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# A GRA1 DNA Vaccine Primes Cytolytic CD8$^+$ T Cells To Control Acute *Toxoplasma gondii* Infection

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**Protective immunity against *Toxoplasma gondii* is known to be mediated mainly by T lymphocytes and gamma interferon (IFN-γ). The contribution of CD4$^+$ and CD8$^+$ T-lymphocyte subsets to protective immune responses against *T. gondii* infection, triggered by a GRA1 (p24) DNA vaccine, was assessed in this study. In vitro T-cell depletion experiments indicated that both CD4$^+$ and CD8$^+$ T-cell subsets produced IFN-γ upon restimulation with a *T. gondii* lysate. In addition, the GRA1 DNA vaccine elicited CD8$^+$ T cells that were shown to have cytolytic activity against parasite-infected target cells and a GRA1-transfected cell line. C3H mice immunized with the GRA1 DNA vaccine showed 75 to 100% protection, while 0 to 25% of the mice immunized with the empty control vector survived challenge with *T. gondii* cysts. In vivo T-cell depletion experiments indicated that CD8$^+$ T cells were essential for the survival of GRA1-vaccinated C3H mice during the acute phase of *T. gondii* infection, while depletion of CD4$^+$ T cells led to an increase in brain cyst burden during the chronic phase of infection.**

In immunocompetent individuals, *Toxoplasma gondii* generally induces a mild asymptomatic infection that is associated with the rapidly dividing tachyzoite form of the parasite. Resolution of the infection in the host occurs through induction of strong and persistent cell-mediated immunity that results in the control of *T. gondii* tachyzoites (11, 46). In humans, this relatively benign infection may reactivate under conditions of immunosuppression, resulting in toxoplasma encephalitis and other complications (11). A primary *T. gondii* infection and subsequent transplacental transmission during pregnancy can result in miscarriage or in severe disease in the infant (26). These pathological consequences associated with congenital toxoplasmosis not only represent a threat to humans but also are a cause of economic losses due to abortions in farm animals (17). Therefore, a vaccine capable of controlling the tachyzoite multiplication associated with the acute primary infection is important and has been the subject of study in our laboratory.

Reports on DNA vaccination against experimental *T. gondii* infection in mice have been accumulating, and the antigens that have been tested now include *T. gondii* membrane-associated surface antigen SAG1 (1, 33), excreted-secreted dense granule proteins GRA1 (43), GRA4 (16), and GRA7 (43), and rhoptry proteins ROP1 (23) and ROP2 (30, 43). For all these antigens, immunity was associated with Th1-type responses, which are characterized by production of gamma interferon (IFN-γ). It was reported previously that DNA vaccination with three distinct immunogenic *Toxoplasma* antigens (GRA1, GRA7, and ROP2) induced partial immunity in C3H/HeN mice and that the responses were associated with a Th1-type profile (43). The GRA1 antigen (p24), a product of *T. gondii* tachyzoites and bradyzoites, is a promising vaccine candidate (3, 7, 18, 43). This antigen induces humoral and cellular immune responses in mice and humans in the chronic phase of the infection (7, 19). Vaccination with GRA1 has been shown to be protective in two animal models of infection (18, 38). Adoptive transfer of T cells from rats vaccinated with GRA1-expressing vaccinia virus partially protected nude rats against lethal challenge with the virulent *T. gondii* RH strain (18). In addition, immunization of sheep with recombinant *Mycobacterium bovis* BCG producing and secreting GRA1 resulted in specific, partially protective cellular immune responses characterized by the production of IFN-γ (38).

It is well established that IFN-γ and T cells play a central role in host resistance to *T. gondii* during both the acute and chronic phases of infection (11, 46). However, the concerted interplay of several other cytokines may be necessary to maintain the delicate balance between protection and immunopathology caused by excessive inflammation (22, 32, 35). Another crucial interaction for protection against a *T. gondii* infection is the one between CD8$^+$ and CD4$^+$ T cells (21). Although some reports have suggested that CD8$^+$ T cells mediate their effect through IFN-γ production (5, 14, 27), CD8$^+$ T cells with cytotoxic activity against infected cells have been described in humans (9, 31, 34, 45) and mice (8, 12, 25, 27, 37).

In the present study we compared the contributions of

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$CD4^+$ and $CD8^+$ T cells in the protection conferred by the pVR1020-GRA1 DNA vaccine. Both $CD4^+$ and $CD8^+$ T cells produced IFN-γ, and the $CD8^+$ T-cell subset had *T. gondii*-specific cytolytic activity. Our results suggest that although $CD4^+$ T cells are the strongest producers of IFN-γ, $CD8^+$ T cells are the major effectors of the vaccine-induced protection against acute toxoplasmosis.

## MATERIALS AND METHODS

**Plasmid construction.** The DNA construct used for vaccination was based on the plasmid vector VR1020, obtained from Vical, Inc. (San Diego, Calif.). The gene encoding GRA1 was amplified by PCR from cloned DNA fragments and inserted into pVR1020 as described previously (43). Briefly, sense and antisense primers were designed to contain a *Bam*HI restriction site. The gene was cloned into the *Bam*HI site of the expression vector pVR1020 to generate an in-frame fusion with the vector-encoded signal sequence of human tissue plasminogen activator. All plasmids were propagated in *Escherichia coli* DH1. DNA for vaccination was purified by anion-exchange chromatography (EndoFree plasmid giga kits; Qiagen GmBH, Hilden, Germany) and was dissolved in sterile endotoxin-free phosphate-buffered saline (PBS) (BioWhittaker Europe, Verviers, Belgium). Plasmid integrity was checked by agarose gel electrophoresis after digestion with appropriate restriction enzymes. The DNA concentration was determined by absorbance at 260 nm.

