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Determination of a Key Antigen for Immunological Intervention to Target the Latent Stage of *Toxoplasma gondii*

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Abstract

Toxoplasma gondii, an obligate intracellular protozoan parasite, establishes a chronic infection by forming cysts preferentially in the brain, and up to one third of human population worldwide is estimated to be chronically infected with this parasite. However, there are currently no drugs effective against the cyst form of the parasite. In addition, the protective immunity against the cysts remains largely unknown. We analyzed the molecular mechanisms by which the immune system detects host cells harboring the cysts to eliminate the latent stage of the parasite using mice with the H-2^d haplotype, which are genetically resistant to the infection. Our study revealed that CD8⁺ immune T cells bearing T cell receptor V β 8.1, 8.2 chain have a potent activity to remove *T. gondii* cysts from the brain. Our studies also uncovered that the H-2L^d is the major antigen-presenting molecule to CD8⁺ T cells for initiating the cyst elimination, and that the CD8⁺V β 8.1, 8.2⁺ immune T cells recognize the amino-terminal region (amino acids 41 to 152) of dense granule protein 6 (GRA6Nt) of the parasite presented by the H-2L^d molecule. Furthermore, CD8⁺ immune T cells induced by immunization with recombinant GRA6Nt were eventually capable of removing the cysts from the brain when transferred to infected immunodeficient mice lacking T cells. Thus, GRA6Nt is a novel and potent antigen to activate CD8⁺ T cells capable of removing *T. gondii* cysts. These observations offer the basis for immunological intervention to combat the chronic infection with *T. gondii* by targeting the persistent cysts of the parasite.

Introduction

Toxoplasma gondii is one of the pathogens that can establish a chronic infection in the brain. Acute infection with the parasite is characterized by proliferation of tachyzoites in various organs, and it can cause various diseases including lymphadenitis and congenital infection of

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the fetuses (1). Although IFN- γ -mediated immune responses and, to a lesser extent humoral immunity, inhibit the tachyzoite growth (2–4), the parasite establishes a chronic infection by forming tissue cysts, which can contain hundreds to thousands of bradyzoites, preferentially in the brain. This infection is ubiquitous and one of the most common parasitic infections in humans worldwide (1). It is well recognized that chronic *T. gondii* infection can reactivate and develop life-threatening toxoplasmic encephalitis in immunocompromised individuals such as those with AIDS and organ transplants (1, 5, 6). Even in immunocompetent individuals, recent epidemiological studies demonstrated that *T. gondii* infection is associated with a 1.8-fold increase in the risk of brain cancers (7), and that brain cancer mortality rates increase with seroprevalence of IgG antibodies to *T. gondii* (8). Therefore, for improving public health, it is important to develop a method to eliminate *T. gondii* cysts from chronically infected individuals. However, there is no current drug that can target the cyst stage of the parasite.

Although it had generally been considered that the immune system of infected hosts is unable to recognize or remove *T. gondii* cysts, our recent studies demonstrated that CD8⁺ immune T cells of chronically infected BALB/c mice (the H-2^d haplotype), which are genetically resistant to the infection, possess a potent activity to initiate the immune process to remove the tissue cysts from the brain when these T cells are transferred to infected immunodeficient (athymic nude or SCID) mice that have already developed large numbers of cysts (9). This anti-cyst activity of CD8⁺ immune T cells does not require their IFN- γ production, which is essential for controlling tachyzoites as mentioned earlier, but does require their perforin (9). Perforin is critical for cytotoxic activity of CD8⁺ T cells. Therefore, it is most likely that cytotoxic activity of CD8⁺ T cells initiates the immune process to remove the cysts. *T. gondii* cysts are formed within infected host cells (10, 11). However, the mechanisms by which the CD8⁺ T cells recognize the cyst-containing cells are currently unknown. For developing a novel method to activate CD8⁺ T cells capable of removing *T. gondii* cysts, it is crucial to understand the molecular mechanisms by which the T cells recognize the cyst-containing cells to initiate the immune process for eliminating the cysts. The present study revealed that the H-2L^d is the major antigen-presenting molecule required for CD8⁺ immune T cells to initiate the T cell-mediated cyst elimination against cyst-containing cells. The present study also determined that the CD8⁺ immune T cells bearing TCR V β 8.1, 8.2⁺ chain have a potent activity to remove the cysts, and this T cell population recognizes the amino-terminal region (amino acids 41 to 152) of the dense granule protein 6 (GRA6Nt) of *T. gondii* presented by the H-2L^d molecule. Furthermore, CD8⁺ T cells activated by an immunization with GRA6Nt were able to remove *T. gondii* cysts. These findings provide the foundation required for immunological intervention targeting the latent stage of *T. gondii* to eradicate chronic infection with this parasite.

Materials and Methods

Mice, infection with *T. gondii*, and study design

BALB/c, BALB/c-background SCID, and BALB/c-H-2^{dm2} (dm2) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Swiss-Webster mice were from Taconic (Germantown, NY). Female mice were used for all studies. In each experiment, all mice in

the same strain were mixed together once and then randomly divided into experimental groups prior to applying them to the experiment. There were three to six mice in each experimental group. Cysts of the ME49 strain (a type II strain) of *T. gondii* were obtained from brains of chronically infected Swiss-Webster mice (9, 12). Type II *T. gondii* is the genotype of the parasite that has been recognized as predominant in infections in North American and European human populations (13, 14). All animals were infected with 10 cysts (BALB/c mice) or 20 cysts (SCID and dm2 mice) perorally by gavage. SCID and dm2 mice were treated with sulfadiazine in the drinking water (400 mg/L) beginning at 7–10 days after infection for 3 weeks to control the proliferation of tachyzoites and establish a chronic infection in their brains (9, 12). All animal experiments were conducted with the approval of the University of Kentucky Institutional Animal Care and Use Committee in accordance with the “Guide for the Care and Use of Laboratory Animals,” published by the National Research Council and endorsed by the NIH Office of Laboratory Animal Welfare. There were three to six mice in each experimental group.

Two independent replicates were performed in most of *in vivo* studies and the data from the independent replicates were combined for the results presented in Figures and for statistical analyses (see Figure Legends for the details for each experiment). Most of *in vitro* experiments were performed once, but the reproducibility of the results was confirmed by using multiple clones of V β 8.1,8.2⁺ T cell hybridoma for evaluating their responses, using both the T cell hybridoma and the primary V β 8.1,8.2⁺ immune T cells purified from the spleens of infected mice, and using antigen-presenting cells from dm2 mice lacking the *H-2L^d* gene and applying anti-H-2L^d antibody to antigen-presenting cells from wild-type mice with the *H-2L^d* gene.

Purification and transfer of CD8⁺ and CD8⁺V β 8.1, 8.2⁺ immune T cells

Spleen cells were obtained from chronically infected BALB/c mice and suspended in HBSS (Irvine Scientific, Santa Ana, CA) with 2% FBS (Sigma, St. Louis, MO). CD8⁺ immune T cells were purified from the spleen cells using magnetic bead-conjugated anti-mouse CD8 (clone 53–6.7) mAb (Miltenyi Biotech, Auburn, CA) as described previously (9, 15). CD8⁺V β 8.1, 8.2⁺ immune T cells were purified by sorting after incubating the CD8⁺ immune T cells with PE-conjugated mAb to mouse CD8 α (clone 53–6.7) and FITC-conjugated mAb to mouse TCR V β 8.1,8.2 chain (clone MR5-2). The mAb for sorting were from BD Bioscience (San Jose, CA). A total of 2.7–6.7 $\times 10^6$ CD8⁺ immune T cells or 0.9 $\times 10^6$ CD8⁺V β 8.1, 8.2⁺ immune T cells were injected intravenously from a vein in the tail into infected, sulfadiazine-treated SCID mice at 3 weeks after infection (9). In one experiment, the CD8⁺ immune T cells were also injected into infected, sulfadiazine-treated dm2 mice in the same manner.

