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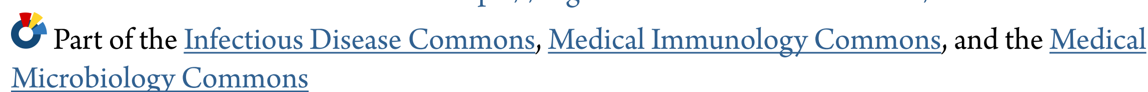
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INFECTION AND IMMUNITY, Dec. 2009, p. 5380–5388 Vol. 77, No. 12
0019-9567/09/$12.00 doi:10.1128/IAI.00649-09
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Long-Term Immunity to Lethal Acute or Chronic Type II *Toxoplasma gondii* Infection Is Effectively Induced in Genetically Susceptible C57BL/6 Mice by Immunization with an Attenuated Type I Vaccine Strain[▽]

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Received 8 June 2009/Returned for modification 7 July 2009/Accepted 15 September 2009

C57BL/6 (B6) mice are genetically highly susceptible to chronic type II *Toxoplasma gondii* infections that invariably cause lethal toxoplasmic encephalitis. We examined the ability of an attenuated type I vaccine strain to elicit long-term immunity to lethal acute or chronic type II infections in susceptible B6 mice. Mice immunized with the type I *cps1-1* vaccine strain were not susceptible to a lethal (100-cyst) challenge with the type II strain ME49. Immunized mice challenged with 10 ME49 cysts exhibited significant reductions in brain cyst and parasite burdens compared to naive mice, regardless of the route of challenge infection. Remarkably, *cps1-1* strain-immunized B6 mice chronically infected with ME49 survived for at least 12 months without succumbing to the chronic infection. Potent immunity to type II challenge infections persisted for at least 10 months after vaccination. While the *cps1-1* strain-elicited immunity did not prevent the establishment of a chronic infection or clear established brain cysts, *cps1-1* strain-elicited CD8^{+} immune T cells significantly inhibited recrudescence of brain cysts during chronic ME49 infection. In addition, we show that uracil starvation of the *cps1-1* strain induces early markers of bradyzoite differentiation. Collectively, these results suggest that more effective immune control of chronic type II infection in the genetically susceptible B6 background is established by vaccination with the nonreplicating type I uracil auxotroph *cps1-1* strain.

Toxoplasma gondii is a common and significant obligate intracellular pathogen of humans and animals. There are three clonal types that exist which are also thought to be derived when *T. gondii* acquired oral infectivity (50). Virulence in mice is strain specific where type I clones are universally virulent, type II clones are of intermediate virulence, and type III clones are avirulent. Ingestion of contaminated food sources is the most common route of human infection, resulting in systemic disease that can be divided into two stages: the acute disseminating tachyzoite stage and the chronic encysted bradyzoite stage (12). Recrudescent infections arising from reactivation of preexisting chronic latent cyst stages are particularly severe in the context of immune deficiency such as AIDS (38), and improved treatments and the development of vaccines to reduce disease burden are important therapeutic objectives. Strategies with the potential to eradicate the latent cyst stages present in already-infected individuals could be helpful but, unfortunately, the biology of cyst development, as well as the immune control mechanisms of latent stages, are relatively poorly understood at this time. Clearly, CD8^{+} T cells and gamma interferon (IFN-γ) are significant effectors in mediating resistance to acute and chronic *T. gondii* infection (17, 19).

Numerous studies have evaluated responses to vaccines based on protein or DNA components of *T. gondii* with various degrees of success (1, 2, 4, 5, 9, 11, 22, 24, 25, 28, 36, 37, 40, 44, 46). Virulent parasite strains, as well as attenuated *T. gondii* strains, have been paramount in dissecting the immunobiology of host response in regard to understanding adaptive immune responses that may be helpful in vaccine design. Dense granule protein 6 (GRA6), GRA4, and rhoptry bulb protein 7 (ROP7) were recently identified as parasite antigens possessing a H-2L^{d}-restricted major histocompatibility complex class I (MHC-I) epitope that correlates with stage-specific expression and resistance to lethal chronic type II infections in the H-2L^{d} background (BALB/c). These data further define a potential molecular basis for genetic susceptibility to lethal type II chronic infections in the C57BL/6 H-2^{b} MHC-I-restricted background (6, 16). Vaccine models using either live attenuated parasites, such as type I strain ts-4, or irradiated tachyzoites, have had the greatest success in providing complete protection against lethal type I challenges. These studies also report more significant reductions in type II cyst burdens than component vaccines or whole-dead parasite vaccines (42, 48, 53, 54). However, live parasite-based vaccines such as strain ts-4 are still slowly replicating and retain a significant potential for virulence in the immunocompromised host. Furthermore, immune protection elicited by strain ts-4 is not long-lasting and significantly decreases within months after immunization (27).

From the same parental RH strain that strain ts-4 was developed (45), our laboratory developed a fully attenuated nonreplicating type I *cps1-1* strain that exhibits a severe uracil auxotrophy. The *cps1-1* strain in a single immunization elicits complete immune protection and is able to clear high lethal

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▽ Published ahead of print on 21 September 2009.

dose virulent type I infection (14). Significantly, this highly attenuated strain is completely avirulent at extreme doses in immunocompromised hosts, such as in Tyk2$^{-/-}$ mice (49), which cannot control inflammation, and also in IFN-$\gamma^{-/-}$ mice (14, 20). The *cps1-1* strain elicits potent Th1 immunity to lethal type I challenge infection after immunization of BALB/c, C57BL/6, Tyk2$^{-/-}$ (C57BL/6), or MyD88$^{-/-}$ (C57BL/6) mice (13, 14, 20, 49, 51, 56). Immunity to lethal type I challenge infection induced by the *cps1-1* strain is dependent on CD8^{+} T cells (20), local production of IFN-γ (20), and interleukin-12 (IL-12) p70 (20, 51, 56). Remarkably, the potent immunity elicited by vaccination with the *cps1-1* strain does not require systemic IFN-γ (20).