**Vaccination of experimental animals.** Female inbred C3H/HeN mice ($H\text{-}2^k$) that were 6 to 8 weeks old were purchased from Harlan (Horst, The Netherlands). The C3H/HeN mouse model is relevant for investigation of the induction of protective anti-*T. gondii* immune responses because the mice are moderately resistant to acute infection and develop *T. gondii* brain cysts and progressive toxoplasma encephalitis (39). These features allow workers to monitor the acute phase as well as the chronic phase of infection in response to vaccination. The mice received three injections of 100 μg of pVR1020-GRA1 DNA (separated by 2-week intervals) in both tibialis anterior muscles administered with a 0.3-ml syringe (BD Biosciences, San Diego, Calif.). Mice injected with the empty vector pVR1020 were used as negative controls. Three weeks after the third DNA injection, the vaccinated mice were bled from the tail vein, and sera were analyzed with a GRA1-specific enzyme-linked immunosorbent assay (ELISA) as described previously (43). All mice vaccinated with pVR1020-GRA1 were shown to have GRA1-specific antibodies after the third injection. This study was conducted in compliance with the regulations concerning the use of laboratory animals at the Pasteur Institute, Brussels, Belgium.

**Parasite and antigen preparation.** The *T. gondii* type II IPB-G and IPB-M strains (43) were isolated from the placentas of women who gave birth to infants with congenital toxoplasmosis. Tachyzoites of the IPB-M and IPB-G strains were obtained from the ascites of C3H mice infected by intraperitoneal injection and subcutaneously treated with hydrocortisone acetate (25 μg; Laboratoires Roussel, Paris, France) at 2-day intervals for 1 week. Expression of GRA1 in both strains was confirmed by Western blotting.

To prepare toxoplasma lysate (TLA), tachyzoites from the virulent *T. gondii* RH strain were obtained from the peritoneal fluid of infected Swiss mice as described previously (43). The material was passed twice through a 26-gauge needle. The parasites were washed, resuspended in PBS, and sonicated (1-min burst, 1 min of cooling, 150 W) with an Ultrasonic disintegrator (MSE, Leicester, United Kingdom). The protein concentration of TLA was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, Calif.).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (28) by using a 12% polyacrylamide gel and the Bio-Rad minigel system (Bio-Rad Laboratories). The BenchMark prestained protein ladder (Life Technologies, Grand Island, N.Y.) was used for molecular weight standards. Electrophoretic transfer onto nitrocellulose membranes (Hybond-C; Amersham Biosciences, Uppsala, Sweden) was done with a mini Trans-Blot electrophoretic cell system (Bio-Rad Laboratories) as instructed by the manufacturer. The membrane was blocked by incubation with 3% bovine serum albumin (Merck, Darmstadt, Germany) in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. For detection of recombinant GRA1 in BW-Sp3 transfectants, a polyclonal pool of sera from five *T. gondii*-infected C3H mice, diluted 1:200, was used as the primary antibody; it was incubated overnight at 4°C and subsequently with a peroxidase-labeled rat anti-mouse immunoglobulin G (IgG) (Amersham Biosciences) as the secondary antibody for 1 h at room temperature. The chemiluminescent compound Supersignal (Pierce, Rockford, Ill.) was used as the substrate according to the manufacturer's instructions. For detection of native GRA1 in *T. gondii* IPB-M- and IPB-G-derived TLA, a monoclonal antibody (MAb) against GRA1, MAb BATO 35 (36), was used at a dilution of 1:1,000, and pools of sera from three seropositive pVR1020-GRA1-vaccinated C3H mice or pVR1020-vaccinated C3H mice, diluted 1:300, were also used. These primary antibodies were incubated overnight at 4°C with the membrane strips. A peroxidase-labeled rat anti-mouse IgG (Sigma, St. Louis, Mo.) was used as the secondary antibody for 1 h at room temperature. The chromogenic reaction was performed with 4-chloro-naphthol substrate tablets (Sigma) according to the manufacturer's instructions. The reaction was stopped by washing the preparations in water.

**In vitro spleen cell cultures.** Animals were sacrificed 2 months after the third DNA injection. Single-cell suspensions of splenocytes were prepared, and red blood cells were lysed with RBC Lysing buffer (Sigma). The cell suspension recovered was washed in RPMI 1640 (GIBCO, Life Technologies, Paisley, United Kingdom) and plated on RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine 1640 (GIBCO, Life Technologies), 0.05 mM 2-mercaptoethanol (Sigma), 1× nonessential amino acids (GIBCO, Life Technologies), 1 mM sodium pyruvate (GIBCO, Life Technologies), and 100 U of penicillin-streptomycin (Life Technologies) per ml. Splenocytes ($3 \times 10^6$ cells/ml) were stimulated with TLA (25 μg/ml) and cultured for 4 days in 24-well plates (Nunc, Roskilde, Denmark). The optimal TLA concentration (25 μg/ml) and the optimal time of culture were determined previously on the basis of a kinetics experiment (data not shown).

**In vitro depletion of $CD4^+$ and $CD8^+$ T cells and IFN-γ measurement.** For in vitro and in vivo depletion experiments, culture supernatants of the anti-mouse CD4-producing GK1.5 hybridoma (American Type Culture Collection) and the anti-mouse CD-8α-producing H35-17.2 clone (kindly provided by Anja Geldhof, Free University of Brussels) were obtained and concentrated by passage through protein G-Sepharose columns (Amersham Biosciences) by using an Econopump and a UV monitor (Bio-Rad Laboratories). The concentrates were exhaustively dialyzed against PBS, and the protein content was measured by the Bio-Rad DC protein assay (Bio-Rad Laboratories). The MAbs were divided into aliquots and stored at −80°C until they were used. Selective in vitro depletion of $CD4^+$ or $CD8^+$ T cells was achieved by incubating splenocytes ($2 \times 10^7$ cells) either with 20 μg of the anti-mouse CD4 MAb per ml or with 20 μg of the anti-mouse CD8α MAb per ml at 4°C for 30 min. The cells were washed, the cell concentration was adjusted to the original concentration in medium containing 5% FCS, and each preparation was incubated by using a ratio of sheep anti-rat IgG M-450 dynabeads (Dynal Biotech, Oslo, Norway) to cells of 4:1 for 1 h at 37°C with constant rotation. $CD4^+$ and/or $CD8^+$ T cells were removed by magnetic sorting. Finally, the cells were washed twice and resuspended at a concentration of $3 \times 10^{54}$ cells/ml in complete medium (RPMI 1640 containing 2 mM glutamine, 1× nonessential amino acids [GIBCO, Life Technologies], 1 mM sodium pyruvate [GIBCO, Life Technologies], 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM 2-mercaptoethanol). The efficiency of depletion of each T-cell subset was monitored by cytofluorometric analysis before and after depletion, as well as after the 4 days of in vitro stimulation with TLA, and was found to be at least 90% for each T-cell subset. Both T-cell subsets remained at these levels on day 4 of the experiment (data not shown), when the supernatants were harvested. The IFN-γ content was measured by ELISA, as previously described (43).

