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γδ T Cell-Deficient Mice Have a Down-Regulated CD8+ T Cell Immune Response Against Encephalitozoon cuniculi Infection

Magali Moretto,†§ Brigit Durell, † Joseph D. Schwartzman, ‡ and Imtiaz A. Khan²*†§

Recent studies have suggested that the γδ T cell subtype may also be important for the induction of adaptive immune response against certain microbial pathogens. In the present study, an early increase of γδ T cells during murine infection with Encephalitozoon cuniculi, an intracellular parasite, was observed. The role of γδ T cells against E. cuniculi infection was further evaluated by using gene-knockout mice. Mice lacking γδ T cells were susceptible to E. cuniculi infection at high challenge doses. The reduced resistance of δ−/− mice was attributed to a down-regulated CD8+ immune response. Comparison with parental wild-type animals, suboptimal Ag-specific CD8+ T cell immunity against E. cuniculi infection was noted in δ−/− mice. The splenocytes from infected knockout mice exhibited a lower frequency of Ag-specific CD8+ T cells. Moreover, adoptive transfer of immune TCRαβ+ CD8+ T cells from the δ−/− mice failed to protect naive CD8−/− mice against a lethal E. cuniculi challenge. Our studies suggest that γδ T cells, due to their ability to produce cytokines, are important for the optimal priming of CD8+ T cell immunity against E. cuniculi infection. This is the first evidence of a parasitic infection in which down-regulation of CD8+ T cell immune response in the absence of γδ T cells has been demonstrated. The Journal of Immunology, 2001, 166: 7389–7397.

During recent years, several cases of HIV-infected patients with Encephalitozoon cuniculi infection have been reported (1, 2). Cell-mediated immunity has been shown to be critical for the host resistance against E. cuniculi infection (3). Studies involving SCID mice have shown that these immunodeficient animals are unable to survive E. cuniculi infection (4). However, an adoptive transfer of a sensitized syngeneic T cell population to these immunodeficient mice protects them from E. cuniculi infection (5). In contrast, transfer of naive T lymphocytes or hyperimmune anti-serum failed to protect or prolong the survival of the SCID mice. Previous studies from our laboratory have suggested that, among the T cell population, the CD8+ T cell subset plays a predominant role during E. cuniculi infection (6). Mice lacking CD8+ T cells are unable to survive E. cuniculi infection. The cytotoxic response of CD8+ T cells is essential for protective immunity against the parasite.

A majority of T cells in an adaptive immune response bear the αβ TCR (7). However, minor populations of T cells carrying the γδ TCR are higher in a number of intracellular infections. Studies with human malarial infection have shown that γδ T cells inhibit the replication of blood-stage Plasmodium in vitro and in vivo (8). Similarly, higher parasite levels as compared with parental controls were maintained in the mice lacking γδ T cells (9). Preferential expansion of the Vγ9 subset of γδ T cells in human malarial infection has been reported (10). Mice infected with Leishmania major show a rise in the γδ T cell population, which may be involved in the host protection (11). Lack of γδ T cells in Listeria monocytogenes-infected mice results in exacerbation of the infection (12, 13). Adoptive transfer of γδ T cells from Toxoplasma gondii-infected animals protects the recipient immunodeficient mice against a lethal challenge (14).

Although γδ T cells can act as effector cells in a number of disease models (15), they also have been reported to play a role in the regulation of immune functions (16, 17). Recent studies by Ferrick et al. (18) have shown that γδ T cells may be important in the outcome of CD4+ T cell response during acute parasite infection. T cells bearing γδ TCRs discriminate early during infection with L. monocytogenes and Nippostrongylus brasiliensis by producing cytokines associated with either a Th1 (IFN-γ) or Th2 (IL-4) pattern that is appropriate to the Th cell response (18). In a recent study with L. monocytogenes, it has been demonstrated that γδ T cells are responsible for the establishment of protective immunity against the bacteria by priming Ag-specific CD8+ T cells (12).

In the present study, the role of γδ T cells in E. cuniculi infection was evaluated. A very early and significant systemic rise of this T cell subset in the infected animals was observed. Gene-knockout mice lacking γδ T cells succumbed to high doses of E. cuniculi infection. These mutant animals exhibited suboptimal levels of CD8+ T cell immunity against the infection.

Materials and Methods

Mice

Dr. T. W. Mak (Amgen Institute, Ontario Canada) kindly provided a breeding pair of CD8−/− mice on C57BL/6 background. Animals were bred under approved conditions at the Animal Research Facility at Dartmouth Medical School (Lebanon, NH) and the Louisiana State University Medical Center (New Orleans, LA). δ−/− and α−/− mice on the same genetic background were obtained from The Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched C57BL/6 mice were used as wild-type (WT) controls.