We show here that the immunity induced in C57BL/6 mice after vaccination with the *cps1-1* strain provides a surprisingly effective and complete protection from a lethal oral or intraperitoneal (i.p.) challenge infection of type II cysts from the ME49 strain. We address the durability of *cps1-1* strain-induced immunity to lethal type II cyst challenge infection by different routes and find that immunization with the *cps1-1* strain provides long-term protective immunity to lethal type II challenge. Vaccination with the *cps1-1* strain also markedly reduces the cyst burden and protects susceptible C57BL/6 mice from succumbing to chronic infection. CD8^{+} immune T cells elicited by vaccination with the *cps1-1* strain prevent cyst recrudescence during chronic infection.

MATERIALS AND METHODS

Mice. Adult 6- to 8-week-old C57BL/6 (B6) mice were obtained from the National Cancer Institute and mice were maintained in Tecniplast Seal Safe mouse cages on vent racks at the Dartmouth-Hitchcock Medical Center mouse facility. All mice were cared for and handled according to the Animal Care and Use Program of Dartmouth College using National Institutes of Health-approved institutional animal care and use committee guidelines.

Tachyzoite culture and immunization with the *cps1-1* strain. Tachyzoites of the attenuated *cps1-1* strain were obtained by uracil supplemented tissue culture (14). Tachyzoites were isolated from freshly lysed human foreskin fibroblast (HFF) monolayers by filtration through 3.0-μm-pore-size Nucleopore membranes, washed with phosphate-buffered saline (PBS), centrifuged, and then resuspended in PBS at defined numbers after determination of the tachyzoite concentration using a hemacytometer. The viability of tachyzoite preparations was tested in plaque assays to confirm that 30 to 50% of tachyzoites were infectious. Mice were immunized with 10^6 freshly isolated tachyzoites of the *cps1-1* strain twice 14 days apart. At 1 or 10 months after final immunization, the mice were infected i.p. or perorally (i.g.) via gavage needle with either 10 or 100 brain-derived cysts of the ME49 strain.

ME49 cyst maintenance, isolation, and enumeration. Brain cysts of the strain ME49 were obtained by continuous passage every 4 to 5 weeks in B6 mice infected i.p. with 10 cysts. Brains from mice infected with ME49 were harvested and homogenized by using a Dounce homogenizer in 2 ml of sterile 1× PBS. Cysts were then counted by examining 10 μl of the brain homogenate under a coverslip using light microscopy under ×40 magnification. Total cyst numbers were determined per brain based on counting 5 to 50 slides prepared from each brain homogenate.

Adoptive transfer of total splenocytes and purified T-cell subsets. Spleens from *cps1-1* strain-immunized or naive B6 mice were harvested and splenocytes released by grinding the spleen through a 70-μm-pore-size nylon screen in 5 ml of Hanks balanced salt solution. Splenocytes were pelleted then subject to erythrocyte lysis for 3 min at room temperature with sterile ACK buffer prepared in house using 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2 EDTA in distilled H_2O. Splenocytes were then washed extensively in sterile PBS, and live cells were identified via trypan blue exclusion and enumerated by using a hemacytometer. Then, 5×10^7 whole naive strain or *cps1-1* strain immune splenocytes were then transferred to chronically infected mice via tail vein injection. Separately, from naive or *cps1-1* strain immune splenocytes, purified CD4^{+} and CD8^{+} T cells were isolated by using EasySep positive selection (Stem Cell Technologies). Either 9×10^6 purified CD4^{+} T cells, 4.5×10^6 purified CD8^{+} T cells, or a combination of 9×10^6 CD4^{+} and 4.5×10^6 CD8^{+} T cells were injected via tail vein injection into chronically infected recipient mice.

Parasite burden measured by quantitative real-time PCR. Infected animals were euthanized via CO_2 overdose, and brain tissue was harvested and flash frozen in liquid nitrogen. DNA was extracted from the entire organ by using a DNeasy tissue kit (Qiagen, Inc., Germantown, MD), and samples were pooled. Amplification of parasite DNA from 400 ng of purified tissue DNA was performed using primers specific for the *T. gondii* B1 gene at 10 pmol of each per reaction (29, 31) (Integrated DNA Technologies, Coralville, IA) and amplified by real-time PCR using SMartMix HM (Cepheid, Sunnyvale, CA) on a Cepheid Smart Cycler. Each reaction contained one lyophilized SMartMix HM bead and SYBR green I (Cambrex BioScience, Inc., Rockland, ME). Known parasite DNA equivalents were used to establish a standard curve, and then parasite numbers in tissue samples were calculated via extrapolation from the standard curve.

