work (3). Briefly, unpulsed or OTI peptide-pulsed cells from congenic CD45.1 mice were CFSE labeled, mixed at a 1:1 ratio, and injected into immunized (CD45.2) mice. When immunized recipient mice were analyzed, loss of the OTI-pulsed $CFSE^{hi}$ population indicated that CPS-OVA induced robust cytolytic activity in $CD8^+$ T cells (Fig. 5B). While cytolytic activity could be detected as soon as 4 h after target cell transfer in the recipient spleens, maximum lysis was achieved after a 16-hour incubation of the target cells in the recipient mice (Fig. 5C). When multiple experiments were combined from various time points from 7 to 30 days following immunization, there was a correlation between the level of cytotoxic activity and the frequency of antigen-specific cells present at the time of the CTL assay (Fig. 5D) (all mice were analyzed at 4 h posttransfer of target cells).

To better understand the requirements for cytotoxic activity, WT and perforin-deficient mice were immunized with a single dose of 10^5 CPS-OVA. As seen in other infection models (2), perforin-deficient mice generated increased frequencies of antigen-specific $CD8^+$ T cells (Fig. 6A). Despite this phenotype, the perforin-deficient mice demonstrated significantly lower levels of cytolytic activity against peptide-pulsed target cells when analyzed at 4 or 16 h following target cell transfer (Fig. 6B). In addition to granule-mediated cytotoxicity, $CD8^+$ T cells can also mediate cytolysis via Fas-FasL interactions (4). To examine perforin-independent pathways of cytotoxicity in this model, Fas knockout (KO) peptide-pulsed target cells were mixed with WT target cells and unpulsed cells and transferred into immunized mice. WT and Fas KO target cells were killed equally well, indicating that the cytotoxic activity generated in response to immunization is not mediated via the Fas-FasL pathway (data not shown). Together, these findings indicate that immunization induces a perforin-dependent pathway but that a perforin-independent pathway of cytolysis also exists.

Depletion of $CD4^+$ T cells abrogates effector $CD8^+$ T-cell response to CPS-OVA. Different models of infection have shown diverse requirements for CD4 help in the generation of $CD8^+$ T cells. Following infection with a replicating strain of *T. gondii*, $CD4^+$ T-cell help is not required for the generation of $CD8^+$ effector responses in the spleen (26). To address whether CD4 help was required in the CPS-OVA experimental system, mice were depleted of $CD4^+$ T cells 1 week prior to immunization with two doses of 0.5 mg anti-CD4 antibody. At 7 days following immunization, CD4-depleted mice demonstrated a decrease in the frequency (Fig. 7A) as well as total numbers (Fig. 7B) of OVA-specific $CD8^+$ T cells that were generated. However, the tetramer-positive T cells generated in