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Abbreviations used in this paper: WT, wild type; p.i., postinfection; pfp, precursor proliferation frequency; LDA, limiting dilution assay; pCTL, CTL precursors.
Parasites and infection

A rabbit isolate of E. cuniculi, kindly provided by Dr. E. Didier (Tulane Regional Primate Research Center, Covington, LA) was used throughout the study. The parasites were maintained by continuous passage in rabbit kidney (RK-13) cells, obtained from American Type Culture Collection (Manassas, VA). The RK-13 cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FCS (HyClone Laboratories, Logan UT). Organisms were collected from the culture medium and centrifuged at 325 × g for 10 min. After two washes with PBS, the parasites were resuspended and injected i.p. Mice were infected with a dose of 1 × 10^7 spores/mouse unless indicated otherwise.

Phenotypic analysis

Following euthanasia, the spleens from C57BL/6 mice were removed and homogenized in a petri dish. The contaminating red blood cells were lysed with RBC lysis buffer (Sigma, St. Louis, MO). Splenocytes were washed, suspended in 1% PBS-BSA (Sigma), and analyzed by FACS (BD Biosciences, San Jose, CA) for CD8^+ cells using a direct immunofluorescence assay. Cells (1 × 10^6/mL) were incubated with 1 μg of FITC-labeled anti-TCR γδ (clone GL3; BD Pharmingen, San Diego, CA) in 1% PBS-BSA. After a 1-h incubation at 4°C, the cells were washed several times in buffer, fixed in 1% methanol-free formaldehyde and stored at 4°C for FACS analysis.

Lymphoproliferation assay

The frequency of E. cuniculi-specific proliferative response of purified CD8^+ T cells was measured by performing a precursor proliferation frequency (pPF) analysis. The splenocytes from day 15-postinfection (p.i.) mice were homogenized, and RBCs were lysed as mentioned above. After two to three washes in PBS containing 3% FCS (HyClone Laboratories), the CD8^+ T cell population was separated by MicroBeads (Miltenyi Biotec, Auburn, CA). The separation procedure was conducted according to the manufacturer’s instructions. The purity of separated cells was >95% as determined by FACS analysis. Limiting dilution assay (LDA) was performed on purified CD8^+ T cells by plating spleen cells in five serially diluted dilutions starting at 5 × 10^4 cells/well in U-shaped round-bottom 96-well plates. For each dilution, there were 24 replicates. A total of 1 × 10^5 irradiated syngeneic feeder cells and 5 × 10^4 spores were added to each well. Twelve control wells were set as described above by replacing spores with extract from host cell lysate. The lysate was prepared from RK-13 cells, which were sonicated and centrifuged at 10,000 × g for 15 min. The concentration of proteins was determined by a bicinchonic acid assay (Pierce, Rockford, IL). A total of 15 μg soluble Ag/ml was added to each control well. After 5 days, 1 μCi tritiated thymidine/well (Amersham, Arlington Heights, IL) was added for 12 h to determine the DNA synthesis. Wells were scored as positive if the cpm from the sample wells were greater than 3 × SD above the mean cpm from the control wells. The pPFs were calculated by a standard method (19).

Cytotoxic assays

Bulk cytotoxic assay. A CTL assay was performed to detect the cytotoxic activity of the splenocytes from mice lacking αβ or γδ T cells. The assay was conducted by the protocol previously standardized in our laboratory (20). Briefly, peritoneal macrophages from thioglycollate-treated mice were collected, washed, and dispensed at the concentration of 5 × 10^6 cells/well in U-bottom 96-well plates. After overnight incubation, the cells were infected with 2 × 10^3 spores of E. cuniculi/well for 48 h. The wells were extensively washed with PBS to clear extracellular parasites. Macrophages were incubated with 0.5 μCi/well for 2 h at 37°C. Macrophages were washed 5 times with PBS and incubated with cultured spleen cells at various E:T ratios in a final volume of 200 μl of culture medium. The microtiter plates were centrifuged at 200 × g for 3 min and incubated at 37°C for 4 h. One hundred-microliter samples were removed and assayed for released cpm by scintillation counting. The percentage of lysis was calculated as (mean cpm of test sample − mean cpm of spontaneous release) / (mean cpm of maximal release − mean cpm of spontaneous release).%)

A cytotoxic assay was also performed on the purified γδ-positive T cells. Purified γδ T cells were isolated from the pooled spleen cells of E. cuniculi-infected mice (six animals) at day 15 p.i. The splenocytes were incubated for 1 h at 4°C with biotin-conjugated anti-γδ Ab (BD Pharmingen). After two washes in 1% BSA-PBS, the cells were incubated for 15 min at 4°C with streptavidin-coated MicroBeads according to the manufacturer’s instructions (Miltenyi Biotec). Subsequently, the cells were washed and eluted from the magnetic columns. The purity of the γδ T cells was >97% as determined by FACS analysis.