Flow cytometry to determine TCR V β chain usage in CD8⁺ immune T cells that infiltrated into the brains of infected SCID mice after a systemic T cell transfer

Mononuclear cells were purified from brains of infected, sulfadiazine-treated SCID mice at 7 days after a systemic transfer of CD8⁺ immune T cells as described previously (16, 17). To determine TCR V β chain usage in the CD8⁺ immune T cells that had infiltrated into the brains of the recipient SCID mice, the purified mononuclear cells were first pretreated with

anti-Fc γ II/III receptors mAb (clone 2.4G2) and then incubated with a PE-conjugated mAb to CD8 α (clone 53–6.7) and FITC-conjugated mAb to various TCR V β chains as described previously (18). The mAbs were from BD Bioscience, and the staining was performed using pooled cells from the mice due to availability of only limited numbers of cells from the brain of each animal. Because of the limited availability of the cells and the requirement of measuring usage of 15 different TCR V β chains on the CD8 $^+$ T cells purified from the brains of the animals, only one staining was performed for each of TCR V β chain. The stained cells were applied to a FACSCalibur and the data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR).

Quantification of *T. gondii* cysts and amounts of mRNA for bradyzoite-specific BAG1, perforin, and granzyme B in the brains of mice following the T cell transfer

Seven to twelve days after a transfer of CD8 $^+$ or CD8 $^+$ V β 8.1, 8.2 $^+$ immune T cells, the brains of SCID or dm2 mice with and without the cell transfer were removed, and a half of each brain was triturated in 0.5 ml of phosphate-buffered saline (9, 19). Numbers of cysts in 3–6 aliquots (20 μ l each) of the brain suspensions were counted microscopically. The counting was performed by multiple people in most of the experiments and the average of the counting from the multiple people was used for each mouse. RNA was purified from a half of each brain, and amounts of mRNA for BAG1, perforin, and granzyme B were measured by reverse transcription real-time PCR using StepOnePlus real-time PCR system with TaqMan reagents (Applied Biosystems, Branchburg, New Jersey) (9, 16). Primers and probe for BAG1 were as follows: TCACGTGGAGACCCAGAGT (Forward), CTGGCAAGTCAGCCAAAATAATCAT (reverse), TTTGCTGTGCAACTCC (probe) (20). Ready-made primers and probes for perforin, granzyme B, and β -actin were from Applied Biosystems.

Generation of CD8 $^+$ V β 8.1, 8.2 $^+$ T cell hybridomas and stimulation of the hybridomas and primary CD8 $^+$ V β 8.1, 8.2 $^+$ immune T cells with *T. gondii* antigens

CD8 $^+$ V β 8.1,8.2 $^+$ immune T cells purified from the spleens of chronically infected BALB/c mice were stimulated with 5 ng/mL PMA (Sigma) and 500 ng/mL ionomycin (Sigma) and fused with TCR α - β - fusion partners, BWZ.36 lymphoma cells (21) as described previously (15). To analyze the *T. gondii* antigens that stimulate the CD8 $^+$ V β 8.1/8.2 $^+$ immune T cells, the CD8 $^+$ V β 8.1,8.2 $^+$ T cell hybridomas and primary CD8 $^+$ V β 8.1,8.2 $^+$ immune T cells purified from the spleens of infected BALB/c mice were suspended in RPMI 1640 medium (GIBCO) containing 10% FBS (Hyclone), 1 mM sodium pyruvate (GIBCO), 50 μ M 2-mercaptoethanol (Sigma) and 100 U/mL penicillin (GIBCO), and placed into wells of 96-well culture plates (5×10^4 cells/well for the hybridomas and 1×10^5 cells/well for the primary CD8 $^+$ V β 8.1,8.2 $^+$ immune T cells). The cells were stimulated with either *T. gondii* epitope peptides previously reported (22–24) or recombinant dense granule proteins (rGRAs) of *T. gondii* (25) at a concentration of 1 μ g/ml, or a total lysate antigens of tachyzoites (TLA) (26) at a concentration of 20 μ g/ml for 72 hrs in the presence of APCs prepared as plastic-adherent cells from the spleens of normal BALB/c mice (15). The epitope peptides were from Synthetic Biomolecules (San Diego, CA). The rGRAs are those of a genotype II strain (the 76K strain) of *T. gondii*. The type II of the parasite includes the ME49 strain used for infection of mice in the present study, and all of the rGRAs are pUET

fusion proteins. rGRA2 contains the amino acids 21 to 185 of GRA2 (protein deleted from its signal peptide). rGRA6Nt and rGRA6Ct contain the amino acids 41–152 and 174–224 of GRA6, respectively. The TLA was prepared with tachyzoites of the ME49 strain (26). After the incubation of the T cells with *T. gondii* antigens, the plates were centrifuged at 800 rpm for 10 min, and the culture supernatants were collected. In some experiments, to examine the role of the H-2L^d molecule for presentation of *T. gondii* antigens to CD8⁺ Vβ8.1,8.2⁺ immune T cells, the hybridomas were cultured with the antigens in the presence of APCs from normal BALB/c and dm2 mice that have a complete deletion of the *H-2L^d* gene. The role of the H-2L^d molecule for presentation of *T. gondii* antigens to CD8⁺ Vβ8.1,8.2⁺ immune T cells were also examined by adding anti-H-2L^d mAb or an isotype control at a concentration of 25 µg/ml to the cultures of primary CD8⁺ Vβ8.1,8.2⁺ immune T cells purified from the spleens of chronically infected BALB/c mice with J774 macrophage cell line (ATCC, Manassas, VA) infected with tachyzoites of the ME49 strain (26).

Measurement of amounts of perforin and granzyme B in the culture supernatants

The concentrations of perforin and granzyme B in the culture supernatants of various CD8⁺ immune T cell populations and CD8⁺Vβ8.1, 8.2⁺ T cell hybridomas were measured by using commercial ELISA kits from Cloud-Clone Corp (Houston, TX) and R&D Systems (Minneapolis, MN), respectively.

Immunization of BALB/c mice with rGRA6Nt-primed BM-DC and a transfer of CD8⁺ T cells from the immunized mice to infected SCID mice

Bone marrow cells were harvested from tibia and femur bones of normal BALB/c mice and suspended in RPMI 1640 medium containing 10% FBS, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 0.1 mM nonessential amino acids (GIBCO), 20 µg/mL gentamicin (Gemini Bio-Products, West Sacramento, CA) and 20 ng/mL GM-CSF (R&D Systems) and 10 ng/mL IL-4 (BD Biosciences). The cells were seeded onto 10 cm-diameter petri dishes at a cell density of 0.5×10^6 cells/ml. A half of culture medium was replaced with fresh medium containing GM-CSF and IL-4 every other day. The cells were expanded into new petri dishes on Day 3 of the culture, and 5 days later the BM-DC were harvested with cold PBS and stimulated with 1 µg/mL of rGRA6Nt in the culture medium without GM-CSF and IL-4 for 24 hrs. As a control the BM-DC were cultured without any antigens added in the same manner. After the incubation with and without rGra6Nt, the BM-DC were harvested, washed and resuspended in PBS (Amresco, Solon, OH). Normal BALB/c mice were injected intraperitoneally with the rGra6N-primed BM-DC and control BM-DC ($1.0\text{--}1.2 \times 10^6$ cells) once weekly for two weeks. Fifteen days after the second injection of the BM-DC, CD8⁺ T cells were purified from the spleens of the immunized mice by using magnetic beads-conjugated anti-CD8 mAb as described earlier. The purities of the CD8⁺ immune T cell preparations were > 98%. A total of $2.7\text{--}3.3 \times 10^6$ CD8⁺ immune T cells were injected intravenously into infected and sulfadiazine-treated SCID mice at 3 weeks after infection.