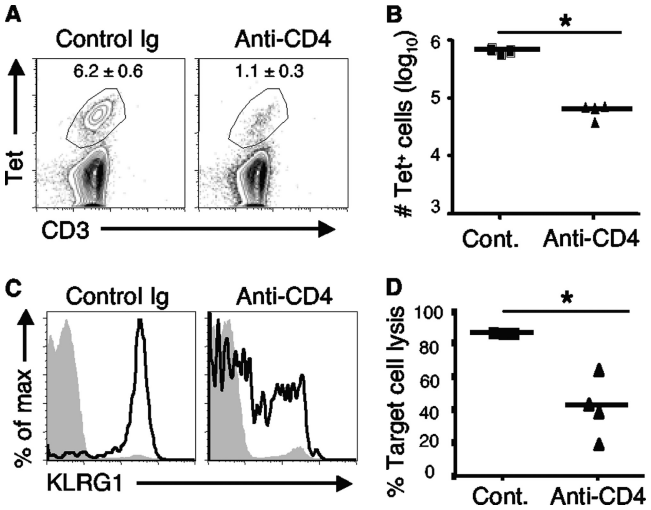

FIG. 7. $CD4^+$ T cells are required for the generation of an effector $CD8^+$ T-cell response. (A and B) Mice were depleted at 1 week prior to immunization (anti-CD4) or treated with control antibody. Both the frequency (A) and numbers (B) of antigen-specific $CD8^+$ T cells were decreased in the spleen ($P < 0.001$ for both, Student's *t* test). (C) Expression of KLRG1 on antigen-specific cells is decreased in the absence of $CD4^+$ help (dark line, tetramer-positive cells; shaded gray, $CD3^+$ $CD8^+$ tetramer-negative cells). (D) The frequency of target cell lysis is reduced in mice depleted of $CD4^+$ T cells prior to immunization ($P < 0.01$, Student's *t* test). Representative data from one experiment with three (control) or four (anti-CD4) mice per group are shown; similar results were obtained in three separate experiments.

the absence of $CD4^+$ help were still capable of making IFN-γ in response to OTI peptide stimulation (data not shown). Expression of KLRG1 on antigen-specific $CD8^+$ T cells generated in the absence of $CD4^+$ T-cell help was decreased (Fig. 7C) (76.3% ± 3.2%, versus 21.77% ± 4% for CD4-depleted mice; $P = 0.0004$). Additionally, tetramer-positive cells generated without $CD4^+$ help were capable of killing peptide-pulsed target cells as demonstrated by an in vivo CTL assay, though the rates of cytolysis were decreased (Fig. 7D). Based on the results shown in Fig. 4, it seems likely that the decreased levels of cytotoxicity seen in the absence of CD4 help are due to lower overall OVA-specific cell numbers, since these cells were capable of expressing granzyme B (data not shown).

DISCUSSION

The studies presented here build on the original report using the cps1-1 parasites and establish that this replication-deficient strain induces a protective response that is dependent on $CD4^+$ and $CD8^+$ T cells. Given the important role of $CD8^+$ T cells in the immune response to *T. gondii* (28), we took advantage of a parasite expressing OVA, which allowed detailed characterization of the numbers and frequencies of these cells and provided new information about the phenotype and cytolytic function of parasite-induced $CD8^+$ T cells. While these studies were in progress, another group proposed that antigen-specific T cells induced by cps1-1 immunization can be subdivided into four subpopulations based on their expression of KLRG1 and CD62L (35). Our results using a model antigen, where *T. gondii*-specific cells can be precisely identified using a tetramer reagent, suggest that tetramer-positive cells are almost entirely $CD62L^{lo}$ and $KLRG1^+$, i.e., falling entirely into fraction III as defined by Wilson et al. (35). Interestingly, the kinetics of fraction III ($CD62L^{lo}$ $KLRG1^+$) cells most closely resembled the expansion of the tetramer-positive $CD8^+$ population as observed here, and further work will be needed to examine whether these subpopulations are truly parasite specific and, if so, the factors that influence their development. The identification of additional endogenous $CD8^+$ T-cell epitopes would facilitate this comparison. Recent work from the Shastri laboratory has identified endogenous epitopes of *T. gondii* in the B10.D2 strain of mice restricted by the major histocompatibility complex class I molecule $H\text{-}2L^d$ (7); additional endogenous epitopes from $H\text{-}2L^d$-restricted BALB/c mice have also been reported (14). In both of those studies, the immunodominant antigens were part of secreted molecules and so are likely to be presented in a fashion similar to that for the model antigen used here and by others (28).

Examination of antigen-specific cells in the spleen demonstrated that as early as 7 and as late as 75 days following immunization, these cells primarily resembled effector or effector-memory phenotype cells, with high levels of KLRG1 and low levels of CD62L and IL-7Rα. The pattern of expression of markers such as IL-7Rα differed from what has been seen in other models where the duration of antigen exposure is limited, such as in a model of acute lymphocytic choriomeningitis virus infection. In that system, antigen-specific cells downregulate expression of the IL-7Rα quickly following activation, and 1 week after infection all antigen-specific cells are negative for IL-7Rα (21). Similar phenotypic characterizations for parasitic infections have been limited, though one recent report on antigen-specific $CD8^+$ T cells in *Trypanosoma cruzii*-infected drug-cured mice suggests that the conversion of $CD8^+$ T cells to a memory phenotype occurs very slowly (6, 8). In the CPS-OVA model system used in these studies, the majority of antigen-specific cells still had low expression of IL-7Rα at 30 days following immunization, in contrast to the pattern of IL-7Rα expression on lymphocytic choriomeningitis virus-specific cells. These studies provided phenotypic information for antigen-specific cells at 75 days following immunization, and it is surprising that they had not yet converted to a more memory-like phenotype even at this time. One implication of this observation is that the combination of markers commonly used to track memory populations may have to be tailored to individual pathogens and may not be applicable to every infection.

