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In Vitro Correlates of Ld-Restricted Resistance to Toxoplasmic Encephalitis and Their Critical Dependence on Parasite Strain[1]

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Resistance to murine toxoplasmic encephalitis has been precisely and definitively mapped to the L^d class I gene. Consistent with this, CD8$^+$ T cells can adoptively transfer resistance to toxoplasmic encephalitis. However, cytotoxic CD8$^+$ T cells, capable of killing class I-matched, infected target cells, are generated during the course of *Toxoplasma gondii* infection even in mice lacking the L^d gene. L^d-restricted killing could not be demonstrated, and the functional correlate of the L^d gene has therefore remained elusive. Herein, L^d-restricted killing of *T. gondii*-infected target cells is demonstrated for the first time. L^d-restricted killing is critically dependent on the strain of *T. gondii* and is observed with all the derivatives of type II strains tested, but not with a type I strain. These results have important implications for vaccine development. *The Journal of Immunology*, 2002, 169: 966–973.

*T*oxoplasma gondii tachyzoites cause destruction of tissues and disease in congenitally infected and immunocompromised individuals and occasionally in immunologically normal individuals (1, 2). The latent, slowly growing, encysted *T. gondii* bradyzoites are released when cysts rupture and can revert to tachyzoites, leading to tissue destruction (1, 2). Earlier immunogenetic studies using inbred, congenic, mutant, knockout, and transgenic mice demonstrated definitively that the L^d gene confers protection against *T. gondii* parasite burden in the brain and against encephalitis following peroral infection with encysted bradyzoites of the Me49 strain of *T. gondii* (3–7). These studies strongly implied that CD8$^+$ T cells, through interaction with the MHC class I L^d molecule, are of critical importance in preventing toxoplasmic encephalitis. Consistent with this, abrogation of CD8$^+$ T cells in mice of the H-2^d haplotype eliminated protection mediated by the L^d gene (5). However, in vitro studies have demonstrated that both mice expressing the L^d gene and mice lacking the L^d gene can generate CTLs capable of killing class I-matched cells infected with *T. gondii* by a mechanism independent of the L^d gene (8, 9). A functional correlate for the protection mediated by the L^d gene against toxoplasmic encephalitis has thus remained elusive.

The above-mentioned in vitro studies all examined the response of splenic effector cells derived from mice immunized with a temperature-sensitive mutant of the non-cyst-forming RH strain of *T. gondii* and expanded these effectors using cells infected with UV- or gamma-

irradiated attenuated RH tachyzoites. It is noteworthy that the L^d gene has only been demonstrated to mediate resistance to the cyst-forming Me49 strain of *T. gondii* and has never been shown to play any role during infection with the RH strain of *T. gondii*. This raised the possibility that either the L^d gene effector function is only important in resistance against certain strains of *T. gondii* or, alternatively, that the L^d gene exerts its effect during cyst formation.

Herein, we examined both of these possibilities using C3H.L^d transgenic mice and their wild-type parental control strain, C3H/HeJ. C3H.L^d mice have been genetically altered to express the L^d gene in addition to their own MHC genes and thus provide a uniquely informative tool for these studies (10). As predicted and as discussed above, these mice are resistant to the development of high numbers of parasites in their brains and toxoplasmic encephalitis (6). In addition we compare the early immune responses in C3H.L^d and C3H/HeJ mice infected with the Me49 strain of *T. gondii* to gain insight into other possible effector functions, such as IFN-γ production, which may be determined by the L^d gene. Our results provide the first evidence of a functional correlate of L^d-restricted resistance to *T. gondii* infection and demonstrate that the generation of L^d-restricted CTLs specific for *T. gondii*-infected cells occurs during the course of infection. Their generation, however, is critically dependent on the strain of *T. gondii* used to infect the donor mouse, to drive the expansion of effector cells, and to infect the target cells. Thus, this can be achieved by using the Me49 strain or derivatives thereof, but not the RH strain or its derivative strains. These results suggest that the Me49 strain of *T. gondii* has a peptide that interacts with the L^d molecule to facilitate CTL recognition and killing of infected cells. This peptide would appear to be absent in the RH strain of *T. gondii*. These results have important implications for vaccine design, as they indicate that an important protective effector mechanism may be strain specific.

Materials and Methods

Mice

C3H.L^d transgenic mice were constructed by J. Forman (10) at University of Texas Southwestern Medical School and were bred at the McLeod Laboratory. The C3H.L^d mice used in experiments to study CTLs and in ELISAs to measure IFN-γ were adult males that were age-matched with uninfected controls. BALB/c female mice were used for experiments with ts-4 immunization and analysis of strains of *T. gondii* involved in stimulating and rendering P815 cells CTL targets. C3H/HeJ mice, purchased

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from The Jackson Laboratory (Bar Harbor, ME), were used as controls in experiments that measured mRNA (RT-PCR IFN-γ) and protein (ELISA). For experiments in which IFN-γ protein was measured using an ELISA, C3H/HeJ mice used as controls were age- and sex-matched to the C3H.L^d mice. Both adult male and female C3H.L^d mice of different ages were used for experiments in which cytokine message was semiquantitated. ND4 mice, purchased from Harlan Sprague Dawley (Indianapolis, IN), were used as hosts for the passage of RH strain *T. gondii*.

T. gondii

The RH (11) strain was passaged every 2–3 days in mice as previously described (12). The temperature-sensitive mutant ts-4 derived from the RH strain (13, 14) was passaged every 3–5 days in tissue culture in human foreskin fibroblasts. Me49, a less virulent (type II) (15) strain that results in chronic infection with encysted bradyzoites, was passaged every 3–5 mo in Swiss-Webster mice as previously described (12). PTg, a clonal derivative of Me49(P) strain *T. gondii* (16), was passaged every 3–5 days in tissue culture. R5 was provided by L. Weiss (Einstein University, New York, NY). It originally was produced by treatment of tachyzoites with ethylnitrosourea and was selected for resistance to 1,4-hydroxynaphthoquinone (17). At physiologic pH, R5 has been demonstrated by immunofluorescent Ab assay using Ab to bradyzoite Ag 1/5 (BAg1/5) to express 50% tachyzoite Ags and 50% bradyzoite Ags as a population (L. Weiss, unpublished observations). The presence of two other bradyzoite-specific Ags, P36 and P18, was detected in R5 *T. gondii* as well (S. Tomavo and J. C. Boothroyd, unpublished observations). PTg, R5, and ts-4 strains were passaged in human foreskin fibroblasts (Viromed, Minneapolis, MN) as described previously (18).