**Generation of BW-Sp3(GRA1) for CTL assays.** In order to obtain target cells expressing GRA1 in an $H\text{-}2^k$ context, expression vector pcDNA3.1 (Invitrogen, Life Technologies) containing the GRA1 gene was transfected into BW-Sp3, a subline of the BW5147T lymphoma cell line of AKR mice (42). BW-Sp3 was obtained by in vivo selection and has higher levels of expression of both $K^k$ and $D^k$ antigens than the parental line. The full-length GRA1 fragment was recovered from the pVR1020-GRA1 construct by restriction with *Sal*I and *Bgl*II and was ligated into plasmid pcDNA3.1 digested with *Bam*HI and *Xho*I. The resulting construct was electroporated into BW-Sp3 cells, and positive clones were selected by using neomycin. Expression of the GRA1 gene was checked by reverse transcription (RT)-PCR by using a sense primer (AGATGATGGGGA ACACGTATCG) starting at position 185 from the ATG codon and an antisense primer (AGGAACCCAATGTCATCC) ending at position 555 from the ATG codon. Thus, the PCR product contained a 370-bp fragment from the carboxy-terminal end of the mature GRA1 gene. GRA1 expression of a PCR-positive clone was confirmed at the protein level by Western blotting. Clone BW-GRA1 was used as the target for cytotoxic T-lymphocyte (CTL) assays.

**Preparation of bone marrow macrophages as CTL targets.** Bone marrow was obtained from the femurs and tibias of 8- to 12 week-old female C3H mice in Hanks balanced salt solution (GIBCO, Life Technologies) supplemented with 10 mM HEPES and 100 U of penicillin-streptomycin (GIBCO, Life Technologies) per ml. Cells were recovered, washed, and plated on 100-mm tissue culture petri

dishes at a concentration of $10 \times 10^6$ cells to $15 \times 10^6$ cells/10 ml in each plate (Falcon, Becton Dickinson, San Diego, Calif.). The culture medium consisted of Dulbecco modified Eagle medium (GIBCO, Life Technologies) supplemented with 10% FCS, sodium pyruvate, and penicillin-streptomycin, as well as 30% supernatant from confluent cultures of L929 fibroblasts (grown in 45% RPMI 1640–45% Dulbecco modified Eagle medium–10% FCS) as a source of macrophage colony-stimulating factor. After 6 days of incubation at 37°C in the presence of 5% $CO_2$, the plates were gently washed to remove nonadherent cells. The plates each contained $3 \times 10^6$ to $5 \times 10^6$ macrophages that were >95% pure. Adherent macrophages were infected overnight with RH tachyzoites attenuated by exposure to UV light. The dose and efficiency of attenuation were determined previously (data not shown). Two hundred microcuries of $Na_2{}^{51}CrO_4$ was simultaneously added to each plate of macrophages. The following day, extracellular tachyzoites, as well as unincorporated radioactivity, were removed by four gentle washes with RPMI 1640. The adherent macrophages were detached with a rubber policeman, washed, counted, and used as targets in a Cr release assay. Noninfected macrophages were included as a control.

**Generation of effector cells for CTL assays.** To generate effector cells for CTL assays, two different experiments were performed. In the first experiment, suspensions containing $6 \times 10^6$ splenocytes from pVR1020-GRA1- and pVR1020-vaccinated mice per 2 ml were stimulated in vitro with irradiated BW-Sp3(GRA1) cells (7,000 rads; ratio of effectors to stimulators, 50:1) for 6 days. The cells were harvested, layered over a Ficoll-Paque gradient (Amersham Biosciences), and centrifuged at $300 \times g$ for 25 min. The cells in the interface were collected, washed twice, and used as effectors in a standard $^{51}Cr$ release assay with BW-Sp3(GRA1) cells ($1 \times 10^5$ cells/ml) as the target cells. In the second experiment, splenocytes from pVR1020-GRA1- and pVR1020-vaccinated mice were stimulated with live tachyzoites from the *T. gondii* IPB-M strain at a multiplicity of infection of 1:10 (ratio of parasites to splenic cells). The *T. gondii* IPB-M strain is characterized by its mild virulence in mice and slow growth in monolayers of Vero cells (unpublished data). Following 6 days of in vitro culture, the viable splenic T cells were used as effectors and RH-infected syngeneic bone marrow macrophages were used as targets in a standard $^{51}Cr$ release assay. In order to confirm the identity of the CTL population, immediately before use in cytotoxicity assays $CD8^+$ T cells were depleted in vitro by magnetic sorting as described above, and the remaining cells were used as effectors.

**In vivo depletion of $CD4^+$ and $CD8^+$ cells.** Mice received 0.5 mg of anti-mouse CD4 depleting MAb GK1.5 or anti-mouse CD8α depleting MAb H35.17.2 intraperitoneally (i.p.) 1 day prior to i.p. challenge with 40 cysts of the IPB-G strain and then 0.25 mg every 6 days for 3 weeks. Depletion had a long-lasting effect (at least 7 days) for a dose of 0.25 mg (data not shown). There was a period of at least 3 weeks between the last DNA vaccination and the onset of the in vivo depletion. Survival of undepleted mice was evaluated in parallel. In a previous study (43), peroral challenge studies were performed with vaccinated C3H mice. In further studies, we did not observe any differences in the susceptibilities of vaccinated C3H mice challenged by the oral and i.p. routes. As the i.p. route allows better control over the dose of cysts administered, this challenge route was used in the experiments. The experiments were repeated twice with experimental groups consisting of four mice. The extent of in vivo depletion was monitored prior to challenge by cytofluorometric analysis of blood samples. In earlier depletion experiments fluorescence-activated cell sorting (FACS) analysis showed that depletion of blood lymphocytes was highly correlated with the depletion of splenic lymphocytes (unpublished data).