Unexpectedly, these studies revealed that CPS-OVA induced an adaptive $CD8^+$ T-cell response with faster kinetics than reported with replicating parasites (24, 26). The initial expectation was that a replicating form *of T. gondii* that induces tissue damage and produces increasing amounts of antigen would promote a more rapid and quantitatively greater $CD8^+$ T-cell response than that generated by the CPS parasites. One potential explanation for this difference has been suggested by other groups who have found that replicating parasites produce factors that limit host cell proliferation and cytokine responses (9, 23); obviously, these effects would be muted if the parasite cannot replicate. Further studies will be needed to characterize the early responses to the parasite, including how

the early innate and inflammatory response contributes to the robust generation of the $CD8^+$ T-cell response following CPS-OVA infection. Indeed, recent work demonstrated that CPS parasites can induce high levels of IL-12p70 at the site of infection as well as the spleen (18), consistent with the idea that IL-12 production is a major determinant that promotes $CD8^+$ T-cell responses during *T. gondii* infection (35).

Previous analysis of cytotoxic responses induced by *T. gondii* required that splenocytes undergo expansion for 6 to 7 days in the presence of exogenous cytokines, which may have influenced their effector function (19). In contrast, direct ex vivo staining at 7 days following CPS-OVA immunization showed that high frequencies of OTI-specific cells expressed the cytolytic effector molecule granzyme B (Fig. 5A) and that these cells were cytolytic. Perforin-deficient mice were able to generate a robust antigen-specific $CD8^+$ T-cell response, but in spite of this, cytolytic activity was decreased (Fig. 6B). After overnight incubation with target cells, approximately 70% of target cells were lysed in perforin-deficient mice, compared to 100% in the WT mice, suggesting that another mechanism of cytolysis is acting on the target cells in the absence of perforin. Further, we found that Fas-FasL interactions are not required for CTL activity, implying that other pathways such as those mediated via tumor necrosis factor family members may be playing a role in the destruction of target cells. It still remains possible that a role for the Fas-FasL pathway might be detected in the absence of perforin-dependent cytolysis.

The requirement for $CD4^+$ T-cell help in the generation of a $CD8^+$ T-cell response has been addressed in other viral and bacterial models. The general consensus is that $CD4^+$ T cells are not required for the generation of a $CD8^+$ T-cell effector response but are required for the maintenance of memory (32, 33). In studies with *T. gondii*, it was previously shown that CD4 KO mice had decreased numbers of antigen-specific $CD8^+$ T cells as demonstrated by a precursor CTL assay (11). This defect was seen only at late time points following *T. gondii* infection; it is possible that defects were also present earlier during infection but were not detected by the methods available at that time. In another model of *T. gondii* infection, $CD4^+$ T-cell help was required to maintain antigen-specific $CD8^+$ T cells in the brain during chronic infection (26). Other parasite models have demonstrated that $CD4^+$ T-cell help is required during the primary response to immunodominant epitopes (27). The findings presented here might thus be influenced by the fact that the H-$2K^b$-restricted SIINFEKL epitope is known to be immunodominant (10). While $CD4^+$ T-cell production of IL-2 has been regarded as the most likely mechanism whereby these cells can help the development of $CD8^+$ T-cell responses, the observation that depletion of $CD4^+$ T cells leads to decreased expression of KLRG1 on antigen-specific $CD8^+$ T cells may provide an alternative explanation. The expression of KLRG1 has been linked to IL-12 production and expression of the Th1 transcription factor T-bet (19). How $CD4^+$ T cells may influence these events is uncertain. Regardless, these results suggest that in a vaccine setting where inflammation might be limited, both $CD4^+$ and $CD8^+$ epitopes should be targeted in order to drive a protective response.