Infection of mice

One hundred cysts of the Me49 strain per mouse were administered perorally, as described previously (6).

Immunization of mice with ts-4

Mice were immunized i.p. as previously described (3, 9). A total of 2×10^4 ts-4 tachyzoites were administered initially, and 2×10^5 tachyzoites were administered 2 and 4 wk following the first inoculation. Mice were used for experiments 4–10 mo after the last inoculation.

Media used for culture of T. gondii and cell lines

Uninfected and ts-4-infected human foreskin fibroblasts were cultured in IMDM (Life Technologies, Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM glutamine (Life Technologies), and antibiotic-antimycotic containing (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B; Life Technologies). R5 and PTg *T. gondii* strains were passaged in high glucose DMEM (Life Technologies) supplemented with 10% FCS, 0.055 mM 2-ME (Life Technologies), 2 mM glutamine, and antibiotic-antimycotic. All spleen cell suspensions and target cells were cultured in IMDM containing 10% FCS, 0.055 mM 2-ME, 1 mM sodium pyruvate (Life Technologies), 1× (0.1 mM) MEM nonessential amino acids (Life Technologies), 1× MEM amino acids (Life Technologies), 2 mM glutamine, and antibiotic-antimycotic.

Cytolytic T cell assay: primary culture of splenocytes

Spleens from uninfected control mice and from mice infected perorally 11–13 days previously were pressed through wire mesh and pipetted into 24-well plates at 1×10^7 cells/well in a total volume of 2 ml. Tachyzoites of the RH strain of *T. gondii*, harvested from mouse peritoneum in saline, were passed through a 25-gauge needle and then through a 3-μm pore size filter. To harvest *T. gondii* strains passaged in tissue culture, medium was decanted to remove extracellular *T. gondii*, and a rubber policeman was used to scrape the cell layer into saline. The cell suspension was passed twice through a 25-gauge needle and once through a 27-gauge needle, followed by passage through a 3-μm pore size filter. All strains of *T. gondii* were centrifuged at $500 \times g$ for 15 min and resuspended at a concentration of 1×10^7/ml. The *T. gondii* was attenuated by either gamma irradiation with 20,000 rad or UV irradiation with 144,000 erg/cm^2 (19–21), which corresponded to a 90-s exposure under a UV lamp. UV irradiation was performed with constant rotation of a 4-ml suspension in a 100-mm petri dish (C. S. Subauste, unpublished observations). Two million attenuated *T. gondii* were added to each well of splenocytes, and the cultures were incubated for 5–7 days at 37°C in 5% CO$_2$.

Preparation of effector and target cells

Effector cells, unless stated otherwise, were purified using Ficoll-Hypaque gradients (Nycomed, Oslo, Norway; ρ1.083), washed in 1× PBS contain-

ing 5% FCS, and placed over a T cell enrichment column (R&D Systems, Minneapolis, MN). Approximately 74% of the cells eluted from each column were CD3$^+$, as determined by FACS analysis (specifications from R&D Systems indicate an expected purification efficiency of ~80% or higher for CD3$^+$ T cells). Target cells (P815 mastocytoma, R1.1 lymphoma, EL4 lymphoma, or L5 MF22 cells) were obtained from American Type Culture Collection (Manassas, VA) or were provided by I. Nakamura (State University of New York, Buffalo, NY) and were either uninfected or infected overnight at a multiplicity of infection of six *T. gondii* organisms per target cell.

Chromium release

One million target cells were labeled with 100 μCi Na^{51}CrO$_4$ (ICN, Costa Mesa, CA) for 1 h at 37°C, then washed twice with 1× HBSS (Life Technologies) containing 5% FCS, 10 mM HEPES (Mediatech, Washington, D.C.), and 0.035% sodium bicarbonate (Mediatech) and once with supplemented IMDM. The percentages of infected target cells were determined by cytocentrifuge preparations stained with Giemsa (Fisher, Pittsburgh, PA). The assay was performed using triplicate samples, with 1×10^4 target cells/well of a round-bottom microtiter plate, and E:T cell ratios of 20:1, 10:1, 5:1, and 2.5:1. Chromium release was measured, and a mean was calculated, after 4 h in culture at 37°C in 5% CO$_2$. Specific lysis was calculated by subtracting the amount of spontaneous lysis (chromium release of target cells alone) from the amount of chromium release in the experimental sample and dividing by the difference between the maximum amount of lysis (chromium release of target cells lysed with an equal volume 2% Triton X-100) and the spontaneous lysis and multiplying by 100 (i.e., (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100).

Treatment with Ab to L^d or isotype control

Experiments involving blocking CTL activity with Ab were performed using Ab 30-5-7, to L^d (22) (a gift from T. Hanson) purified with protein A-Sepharose (23) or isotype control purified from lyophilized ascites (IgG2a; Sigma, St. Louis, MO) by ammonium sulfate precipitation. Target cells were incubated with 1.5–2 μg Ab to L^d or isotype control/1 × 10^4 cells for 30–60 min before and during incubation with effectors. In each such experiment maximum and spontaneous lysis were measured with and without Ab to L^d or isotype control Ab.

T cell proliferation assay

T cell proliferation assays were conducted by a modification of the procedure described previously (24) with groups of three to five C3H/HeJ and C3H.L^d mice before infection and on days 6 and 12 after infection. Spleen cell suspensions were prepared by forcing spleens through a wire mesh. Erythrocytes were removed from spleen cell suspensions by treatment with Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride) for 3 min at 37°C. Following washing in IMDM, viable cells were counted, and cell suspensions were adjusted to 5×10^6/ml. One hundred-microliter aliquots of cell suspensions were added to the wells of a 96-well flat-bottom tissue culture-treated plate containing 100 ml medium and *Toxoplasma* lysate Ag (TLA;[7] 10 μg/ml). Cells were incubated for 60 h at 37°C in 5% CO$_2$, after which 0.25 μCi [^{3}H]thymidine (5 Ci/mmol; Amersham Life Science, Arlington Heights, IL) was added to each well. At this time supernatants were removed from parallel cultures and stored at −70°C for measurement of IFN-γ. [^{3}H]Thymidine-pulsed wells were cultured for an additional 12 h, after which they were harvested onto glass-fiber filter strips (Cambridge Technology, Watertown, MA) using an automated PHD cell harvester (Cambridge Technology) and processed as previously described (25). Results are expressed as the mean stimulation index ± SE for each group of animals.