CTL precursors (pCTL). The cytolytic activity was quantitated by determining the pCTL response of the infected mice using LDA. CD8^+ T cells were purified by magnetic depletion using a direct immunofluorescence assay. Cells (1 × 10^7 spores) were washed and eluted from the magnetic columns. The purity of the splenocytes from the mice was analyzed by the protocol previously standardized in our laboratory. The purity of isolated cells was >95% as determined by FACS analysis. The purity of the splenocytes population was separated at day 15 p.i. as described above. Purified CD8^+ T cells were cultured by limiting dilution in 96-well round-bottom plates. Dilution of cells ranging between 1,250 to 25,000 cells/well were grown in RPMI 1640 medium containing appropriate growth factors including 15 U/ml of IL-2 (R&D Systems, Minneapolis, MN) and 5 × 10^4 irradiated spores/well (3000 rad). For each dilution, there were 24 replicates. Irradiated splenocytes (3000 rad) obtained from naive syngeneic mice were used as feeder cells at a concentration of 1 × 10^5 cells/well. Wells containing only irradiated parasites and feeder cells, without effector cells, served as controls. After 1 wk, the cells were harvested and incubated with 3^11Cr-labeled parasite-infected and uninfected macrophages. Macrophages were collected and labeled as described above and incubated with purified CD8^+ T cells. The amount of radiolabeled released was measured following a 4-h incubation. The wells were considered to be positive for lytic activity if the mean cpm release of infected cells was greater than 3 × SD above control wells (mean cpm released by the target cells incubated with feeder cells and irradiated parasites alone). The pCTL frequency was calculated according to a standard formula (21).

Histopathological analysis

Tissues from infected δ^−/− and parental control animals were fixed in 10% buffered formalin at day 15 p.i. The tissues were processed for 5-μm histological sections, which were stained with hematoxylin and eosin.

Detection of cytokines

Quantitation of mRNA by PCR. Splenocytes from E. cuniculi-infected animals were collected on days 0, 7, 14, and 21 p.i. RNA from spleen cells was isolated using TRIzol (Life Technologies) according to the manufacturer’s instructions. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) and random hexamer primers (Promega, Madison, WI). Expression of mRNA for IFN-γ, IL-10, and IL-4 was performed by quantitative PCR using the PFRS quantitative method (22). The splenocytes from uninfected mice were used to establish a baseline value of 1.0, against which the level of message for cytokine in the test mice was quantitated.

Protein analysis by fluorescent assay. Intracellular cytokine staining was performed to determine IFN-γ, IL-4, and IL-10 production by γδ TCR-bearing cells as previously described (23). Splenocytes from day 7- and day 15-infected mice were isolated and resuspended in RPMI 1640 containing 10% FCS. The cells were cultured at the concentration of 10^5 cells/well in a 96-well plate and stimulated with 10 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma), and 2 μM monensin (GolgiStop; BD Pharmingen). Cultures were incubated for 4 h at 37°C in 5% CO2 in a humidified incubator. After incubation, cells were washed with PBS and 1% FCS and stained with anti-γδ T cell conjugated with fluorescein (BD Pharmingen) for 30 min at 4°C. Intracellular staining was performed using the Cytofix/ CyPerm kit (BD Pharmingen) in accordance with the manufacturer’s recommendations. Briefly, following cell surface staining, cells were washed and then treated with formaldehyde and saponin to fix and permeabilize the cells. Intracellular staining was then performed using anti-IFN-γ, anti-IL-4, or anti-IL-10 conjugated with PE (BD Pharmingen). Samples were resuspended in PBS containing 1% formaldehyde, acquired on a FACScan flow cytometer, and analyzed using CellQuest software (BD Biosciences).

In vivo cytokine administration

Mice lacking γδ TCR were treated with recombinant mouse IFN-γ (R&D Systems). The cytokine treatment was started 1 day prior to i.p. infection with 1 × 10^7 spores, and each animal received 2 μg of cytokine alternatively for a period of 8 days.