Statistics

Differences between experimental groups were compared with one-way ANOVA with Newman-Keuls Post test or two-tailed Mann-Whitney test using GraphPad prism 6 software. The latter was used when the variations of the data within experimental groups were large.

When Mann-Whitney test was used, a corrected P value (P_c) was calculated by multiplying the P value with the number of comparisons performed within the experiment. Two-tailed Student's t test was also used for one experiment with only two groups ($n=3$ for each group) for comparison because the sample number in each group was too small for Mann-Whitney test to reach statistical significance. A P value in the one-way ANOVA and Student's t test or P_c value in Mann-Whitney test of <0.05 was considered significant.

Results

TCR V β 8.1, 8.2⁺ cells are most abundant among the CD8⁺ immune T cells that infiltrated into the brain during removal of *T. gondii* cysts

CD8⁺ T cells recognize the target antigens by TCR composed of α and β chains. To begin understanding the molecular mechanisms of recognition of the cyst-containing cells by anti-cyst CD8⁺ immune T cells, we first analyzed TCR V β chain usage of CD8⁺ immune T cells that infiltrated into the brain during cyst removal. For this purpose, SCID mice, which lack T and B cells, were infected and treated with sulfadiazine to control tachyzoite proliferation and establish a chronic infection by forming cysts in their brains (9, 12). CD8⁺ immune T cells purified from the spleens of infected BALB/c mice, which are genetically resistant to the infection (27–29), were systemically transferred into the infected SCID mice. Since our previous study demonstrated that one week is sufficient to detect marked decreases in numbers of cysts in the brains of the recipient SCID mice after the cell transfer (9), we examined TCR V β chain usage in the CD8⁺ T cells, which had infiltrated into the brains of the recipients, at 7 days after the cell transfer. CD8⁺ T cells bearing TCR V β 8.1, 8.2 chain were found to be most abundant (21.1 %) among the T cells detected in their brains (Fig 1A). Our previous study showed that the activity of CD8⁺ T cells to remove cysts is mediated by perforin (9). Among the CD8⁺ T cells that infiltrated into the brains, the frequencies of perforin-expressing cells were approximately twice as high in V β 8.1, 8.2⁺ population than in a total population of CD8⁺V β 8.1, 8.2⁻ cells (Fig. 1B). These results strongly suggest that V β 8.1, 8.2⁺ cells play an important role in the activity of CD8⁺ immune T cells to remove *T. gondii* cysts from the brain.

CD8⁺V β 8.1, 8.2⁺ immune T cells have a potent activity to remove *T. gondii* cysts from the brain

In order to examine whether CD8⁺ immune T cells bearing TCR V β 8.1, 8.2 chain are actually capable of removing *T. gondii* cysts from the brain, this T cell population was purified from infected BALB/c mice and transferred to infected SCID mice. At 12 days after the cell transfer (Day 12), numbers of cysts in the brains of the mice that had received the CD8⁺V β 8.1, 8.2⁺ immune T cells were markedly fewer than those in the brains of the control animals without the cell transfer (Fig 1C). Consistent with the difference in cyst numbers, amounts of mRNA for bradyzoite-specific BAG1 were 35 times less in the brains of the former than the latter (Fig 1D). This reduction of cyst numbers in the recipients of CD8⁺V β 8.1, 8.2⁺ immune T cells is not due to inhibition of tachyzoite growth by the T cell population because all groups of mice were under treatment with sulfadiazine to inhibit tachyzoite proliferation. In agreement, amounts of mRNA for tachyzoite-specific SAG1 were consistently very low (mostly close to detection limit) in all of the experimental groups

with and without the T cell transfer (data not shown). In addition, anti-cyst activity of CD8⁺ T cells does not require their production of IFN- γ (9), the essential molecule for controlling tachyzoite growth. Thus, the marked reduction of cyst numbers in the recipients of CD8⁺V β 8.1, 8.2⁺ immune T cells is due to removal of *T. gondii* cysts by the immune process initiated by the T cells. Since anti-cyst activity of CD8⁺ T cells requires their perforin (9) that is critical for their cytotoxic activity, the cytotoxic activity of CD8⁺ T cells most likely initiates the immune process to remove *T. gondii* cysts. Granzyme B also plays an important role in cytotoxic activity of CD8⁺ T cells. Consistently, amounts of mRNA for perforin and granzyme B were 3.4 and 7 times greater, respectively, in the brains of the mice that had received CD8⁺V β 8.1, 8.2⁺ immune T cells than those of the control animals without the cell transfer (Figs 1E and 1F). These results indicate that CD8⁺ immune T cells bearing TCR V β 8.1, 8.2 chain have a potent activity to induce removal of *T. gondii* cysts from the brains of infected hosts, and that this T cell population alone without any other T cells is capable of inducing the cyst removal.

CD8⁺V β 8.1, 8.2⁺ immune T cells do not recognize epitopes of *T. gondii* previously reported

To address *T. gondii* antigens that activate the CD8⁺V β 8.1, 8.2⁺ immune T cells, we generated CD8⁺V β 8.1, 8.2⁺ T cell hybridomas by fusing CD8⁺V β 8.1, 8.2⁺ immune T cells purified from the spleens of infected BALB/c mice with BWZ.36, a TCR $\alpha\beta$ -negative fusion partner that contains an inducible receptor gene encoding β -galactosidase. Previous studies by us (28) and by others (29) demonstrated that BALB/c-H-2^{dm2} (dm2) mice that have a complete deletion of the H-2L^d gene of MHC class I molecules have markedly greater numbers of *T. gondii* cysts in their brains than wild-type BALB/c mice during the later stage of infection. In these studies, the reasons for the presence of the large numbers of cysts in the brains of dm2 mice were unclear, but it was considered that these animals could have an accelerated proliferation of tachyzoites during the acute stage of infection, which results in formation of larger numbers of cysts. However, it is also possible that dm2 mice lack the immune responses that can remove the cysts and thereby harbor the large numbers of cysts in their brains. Five epitopes of *T. gondii* antigens (GRA6, GRA4, ROP7, SAG1, and SAG3) presented by the H-2L^d molecule have been reported (22–24). These epitopes were shown to induce IFN- γ production by CD8⁺ T cells and/or confer a resistance against an acute acquired infection. In contrast, anti-cyst activity of CD8⁺ T cells requires their perforin (9) that is critical for their cytotoxic activity, as mentioned earlier. Therefore, we first examined whether any of these previously identified epitopes stimulate the CD8⁺V β 8.1, 8.2⁺ T cell hybridoma to induce their perforin and granzyme B secretion. Of interest, none of the five epitopes induced any significant secretion of either perforin or granzyme B from the CD8⁺V β 8.1, 8.2⁺ T cell hybridoma when compared to the negative control cultures without any *T. gondii* antigens added (Figs 2A and 2B). This is a clear contrast to stimulation with total lysate antigens of tachyzoites, which induced a secretion of increased amounts of both perforin and granzyme B from the T cell hybridoma into the culture supernatants (Figs 2A and 2B). Therefore, it is most likely that CD8⁺V β 8.1, 8.2⁺ immune T cells recognize a novel epitope(s) of *T. gondii* molecule(s) to initiate their cytotoxic activity.