Immunohistochemistry of the *cps1-1* tachyzoite-to-bradyzoite differentiation. HFF monolayers were infected with the *cps1-1* strain in the presence or absence of uracil for 2 days, and immunohistochemistry analysis of infected HFF monolayers was performed (15). Briefly, confluent HFF cultures on glass coverslips were infected with tachyzoites and then fixed with Histochoice tissue fixative (Amresco) as specified by the manufacturer. Fixed infected cells were treated in 0.2% Triton X-100 for 20 min and blocked with 3% bovine serum albumin overnight. Infected HFF cells on coverslips were incubated with appropriate dilutions of primary antibody or biotinylated Dolichos biflorus lectin (DBA; Vector Laboratories) for 90 min at 37°C, washed, and incubated with the secondary antibody or Strepavidin-Alexa 568, respectively. Monospecific polyclonal rabbit antibodies (immunoglobulin G) to SAG1 (43) were used and stained with anti-rabbit secondary antibody coupled to Alexa 488 (Molecular Probes). Biotinylated DBA was stained by using streptavidin-Alexa 568 (Molecular Probes) (32). Coverslips were mounted by using Vectashield (Vector Laboratories) or a SlowFade Light Antifade kit with DAPI (4′,6′-diamidino-2-phenylindole) for staining nucleic acid (Molecular Probes). Images were captured by using a Zeiss AxioPhot upright photomicroscope with a slow-scan, cooled charge-coupled device CH250 detector (Photometrics). The computer with this microscope uses IPLab software (Scanalytics), which provides control of excitation filters and image acquisition. The brightest pixels in an image were below saturation, and some images were contrast enhanced for display purposes but were qualitatively reflective of unprocessed data collected from the original image(s).

Statistical analysis. The Kaplan-Meier product limit test was used to measure significant differences between survival curves (GraphPad Prism software). All other samples were subject to a Student *t* test and are represented as the means ± the standard errors of the mean (SEM).

RESULTS

Immunization with the type I *cps1-1* strain elicits long-term immunity to lethal type II infection in genetically susceptible C57BL/6 mice. We measured the ability of the *cps1-1* vaccine strain to elicit immune protection against lethal type II infection. One month after immunization, immunized and age-matched naive mice were infected with either a nonlethal (10-cyst) or lethal (100-cyst) dose of the type II strain ME49 by the i.p. or i.g. route, and survival was monitored. Mice immunized with the *cps1-1* strain completely survived both doses of challenge regardless of route (Fig. 1A and B). As expected, naive mice infected with the nonlethal challenge of 10 cysts survived and naive mice infected with the lethal 100 cyst dose succumbed to infection (Fig. 1A and B).

Due to the high potency of immunization with the *cps1-1* vaccine in protecting mice against high-dose type I and type II lethal challenge infections and induction of long-lasting immunity to type I challenge (14, 20), we measured the durability of *cps1-1* strain-induced protective immunity against type II challenge. Ten months after immunization, immunized and age-matched naive mice were infected with either a nonlethal (10-cyst) or lethal (100-cyst) dose of the type II strain ME49 by the i.p. or i.g. route, and survival was monitored. *cps1-1* strain-

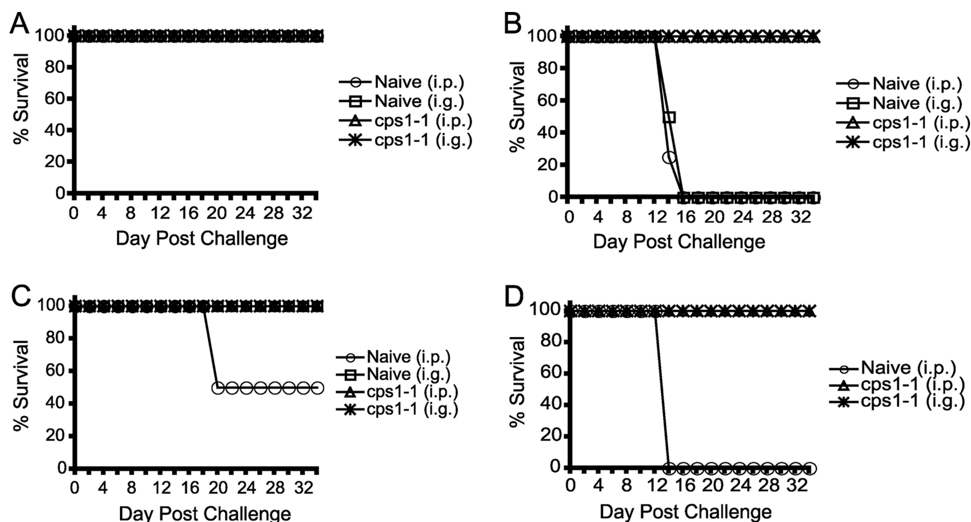

FIG. 1. Immunization with the *cps1-1* vaccine elicits long-lasting immunity against a lethal type II challenge regardless of the immunization route. C57BL/6 mice were either not immunized or immunized i.p. with 10^6 *cps1-1* tachyzoites given twice 14 days apart. One month later (A and B) or 10 months later (C and D) naive mice were challenged i.p. (○) or i.g. (□), and *cps1-1* strain-immunized mice were challenged i.p. (△) or i.g. (✳) with either 10 (A and C) or 100 (B and D) ME49 cysts. The percent survival was monitored for 5 weeks, at which time the experiment was stopped. The data represent the results of one experiment with six mice per group.

immunized mice completely survived both doses of challenge regardless of the challenge route (Fig. 1C and D). In contrast, only 50% of nonimmunized mice that were age matched to those that had been immunized survived the 10-cyst challenge when given i.p., most likely due to the loss of immune function with age (Fig. 1C). Age-matched 10-month-old unimmunized mice did not survive the lethal 100-cyst challenge (Fig. 1C and D).