**Cytofluorometric analysis.** For intracellular IFN-γ staining of effector $CD8^+$ T cells amplified for the CTL assays, Golgi Plug (BD Biosciences Pharmingen, San Diego, Calif.) was added to cultures of stimulated splenocytes. Five hours later, the cells were harvested and washed twice in FACS wash buffer (5% FCS in PBS). A total of $10^6$ cells for each condition were incubated with anti-CD8 MAb (clone 53.6.7; BD Biosciences) for 30 min. on ice. The cells were washed and fixed with 4% paraformaldehyde for 20 min at 4°C, washed twice with FACS wash buffer containing 0.1% saponin (Sigma) to permeabilize the cell membrane, and incubated with R-phycoerythrin (PE)-labeled anti-IFN-γ MAb (clone XMG1.2; BD Biosciences) for 30 min at 4°C. Prior to cytofluorometric analysis, the lymphocytes were washed and resuspended in 300 μl of PBS.

For ex vivo determination of $CD4^+$ and $CD8^+$ T-cell populations 4 days after the last in vivo $CD4^+$ T-cell depletion, whole blood was collected in 2% EDTA to prevent coagulation. Red blood cells were lysed by incubation with ammonium chloride lysis buffer (150 mM $NH_4Cl$, 10 mM $NaHCO_3$, 0.4% EDTA). Blood lymphocytes were washed twice in FACS wash buffer and directly labeled with anti-CD4 fluorescein isothiocyanate (FITC) (clone RM4-4) or anti-CD8 FITC (clone 53.6.7) and anti-CD3 PE (clone 145-2C11) MAbs (BD Biosciences).

Background fluorescence was measured by using FITC- or PE-labeled isotype control antibodies (BD Biosciences). Cytofluorometric analysis was performed with a FACScalibur cytofluorometer (Becton Dickinson). Calculations were performed on the basis of an appropriate lymphocyte gate for $5 \times 10^4$ counts.

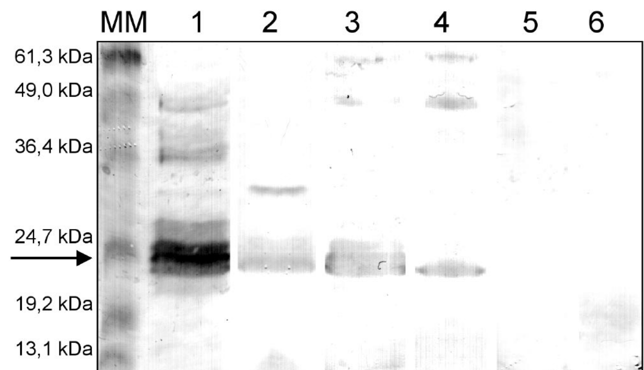


FIG. 1. Presence of GRA1 in total lysates of *T. gondii* strains IPB-G and IPB-M confirmed by detection with a MAb against GRA1 (MAb BATO 35). In addition, serum from pVR1020-GRA1-vaccinated C3H mice was shown to recognize GRA1 in these lysates, whereas serum from C3H mice vaccinated with the empty vector did not. Lanes 1, 3, and 5, IPB-G TLA; lanes 2, 4, and 6, IPB-M TLA. GRA1 was detected with MAb BATO 35 (lanes 1 and 2), serum from pVR1020-GRA1-vaccinated mice (lanes 3 and 4), and serum from control pVR1020-vaccinated mice (lanes 5 and 6).

**Enumeration of *T. gondii* cysts in mouse brains.** Mice challenged with *T. gondii* parasites were sacrificed 6 weeks after infection, and their brains were homogenized in 2 ml of PBS. Four samples of the suspension were counted by using a phase-contrast microscope at a magnification of ×40.

**Statistical analysis.** For statistical evaluation of data obtained from IFN-γ production and brain cyst counting analysis, the data from pVR1020-GRA1-vaccinated mice were compared to control data by using a two-sided Student *t* test. The survival curves for vaccinated mice were compared to those for controls by the Mantel-Haenszel test. Statistical analysis and graphics were carried out by using the Prism 3 software (GraphPad, San Diego, Calif.).

## RESULTS

**GRA1 expression in the *T. gondii* IPB-G and IPB-M strains.** To confirm that GRA1 is expressed in the IPB-G and IPB-M strains, TLA was prepared from tachyzoites isolated from ascites fluid obtained after cortisone treatment of IPB-G- and IPB-M-infected mice and analyzed by Western blotting. In both strain IPB-G and strain IPB-M, the 23-kDa GRA1 antigen could be detected with the anti-GRA1 MAb BATO 35 and serum from pVR1020-GRA1-vaccinated mice (Fig. 1).

**Production of IFN-γ by $CD4^+$ and $CD8^+$ T cells from mice vaccinated with GRA1 DNA.** In order to assess the relative contribution to the production of IFN-γ by the $CD4^+$ or $CD8^+$ T-cell subset, in vitro depletion experiments were performed. Splenocytes from pVR1020-GRA1-seropositive and pVR1020-vaccinated mice were cultured in the presence of TLA. As reported previously (43), production of IFN-γ by splenocytes from pVR1020-GRA1-vaccinated mice was maximal after 4 days, while IFN-γ production by splenocytes from control pVR1020-vaccinated mice remained at the background level (data not shown). Compared with the results obtained with undepleted splenocyte cultures from pVR1020-GRA1-vaccinated mice, $CD4^+$ T-cell depletion resulted in a more-than-fourfold decrease in IFN-γ production, whereas after depletion of $CD8^+$ T cells IFN-γ production decreased less than twofold (Fig. 2).