Determination of IFN-γ production

Assays were performed on supernatants from TLA-stimulated splenocyte cultures by capture ELISA as previously described (24). Briefly, microtiter plates were coated overnight at 4°C with capture Ab (clone R4-6A2; BD PharMingen, San Diego, CA) in PBS (pH 9.0). Following three washes in PBS (pH 7.0) containing 0.05% Tween 20, plates were blocked for 1 h at 37°C with PBS (pH 7.0) containing 10% FCS. Samples and standards consisting of rIFN-γ (0–7000 pg/ml; BD PharMingen) were applied in duplicate and incubated for 2 h at 37°C. After an additional three washes, biotinylated detection Ab (clone XMG1.2; BD PharMingen) was added in PBS (pH 7.0) containing 10% FCS, and the plates were incubated for 45

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[7] Abbreviations used in this paper: TLA, *Toxoplasma* lysate Ag; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

min at 37°C before washing. Streptavidin-alkaline phosphatase (Sigma) was added to each well (0.5 μg/ml) for 30 min, followed by three more washes. Binding was visualized with substrate consisting of *p*-nitrophenyl-phosphate in 10% diethanolamine buffer. Absorbances at 405 nm were measured on a microplate Autoreader (Bio-Tek Instruments, Winooski, VT) after a 90-min incubation. IFN-γ concentrations were determined from a standard curve and are expressed as the mean IFN-γ concentration and SE for each group of animals.

Preparation of TLA

TLA was prepared from tachyzoites of RH strain *T. gondii* grown in the peritoneum of ND4 outbred mice. Tachyzoites were harvested from the peritoneum of mice infected 3 days previously and purified by filtration through a 3-μm pore size filter. Following washing in PBS (pH 7.2), tachyzoites were resuspended in water and frozen and thawed three times (-135 to 37°C). After the addition of a 0.1 vol of 10× PBS (pH 7.2), the resulting suspension was filtered through a 0.2-μm filter, and the protein concentration was determined as previously described (26).

RT-PCR

RNA was isolated from splenic tissue by grinding frozen tissue with a mortar and pestle in liquid nitrogen and extracting the RNA using RNAzol or Ultraspec RNA (Biotecx, Houston, TX). cDNA was reverse transcribed in 90-μl reactions containing 6 μg RNA, 500 ng random primer (Promega, Madison, WI), 90 U RNase inhibitor (Life Technologies), 2 mM dNTPs (Promega), and 1200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies). RT was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) at 22°C for 10 min, then at 42°C for 1 h, followed by 99°C for 5 min. PCR was performed in the presence of a competitor plasmid, PQRS, provided by S. Reiner (University of Pennsylvania, Philadelphia, PA) (27). The amount of PQRS varied with each cytokine measured; the amount used was that established empirically to be required to amplify in each sample a band from both the wild-type template and the PQRS plasmid template. PCR (27) were performed in a 25-μl total volume, containing 1.5 mM MgCl$_2$, 0.4 μM of each primer (Operon, Alameda, CA), 0.2 mM dNTPs (Promega), and 0.6 U Taq polymerase (Promega) in 1× buffer supplied with the enzyme. PCR was performed in a PTC-100 thermal cycler. The program consisted of 3 min at 95°C, followed by 40 cycles of 40 s at 94°C, 40 s at 60°C, and 40 s at 72°C, followed by 10 min at 72°C.

Semiquantitation and normalization of PCR results

PQRS, encoding cDNA sequence of IFN-γ, IL-10, and TGF-β as well as other cytokines, was constructed to generate a PCR product larger than the product generated from reverse transcribed wild-type message (27). For each sample the amount of wild-type cytokine message amplified from the tissue sample was calculated as follows. Densitometry was performed to determine the relative amounts, as measured by band intensity on an agarose gel, of wild-type and PQRS PCR products. The band intensity of the wild type was divided by that of PQRS. Band intensity was determined by scanning a photographic negative of the ethidium bromide-stained gel with a densitometer (Molecular Dynamics, Sunnyvale, CA). Variation in PCR results was addressed by performing PCR in duplicate and if there was a difference of >0.2 or >20% between the duplicates, the PCR for those samples was repeated. The PQRS plasmid also contained sequence of a constitutively expressed gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT), to which message was normalized by dividing the amount of cytokine message in the tissue relative to the amount of message amplified from PQRS (wild-type cytokine message/PQRS amplification) by the amount of HPRT message in the same sample relative to PQRS (wild-type HPRT message/PQRS HPRT). This value was calculated for each mouse in a treatment or control group, and the mean ± SD were calculated from a total of two or three mice studied per time point.

Presentation of data and statistics

Each experiment was performed at least twice. The data shown are representative of a minimum of two experiments. Every experiment included uninfected control target cells. Cytolysis was <10% for almost all these uninfected target cells. Therefore, data are only shown for uninfected targets if cytolysis exceeded 10%. Statistical analysis was by two-tailed Student's *t* test.

Results

L^d-restricted CTLs in spleens of Me49-infected C3H.L^d mice

C3H.L^d mice were infected perorally with 100 Me49 *T. gondii* cysts. Twelve days later their spleens were removed and stimulated in vitro for 7 days with UV-attenuated organisms of the R5 strain of *T. gondii*. R5 *T. gondii* is a mutant Me49 strain that has been demonstrated to express a population 50% tachyzoite and 50% bradyzoite Ags as described in *Materials and Methods* (17). Effector cells were tested against infected and uninfected target cells of different haplotypes. The data in Fig. 1*A* demonstrate L^d-restricted lysis of R5-infected P815 (H-2^d) target cells by splenocyte effector cells derived from C3H.L^d mice infected with the Me49 strain of *T. gondii*. The control cell lines, EL4 (H-2^b) and L5 MF22 (H-2^b) target cells, which have mismatched MHC class I alleles, were not lysed. There was a small amount of H-2^k-restricted lysis of infected R1.1 (H-2^k) target cells. In certain instances CTL were generated from the in vitro culture of splenocytes from control uninfected mice that demonstrated increased nonspecific background lysis. For example, splenocyte effectors from uninfected
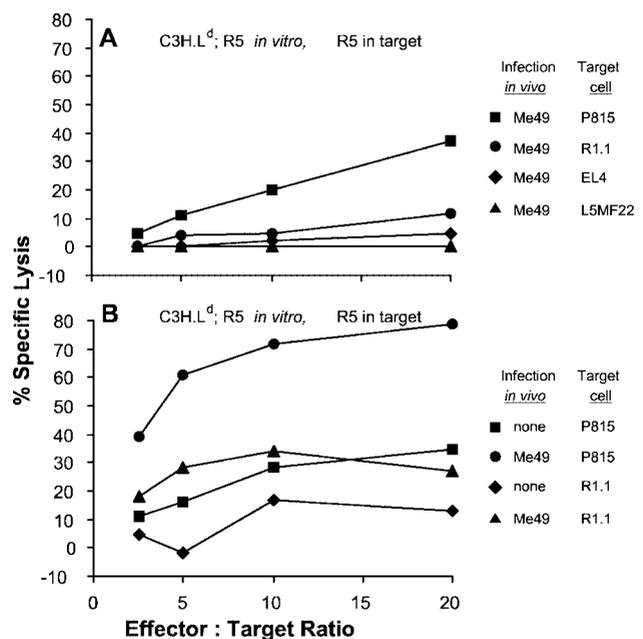