Vaccination with the *cps1-1* strain significantly reduces the brain cyst and parasite burden after type II challenge infection but does not prevent establishment of chronic infection. C57BL/6 mice are genetically highly susceptible to type II infections in that they acquire higher brain cyst burdens during acute infection, and these cysts ultimately recrudesce and cause lethal toxoplasmic encephalitis during chronic infection (3, 7, 10, 23, 41, 52). Thus, we measured cyst burdens and parasite burden in brains of *cps1-1* strain-immunized or naive mice 5 weeks after ME49 challenge infection. Naive mice (1 month age-matched), challenged with 10 cysts i.p. or i.g., contained 4,130 ± 490 cysts and 2,730 ± 380 cysts per brain, respectively (Fig. 2A). Immunized mice, challenged with 10 cysts either i.p. or i.g., had significantly (P = 0.0001 and P = 0.0001) fewer cysts per brain compared to naive mice (170 ± 30 and 10 ± 6, respectively) (Fig. 2A). Immunized mice challenged at 10 months postimmunization showed similar reductions in cyst burden and exhibited brain cyst burdens of 106 ± 48.3 cysts (i.p.) and 275 ± 140 cysts (i.g.), respectively, compared to naive mice challenged with 10 cysts i.p. or i.g. that exhibited brain cyst burdens of 10,633 ± 638 and 3,800 ± 1625, respectively (Fig. 2C). Interestingly, *cps1-1* strain-immunized mice challenged with a lethal high cyst dose (100 cysts) at either 1 month or 10 months postimmunization survived acute infection and exhibited a decrease in brain cysts similar to that observed with the 10-cyst dose challenges.

During the course of our cyst enumeration, we observed brain cysts of various sizes under light microscopy. Consequently, a real-time PCR assay was used to determine absolute parasite burdens in brain tissue based on measurement of genomes or parasite equivalents (PE) (see Materials and Methods). As previously observed (20, 26), unchallenged *cps1-1* strain-immunized mice exhibited no detectable PE in the blood, spleen, and brain, indicating that the *cps1-1* strain does not replicate or develop mature cysts and is cleared before challenge is administered (data not shown). Naive mice contained 871.6 ± 13.7 or 626.9 ± 292.8 PE per 400 ng of total brain DNA when challenged at 1 month by either the i.p. or the i.g. route, respectively (Fig. 2B). Naive mice challenged at 10 months contained 881.9 ± 75.8 (i.p.) and 593.1 ± 151.2 (i.g.) PE, respectively, per 400 ng of brain DNA (Fig. 2D). In contrast, *cps1-1* strain-immunized mice challenged 1 month after immunization contained 28.8 ± 6.2 (i.p.) PE (P = 0.001) and 30.8 ± 16.5 (i.g.) PE (P = 0.001) (Fig. 2B). Mice challenged 10 months after immunization contained 69.3 ± 7.3 (i.p.) PE (P = 0.001) and 12.4 ± 4.9 (i.g.) PE (P = 0.01) (Fig. 2D). Despite the significant immune protection afforded after immunization with *cps1-1*, these observations revealed that a population of ME49 parasites was still capable of trafficking to the brain and establishing a chronic infection with a reduced cyst burden.

Adoptive transfer of immune splenocytes to a type II *T. gondii*-infected mouse enhances control of chronic infection. Vaccination with the *cps1-1* strain is highly effective at promoting survival to acute infection, as well as reducing cyst burdens and parasite numbers in chronic infection in mice challenged with type II ME49 cysts. The immune control mechanisms elicited by *cps1-1* strain vaccination could prevent tachyzoites from trafficking to brain, could promote bradyzoite or cyst development, could eradicate existing brain cysts, or could prevent parasite access to the brain. In type I RH challenge infection, immunity elicited by the *cps1-1* strain shows a powerful effect and rapidly clears tachyzoites (20, 26). To establish

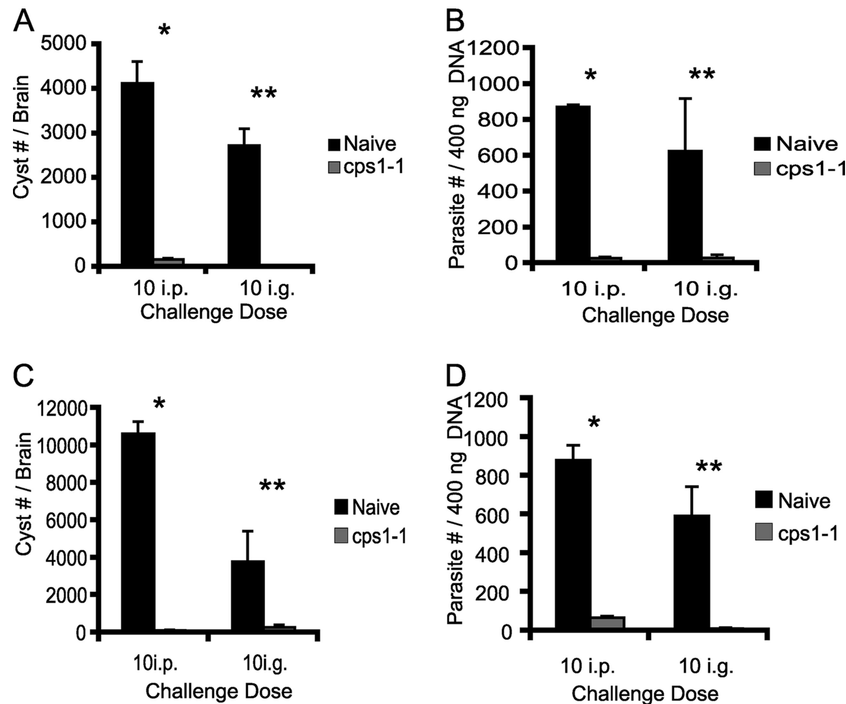

FIG. 2. Short-term and long-term immunity induced by the *cps1-1* strain prevents cyst formation. C57BL/6 mice were either not immunized or immunized i.p. with 10^6 *cps1-1* tachyzoites twice 14 days apart. One month and ten months after *cps1-1* vaccine administration, age-matched naive (■) and immune (▨) mice were challenged with 10 cysts of ME49 i.p. or i.g. At 5 weeks after challenge infection, cyst enumeration was performed in the brains of the mice challenged 1 month (A) and 10 months (C) after final immunization. Brain parasite burdens were measured by real-time quantitative PCR of the *T. gondii* B1 gene in mice challenged 1 month (B) and 10 months (D) after final the immunization. The data represent the means ± the SEM from one experiment with six mice per group. Statistical differences were calculated by using the Student *t* test, and $P < 0.001$ was considered significant (* for the i.p.-challenged group and ** for the i.g.-challenged group).