**Induction of GRA1-specific cytotoxic T lymphocytes after**

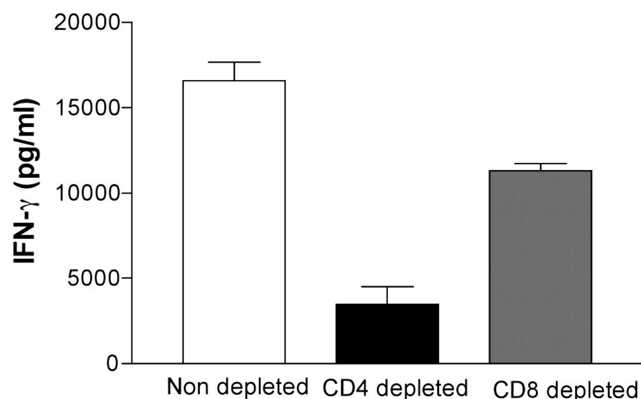


FIG. 2. IFN-γ production in $CD4^+$ and $CD8^+$ T-cell-depleted splenocyte cultures from GRA1 DNA-vaccinated mice. Spleen cells were depleted in vitro with anti-CD4 or anti-CD8 MAb and cultured for 96 h in the presence of TLA. IFN-γ levels in the culture supernatants were measured by ELISA. The data are the averages of three independent depletion experiments. For undepleted mice, the average IFN-γ level reached 16,600 ± 1,852 pg/ml, while the average IFN-γ level dropped after $CD4^+$ and $CD8^+$ T-cell depletion to 3,467 ± 1,815 and 11,300 ± 755 pg/ml, respectively ($P < 0.001$ and $P < 0.02$, respectively).

**vaccination with pVR1020-GRA1.** As our in vitro depletion experiments indicated that $CD8^+$ T cells could be primed by GRA1 DNA vaccination, it was of interest to determine whether these cells had *T. gondii*-specific cytolytic activity.

In a first approach, a GRA1-transfected lymphoma cell line was generated. The presence of GRA1 mRNA and the presence of protein in the BW-Sp3 cell line were verified by RT-PCR (Fig. 3A) and Western blotting with pooled sera from *T. gondii*-infected C3H mice (Fig. 3B), respectively. Splenocytes from pVR1020-GRA1-vaccinated and control pVR1020-vaccinated mice were amplified with irradiated BW-Sp3(GRA1) cells. Radioisotope-labeled BW-Sp3(GRA1) cells were then used as targets in cytotoxic assays. When a ratio of effectors to targets of 60:1 was used, the percentage of specific lysis was more than 70%, and significant lytic activity was observed even at a ratio of 10:1 (Fig. 4A). The magnitude of the lysis exerted by vaccine-induced CTLs was the same as the magnitude of the lysis observed with splenocytes from chronically infected mice (data not shown). The levels of lysis of untransfected control BW-Sp3 cells remained at background levels (data not shown).

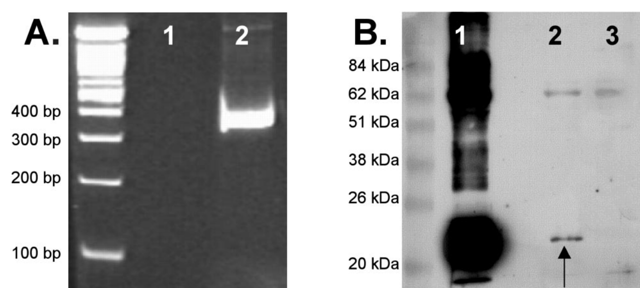


FIG. 3. BW-Sp3(GRA1) transfectants were generated by electroporation of pcDNA3.1 containing the GRA1 gene into BW-Sp3, and the positive clones were selected with neomycin. Expression of the GRA1 gene was further confirmed by RT-PCR (A) and Western blotting (B). The size of the cDNA generated corresponds to the calculated size (370 bp) of the amplified fragment from pCDNA3.1-GRA1, while sera from *T. gondii*-infected mice detected GRA1 corresponding to the known molecular weight. (A) RT-PCR. Lane 1, BW-Sp3; lane 2, BW-Sp3(GRA1). (B) Western blotting. Lane 1, TLA; lane 2, lysate of BW-Sp3(GRA1); lane 3, lysate of BW-Sp3.

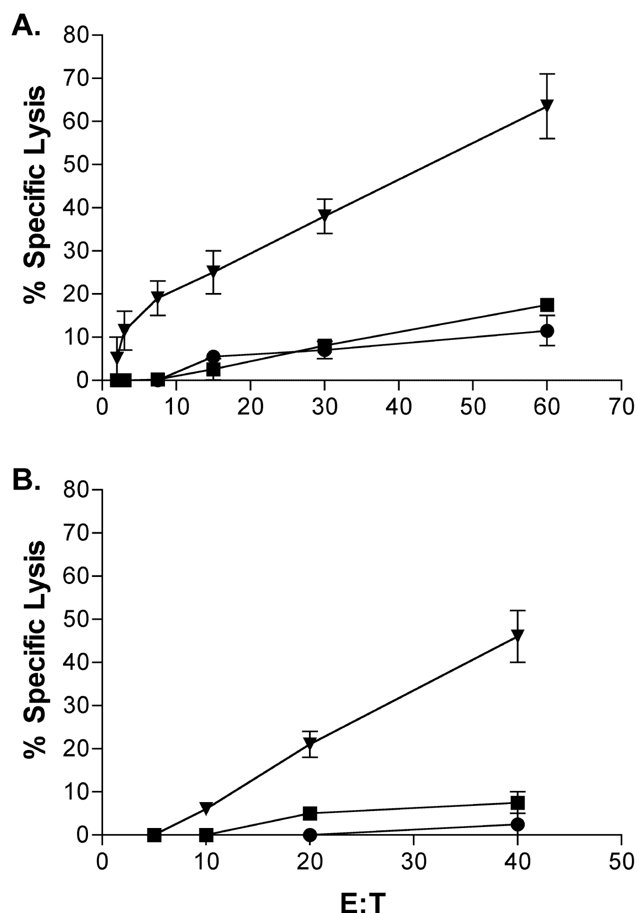


FIG. 4. Vaccination with pVR1020-GRA1 induced $CD8^+$ T cells with specific GRA1 cytolytic activity. (A) Cytotoxic activities of effectors from pVR1020-vaccinated (■), pVR1020-GRA1-vaccinated (▼), and pVR1020-GRA1-vaccinated $CD8^+$ T-cell-depleted (●) C3H mice against BW-Sp3(GRA1) targets. Effector cells were stimulated for 6 days with irradiated BW-Sp3(GRA1) cells. The data are the means from three independent experiments. (B) Cytotoxic activities of effectors from pVR1020-vaccinated (■), pVR1020-GRA1-vaccinated (▼), and pVR1020-GRA1-vaccinated $CD8^+$ T-cell-depleted (●) C3H mice against *T. gondii*-infected syngeneic bone marrow macrophages. Effector cells were stimulated for 6 days with tachyzoites from the *T. gondii* IPB-M strain. The data are the means from three independent experiments. E, effector cells; T, target cells.