FIGURE 1. Genetic restriction and comparison of effector CTL from uninfected and infected C3H. L^d mice. *A*, L^d-restricted CTLs in spleens of *T. gondii*-infected C3H.L^d mice. Splenocytes were harvested from C3H.L^d mice infected with cysts of the Me49 strain of *T. gondii* 12 days earlier and stimulated in culture for 7 days with UV-attenuated organisms of the R5 strain of *T. gondii*. Effectors were tested against uninfected (data not shown) or R5-infected (■, ●, ▲, and ◆) target cells. Target cells were P815 (H-2^d; ■), R1.1 (H-2^k; ●), EL4 (H-2^b; ◆), and L5 MF22 (H-2^b; ▲). Spontaneous lysis of target cells was 20% or lower for all target cells, with the exception of 30% for infected R1.1 cells. The percentages of infected cells were: P815, 70%; R1.1, 76%; EL4, 48%; and L5 MF22, 90%. All cultures with uninfected targets had <5% lysis at all E:T cell ratios (data not shown). *B*, Specific lysis of infected target cells by C3H.L^d splenocyte effectors. Splenocytes were harvested from uninfected C3H.L^d mice (■ and ◆) or C3H.L^d mice 12 days following infection with Me49 strain of *T. gondii* (● and ▲) and were stimulated for 6 days in vitro with organisms of the R5 strain *T. gondii* attenuated with gamma irradiation. Effectors were tested against uninfected (data not shown) and R5-infected (■, ●, ▲, and ◆) P815 (H-2^d; ■ and ●) and R1.1 (H-2^k; ◆ and ▲) target cells. Spontaneous lysis of target cells was 16% or less for all target cells, with the exception of 42% for infected R1.1 cells. Seventy to 87% of target cells were infected. All cultures with uninfected targets had <10% lysis at all E:T cell ratios.

mice (data not shown) could be produced that killed both uninfected (30% lysis at an E:T cell ratio of 20:1) and infected (60% at an E:T cell ratio of 20:1) P815 target cells, but not infected or uninfected R1.1, EL4, or L5 MF22 target cells (<10% at an E:T cell ratio of 20:1).

L^d restriction also was demonstrated by the abrogation of cytolytic activity with Ab to L^d. Fig. 1B demonstrates cytolytic activity in the absence of Ab in these experiments. In the data shown in Table I, Ab to L^d, but not isotype control Ab, decreased lysis of infected P815 target cells from 72% without the Ab at an E:T cell ratio of 10:1 to 43% with the Ab. When the lysis of uninfected target cells was subtracted from the lysis of infected target cells, there was a 50% reduction in lysis by Ab to L^d compared with a 16% reduction by the isotype control Ab. Lysis of R1.1 cells was not reduced by either Ab to L^d or the isotype control.

Cytolytic activity by splenocytes stimulated by culture with either of two Me49-derived T. gondii *strains, but not with the virulent RH strain*

We investigated whether the cytolytic activity shown in Fig. 1 was specific to organisms of the Me49 strain or to bradyzoite Ags by using the PTg and RH strains of *T. gondii*. PTg is clonally derived from Me49 (type II strain), whereas RH is a type I strain that is highly virulent and lethal in mice and does not naturally persist to form bradyzoites in mice without antimicrobial treatment (28). Both strains are maintained as tachyzoites in the laboratory. The data in Fig. 2 demonstrate that such cytolytic activity was present not only in splenocyte cultures stimulated with organisms of the R5 strain of *T. gondii* (Fig. 2A), but also in splenocyte cultures stimulated with tachyzoites of the PTg strain of *T. gondii* (Fig. 2B). Culture of splenocytes from Me49-infected C3H.L^d mice with irradiated tachyzoites of the RH strain did not yield L^d-restricted CTL (Fig. 2C), suggesting that the CTL response was specifically elicited by Me49-derived strains or, more generally, by type II strains. However, P815 target cells infected with the RH strain of *T. gondii* were killed by splenocytes from BALB/c (H-2^d) mice immunized with ts-4, a clonal, temperature-sensitive mutant of the RH strain of *T. gondii* (14) (Fig. 3A). Splenocytes from ts-4-immunized C3H.L^d mice cultured with irradiated RH tachyzoites were unable to lyse the RH strain-infected P815 (H-2^d) target cells (Fig. 3B), demonstrating that the CTL response was not elicited by those peptides presented by L^d. There was a small amount of lysis of R1.1 (H-2^k) target cells infected with the RH strain by splenocyte effectors from ts-4-immunized C3H.L^d mice (Fig. 3C).

Cytolytic activity present in R5-stimulated cultures from mice infected 11–13 days, but not 6 days earlier

We investigated whether CTLs were produced earlier in the Me49 infection as well as 11–13 days thereafter. Splenocytes from C3H.L^d mice were stimulated in vitro with irradiated R5 or RH strain *T. gondii* 6 or 12 days following peroral infection with Me49 strain *T. gondii*. Despite the presence of a CTL response in R5-stimulated splenocyte cultures from mice infected 12 days earlier, there was very minimal or no response in cultures from mice infected 6 days earlier (Fig. 4A). Fig. 4B demonstrates the absence of CTLs in RH-simulated splenocyte cultures from mice infected perorally with the Me49 strain of *T. gondii* either 6 or 12 days earlier.