a kinetic of parasite recrudescence, genetically susceptible B6 mice were infected with 10 ME49 cysts i.p., the brains were harvested, and the cysts were enumerated at weeks 5, 5.5, and 7 postinfection. As shown in Fig. 3A, cyst burdens decreased overtime and higher numbers were found at week 5 compared to week 7. To measure the ability of *cps1-1* strain-induced immunity to target established brain cysts, total splenocytes were adoptively transferred into mice with established ME49 chronic infections. B6 mice were infected with 10 ME49 cysts i.p., and then at 3.5 or 5 weeks after infection mice received either no treatment, 5×10^7 whole naive splenocytes, or 5×10^7 whole *cps1-1* strain-immune splenocytes. At 2 weeks after adoptive transfer of total splenocytes, the brains were harvested, and the cysts were enumerated. Treatments conducted at 3.5 weeks after ME49 infection showed no significant differences in the brain cyst burden (Fig. 3B) or parasite burden (Fig. 3C). In contrast, treatments conducted at 5 weeks after ME49 infection showed significant differences in brain cyst burden (Fig. 3B) and parasite burden (Fig. 3C). Mice receiving naive splenocytes or no treatment showed nearly identical cyst and parasite burden reductions compared to week 3.5 treatment groups. Remarkably, mice receiving *cps1-1* strain-immune splenocytes exhibited significantly higher brain cyst burden (Fig. 3B), as well as higher parasite burdens compared to naive or no treatment groups (Fig. 3C). However, compared to the week 5 cyst burdens shown in the kinetic analysis (Fig. 3A), the cyst numbers were only slightly reduced. The parasite burden (Fig. 3C) correlated well with the cyst burden (Fig. 3B), but we observed an increase in the average cyst diameter in light microscopy (data not shown) and an increase in parasite units (Fig. 3D). These observations indicated that adoptive transfer of the *cps1-1* strain-immune splenocytes could delay or prevent cyst recrudescence in chronic infection and cysts continue to develop.

To define which cell type in *cps1-1* strain-immune splenocytes was preventing recrudescence of brain cysts, purified $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$ T cells from *cps1-1* strain-immunized or naive mice were adoptively transferred into mice infected with 10 ME49 cysts 5 weeks earlier, and then the brains were harvested 2 weeks later to enumerate the cyst burden. Mice receiving naive $CD4^+$, $CD8^+$, $CD4^+$ $CD8^+$, or immune $CD4^+$ T cells were not statistically different in brain cyst burden of 1,945 ± 254, 2,200 ± 826, 1,519 ± 298, or 2,531 ± 767, respectively (Fig. 4). In contrast, mice receiving immune $CD8^+$ T cells or a combination of immune $CD4^+$ and $CD8^+$ T cells had significantly higher cyst burdens (5,006 ± 705 and 3,891 ± 368, respectively) (Fig. 4) compared to naive controls. The cyst burdens in mice receiving immune $CD8^+$ T cells were not different from those measured at week 5 as shown in Fig. 3A. These results suggest that *cps1-1* strain-immune $CD8^+$ T cells delay or prevent cyst recrudescence in chronic infection.

C57BL/6 mice immunized with *cps1-1* strain do not succumb to chronic type II ME49 infection. Type II strain *T. gondii*-