CD8⁺Vβ8.1, 8.2⁺ immune T cells recognize the amino-terminal region of dense granule protein (GRA) 6 of *T. gondii*

Both tachyzoites and bradyzoites express GRAs, and these molecules are known to be a component of cyst wall of *T. gondii* (30). Therefore, it is possible that CD8⁺Vβ8.1, 8.2⁺ T cells recognize one of these GRAs. To address this possibility, we examined whether the CD8⁺Vβ8.1, 8.2⁺ T hybridoma recognizes recombinant amino-terminal region (amino acid 41-152) or carboxyl-terminal region (amino acid 174-224) of GRA6 (namely rGRA6Nt, and rGRA6Ct, respectively), or recombinant GRA2 (rGRA2) (Supplemental Fig. 1). These recombinant molecules were used because they were the only recombinant GRA proteins of the type II *T. gondii* (that includes the ME49 strain used in the *in vivo* studies in the present study) available at that time. rGRA6Ct contains one of the negative epitope (HF10) tested in the study shown in Figs. 2A and 2B (Supplemental Fig. 1). Concentrations of perforin and granzyme B in the culture supernatants of the CD8⁺Vβ8.1, 8.2⁺ T cell hybridoma stimulated with rGRA6Nt were significantly higher than those in control cultures without any antigens (Figs. 2C and 2D). In contrast, perforin and granzyme B levels in the culture supernatants of the hybridoma stimulated with rGRA2 did not differ from those of the control cultures without antigens (Figs 2C and 2D). rGRA6Ct also induced secretion of perforin and granzyme B from the CD8⁺Vβ8.1, 8.2⁺ T cell hybridoma, but the concentrations of each of perforin and granzyme B were significantly higher in the cultures with rGRA6Nt than those in the cultures with rGRA6Ct (Fig 2C and 2D). Secretion of perforin and granzyme B in response to rGRA6Nt was also observed in another clone of CD8⁺Vβ8.1, 8.2⁺ T hybridoma (perforin: 3.41 ± 0.35 ng/ml with no antigen [n=3] vs. 4.12 ± 0.06 ng/ml with GRA6Nt [n=3], $P < 0.05$ using unpaired Student's *t* test; granzyme B: 0.82 ± 0.15 ng/ml with no antigen [n=3] vs. 2.55 ± 0.07 ng/ml with GRA6Nt [n=3], $P < 0.01$ using unpaired Student's *t* test). These results indicate that GRA6Nt is a novel and effective target of CD8⁺Vβ8.1, 8.2⁺ T cells to recognize *T. gondii* for their cytotoxic activity.

To further confirm that rGRA6Nt is a potent stimulator of the cytotoxic activity of the CD8⁺Vβ8.1, 8.2⁺ immune T cells, we purified this T cell population from the spleens of infected BALB/c mice and stimulated the purified T cell population with rGRA6Nt. Concentrations of both perforin and granzyme B in their culture supernatants were significantly higher than those in control cultures without any antigens added (Figs 2E and 2F). Consistent with the results of the T cell hybridomas shown in Figs. 2B and 2C, concentrations of each of perforin and granzyme B in the cultures of the primary CD8⁺Vβ8.1, 8.2⁺ immune T cells with rGRA6Nt were significantly higher than those in the cultures with rGRA6Ct (Figs 2E and 2F). Notably, the concentrations of perforin and granzyme B in the former were also significantly higher than those in the cultures with total lysate antigens of tachyzoites (Figs 2E and 2F), suggesting that the GRA6Nt is a dominant stimulator in *T. gondii* to activate perforin- and granzyme B-mediated cytotoxic activity of CD8⁺Vβ8.1, 8.2⁺ T cells.

We also compared the amounts of perforin and granzyme B secreted from CD8⁺Vβ8.1, 8.2⁺ T cells and a total population of CD8⁺Vβ8.1, 8.2⁻ T cells purified from the spleens of infected BALB/c mice in response to GRA6Nt. Amounts of perforin in the culture supernatants in both of these two T cell populations were greater in their cultures with

rGRA6Nt than in their control cultures with PBS (Fig. 2G). However, the perforin levels in the cultures with rGRA6Nt were significantly greater with CD8⁺Vβ8.1, 8.2⁺ T cells than with a total population of CD8⁺Vβ8.1, 8.2⁻ T cells (Fig. 2G). Granzyme B levels in the culture with rGRA6Nt were also significantly and markedly greater with the former than the latter (Fig. 2H). These results indicate that in addition to CD8⁺Vβ8.1, 8.2⁺ T cells, CD8⁺ T cells bearing TCRs other than Vβ8.1, 8.2 are also able to recognize GRA6Nt and display cytotoxic activity, but their cytotoxic activity in this response is significantly weaker than that of Vβ8.1, 8.2⁺ population. These results along with the abundance of CD8⁺Vβ8.1, 8.2⁺ T cells in the brain and their superior anti-cyst activity strongly suggest that the recognition of GRA6Nt by CD8⁺Vβ8.1, 8.2⁺ immune T cells plays a crucial role in the protective immunity against *T. gondii* cysts.

The H-2L^d presents GRA6Nt of *T. gondii* to CD8⁺Vβ8.1, 8.2⁺ immune T cells

We next addressed the MHC class I molecule that presents rGRA6Nt to CD8⁺Vβ8.1, 8.2⁺ T cells. BALB/c mice have three main MHC class I molecules, the H-2K^d, H-2D^d, and H-2L^d. However, based on the presence of large numbers of cysts in dm2 mice that have a complete deletion of the *H-2L^d* gene (28, 31), as mentioned earlier, it is possible that the H-2L^d molecule plays a crucial role in presenting *T. gondii* antigens to CD8⁺ T cells for inducing cyst removal. When the CD8⁺Vβ8.1, 8.2⁺ T cell hybridoma was stimulated with rGRA6Nt in the presence of antigen-presenting cells (APC) from uninfected wild-type BALB/c or dm2 mice, the hybridoma clone secreted increased amounts of perforin and granzyme B only when stimulated with the antigen in the presence of the wild-type BALB/c APC (Figs 3A and 3B). Whereas dm2 mice lack the *H-2D2^d*, *H-2D3^d*, and *H-2D4^d* genes in addition to the *H-2L^d* gene, only the *H-2L^d* gene among these four genes encodes a cell surface antigen. Thus, CD8⁺Vβ8.1, 8.2⁺ immune T cells recognize the epitope(s) present within GRA6Nt of *T. gondii* through the presentation by the H-2L^d molecule.

To further examine the importance of the H-2L^d molecules for recognition of not only GRA6Nt but also *T. gondii* antigens in general by CD8⁺Vβ8.1, 8.2⁺ immune T cells, this T cell population purified from the spleens of infected BALB/c mice was stimulated with infected J774 macrophage cell line in the presence and absence of a blocking antibody to the H-2L^d molecule. The secretion of both perforin and granzyme B by the primary CD8⁺Vβ8.1, 8.2⁺ immune T cells was markedly (68% and 89 %, respectively) inhibited in the presence of the anti-H-2L^d antibody (Figs 3C and 3D). Thus, the recognition of *T. gondii* infection in J774 macrophages by CD8⁺Vβ8.1, 8.2⁺ immune T cells is mostly mediated by presentation of the parasite antigen(s) by the H-2L^d molecule. Since GRA6Nt, the potent stimulator of the cytotoxic activity of this T cell population, is presented by the H-2L^d molecule as described earlier, GRA6Nt appears to be a dominant *T. gondii* antigen that stimulate the cytotoxic activity of CD8⁺Vβ8.1, 8.2⁺ T cells in response to the parasite.