IFN-γ production and lymphocyte proliferation in splenic cultures stimulated with T. gondii *Ags in vitro*

To determine whether the kinetics of the CTL response were associated with the production of IFN-γ, splenocytes from C3H.L^d and C3H/HeJ mice infected perorally 6 or 12 days earlier with the Me49 strain *T. gondii* were cultured in vitro for 2 days with TLA (Fig. 5). Splenocytes from uninfected mice of each strain served as controls. The ability of these splenocytes to produce IFN-γ and proliferate in response to TLA was assessed in vitro by capture ELISA and [^{3}H]thymidine uptake, respectively (Fig. 5, A and B). Splenocytes from uninfected C3H/HeJ or C3H.L^d mice did not produce detectable amounts of IFN-γ within the limits of the assay. At 6 days postinfection C3H.L^d splenocytes produced ~2-fold more IFN-γ than did C3H/HeJ splenocytes ($p < 0.05$). However, at 12 days after infection IFN-γ production by C3H.L^d splenocytes had decreased, while production by C3H/HeJ splenocytes had increased to a level higher than that of splenocytes from C3H.L^d mice 12 days or 6 days after infection ($p < 0.05$). The splenocytes of C3H/HeJ and C3H.L^d mice proliferated at nearly equivalent levels when measured at 6 days of infection, but at 12 days C3H.L^d splenocytes proliferated at a significantly higher level than C3H/HeJ splenocytes ($p < 0.05$).

IFN-γ mRNA production was assessed in vivo with C3H/HeJ and C3H.L^d mice using RT-PCR. In three separate experiments three C3H/HeJ and three C3H.L^d mice for each time point were infected perorally with cysts containing bradyzoites of the Me49 strain of *T. gondii*. Splenic lymphocytes were tested for the amount of cytokine message produced before infection and 3, 4, 5, 6, 7, 8, and 14 days after infection. At the earlier times spleens from all three C3H/HeJ and three C3H.L^d mice were studied. However, in some experiments only two, rather than three, mice survived to the later days; therefore, results are in duplicate or triplicate depending upon numbers of mice surviving the Me49 infection. RNA was isolated from frozen tissue and reverse transcribed, and PCR was performed using the cDNA. mRNA for the expression of IFN-γ was semiquantitated as described in *Materials and Methods* by adding a PCR competitor to each reaction and was normalized by comparison of cytokine message to HPRT message. IFN-γ mRNA levels were raised from days 4–8 in the spleens of all mice compared with control mice. In initial experiments the production of IFN-γ mRNA was greater in C3H.L^d splenocytes, but no reproducible statistically significant difference in IFN-γ mRNA levels

Table I. *Ab to* L^d *abrogates cytolytic activity of Me49-infected C3H.L^d effectors*

Treatment of Cultures	P815 Cells				R1.1 Cells			
	U[a]	I[b]	% lysis[c]	% reduction[d]	U[a]	I[b]	% lysis[c]	% reduction[d]
No Ab	10	72	62		0	34	34	
Isotype control	20	72	52	16	1	36	35	0
Antibody to L^d	43	43	31	50	11	45	34	0

[a] Percent lysis of uninfected cells.
[b] Percent lysis of R5-infected cells.
[c] Percent lysis of infected cells minus percent lysis of uninfected cells.
[d] Percent lysis of Ab-treated cells/percent lysis of untreated cells (100%). Spontaneous lysis of Ab-treated cells was 18% or lower for all target cells, with the exception of 41% for infected R1.1 cells.
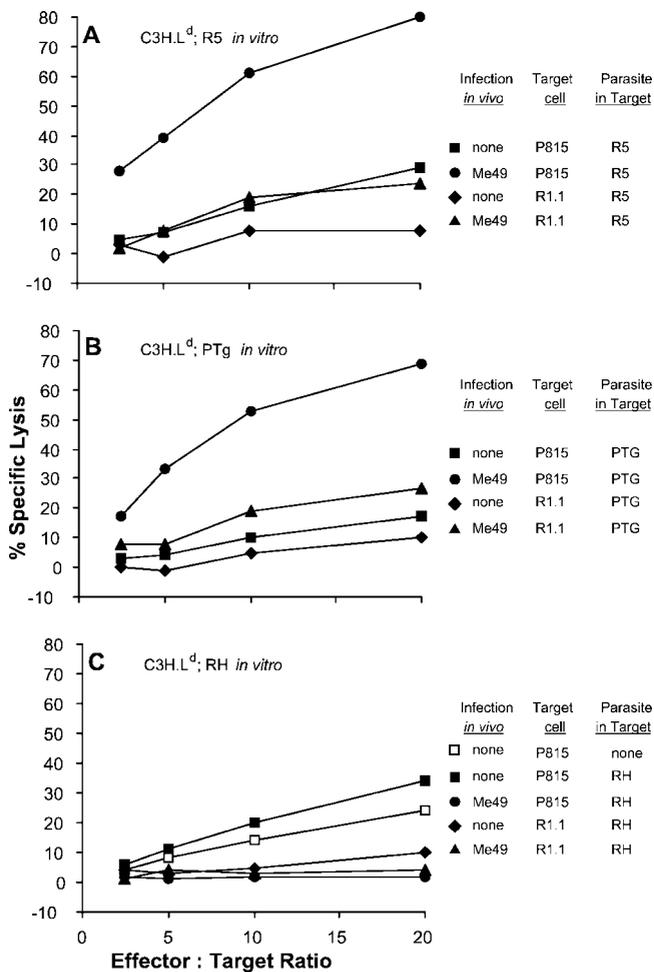
FIGURE 2. Splenocytes from *T. gondii*-infected C3H.L^d mice exhibit L^d specific lysis when cultured in vitro with either of two type II strains, but not a type I strain of *T. gondii*. *A* and *B*, Splenocytes were harvested from uninfected C3H.L^d mice or from mice infected 12 days earlier with cysts of the Me49 strain *T. gondii* and stimulated for 6 days in vitro with R5 (*A*) or PTg (*B*) organisms attenuated by gamma irradiation. Effectors were tested against uninfected targets (data not shown, as lysis was <10% at all E:T cell ratios) and against R5 strain (*A*) and PTg strain (*B*) *T. gondii*-infected P815 (H-2^d) and R1.1 (H-2^k) target cells. Spontaneous lysis of all target cells was 23% or less, with the exception of 40% for R5-infected R1.1 target cells. R1.1 target cells were 38–55% infected. P815 target cells were 65% infected. *C*, Splenocytes were harvested as in described in *A* and *B* from mice infected 11 days and stimulated with the UV-attenuated RH strain *T. gondii*. Effectors were tested against uninfected (□; data not shown when lysis was <10% at all E:T cell ratios) and RH-infected (■) P815 and R1.1 target cells. Spontaneous lysis of targets was 14% or less for all target cells. Target cells were 50–55% infected.

was found in splenocytes from C3H.L^d compared with C3H/HeJ mice in the repeat studies.