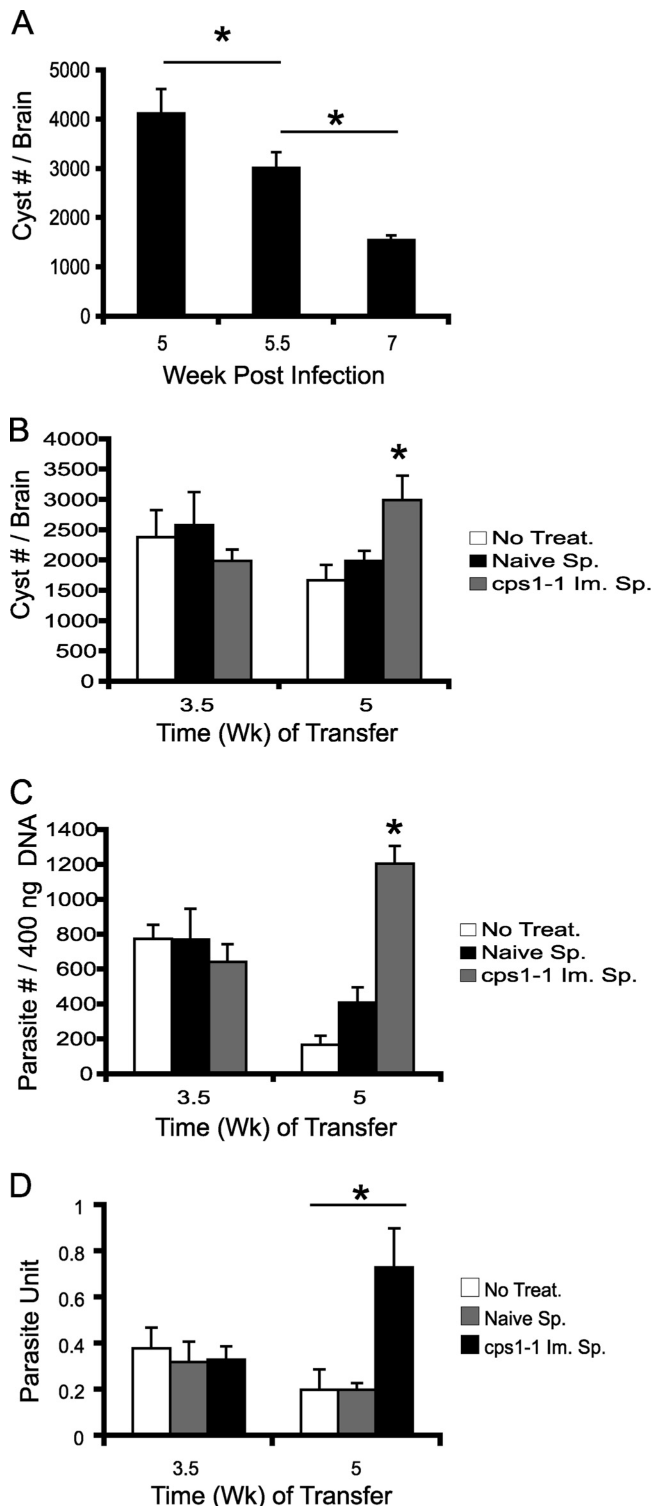

FIG. 3. Immune splenocytes from *cps1-1* strain-immunized mice prevents cyst recrudescence. C57BL/6 mice were either not immunized or immunized i.p. with 10^6 *cps1-1* tachyzoites twice given 14 days apart. Separately, susceptible C57BL/6 mice were infected i.p. with 10 cysts of ME49 to establish chronic infection. (A) Brains were harvested from chronically infected mice at weeks 5, 5.5, and 7 postinfection as indicated, and the cysts were enumerated. (B, C, and D) Chronically infected mice received no treatment (□), naive splenocytes (■), or immune splenocytes (▤) from *cps1-1* strain-immunized mice at 3.5 or 5 weeks after ME49 infection. At 2 weeks after adoptive transfer, the cysts were enumerated (B), the parasite burdens (PE) were calculated by using quantitative real-time PCR of the *T. gondii* B1 gene (C), and the parasite units were calculated as PE per 400 ng of brain tissue DNA per cyst (D). The data represent the means ± the SEM from a group of five animals; the experiment was repeated twice. Statistical differences were calculated by using the Student *t* test, and $P < 0.05$ (*) was considered significant.

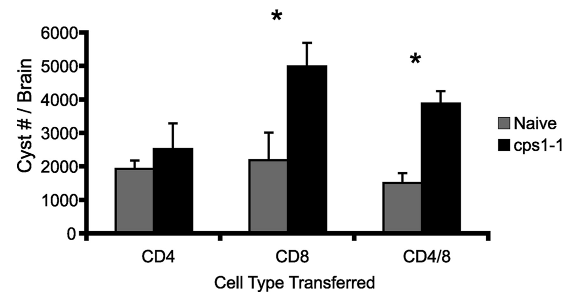

FIG. 4. CD8 T cells from *cps1-1* strain-immunized mice prevent cyst recrudescence. C57BL/6 mice were either not immunized or immunized i.p. with 10^6 *cps1-1* tachyzoites given twice 14 days apart. Separately, genetically susceptible C57BL/6 mice were infected i.p. with 10 cysts of ME49 to establish chronic infection. Chronically infected mice received naive (▤) or immune (■) $CD4^+$, $CD8^+$, or $CD4^+$ $CD8^+$ T cells isolated from *cps1-1* strain-immunized mice at 5 weeks after ME49 infection. At 2 weeks after adoptive transfer, the cysts were enumerated. The data represent the means ± the SEM from a group of five animals; the experiment was repeated twice. Statistical differences were calculated by using the Student *t* test, and $P < 0.05$ (*) was considered significant.

infected C57BL/6 mice invariably succumb to complications associated with either recrudescent disease or the damage incurred during the acute stage of infection typically sometime after 12 weeks of infection (3, 18, 21, 34). Sensitivity to type II *T. gondii* infection is clearly dependent on the mouse genotype (3, 7, 10, 23, 41, 52) and, as recently proposed, may be specifically related to immunodominant H-2L^d-restricted MHC-I epitopes found within GRA6, GRA4, and ROP7 proteins (6, 16). We examined whether *cps1-1* strain immunization would prolong the survival of susceptible C57BL/6 mice challenged long term with 10 cysts of ME49 i.p. The *cps1-1* strain-immunized mice survived chronic infection for at least 12 months after ME49 infection. In contrast, naive mice rapidly succumbed to chronic ME49 infection (Fig. 5).

***cps1-1* tachyzoites begin to differentiate into bradyzoites during uracil starvation.** Recent data show that the nonreplicating *cps1-1* strain elicits a strong local and systemic IL-12 p70 response that is more typical of a type II strain than a type I strain (20), and cytolytic $CD8^+$ T cells develop with faster kinetics compared to infection with replicating parasites (26). Previous studies also suggest that *T. gondii* life-stage-specific $CD8^+$ T-cell responses are important for control of the parasite (16, 30, 39). We examined the expression pattern of tachyzoite-stage marker (SAG1) and an early-bradyzoite-stage marker (CST1) during uracil starvation of the *cps1-1* strain. While replicating *cps1-1* parasites expressed SAG1 at high levels and did not express CST1 bradyzoite marker (Fig. 6, top panels), uracil starvation induced a nearly complete loss of