Significant CTL activity was also measured by using a second approach. Splenocyte cultures from vaccinated mice were infected with live IPB-M tachyzoites to amplify effector cells, while $^{51}Cr$-labeled syngeneic bone marrow macrophages infected with UV radiation-attenuated RH parasites served as target cells. When a ratio of effectors to targets of 40:1 was used, the percentage of specific lysis was more than 45% (Fig. 4B). The levels of lysis of uninfected bone marrow macrophages remained at background levels (data not shown).

Using both approaches, we demonstrated that vaccination

with pVR1020-GRA1 induced genuine CTLs, which could lyse GRA1-transfected lymphoma cells and *T. gondii*-infected syngeneic bone marrow macrophages. In order to identify the T-cell subset with CTL activity, we also included in vitro CD8$^{+}$ T-cell-depleted splenocytes from pVR1020-GRA1-vaccinated mice. The T-cell-mediated cytotoxicity was completely eliminated after in vitro depletion of CD8$^{+}$ T cells (Fig. 4). No significant IFN-γ production by CD8$^{+}$ T effector cells from pVR1020-GRA1-vaccinated mice was detected by cytofluorometric analysis in either of the two approaches used (data not shown). Interestingly, CD8$^{+}$ T cells from splenocyte cultures of *T. gondii*-infected mice produced IFN-γ only when they were stimulated with live IPB-M tachyzoites, not when they were stimulated with the irradiated BW-Sp3(GRA1) target cells (data not shown).

**The protective effect of the GRA1 DNA vaccine was eliminated by in vivo CD8$^{+}$ T-cell depletion.** In order to evaluate the relative contribution of CD8$^{+}$ T cells to the protection conferred by the pVR1020-GRA1 vaccine, in vivo depletion experiments were performed. The induced protection was monitored by comparison of the survival curves of pVR1020- and pVR1020-GRA1-vaccinated mice after i.p. challenge with 40 cysts of the *T. gondii* IPB-G strain. Confirming a previous report (43), we found that GRA1 DNA vaccination resulted in significant protection. Data from two independent experiments are shown in Fig. 5. In the first experiment (Fig. 5A), all control pVR1020-vaccinated mice succumbed to infection, while 75% of the mice vaccinated with pVR1020-GRA1 were protected against infection with IPB-G ($P < 0.02$). In vivo CD8$^{+}$ T-cell depletion at the onset of challenge resulted in 75% mortality of pVR1020-GRA1-vaccinated mice and 100% mortality of pVR1020-vaccinated mice. There was no significant difference between the survival curves of these two groups. ($P > 0.05$). In the second experiment (Fig. 5B), all mice vaccinated with GRA1 DNA survived, while 75% of the mice vaccinated with control DNA succumbed to infection ($P < 0.05$), and again, CD8$^{+}$ T-cell depletion completely eliminated the protective effect of the GRA1 DNA vaccine. No significant differences were observed between the survival curves of GRA1-vaccinated and control mice (50 and 25% survival, respectively; $P > 0.05$) after CD8$^{+}$ T-cell depletion.

**Contribution of CD4$^{+}$ T cells from vaccinated mice to brain cyst development after infection with *T. gondii*.** In contrast to CD8$^{+}$ T-cell depletion, in vivo depletion of CD4$^{+}$ T cells did not influence the survival of pVR1020-GRA1-vaccinated mice during the acute phase of *T. gondii* infection (Fig. 5). All CD4$^{+}$ T-cell-depleted GRA1-vaccinated mice survived infection, and the data were similar to the 75 and 100% survival rates of undepleted GRA1-vaccinated mice (Fig. 5) ($P > 0.05$ for both experiments). As CD4$^{+}$ T-cell depletion was reported to influence the development of brain cysts (6), we assessed the contribution of CD4$^{+}$ T cells to *T. gondii* brain cyst development during the acute and early chronic phases of infection. As described previously (43), after infection with *T. gondii* pVR1020-GRA1-vaccinated mice developed significantly lower numbers of brain cysts than pVR1020-vaccinated mice developed. In additional in vivo CD4$^{+}$ T-cell depletion experiments, mice were challenged with a sublethal dose of IPB-G (20 cysts i.p.). Depletion of CD4$^{+}$ T cells resulted in a significant increase in the numbers of brain cysts in pVR1020-GRA1-vaccinated mice (4,540 ± 932 cysts/brain; $n = 4$) compared to the numbers of brain cysts in undepleted pVR1020-GRA1-vaccinated mice (1,247 ± 195 cysts/brain; $n = 4$) ($P < 0.01$). Furthermore, no significant differences between the numbers of brain cysts in CD4$^{+}$ T-cell-depleted pVR1020-GRA1-vaccinated mice and the numbers of brain cysts in CD4$^{+}$ T-cell-depleted pVR1020-vaccinated mice (5,793 ± 347 cysts per brain; $n = 4$) were detected. Interestingly, in these in vivo CD4$^{+}$ T-cell depletion experiments, decreased numbers of CD8$^{+}$ T cells were observed by cytofluorometric analysis in both GRA1- and control-vaccinated mice. Compared to undepleted splenocytes from pVR1020-GRA1-vaccinated mice, the CD4$^{+}$ T-cell population decreased by 93%, while the CD8$^{+}$