Adaptive transfer of CD8^+ T cells

Parental C57BL/6 mice and δ^−/− mice were infected i.p. with 1 × 10^7 spores of E. cuniculi. At day 15 p.i., the mice were splenectomized, and spleen cells were isolated and collected. Splenic CD8^+ T cells were separated as described above. CD8^+ T cells were adoptively transferred to naïve CD8^+ mice i.v. by tail vein inoculation. A total of 24 h after the adoptive transfer of immune cells, the CD8^+ mice were challenged with 1 × 10^5 spores of E. cuniculi.

Adaptive transfer of CD8^+ TCRαβ^+ T cells

Splenocytes from the infected animals were isolated as described above, and αβ^+ CD8^+ T cells from the spleen cells were isolated by a two-step...
separation procedure using Ab-coated beads (Miltenyi Biotec). Briefly, the splenocytes were incubated with MicroBeads coated with anti-CD4 Ab for 15 min at 4°C as directed by the manufacturer. After two washes with 1% BSA-PBS, CD4+ T cells were removed by magnetic selection. In the second step of separation, cells bearing γδ TCR were isolated from the CD4+ T cell-depleted population. The cells were incubated for 1 h at 4°C with a 1/250 dilution of biotin conjugated anti-β-chain Ab (BD PharMingen). After two washes with PBS, they were subsequently incubated for 15 min at 4°C with streptavidin-coated MicroBeads (Miltenyi Biotec). The positive cells were eluted from the magnetic columns, and the purity was measured by FACS analysis (>95% pure CD8+ TCRαβ+ T cells).

Statistical analysis

Statistical analysis of the data was performed by Student’s t test (24).

Results

E. cuniculi infection induces an increase in γδ T cells

WT C57BL/6 mice were infected i.p. with $1 \times 10^7$ spores of E. cuniculi. Obvious splenomegaly in the infected animals was observed starting at day 7 p.i. and, by day 14, an almost 4-fold increase in the splenic CD8+ T cell population at this time point (6). In the present studies, the kinetics of the γδ T cell response within the splenocytes of E. cuniculi-infected animals was analyzed. At days 3, 7, 14, and 24 p.i., the splenocytes from infected animals were analyzed for the expression of γδ TCR. As shown in Fig. 1, the assay revealed a very early and significant rise in γδ T cell population starting at day 3 p.i. ($p < 0.05$). The rise in γδ T cell population was further enhanced by day 14 p.i. At this time point, a 6-fold increase in the percentage of this cell type (13 ± 3%) over the uninfected controls (2 ± 1%) was noted (Fig. 1). The γδ T cell population remained elevated until day 24 p.i. (11 ± 2%), suggesting that these cells may be important for the protective immunity against the parasite.

γδ T cell-deficient mice are susceptible to high doses of E. cuniculi infection

To determine the role of γδ T cells during E. cuniculi infection, gene-knockout mice were infected i.p. with different doses ($1 \times 10^7$ to $5 \times 10^7$) of E. cuniculi spores. When the animals were infected with $1 \times 10^7$ spores, all the δ−/− mice developed ascites but subsequently recovered from the infection (data not shown). However, when the challenge dose was increased to $5 \times 10^7$ spores, the δ−/− mice developed ascites and died by day 24 p.i. (Fig. 2). The study was performed twice with similar findings.
spores, 60% of the $\delta^{-/}$ animals died by day 22 p.i. (Fig. 2). No mortality was observed in the WT control mice. Conversely, all the $\alpha^{-/}$ mice died in response to E. cuniculi infection when infected with either $10^7$ (data not shown) or $5 \times 10^7$ spores (Fig. 2). Based on these observations, it appears that both $\alpha^{-/}$ and $\gamma^{-/}$ T cells play an important role for clearing E. cuniculi infection. However, the fact that $\alpha^{-/}$ mice died at lower infective doses suggests that, in comparison to $\gamma^{-/}$ T cells, $\alpha^{-/}$ T cells seem to be more crucial for protective immune response against the parasite.

**Histopathology**

In comparison to control tissue, which had evidence of mild inflammation but no evidence of microsporidal multiplication (Fig. 3A), the liver of $\delta^{-/}$ mice showed nodules of lymphocytic inflammatory cells with intracellular microsporidia (Fig. 3B). Sections of spleen from control mice showed evidence of increased cell turnover and phagocytosis but no intracellular parasites (Fig. 3C). The spleen of $\delta^{-/}$ mice showed some lymphocytic depletion of primary follicles and foci of intracellular microsporidia (Fig. 3D). Other organs had no pathologic changes.