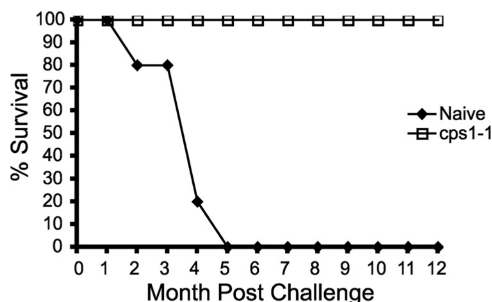

FIG. 5. Immunization with the *cps1-1* strain prevents lethality of chronic infection in the genetically sensitive C57BL/6 mouse strain. C57BL/6 mice were either not immunized or immunized i.p. with 10^6 *cps1-1* tachyzoites given twice 14 days apart. At 1 month after immunization, naive mice (◆) or *cps1-1* strain-immunized mice (□) were challenged with 10 cysts of ME49 i.p., and the percent survival was monitored. The data represent the results of one experiment performed with 10 animals per group.

expression of SAG1 and markedly upregulated the expression of CST1 (Fig. 6, bottom panels). These data suggest that an altered profile of tachyzoite and early bradyzoite antigens occurs within 2 days of initiating uracil starvation of the *cps1-1* strain.

DISCUSSION

These studies extend a series of recent and significant reports that have examined the immune responses elicited by vaccination with the attenuated *cps1-1* strain (13, 14, 20, 35, 49, 51, 56–59). To our knowledge, this is the first report that vaccination of genetically susceptible C57BL/B6 mice with a type I strain can prevent the development of lethal chronic infection.

A potent and long-lasting immunity to lethal type 1 infection is elicited by a single *cps1-1* strain immunization of BALB/c, C57BL/6, Tyk$2^{-/-}$ (C57BL/6), or MyD$88^{-/-}$ (C57BL/6) mice (13, 14, 20, 49, 51, 56). Immunity to lethal type I challenge infection induced by the *cps1-1* strain is dependent on $CD8^+$ T cells (20) and the local production of IFN-γ (20) and IL-12 p70 (20, 51, 56) and, surprisingly, does not require significant production of systemic IFN-γ (20).

Previous studies of the durability of immunity elicited by immunization with type I strain ts-4 or by infection with type II parasite strains in susceptible B6 mice suggest that immunity significantly wanes over time (8, 18, 27, 33, 47). Protecting highly genetically susceptible B6 mice from lethal acute or lethal chronic type II infection by vaccination is problematic. If parasites traffic to the brain or other tissues before the immune response can prevent cyst formation, it is currently unknown whether any immune response can clear preexisting cysts. Many studies have documented that B6 mice (MHC-I-restricted H-2b background) are extremely susceptible to type II infections (10, 23, 52). If these mice survive acute type II infection, then they will invariably succumb from the chronic infection within a few months. Recently, the GRA6 protein of type II strains was shown to possess an immunodominant H-2L^d-restricted MHC-I cell epitope that appears to play a major role in protecting BALB/c mice from lethal chronic infection. In addition, GRA4 and ROP7 have been characterized as containing life-stage-specific dominant epitopes (16). Since these epitopes are not recognized in B6 mice, loss of immune response to these immunodominant epitopes may at least partly explain the increased susceptibility of B6 mice to lethal type II chronic infections (6).

Interestingly, the immunodominant H-2L^d-restricted MHC-I epitope HF10 within the GRA6 protein of type II strains is not present in type I strains such as RH from which the *cps1-1* and ts-4 vaccine strains are derived (14, 45). Our results show that immunization of B6 mice with the *cps1-1* strain completely protects mice from lethal type II cyst challenge (i.p. or i.g. route) at 1 month (short term) and equally well at 10 months (long term). These results suggest that adaptive immunity directed against the immunodominant H-2L^d-restricted MHC-I epitope within the GRA6 protein of type II strains is not

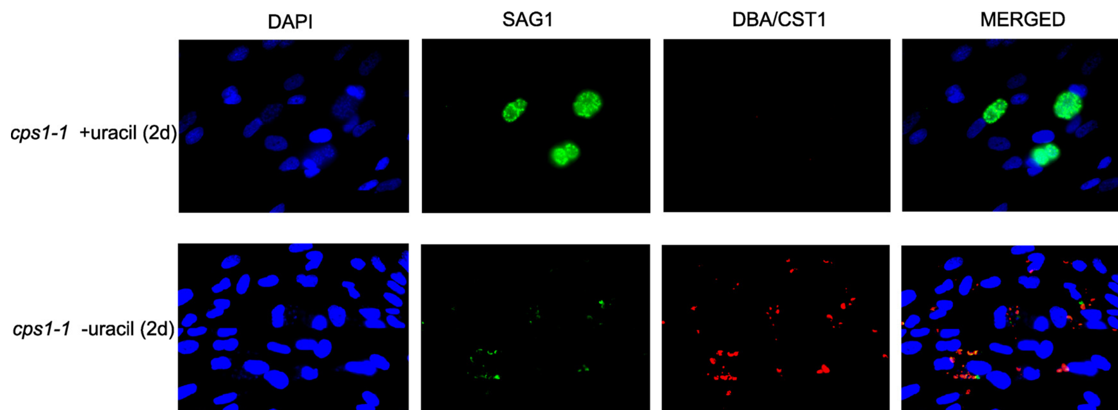

FIG. 6. Uracil starvation of the *cps1-1* strain induces the loss of tachyzoite-stage antigens and the expression of early-bradyzoite-stage antigens. Confluent HFF monolayers were infected with *cps1-1* tachyzoites in the presence (top panels) or absence (bottom panels) of uracil for 2 days. Immunohistochemistry analysis was performed after fixation of infected HFF monolayers with DAPI (blue), anti-P30/SAG1 (green), and DBA/CST1 (red) as indicated. The images show the results with DAPI alone (left panels, top and bottom), anti-P30/SAG1 alone (left center panels, top and bottom), DBA/CST1 alone (right center panels, top and bottom), and merged DAPI/SAG1/DBA (right panels, top and bottom). The images contain the brightest pixels below saturation, are unprocessed, and were collected from the original image(s).

necessary for control of acute lethal type II infections delivered by the i.p. or i.g. route in B6 mice.