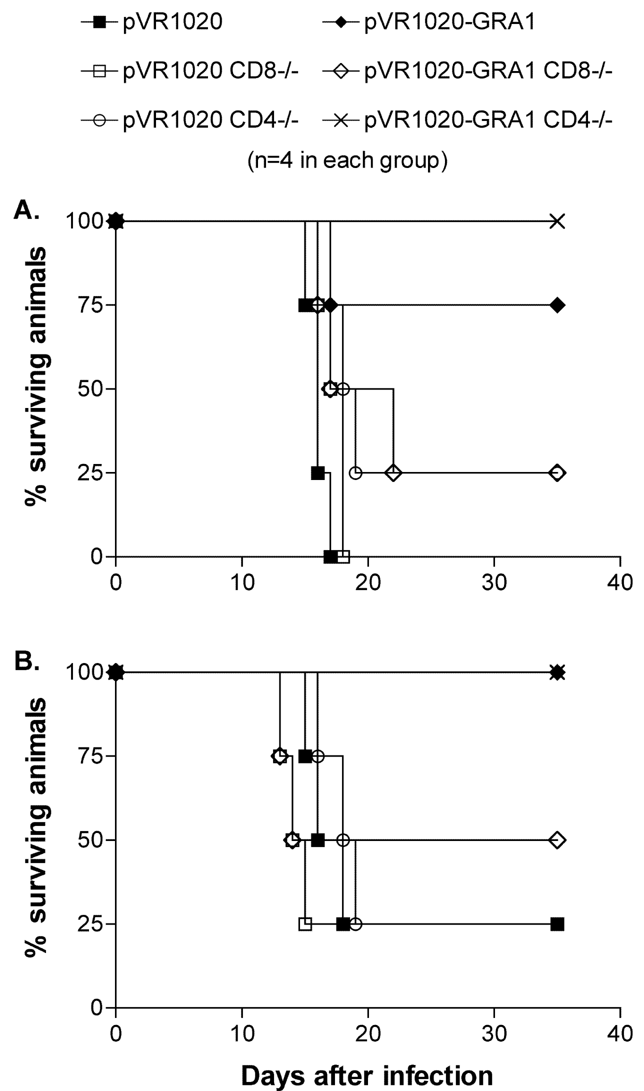


FIG. 5. Survival curves for in vivo T-cell-depleted vaccinated C3H mice from two independent experiments (A and B). Mice were vaccinated with pVR1020 or pVR1020-GRA1 and challenged i.p. with 40 cysts of the *T. gondii* IPB-G strain (solid symbols). Mice that were subjected to CD8$^{+}$ or CD4$^{+}$ T-cell depletion (starting 1 day prior to challenge) are represented by open symbols. The depletion efficiency was monitored by cytofluorometric analysis of blood samples and was found to be 98 to 100%.

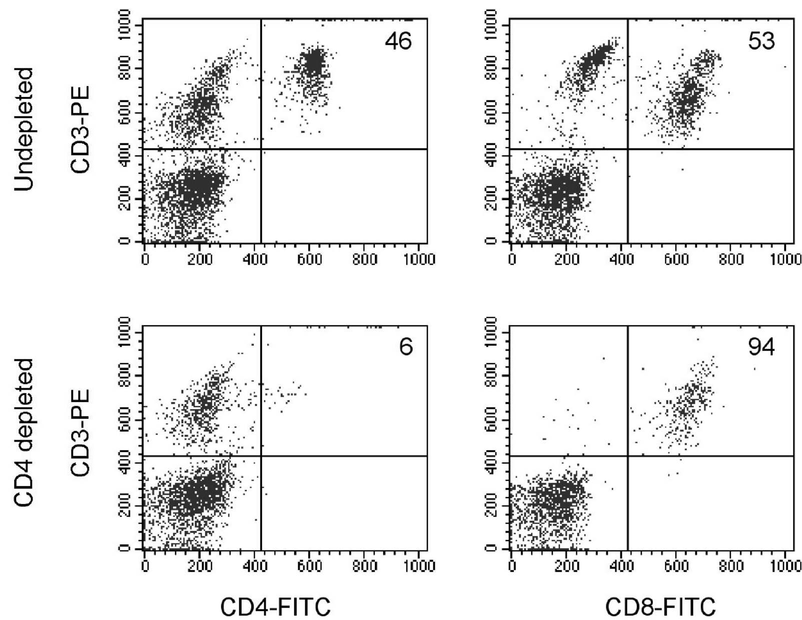


FIG. 6. Cytofluorometric analysis of CD4$^+$ and CD8$^+$ T-cell populations from pVR1020-GRA1-vaccinated mice after in vivo CD4$^+$ T-cell depletion. At least three independent experiments were performed, and dot plots representative of one experiment are shown. A total of $5 \times 10^4$ cells were analyzed, and the upper right quadrant shows the percentage of CD4$^+$ or CD8$^+$ T cells in the CD3$^+$ T-cell population. After CD4$^+$ T-cell depletion, the percentage of CD4$^+$ T cells in the gated population dropped from 21 to 1.5% (a reduction of 93%), and the percentage of CD8$^+$ T cells in the gated population dropped from 24 to 18% (a reduction of 25%). Similar decreases in CD4$^+$ and CD8$^+$ T-cell populations were observed with pVR1020-vaccinated mice (data not shown).

T-cell population decreased by 25% following in vitro CD4$^+$ depletion (Fig. 6). A similar decrease in T-cell subset populations was observed for pVR1020-vaccinated mice after CD4$^+$ depletion (data not shown). Our data thus seem to indicate that CD4$^+$ T-cell depletion during the acute phase of infection may lead either directly or indirectly (through a decreased number of CD8$^+$ T cells) to a greater brain cyst burden, which is maintained throughout the early chronic phase of infection.

## DISCUSSION

In the present study, we compared the relative contributions of CD4$^+$ and CD8$^+$ T cells primed by a GRA1 DNA vaccine to protective immune responses against acute infection with *T. gondii*. It is well known that IFN-γ produced by T cells plays a central role in immune responses against *T. gondii* in both acute and chronic phases of infection and that production of IFN-γ is correlated with protection. By means of in vitro depletion experiments we showed that CD4$^+$ T cells primed by the GRA1 DNA vaccine are the major source of IFN-γ when they are restimulated in vitro with TLA. However, in vivo depletion of CD4$^+$ T cells had no influence on mortality, while depletion of CD8$^+$ T cells eliminated the protective effect of the vaccine against acute toxoplasmosis. More importantly, we identified GRA1 DNA vaccine-induced CD8$^+$ T cells with specific *T. gondii* cytolytic activity. Indeed, following vaccination with pVR1020-GRA1, strong CTL responses against a GRA1-transfected T-cell lymphoma and *T. gondii*-infected syngeneic bone marrow macrophages were detected. T-cell-mediated cytotoxicity was completely eliminated after in vitro CD8$^+$ T-cell depletion. The fact that the GRA1 vaccine-induced CTLs could lyse *T. gondii*-infected target cells suggests that epitopes from GRA1 are processed and presented in major histocompatibility complex class I molecules during infection and that the GRA1 DNA vaccine induces specific CTLs.