The H-2L^d molecule is required for removing *T. gondii* cysts by CD8⁺ immune T cells

To examine whether the H-2L^d molecule is actually crucial for recognition of cyst-containing cells by CD8⁺ immune T cells to remove the cysts, CD8⁺ immune T cells purified from infected BALB/c mice were transferred to infected SCID mice, which have the *H-2L^d* gene, and dm2 mice, which lack the *H-2L^d* gene. The transfer of the CD8⁺ immune T

cells markedly reduced the cyst numbers and BAG1 mRNA levels in the brains of SCID mice as expected (Figs 4A and 4B). In contrast, the T cell transfer did not result in a significant reduction of cyst numbers and BAG1 mRNA levels in the brains of the dm2 mice (Figs 4A and 4B). In agreement, amounts of mRNA for perforin and granzyme B did not differ between the brains of dm2 mice with and without the transfer of CD8⁺ immune T cells (Figs 4C and 4D), whereas amounts of mRNA for these molecules were 12 and 134 times greater in the brains of SCID mice with the T cell transfer than those of control SCID animals without the cell transfer, respectively (Figs. 4C and 4D). These results indicate that the H-2 L^d is the major MHC class I molecule that presents *T. gondii* antigens from cyst-containing cells to CD8⁺ immune T cells to induce removal of the cysts by the T cells. Since CD8⁺Vβ8.1, 8.2⁺ immune T cells, the most abundant CD8⁺ T cell population in the brain with a potent activity to remove *T. gondii* cysts, recognize GRA6Nt presented by the H-2L^d molecule, the recognition of GRA6Nt by Vβ8.1, 8.2⁺ subpopulation appears to play a major role in the H-2L^d-dependent anti-cyst activity of CD8⁺ immune T cells.

CD8⁺ immune T cells activated by immunization with rGRA6Nt are capable of removing *T. gondii* cysts from the brain

To examine whether GRA6Nt eventually activates CD8⁺ T cells capable of eliminating cysts from the brain, uninfected BALB/c mice received bone marrow-derived dendritic cells (BM-DC) pre-incubated with rGRA6Nt to activate GRA6Nt-specific CD8⁺ T cells. As a control, uninfected BALB/c mice received BM-DC pre-incubated with no antigens. CD8⁺ T cells purified from these immunized mice were transferred into infected, sulfadiazine-treated SCID mice at 3 weeks after infection. At 7 days after the cell transfer, numbers of cysts were significantly less in the brains of the SCID mice that had received CD8⁺ T cells from the animals immunized with rGRA6Nt-primed BM-DC than the control mice without receiving any T cells (Fig 5A). In contrast, numbers of cysts in the brains of mice that had received CD8⁺ T cells from animals immunized with un-primed BM-DC did not differ from those of the control animals without receiving any T cells (Fig 5A). Consistently, amounts of mRNA for bradyzoite-specific BAG1 were significantly less in the brains of the SCID mice that had received CD8⁺ T cells from the animals immunized with rGRA6Nt-primed BM-DC than the control mice without receiving any T cells (Fig 5B). Amounts of BAG1 mRNA in the brains of SCID mice that had received the T cells from the animals immunized with un-primed BM-DC also tended to be less than those of the control animals without receiving any T cells, but such difference did not reach statistical significance (Fig 5B). When spleen cells of these recipient animals were cultured with and without rGRA6Nt, only the cells from the recipients of CD8⁺ T cells from donors immunized with rGRA6Nt-primed BM-DC had increased concentrations of both perforin and granzyme B in their culture supernatants following stimulation with rGRA6Nt (Figs 5C and 5D), confirming that the immunization with rGRA6Nt-primed BM-DC indeed activated cytotoxic CD8⁺ immune T cells capable of recognizing GRA6Nt. These results indicate that CD8⁺ T cells activated by rGRA6Nt are capable of removing *T. gondii* cysts from the brains of infected hosts.

Discussion

T. gondii is able to establish a chronic infection in the brain, and the basis of the persistent infection is the formation of tissue cysts. Whereas our recent studies demonstrated that CD8⁺ immune T cells have the capability to remove the cysts from the brains of chronically infected hosts (9, 19), *T. gondii* antigens recognized by CD8⁺ T cells for the cyst removal remained unknown. The present study uncovered that GRA6Nt is a key target antigen of the protective T cells to detect the persistent stage of *T. gondii* for their elimination using a mouse strain genetically resistant to the infection. The cyst stage of the parasite is formed within host cells, and it has the cyst wall, a structure unique to this stage, surrounding large numbers of bradyzoites. GRA6 is one of the components of the cyst wall (30). Therefore, it is possible that host cells containing the cysts are able to present an epitope(s) present within GRA6Nt from the GRA6 molecules composing the cyst wall and initiate their recognition by CD8⁺ T cells to induce removal of the cysts. Another possibility would be that GRA6 released from bradyzoites is able to pass through the cyst wall, and the host cells present the GRA6Nt epitope(s) from the GRA6 molecules released from bradyzoites for recognition by CD8⁺ T cells.

We previously demonstrated that perforin of CD8⁺ T cells is required for their anti-cyst activity, and that the elimination of *T. gondii* cysts by the CD8⁺ T cell-mediated immune process is associated with an accumulation of phagocytes, morphologically microglia and macrophages, around and within the cysts (9). Perforin is the molecule critical for cytotoxic activity of CD8⁺ T cells as mentioned earlier. The present study revealed that GRA6Nt of the parasite is a potent stimulator of both cytotoxic activity and anti-cyst activity of CD8⁺ T cells. Therefore, it is most likely that the protective CD8⁺ T cells induce perforin-dependent lysis of cyst-containing cells by recognizing GRA6Nt presented on their surface, and the cytolysis of the cyst-containing cells initiates an accumulation of the phagocytes that kill the bradyzoites existing within the cysts. This perforin-dependent anti-cyst immunity is a clear contrast to the protective immunity against tachyzoites, which requires IFN- γ production by T cells (2–4). Therefore, the host immune system utilizes two different T cell-mediated mechanisms to control *T. gondii* depending on the acute (tachyzoite) or chronic (cyst) forms of the parasite; IFN- γ -dependent mechanisms against the former and perforin-dependent mechanisms against the latter. Of interest, the C-terminal region of GRA6 contains an epitope that is a potent stimulator of IFN- γ -mediated protective immunity against acute infection with *T. gondii* (22). The present study determined that the N-terminal region of the same molecule is able to activate the cytotoxic activity of CD8⁺ T cells and anti-cyst immunity. Thus, the immune system recognizes multiple regions of a single molecule, GRA6, of *T. gondii* to activate the protective CD8⁺ T cell immunity against both tachyzoites and cysts of the parasite.

In the acute stage of *T. gondii* infection, tachyzoites invade into host cells and quickly proliferate within the cells. The proliferated tachyzoites then egress from the host cells and invade into new host cells located nearby to proliferate. Therefore, when CD8⁺ T cells detect a tachyzoite-infected cell, there is a high probability that multiple cells around the infected cell are also infected with tachyzoites. In this situation, secreting a soluble mediator, IFN- γ , to activate the cells nearby the infected cell to prevent the parasite growth is an effective

strategy of the host protective immunity to control *T. gondii* proliferation. In contrast, the situation of the tissue cysts is different. The cysts are located one by one in a scattered manner, and it is unusual to detect multiple cysts nearby each other. Therefore, attacking the cyst-containing cells by cytotoxic CD8⁺ T cells through a direct cell-cell contact fashion, rather than secreting a soluble factor, could be an efficiently targeted strategy to destroy only the cyst-containing cells without inducing unnecessary activation and functional changes of cells located nearby.