ACKNOWLEDGMENTS

K.A.J. was partially supported by Cancer Research Institute training grant "Predoctoral Emphasis Pathways in Tumor Immunology," and C.A.H. received funding from NIH grant AI 071302.

REFERENCES

1. **Andrews, B. S., R. A. Eisenberg, A. N. Theofilopoulos, S. Izui, C. B. Wilson, P. J. McConahey, E. D. Murphy, J. B. Roths, and F. J. Dixon.** 1978. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. J. Exp. Med. **148:**1198–1215.
2. **Badovinac, V. P., and J. T. Harty.** 2000. Adaptive immunity and enhanced CD8+ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma. J. Immunol. **164:**6444–6452.
3. **Barber, D. L., E. J. Wherry, and R. Ahmed.** 2003. Rapid in vivo killing by memory CD8 T cells. J. Immunol. **171:**27–31.
4. **Barry, M., and R. C. Bleackley.** 2002. Cytotoxic T lymphocytes: all roads lead to death. Nat. Rev. Immunol. **2:**401–409.
5. **Bhopale, G. M.** 2003. Development of a vaccine for toxoplasmosis: current status. Microbes Infect. **5:**457–462.
6. **Bixby, L. M., and R. L. Tarleton.** 2008. Stable CD8+ T cell memory during persistent Trypanosoma cruzi infection. J. Immunol. **181:**2644–2650.
7. **Blanchard, N., F. Gonzalez, M. Schaeffer, N. T. Joncker, T. Cheng, A. J. Shastri, E. A. Robey, and N. Shastri.** 2008. Immunodominant, protective response to the parasite Toxoplasma gondii requires antigen processing in the endoplasmic reticulum. Nat. Immunol. **9:**937–944.
8. **Bustamante, J. M., L. M. Bixby, and R. L. Tarleton.** 2008. Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease. Nat. Med. **14:**542–550.
9. **Candolfi, E., C. A. Hunter, and J. S. Remington.** 1994. Mitogen- and antigen-specific proliferation of T cells in murine toxoplasmosis is inhibited by reactive nitrogen intermediates. Infect. Immun. **62:**1995–2001.
10. **Carbone, F. R., and M. J. Bevan.** 1989. Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization. J. Exp. Med. **169:**603–612.
11. **Casciotti, L., K. H. Ely, M. E. Williams, and I. A. Khan.** 2002. $CD8^+$-T-cell immunity against *Toxoplasma gondii* can be induced but not maintained in mice lacking conventional $CD4^+$ T cells. Infect. Immun. **70:**434–443.
12. **Denkers, E. Y., G. Yap, T. Scharton-Kersten, H. Charest, B. A. Butcher, P. Caspar, S. Heiny, and A. Sher.** 1997. Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. J. Immunol. **159:**1903–1908.
13. **Fox, B. A., and D. J. Bzik.** 2002. De novo pyrimidine biosynthesis is required for virulence of Toxoplasma gondii. Nature **415:**926–929.
14. **Frickel, E. M., N. Sahoo, J. Hopp, M. J. Gubbels, M. P. Craver, L. J. Knoll, H. L. Ploegh, and G. M. Grotenbreg.** 2008. Parasite stage-specific recognition of endogenous Toxoplasma gondii-derived CD8(+) T cell epitopes. J. Infect. Dis. **198:**1625–1633.
15. **Gavrilescu, L. C., and E. Y. Denkers.** 2001. IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence Toxoplasma gondii infection. J. Immunol. **167:**902–909.
16. **Gazzinelli, R., Y. Xu, S. Hieny, A. Cheever, and A. Sher.** 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. J. Immunol. **149:**175–180.
17. **Gazzinelli, R. T., F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher.** 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated Toxoplasma gondii vaccine. J. Immunol. **146:**286–292.
18. **Gigley, J. P., B. A. Fox, and D. J. Bzik.** 2009. Cell-mediated immunity to Toxoplasma gondii develops primarily by local Th-1 host immune responses in the absence of parasite replication. J. Immunol. **182:**1069–1078.
19. **Hakim, F. T., R. T. Gazzinelli, E. Denkers, S. Hieny, G. M. Shearer, and A. Sher.** 1991. CD8+ T cells from mice vaccinated against Toxoplasma gondii are cytotoxic for parasite-infected or antigen-pulsed host cells. J. Immunol. **147:**2310–2316.
20. **Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech.** 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity **27:**281–295.
21. **Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed.** 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat. Immunol. **4:**1191–1198.
22. **Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner.** 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature **369:**31–37.
23. **Khan, I. A., T. Matsuura, and L. H. Kasper.** 1995. IL-10 mediates immunosuppression following primary infection with Toxoplasma gondii in mice. Parasite Immunol. **17:**185–195.
24. **Kwok, L. Y., S. Lutjen, S. Soltek, D. Soldati, D. Busch, M. Deckert, and D. Schluter.** 2003. The induction and kinetics of antigen-specific CD8 T cells