DNA vaccination is a technique with strong potential to elicit cell-mediated immunity, triggering antigen-specific production of IFN-γ and priming CTL responses (24). It has previously been shown that DNA vaccination with the GRA1, GRA7, and/or ROP2 genes elicits a typical type 1 immune response, characterized by an IgG2a-biased antibody response and production of IFN-γ (43). Mice from different genetic backgrounds (C57BL/6, C3H/HeN, and BALB/c) developed high *T. gondii*-specific antibody titers after vaccination, while elevated IFN-γ production was observed in C3H/HeN and BALB/c mice. Partial protection induced by DNA vaccination was observed in C3H/HeN mice upon challenge with moderate doses of *T. gondii* 76K.

Data provided by other laboratories confirm the efficacy of DNA vaccines against *T. gondii* infections. Desolme et al. demonstrated that DNA vaccination with GRA4 induced a protective Th1-type response against acute toxoplasmosis in C57BL/6 mice (16). SAG1, the most prominent antigen, has been assessed and shown to drive Th1-type protective immune responses in both mice (33) and rats (1). In adoptive transfer experiments, naive mice that received CD8$^+$ T cells from SAG1 DNA-vaccinated mice were shown to have extended survival times after challenge with the highly virulent RH strain (33). However, these experiments did not provide conclusive information about whether the DNA vaccine-elicited effector mechanism is due to direct CD8$^+$ T-cell cytolytic activity or is

mediated by the IFN-γ secreted by CD8$^+$ T cells. So far, the only demonstration with SAG1-elicited cytolytic CD8$^+$ T cells was obtained not by DNA vaccination but after immunization with a SAG1-transfected RMA.S cell line (2).

Indeed, both the cytolytic activity and IFN-γ secretion can confer protective properties to CD8$^+$ T cells. In the case of intraepithelial lymphocytes (IELs) elicited after oral infection, it has been shown that the CD8$^+$ IEL subset confers protective immunity to subsequent oral infection (5, 29). The CD8$^+$ IELs produce IFN-γ and have cytotoxic activity (8). It has been assumed that the production of IFN-γ by these cells is crucial for the protective effect since the presence of anti-IFN-γ MAb during adoptive transfer of CD8$^+$ T cells eliminates protection (41). In contrast, CD8$^+$ IELs derived from IFN-$\gamma^{-/-}$ mice display protective activity when they are adoptively transferred to wild-type mice (29). However, the recipient must be able to produce IFN-γ since the protective activity is lost when the CD8$^+$ IELs are adoptively transferred to IFN-$\gamma^{-/-}$ mice (29). Therefore, it is possible that at least for IELs, protective activity results not only from CTL action but also from an ability to promote production of IFN-γ by other cell types.

In the ts-4 *T. gondii* vaccination model, IFN-γ-producing CD4$^+$ and CD8$^+$ T cells have been described, and the latter have been shown to have CTL activity against antigen-loaded or infected cells (21, 25). These CD8$^+$ T cells were considered the major mediators of resistance, increasing survival and reducing the development of *T. gondii* brain cysts (21, 25, 27, 37, 40, 44). The importance of CTL responses in *T. gondii* immunity has been questioned, since ts-4-vaccinated perforin-deficient mice could resist infection with the highly virulent RH strain (15). However, in the same report, the numbers of brain cysts increased at least three- to fivefold when the mice were challenged with the low-virulence ME49 strain. Recently, it was demonstrated by Yamashita et al. that tachyzoites within a cell targeted for CTL lysis are not themselves killed by the cytolytic event (44). This suggests that CTL activity may spread infection by release of tachyzoites. In a hypothetical model suggested by Denkers (10), tachyzoites released after CD8$^+$ T-cell-mediated lysis may be phagocytosed and inactivated by cells such as macrophages, brain astrocytes, and microglial cells; the latter cells are activated for microbicidal function by IFN-γ released from the CTLs.

As reported by Brown and McLeod (4), CD8$^+$ T cells are involved in control of the number of brain cysts. In their studies, in vivo CD8$^+$ T-cell depletion or the use of major histocompatibility complex class I mutants converted mice from cyst resistant to cyst susceptible. In addition, β2-microglobulin-deficient mice, which do not express major histocompatibility complex class I molecules and therefore lack functional CD8$^+$ T cells, could survive acute *T. gondii* infections, although they became more susceptible to the chronic phase of infection (13). In our study, we could not monitor the effect of in vivo CD8$^+$ T-cell depletion on the establishment of chronic toxoplasmosis, since most of the mice succumbed to acute infection. In ongoing studies in our laboratory we aim to address these issues.

The role of GRA1 DNA vaccine-elicited CD4$^+$ T cells in the resolution of acute infection is less clear. Despite the fact that the elicited CD4$^+$ T cells were the major producers of IFN-γ in TLA-stimulated spleen cell cultures, in vivo depletion of these cells did not result in increased mortality. However, in vivo CD4$^+$ T-cell depletion did result in threefold increases in brain parasite burden in both GRA1-vaccinated and control mice. Therefore, our results indicate that CD4$^+$ T cells may participate in the reduction of development of brain cysts, independent of the DNA vaccine administered. These observations are in agreement with other studies in which CD4$^+$ T cells were identified as the major source of IFN-γ (13, 20) and in which depletion of CD4$^+$ T cells increased the number of brain cysts (21).

Interestingly, in additional in vivo CD4$^+$ depletion experiments, we noted that the increase in the number of brain cysts coincided with a decrease in the number of CD8$^+$ T cells in the infected host. This raises the question of whether the increase in brain parasite burden was a direct consequence of the elimination of CD4$^+$ T cells or was indirectly due to a decrease in the size of the CD8$^+$ T-cell subset.

In conclusion, we show here that GRA1 DNA vaccination induces IFN-γ-producing CD4$^+$ and CD8$^+$ T cells. Although CD4$^+$ T cells rather than CD8$^+$ T cells produce most of the IFN-γ in spleen cell cultures, only depletion of vaccine-induced CD8$^+$ T cells had an impact on the survival of *T. gondii*-challenged animals. Most importantly, our data demonstrate for the first time establishment of GRA1 DNA vaccine-induced CD8$^+$ T cells with *T. gondii*-specific cytolytic activity.

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