Discussion

Previous studies definitively demonstrated that the murine L^d gene confers resistance against toxoplasmic encephalitis and cyst burden (5–7). Ab to CD8^{+} T lymphocytes converts H-2^d mice (which are otherwise resistant and form few cysts in their brains) into mice that are susceptible and form many cysts in their brains (5). The results described herein demonstrate that the presence of CTL correlates with protection that the L^d gene confers. There was no lysis of RH-infected targets following peroral infection with the Me49 strain of *T. gondii* (Figs. 2 and 4). Effectors from Me49-infected

C3H.L^d mice lyse H-2^d, but not H-2^b, targets infected with type II parasite strains. There also was some H-2^k-restricted lysis. As the studies herein were with cell lines, not clones, and the percent infection of targets varied, maximum lysis varied between 40 and 80% in assays performed at different times. Ab to L^d and not an isotype control Ab diminished this cytolytic activity, with controls showing some nonspecific inhibition. Thus, L^d restriction was demonstrated by partial inhibition by Ab (with controls showing some nonspecific inhibition; Table I) and by the finding of L^d restriction in CTL assays using effectors from C3H.L^d mice and targets from H-2-matched and -mismatched strains of mice (Fig. 1). We were able to detect L^d-restricted CTL activity in cultures of splenocytes from C3H.L^d mice infected 11–13 days earlier when cultures were driven with organisms of the R5 strain or tachyzoites of the PTg strain (i.e., derivative of the type II Me49 strain of *T. gondii*). Chardes et al. (29) also described optimal CTL activity in intestinal intraepithelial lymphocytes when mice were infected 11–13 days earlier with the 76K strain (type II) of *T. gondii*.

In some experiments (e.g., Fig. 1*A*) control splenocyte effectors from uninfected mice lysed both uninfected and infected P815 target cells. Our in vitro stimulation conditions occasionally appear to
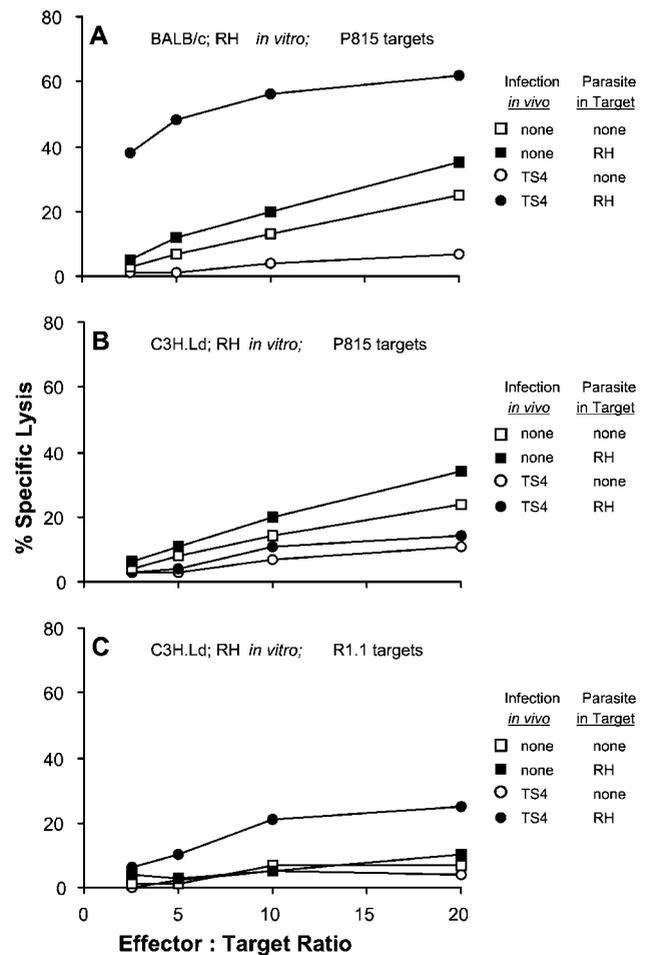

FIGURE 3. Genetic restriction and strain specificity of CTL following ts-4 immunization. Immunization with the ts-4 strain of *T. gondii* elicits CTL production in H-2^d mice that are not L^d restricted. Splenocytes were harvested from nonimmunized (□ and ■) and ts-4-immunized (○ and ●) BALB/c (*A*) and C3H.L^d (*B* and *C*) mice and were stimulated for 6 days in vitro with UV-attenuated RH strain *T. gondii*. Effector cells were tested against uninfected (○ and □) and RH-infected (● and ■) P815 (H-2^d) or R1.1 (H-2^k) target cells. Spontaneous lysis of target cells was 14% or less. Target cells were 50–55% infected.
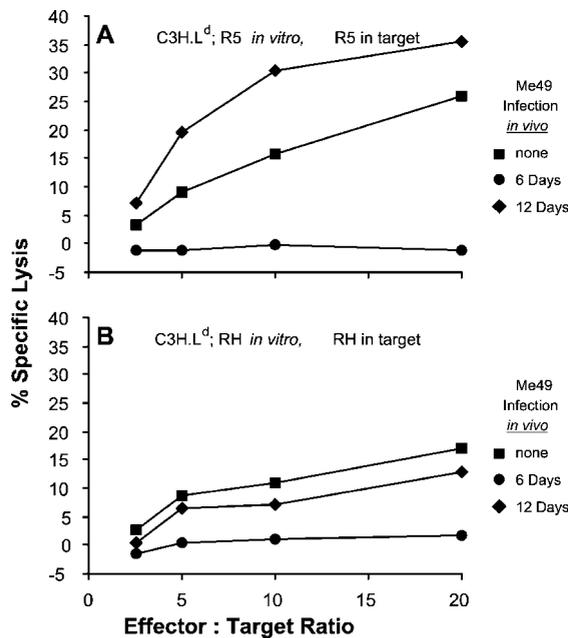
FIGURE 4. Kinetics and strain specificity of CTL following peroral infection with the Me49 strain of *T. gondii*. Specific lysis by C3H.L^d effector cells is elicited by organisms of the R5 (*A*), but not the RH (*B*), strain of *T. gondii* at 12 days following infection, but by neither the R5 nor the RH strain of *T. gondii* at 6 days following infection. Splenocytes were harvested from uninfected C3H.L^d mice (■) and from mice infected with cysts of the Me49 strain of *T. gondii* 6 days (●) or 12 days (♦) earlier and stimulated for 7 days in culture with either R5 (*A*) or RH (*B*) strain-irradiated *T. gondii*. Effector cells were not purified using Ficoll-Hypaque gradients or T cell enrichment columns. Effector cells were tested against uninfected P815 (H-2^d; data not shown; the percent lysis at all E:T ratios was <10%) target cells or target cells infected with either R5 (*A*) or RH (*B*) strain *T. gondii*. Spontaneous lysis of targets was 24% or less. RH-infected target cells were 80% infected, and R5-infected target cells were 60% infected.
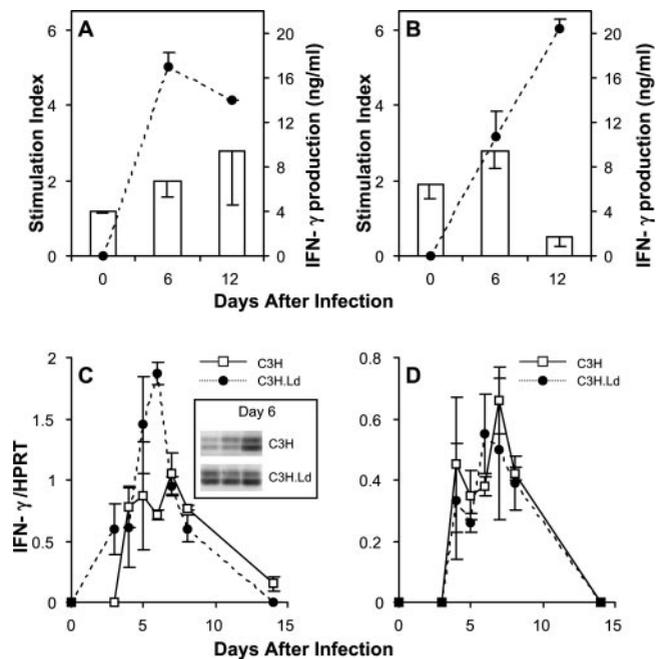