The present study revealed that CD8⁺ immune T cells bearing TCR Vβ8.1, 8.2 chain have a potent anti-cyst activity, and that this T cell population alone in the absence of the other T cell populations is able to remove the cysts. In addition, the present study uncovered that the H-2L^d is the major MHC class I molecule required for CD8⁺ T cell-mediated cyst removal, and that CD8⁺Vβ8.1, 8.2⁺ immune T cells recognize the GRA6Nt presented by the H-2L^d molecule. Therefore, the recognition of GRA6Nt by CD8⁺Vβ8.1, 8.2⁺ immune T cells through antigen presentation by the H-2L^d molecules most likely plays a crucial role in the protective immunity against *T. gondii* cysts and the genetic resistance of BALB/c mice against the infection. However, these results do not exclude a possibility that CD8⁺ T cells bearing the other TCR Vβ chains participate in the anti-cyst protective immune responses in a lesser extent by recognizing *T. gondii* antigens other than GRA6Nt through the H-2L^d or the other MHC class I molecules. GRA6Ct might be one of those antigens, since it stimulated cytotoxic activity of CD8⁺Vβ8.1, 8.2⁺ immune T cells. However, its stimulatory activity was significantly lower than that of GRA6Nt. Importantly, the previously identified epitope (HF10) located within GRA6Ct, which activates IFN-γ-mediated resistance against acute infection, did not stimulate secretion of either perforin or granzyme B from CD8⁺Vβ8.1, 8.2⁺ immune T cells. Therefore, even if GRA6Ct is an additional, but less effective, target of CD8⁺Vβ8.1, 8.2⁺ immune T cells to recognize cyst-containing cells, the anti-cyst cytotoxic T cells recognize a novel epitope(s) other than HF10 located in the C-terminal region of GRA6.

The majority of *T. gondii* isolates from infected people in North America and Europe are one of three genotypes, types I, II, and III (32); type II is predominant and type III is also common in the isolates from immunocompromised individuals (13, 14). Amino acid sequence of GRA6Nt (amino acids 41 to 152 of GRA6) is 96–98% conserved among the three major genotypes I, II, and III of *T. gondii* (Supplemental Fig. 2). Our recent study showed that CD8⁺ immune T cells remove *T. gondii* cysts mostly by recognizing epitopes commonly expressed in type II and III strains or cross-reactive between these two genotypes (19). Thus, the highly conserved amino acid sequence of GRA6Nt further support the possibility that GRA6Nt is a major target of CD8⁺ T cells for their anti-cyst protective activity. The conserved sequence of GRA6Nt among the three genotypes also suggest a high probability that CD8⁺ T cells recognizing GRA6Nt are able to remove cysts of all of these three genotypes of the parasite.

Although the drugs that suppress proliferation of tachyzoites are available, these drugs are not able to prevent an establishment of chronic infection in the individuals that acquired an infection with *T. gondii*. Up to one third of human population worldwide is estimated to be chronically infected with this parasite as mentioned earlier. By considering the fact that there

is currently no drug or any other treatment effective to the cyst stage of *T. gondii*, an immunization of chronically infected hosts with GRA6Nt or the full length GRA6 molecule containing both GRA6Nt and GRA6Ct to activate CD8⁺ T cells capable of removing the persistent cysts appears to be a valuable approach to begin combatting the globally spread chronic infection with the parasite. In addition, an immunization of uninfected individuals to induce GRA6Nt-primed CD8⁺ T cells would be an attractive approach to prevent an establishment of chronic infection by *T. gondii* through the protective activity of the T cells to remove newly formed cysts when these individuals acquire an infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

GRA	Dense granule protein
GRA6Nt	the amino-terminal region (amino acids 41 to 152) of GRA6
GRA6Ct	the carboxy-terminal region (amino acid 174-224) of GRA6
dm2	BALB/c-H-2 ^{dm2}
TLA	total lysate antigens of tachyzoites
rGRAs	recombinant dense granule proteins

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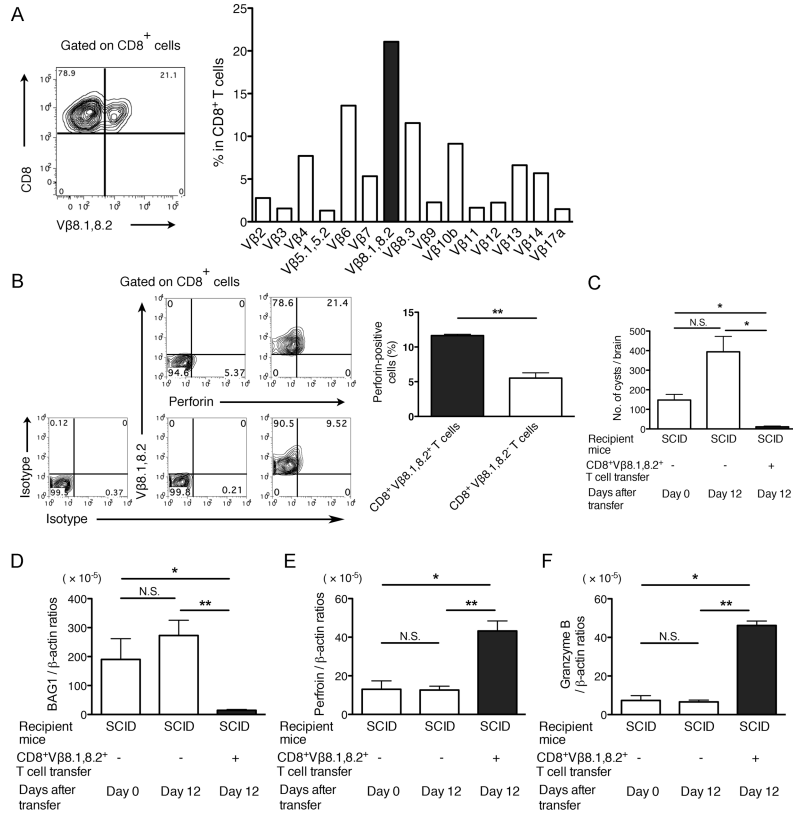
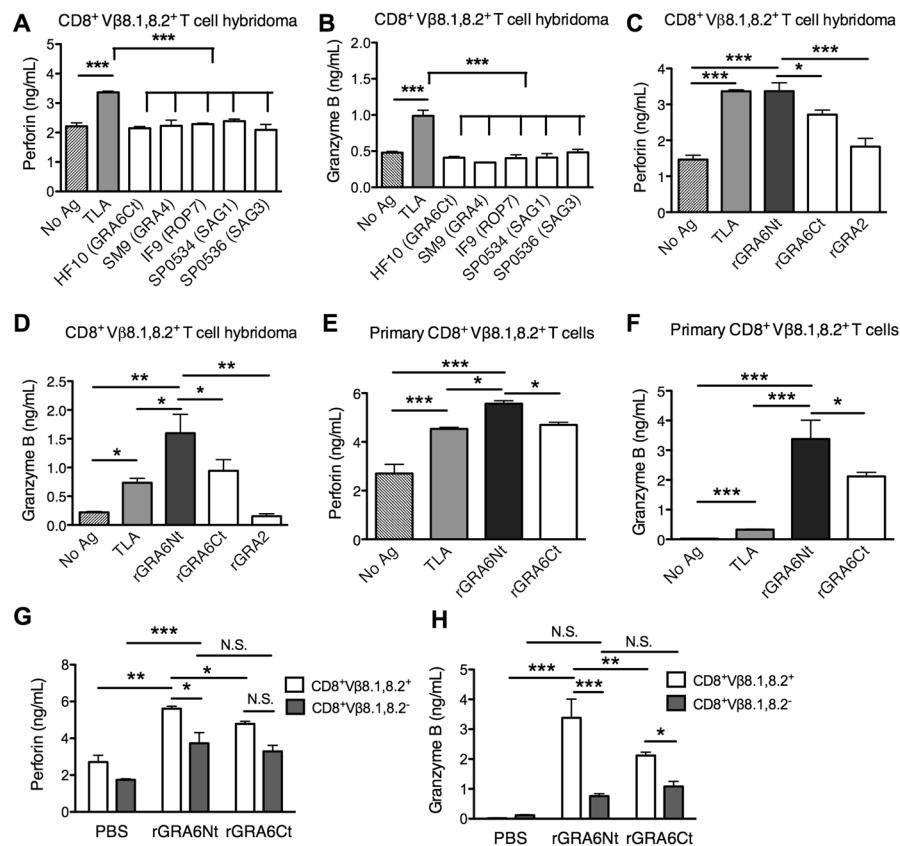


FIGURE 1.