Immune control of chronic infection in regard to whether mice succumb or survive is clearly multifaceted and highly dependent on both genetic factors and parasite genotype. Our results with immunization of genetically susceptible B6 mice with the *cps1-1* strain show that mice challenged with a sublethal acute infection dose of ME49 cysts at 1 month or 10 months postimmunization exhibit remarkably reduced levels of brain cyst burden and brain parasite burden and that these chronically infected B6 mice survive significantly longer than chronically infected naive mice. Our results show that a sterilizing immunity to brain cyst formation is not generated in *cps1-1* strain-immunized mice. The simplest explanation of these results is that type II infections can rapidly elicit new brain cysts early after oral infection and prior to immune destruction of tachyzoite stages. Several studies have suggested that cysts may develop early after infection since cysts containing only a few bradyzoites have been observed (55). Considering the potent immune responses elicited after *cps1-1* strain immunization, it seems likely that parasites emerging from challenge cysts most likely traffic to the brain early during infection or possibly after each wave of cellular infection before immune control of tachyzoite-stage infection is fully reestablished by the recall response to *cps1-1* strain immunity (49).

A significant fraction of *cps1-1* strain-elicited immunity is clearly targeted to tachyzoite stages based on rapid clearance of tachyzoite challenge (20, 26). Our experiments do not specifically address whether *cps1-1* strain-elicited immunity to type II infection is directed against tachyzoite- or bradyzoite-stage antigens. Several studies show that tachyzoite-stage antigens and bradyzoite-stage antigens induce potent stage-specific $CD8^+$ T-cell responses important for the control of *T. gondii* infection (16, 30, 39). Our results suggest that antigens from both stages may play a role in the more potent immunity induced by the *cps1-1* strain compared to the ts-4 strain. The *cps1-1* strain vaccine is delivered in the tachyzoite stage but within 2 days of uracil starvation begins to differentiate to early bradyzoite stages with a corresponding loss of tachyzoite-stage antigens. Although our results show that the *cps1-1* strain may elicit T-cell responses to both stages, our data do not discriminate whether early bradyzoite antigen expression or the loss of tachyzoite-stage antigens contributes to the potent protective immunity induced by the *cps1-1* strain. In addition, our study did not determine whether *cps1-1* strain-induced immunity is solely tachyzoite stage specific or both tachyzoite and bradyzoite stage specific. Because the *cps1-1* strain is completely nonreplicating (compared to ts-4), the immune response elicited by *cps1-1* strain vaccination may favor better control of type II infection. Recent results suggest there is markedly less inflammation induced by the *cps1-1* vaccine model and the cytokine response to the *cps1-1* strain more closely resembles that of a type II strain (20).

Autophagy could also play a role in the more potent immunity elicited by *cps1-1* vaccination. Vaccination with the *cps1-1* strain elicits potent autophagy responses in B6 mice (35, 57–59). Consequently, if the *cps1-1* strain ultimately delivers "self-autophagic" responses and the nonreplicating *cps1-1* parasite and its associated vacuole is digested in an autophagosome, a wider repertoire and/or increased presentation of parasite antigens may occur after immunization with *cps1-1*. Our data do not differentiate between these possibilities or other potential mechanisms that could explain the potent protection against type II infection elicited in C57BL/6 mice by the *cps1-1* vaccine.

Interestingly, our results show that $CD8^+$ T cells elicited by the *cps1-1* vaccine have a direct effect on the outcome of chronic infection in the brain. *cps1-1* strain-elicited immune $CD8^+$ T cells prevent the recrudescence of already-established brain cysts in B6 mice. Cysts that were prevented from recrudescing by *cps1-1* immunity continued to develop. These data do not distinguish whether these T cells recognize tachyzoite- or bradyzoite-stage antigens, and this mechanistic question will be addressed in future studies. Regardless of the mechanism of action, immune $CD8^+$ T cells elicited by *cps1-1* strain vaccination delay or prevent cyst recrudescence, and this clearly correlates with prolonged survival of chronically infected B6 mice. This immune control is independent of the H-2L^d-restricted GRA6, GRA4, and ROP7 derived epitopes and suggests that other factors and antigens are required to control chronic infection in *cps1-1* strain-immunized B6 mice. Our data suggest this immune control may be due to bradyzoite-stage antigens expressed by the *cps1-1* strain during uracil starvation, but we cannot yet rule out the possibility that the increased control of type II infection observed in our study is due to tachyzoite-stage antigens other than GRA6, GRA4, and ROP7.

In summary, we demonstrate that the *cps1-1* vaccine is highly effective at inducing both short-term and long-term protection against type II parasite challenge by significantly inhibiting lethal acute and lethal chronic disease in genetically susceptible C57BL/6 mice. $CD8^+$ T cells elicited by *cps1-1* vaccination delay or prevent cyst recrudescence in chronically infected B6 mice. These studies further establish that the live attenuated *cps1-1* vaccine strain is an excellent vaccine that confers significant protection against both type I and type II strains of *T. gondii*. The *cps1-1* vaccination model represents a valuable tool in which to further dissect the complex biology of how control of chronic infection is won or lost in C57BL/6 and other genetic backgrounds.

ACKNOWLEDGMENTS

This research was supported by NIH grants AI075931 and AI041930 (to D.J.B.).

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Editor: W. A. Petri, Jr.