FIGURE 5. Lymphocyte proliferation, IFN-γ production, and mRNA expression following peroral *T. gondii* infection with the Me49 strain of *T. gondii*. *A* and *B*, TLA-induced proliferation of splenocytes (bars) and IFN-γ production (dotted line) in cultures from C3H.L^d (*A*) or C3H/HeJ (*B*) mice. Splenocytes were isolated from uninfected mice and from mice 6 and 12 days following infection with cysts of the Me49 strain *T. gondii*. Spontaneous IFN-γ production from infected mice was below the detectable level on day 12, but in some cases was detectable, but <1 ng/ml on day 6. IFN-γ mRNA production was greater and occurred earlier in C3H.L^d mice than in C3H/HeJ mice (*C* and *D*). Expt. *C* and *D* represent independent experiments. Three C3H.L^d (●) and three C3H/HeJ (□) mice for each time point were infected perorally with 100 Me49 *T. gondii* cysts. On days 0, 3, 4, 5, 6, 7, 8, and 14 postinfection, their spleens were removed, and RT-PCR was performed on RNA from each mouse. IFN-γ message levels were semiquantitated as described in *Materials and Methods*, and the mean and SE were calculated. The inset in *C* shows the gels on day 6 in this experiment. The *upper band* is the competitor. Where the *lower band* is more prominent than the *upper band*, there is more competition by more IFN-γ in the sample. All PCR was performed within the linear range of amplification.

stimulate a CTL population present in uninfected mice that leads to killing of both uninfected and infected target cells. However, the same splenocytes from infected mice that specifically kill infected target cells do not lyse uninfected target cells. There is evidence (30, 31) that naive human T cells proliferate in response to killed organisms or *T. gondii* Ag, and in other systems sensitization of T cells can be elicited in vitro. Thus, such sensitization of T cells might have occurred with the appropriate cytokine and Ag presentation conditions in some of our experiments.

Interestingly, more lysis resulted from effectors from noninfected mice than when using effectors from mice infected 6 days earlier. Experiments such as those shown in Fig. 4*A*, in which stimulated splenocytes from mice infected 6 days earlier have no CTL activity and cells from uninfected mice can be stimulated in vitro, indicate that there is induction of CTL in vitro. These data also suggest that CTL activity is suppressed by splenocytes from mice infected 6 days earlier. Another report (32) and our own (4) have demonstrated suppression of other lymphocyte functions in infected mice in earlier studies with different models. The mechanism(s) involved in the absence of CTL activity 6 days after infection in our L^d model remain to be determined.

Splenocytes derived from C3H.L^d mice infected orally with the Me49 strain of *T. gondii* 6 or 12 days previously and stimulated in culture with irradiated RH strain tachyzoites did not exhibit L^d-restricted CTL activity. This was not due to experimental limitation in the stimulation with RH strain tachyzoites because cytolytic

activity against target cells infected with tachyzoites of the RH strain of *T. gondii* can be detected in splenocyte cultures from ts-4-immunized BALB/c (H-2^d) mice. Rather, these results highlight an important difference between the clonal strains of *T. gondii*. Our studies with ts-4-immunized H-2^d mice confirm earlier results reported by Denkers et al. (8), in that we demonstrate that although ts-4 immunization of H-2^d ($D^{d, Ld}$) haplotype BALB/c mice elicits CTL activity with homologous P815 target cells (Fig. 3), splenocytes from ts-4-immunized C3H.L^d mice do not have cytolytic activity against RH-infected P815 (H-2^d) target cells (Fig. 3). In our studies C3H.L^d mice, which have all the same genes as C3H with the L^d transgene in addition, were used. Immunization with an attenuated type II strain might produce different results. Denkers et al. (8) demonstrated the MHC restriction conferred by immunization of BALB/c mice with ts-4 *T. gondii* tachyzoites to be D^d restricted.

Our data infer that a *T. gondii*-derived peptide is presented by the L^d molecule on the surface of infected P815 target cells that interacts with a specific TCR, giving rise to cytolytic activity of lymphocytes from spleens of mice infected with the Me49 strain of *T. gondii*. Furthermore, it would also appear likely that class I

processing of Ags from either the R5 or PTg strain *T. gondii* produces this peptide, but processing of Ags from tachyzoites of the RH strain of *T. gondii* does not. Our data indicate that an Me49 strain-derived peptide is presented by APCs eliciting protective L^d-restricted CTL. The identification of protective peptide(s) bound to L^d may reveal important vaccine candidate peptides. RH is a type I strain that is highly virulent in mice, resulting in acute illness, soon followed by death. R5 and PTg strains of *T. gondii* are both derived from the type II Me49 strain, a strain less virulent in mice, which results in a milder acute illness, followed by chronic infection in which encysted organisms are found primarily in the brain. The R5 strain produced by Tomavo and Boothroyd (17) has previously been demonstrated by L. Weiss et al. (unpublished observations) to express 50% tachyzoite Ags and 50% bradyzoite Ags (L. Weiss, unpublished observations), whereas PTg is passaged in tachyzoite form. Both R5 and PTg strains were able to elicit comparable CTL activity, suggesting that activity (Fig. 2) was not elicited by bradyzoite Ags alone. R5 and PTg strains presumably share many common epitopes from their parent Me49 strain. Whether L^d-restricted cytolytic activity is elicited by all type II and not by any type I strains remains to be determined from ongoing experiments. If this does turn out to be the case, then the phenotype of type III strains in this respect will be of interest.