CD8⁺ T cells bearing TCR Vβ8.1, 8.2 chain are most abundant in the brains of *T. gondii*-infected SCID mice following a systemic transfer of CD8⁺ T cells, and this T cell population is capable of removing *T. gondii* cysts from the brains of the recipient SCID mice. (A) SCID mice were infected and treated with sulfadiazine beginning at 10 days after infection to establish a chronic infection by forming cysts in their brains. The animals received CD8⁺ immune T cells (6.7×10^6 cells) purified from the spleens of chronically infected BALB/c mice intravenously at 3 weeks after infection. Seven days later, TCR Vβ usage of the T cells that infiltrated into their brains were examined by flow cytometry. (B) Frequencies of perforin-expressing cells in each of CD8⁺Vβ8.1, 8.2⁺ T cell population and a total population of CD8⁺Vβ8.1, 8.2⁻ T cells. ***P*<0.01 by Student's *t* test. (C–F) Infected and sulfadiazine-treated SCID mice received CD8⁺Vβ8.1, 8.2⁺ immune T cells (0.9×10^6 cells) at 3 weeks after infection. (C) Numbers of *T. gondii* cysts, (D) amounts of mRNA for bradyzoite-specific BAG1, (E) amounts of mRNA for perforin, and (F) amounts of mRNA for granzyme B in their brains were examined on the day (Day 0) and 12 days after the cell transfer (Day 12) (*n*=4 or 6). Data represent the mean ± SEM. (C) **P*<0.05 between the controls without the T cell transfer (Day 0) vs. the experimental group with the T cell transfer (Day 12), and between the controls without the T cell transfer (Day 12) vs. the experimental group with the T cell transfer (Day 12) by Mann-Whitney test. (D–F), **P*<0.05 and ***P*<0.01 by Mann-Whitney test.

**FIGURE 2.**

CD8⁺Vβ8.1, 8.2⁺ immune T cells secrete perforin and granzyme B by recognizing rGRA6Nt of *T. gondii*. (**A** and **C**) Perforin and (**B** and **D**) granzyme B levels of the culture supernatants of CD8⁺Vβ8.1, 8.2⁺ T hybridoma (5×10^4 cells/well) following stimulation with (**A** and **B**) previously identified epitopes of *T. gondii* antigens (epitope HPGSVNEFDF [HF10] of GRA6, epitope SPMNGGYM [SM9] of GRA4, epitope IPAAAGRFF [IF9] of ROP7, epitope TPTENHFHL [SP0534] of SAG1 and epitope YPESGPVNL [SP0536] of SAG3 at 1 μg/ml) (22–24) (n=3), or (**C** and **D**) rGRA6Nt, rGRA6Ct, rGRA2 at 1 μg/ml, or a total lysate antigens of tachyzoites (TLA) at 20 μg/ml in the presence of antigen-presenting cells (APC) from uninfected wild-type BALB/c mice (n=3). (**E**) Perforin and (**F**) granzyme B levels of the culture supernatants of the primary CD8⁺Vβ8.1, 8.2⁺ immune T cells (1×10^5 cells/well) purified from the spleens of infected BALB/c mice following stimulation with rGRA6Nt, rGRA6Ct (1 μg/ml), or TLA (20 μg/ml) in the presence of antigen-presenting cells (APC) from uninfected wild-type BALB/c mice (n=3 except for rGRA6Ct [n=2]). (**G**) Perforin and (**H**) granzyme B levels of the culture supernatants of the primary CD8⁺Vβ8.1, 8.2⁺ immune T cells and a total population of CD8⁺Vβ8.1, 8.2⁻ T cells (1×10^5 cells/well) purified from the spleens of infected BALB/c mice following stimulation with rGRA6Nt (n=3). Data represent the mean ± SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001 by one-way ANOVA with Newman-Keuls Post test. In Fig. 2F, granzyme B levels in the culture with TLA were low when compared to those in the culture with rGRA6Nt but they

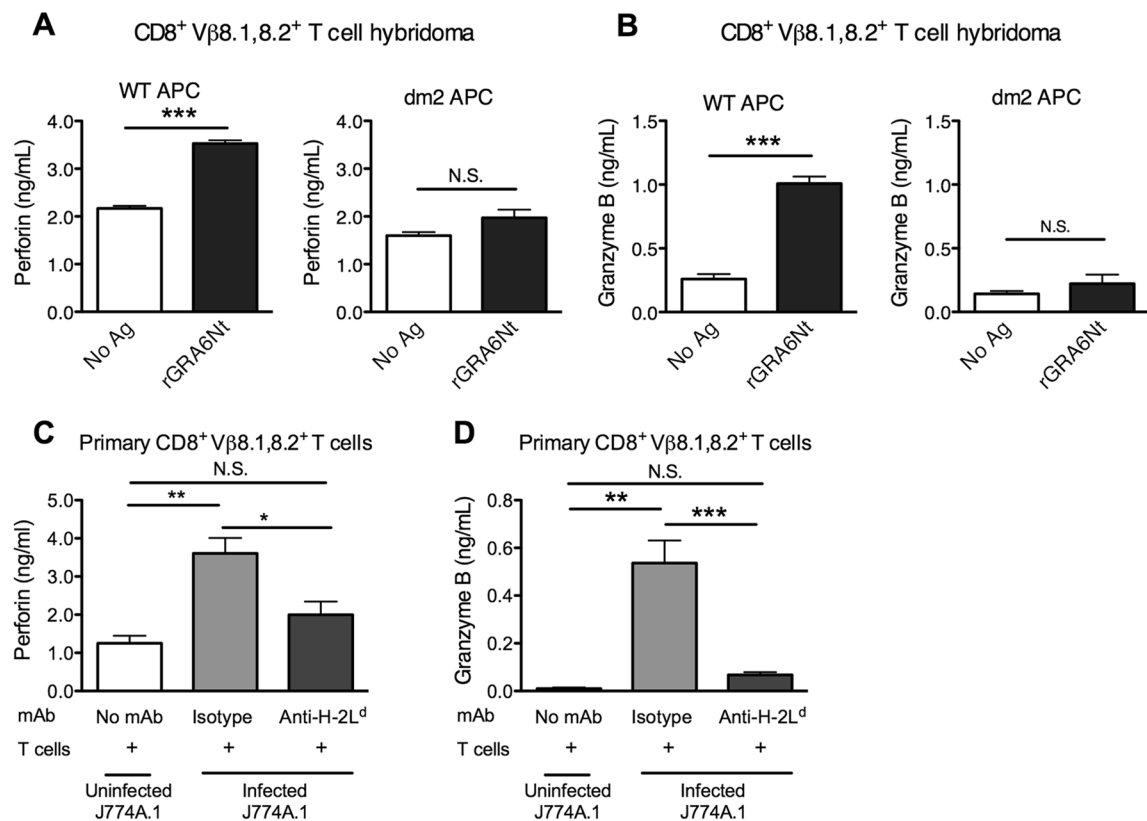
were 14.5 times greater than those in the control culture with PBS ($P < 0.001$ by Student's t test).