are defined by the stage specificity and compartmentalization of the antigen in murine toxoplasmosis. J. Immunol. **170:**1949–1957.
25. **Luft, B. J., R. G. Brooks, F. K. Conley, R. E. McCabe, and J. S. Remington.** 1984. Toxoplasmic encephalitis in patients with acquired immune deficiency syndrome. JAMA **252:**913–917.
26. **Lutjen, S., S. Soltek, S. Virna, M. Deckert, and D. Schluter.** 2006. Organ- and disease-stage-specific regulation of *Toxoplasma gondii*-specific CD8-T-cell responses by CD4 T cells. Infect. Immun. **74:**5790–5801.
27. **Padilla, A., D. Xu, D. Martin, and R. Tarleton.** 2007. Limited role for CD4+ T-cell help in the initial priming of *Trypanosoma cruzi*-specific $CD8^+$ T cells. Infect. Immun. **75:**231–235.
28. **Parker, S. J., C. W. Roberts, and J. Alexander.** 1991. CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to Toxoplasma gondii in mice. Clin. Exp. Immunol. **84:**207–212.
29. **Pepper, M., F. Dzierszinski, A. Crawford, C. A. Hunter, and D. Roos.** 2004. Development of a system to study $CD4^+$-T-cell responses to transgenic ovalbumin-expressing *Toxoplasma gondii* during toxoplasmosis. Infect. Immun. **72:**7240–7246.
30. **Sayles, P. C., and L. L. Johnson.** 1996. Intact immune defenses are required for mice to resist the ts-4 vaccine strain of *Toxoplasma gondii*. Infect. Immun. **64:**3088–3092.
31. **Scorza, T., S. D'Souza, M. Laloup, J. Dewit, J. De Braekeleer, H. Verschueren, M. Vercammen, K. Huygen, and E. Jongert.** 2003. A GRA1 DNA vaccine primes cytolytic $CD8^+$ T cells to control acute *Toxoplasma gondii* infection. Infect. Immun. **71:**309–316.
32. **Shedlock, D. J., and H. Shen.** 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science **300:**337–339.
33. **Sun, J. C., and M. J. Bevan.** 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science **300:**339–342.
34. **Suzuki, Y., and J. S. Remington.** 1988. Dual regulation of resistance against Toxoplasma gondii infection by Lyt-2+ and Lyt-1+, L3T4+ T cells in mice. J. Immunol. **140:**3943–3946.
35. **Wilson, D. C., S. Matthews, and G. S. Yap.** 2008. IL-12 signaling drives CD8+ T cell IFN-γ production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii infection. J. Immunol. **180:**5935–5945.
36. **Wilson, E. H., U. Wille-Reece, F. Dzierszinski, and C. A. Hunter.** 2005. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. J. Neuroimmunol. **165:**63–74.
37. **Yap, G. S., T. Scharton-Kersten, D. J. Ferguson, D. Howe, Y. Suzuki, and A. Sher.** 1998. Partially protective vaccination permits the development of latency in a normally virulent strain of *Toxoplasma gondii*. Infect. Immun. **66:**4382–4388.

Editor: J. F. Urban, Jr.