A family of *T. gondii* surface Ags contains peptide motifs that are specific for type, I, II, or III strains (28, 33) (J. C. Boothroyd, unpublished observations), which suggests that peptide variants between strains may specifically down-regulate the CTL response through altered peptide ligand antagonism, as described recently for malaria parasites (34).

Mice infected with type III strains are protected against challenge with type I strains, and immunization with the ts-4 (type I) strain of *T. gondii* restricts brain parasite burden following peroral challenge with bradyzoites of the type II Me49 strain *of T. gondii* (12, 35). Therefore, it is likely that there are multiple critical effector mechanisms and that they may be redundant. The nonspecific effect of IFN-γ could account for the cross-protection conferred by different strains of *T. gondii*. It will be of interest in future studies to determine the relative roles of IFN-γ and L^d-restricted CTL using C3H.L^d transgenic IFN-γ knockout mice and cell transfer experiments.

In the acute phase of an Me49 infection, one critical window in which survival is linked to five or more genes (4, 35) occurs between 8 and 12 days following infection, with mortality caused by overexuberant IFN-γ production by intraintestinal CD4$^+$ T lymphocytes (36). Despite heightened immunological activity at this time, we were unable to detect any cytolytic activity at 6 days of infection in spleens of Me49-infected C3H.L^d mice using RH, R5, or PTg strains of *T. gondii*. However, we detected a CTL response at 11–13 days after infection.

Cytokines provide another protective effector mechanism (37), and the relative kinetics of their production could account for the differences in susceptibility of C3H.L^d and C3H/HeJ mice. Previous studies (6) demonstrated that 30 days after infection with the Me49 strain *T. gondii*, brains of mice resistant to cyst formation had little detectable cytokine mRNA expression, but brains of mice susceptible to cyst formation had elevated levels of mRNA for a range of cytokines (i.e., TNF-α, TGF-β, IL-10, IL-1α and IL-1β, IL-2, and IFN-γ), associated with inflammation in brains of susceptible C3H/HeJ mice. Six days following Me49 infection, when CTL activity is absent in spleens of C3H.L^d mice, IFN-γ production in vitro is higher in splenocyte cultures from C3H.L^d mice compared with cultures from C3H/HeJ mice. IFN-γ mRNA could be detected in the spleens of all mice from approximately days 4–8 postinfection, but had fallen to baseline levels by day 14

postinfection. No reproducible statistically significant difference in levels between the two strains of mice was observed. At 12 days after infection, when CTL activity is present in C3H.L^d spleens, the converse is true; IFN-γ production is higher in splenocyte cultures from C3H/HeJ mice. The presence of IFN-γ at 6 days after infection may be a critical mechanism for early protection before CTL develops. The higher IFN-γ levels in the spleens of C3H/HeJ mice at 12 days after infection may be due to increased parasite numbers as a result of the inability of these mice to mount an effective CTL response. Splenocyte proliferation was also suppressed in C3H/HeJ, but not C3H.L^d, mice at this time point, a phenomenon previously attributed to IFN-γ production (38, 39). Alternatively, IFN-γ produced by another cell type, such as NK cells, may be a critical effector mechanism, and the L^d molecule may play a role both in producing protective effector CTL at 11–13 days after infection as well as in T cell-NK stimulatory interactions such as those described in other systems (see the discussion below) (40). It is also possible that there are other critical effector cells in anatomic compartments, as our results were obtained with the study of only splenocytes.

We found that a lysate of the RH strain of *T. gondii* (TLA) stimulated the production of IFN-γ from splenocytes of mice infected with the Me49 strain of *T. gondii*, whereas CTL were not elicited by or directed toward epitopes from RH strain tachyzoites. The amount of IFN-γ production at this early time was greater in the L^d transgenic mice. An additional effector mechanism of L^d could be through an early T cell-NK cell interaction (40). At this time NK cells that produce IFN-γ are critical in protection against *T. gondii* infection (41). In studies by others (41) IFN-γ was produced by NK1.1$^+$ cells isolated from ts-4-vaccinated β_2-microglobulin-deficient mice in response to culture with *T. gondii* Ag. Certain NK cell receptors that interact specifically with certain classes of MHC molecules inhibit NK function (killer inhibitory receptors), whereas those without a cytoplasmic inhibitory tail (ITM) appear to stimulate NK function (42). One hypothesis is that perhaps NK cells bind specifically to L^d, stimulating their IFN-γ production and therefore CTL production, ultimately resulting in a restriction of brain cyst number. Alternatively, protective IFN-γ could be produced by NK1.1$^+$ T cells or class I-restricted CD4$^+$ T cells.

Our observations indicate that the early IFN-γ production does not appear to be strain specific, whereas the CTL activity is, and thus suggest that differing epitopes may elicit these two potentially protective effector mechanisms, which could act synergistically. It is also possible that differences between C3H and C3H.L^d mice in IFN-γ recall responses would only be apparent using Me49 as the in vitro Ag, which was not done.

It will be of considerable interest in future studies to determine relative roles of these two effector mechanisms, IFN-γ and CTL mediated by L^d, that correlate with protection in restriction of cyst number and encephalitis in C3H.L^d mice. It is important, especially for vaccine development in future work where strain specificity of protection could require the inclusion of more epitopes, to determine whether the strain specificity (L^d-Me49) applies to all type I, II, and III strains and whether competitor peptides (e.g., motifs described previously (33)) inhibit protective immune responses (34).

Our findings indicate that different clonal types of *T. gondii* may elicit profoundly different immune responses. This may contribute to the differences in virulence noted in the clonal types of *T. gondii* and hybrid crosses between them (43). It also is consonant with recent findings that RH and Me49 strains of *T. gondii* may elicit TH1 immune responses of different magnitudes (44).

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