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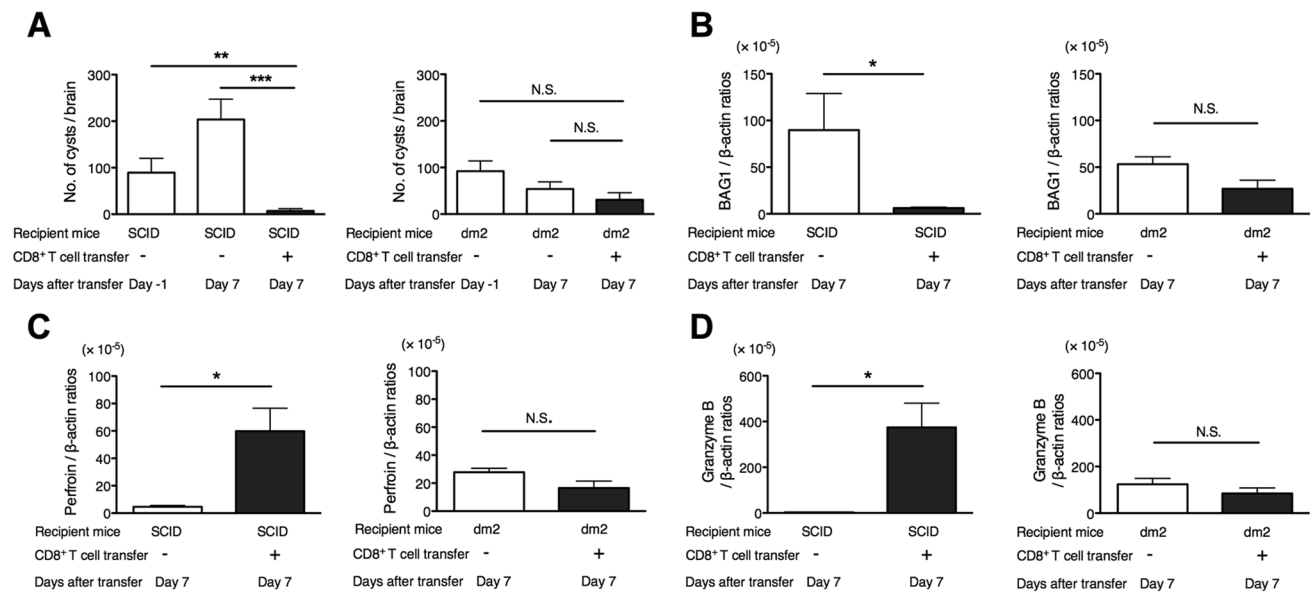
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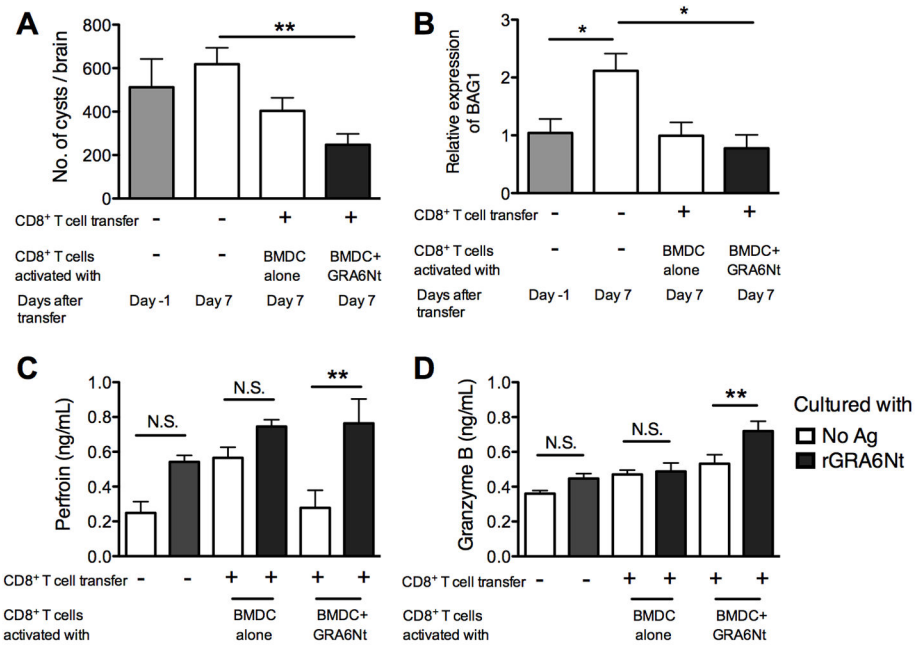
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**FIGURE 3.**

The H-2L^d molecule presents *T. gondii* antigens including rGRA6Nt to CD8⁺Vβ8.1, 8.2⁺ immune T cells. **(A)** Perforin and **(B)** granzyme B levels in the culture supernatants of the CD8⁺Vβ8.1, 8.2⁺ T hybridoma (5×10^4 cells/well) following stimulation with rGRA6Nt (1 μg/ml) in the presence of antigen-presenting cells (APC) from uninfected wild-type BALB/c or dm2 mice (n=3). **(C)** Perforin and **(D)** granzyme B levels in the culture supernatants of the primary CD8⁺Vβ8.1, 8.2⁺ T cells (1×10^5 cells/well) purified from the spleens of infected BALB/c mice following stimulation with tachyzoite-infected J774 macrophage cell line in the presence of anti-H-2L^d mAb (25 μg/ml) or isotype control Ab (n=3). Data represent the mean ± SEM. **(A)** *** $P < 0.0001$ by Student's *t* test. **(B)** *** $P = 0.0004$ by Student's *t* test. **(C and D)** * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by one-way ANOVA with Newman-Keuls Post test.

**FIGURE 4.**

The H-2L^d molecule presents *T. gondii* antigens to CD8⁺ immune T cells for removing cysts of the parasite from the brain. SCID and dm2 mice were infected and treated with sulfadiazine beginning at 7 or 10 days after infection to establish a chronic infection by forming cysts in their brains. The animals received CD8⁺ immune T cells purified from the spleens of chronically infected BALB/c mice intravenously at 3 weeks after infection. (A) Numbers of *T. gondii* cysts, (B) amounts of mRNA for bradyzoite-specific BAG1, (C) amounts of mRNA for perforin, and (D) amounts of mRNA for granzyme B in their brains were examined on the day (Day 0) and 7 days after the cell transfer (Day 7). Data represent the mean \pm SEM. (A) The results from two independent experiments with a transfer of 4.2×10^6 or 5.7×10^6 CD8⁺ immune T cells are combined (n=7–10). ** $P < 0.01$ and *** $P < 0.001$ by Mann-Whitney test. (B–D) The results from one of the two experiments are shown (n=4). * $P < 0.05$ by Mann-Whitney test.

**FIGURE 5.**

CD8⁺ immune T cells induced by immunization with rGRA6Nt-primed BM-DC remove *T. gondii* cysts from the brains of infected SCID mice. SCID mice were infected and treated with sulfadiazine beginning at 10 days after infection to establish a chronic infection by forming cysts in their brains. The animals received CD8⁺ immune T cells from uninfected BALB/c mice, which had received an immunization with BM-DC pre-cultured with and without rGRA6Nt, at 3 weeks after infection. (A) Numbers of *T. gondii* cysts and (B) amounts of mRNA for bradyzoite-specific BAG1 in their brains were examined at one day before (Day -1) and 7 days after (Day 7) the cell transfer. The data from two independent experiments with a transfer of CD8⁺ immune T cells ($2.7\text{--}3.3 \times 10^6$) are combined ($n=8\text{--}10$). (C) Perforin and (D) granzyme B levels of the culture supernatants of the spleen cells of the recipient SCID mice at 7 days after receiving the CD8⁺ immune T cells. There were 4–5 mice in each experimental group, and the culture was performed using pooled cells for each group (5 wells for each group). Data represent the mean \pm SEM. (A) $**P<0.01$ by Mann-Whitney test. (B) $*P<0.05$ between the control group without the T cell transfer (Day 7) and the experimental groups with the T cell transfer from donors immunized with GRA6Nt-primed BM-Dc (Day 7), and between the control groups without the T cell transfer on Day -1 and Day 7 by Mann-Whitney test. (C and D) $**P<0.01$ by one-way ANOVA with Newman-Keuls Post test.