**Cytokine responses in $\delta^{-/}$ mice**

Previous reports from our laboratory have emphasized the importance of CD8$^+$ T cells and IFN-γ in the protection against E. cuniculi infection (6, 25). The role of IFN-γ in the priming of CD8$^+$ T cell response in other infectious disease models has been reported earlier (26). Various studies from other laboratories have demonstrated that $\gamma^{-/}$ T cells can be an important source of cytokines during early infection (14, 18). To determine whether the absence of $\gamma^{-/}$ T cells affects the cytokine production in response to E. cuniculi infection, the kinetics of cytokine message in $\gamma^{-/}$ T cell-deficient and control WT C57BL/6 mice was analyzed. As shown in Fig. 4A, $\gamma^{-/}$ T cell-deficient mice exhibited an almost 10-fold decrease in IFN-γ message at day 7 p.i. in comparison to parental controls. Nevertheless, the levels of IFN-γ message in the knockout mice reached those of WT animals at day 14 p.i. No differences in the production of IL-10 message between the knock-
out (Fig. 4D) and WT mice (Fig. 4C) was observed at all the time points tested. As reported earlier (6), barely detectable levels of IL-4 message in response to *E. cuniculi* infection were observed in WT C57BL/6 mice. However, an almost 6-fold increase in IL-4 message was noted in the *δ* / *δ* animals (Fig. 4F) at day 14 p.i. compared with WT mice (Fig. 4E).

To determine whether γδ T cells from the parental infected mice are responsible for earlier IFN-γ release, cytokine production by γδ T cells was evaluated by intracellular staining. A significant increase (*p*, 0.05) in the percentage of IFN-γ-producing γδ T cells (5 ± 2%) in comparison to uninfected controls (1 ± 1%) was noted starting at day 7 p.i. (Fig. 5). The percentage of IFN-γ-producing γδ T cells continued to rise until day 15 p.i. (22 ± 4%). A very nominal increase in the IL-4-positive γδ T cells (3 ± 2%) was observed at day 15 p.i. as compared with uninfected controls (1 ± 1%) (Fig. 5). IL-10-positive γδ T cells could not be detected at any of the time points tested (Fig. 5). The observations made above suggest that γδ T cells may be an important source of early IFN-γ during *E. cuniculi* infection.

Exogenous treatment with IFN-γ protects δ−/− mice against *E. cuniculi* infection

Next, we determined whether a treatment with rIFN-γ could enable the δ−/− mice to withstand a lethal challenge with *E. cuniculi* infection. A group of gene-knockout mice on a C57BL/6 background was treated exogenously with IFN-γ, and the animals were infected the following day with 5 × 10⁷ spores of *E. cuniculi*. The IFN-γ treatment was continued on an alternate basis for a period of 8 days. All the cytokine-treated mice survived *E. cuniculi* infection until the termination of the experiment (Fig. 6). On the contrary, three of five untreated controls succumbed to the infection.

Splenocytes from γδ T cell-deficient mice are able to lyse *E. cuniculi*-infected macrophages

Previous studies from our laboratory have demonstrated that protective immunity against *E. cuniculi* infection in the WT mice is dependent on the cytotoxic property of the CD8⁺ T cell subset (6). To determine whether γδ T cells are involved in the cytotoxic
response against *E. cuniculi*-infected cells, we compared the cytotoxic activity of the splenocytes from the α−/−, δ−/−, and parental WT mice. At 15 days p.i., spleen cells were harvested and cultured in the presence of irradiated spores. After 5 days of incubation, the cultured splenocytes from the α−/− mice failed to exhibit a cytolytic effect on *E. cuniculi*-infected macrophages at all E:T ratios. On the contrary, splenocytes from both δ−/− and parental WT mice exhibited cytotoxic activity against infected macrophages (Fig. 7). However, the splenocytes from the WT mice exhibited a significantly higher cytotoxic activity than those from the δ−/− mice at all the E:T ratios. For example, at an E:T ratio of 20:1, the cytotoxic activity of the spleen cells from WT mice was 31.8 ± 2.2% vs 22.6 ± 1.7% in the δ−/− mice (p = 0.01). No lysis of the uninfected macrophages was observed (data not shown). As previously reported, the killing was MHC restricted, because the immune splenocytes were unable to lyse the infected macrophages derived from nonsyngeneic BALB/c mice (6). These findings suggest that MHC-restricted cytotoxic function in *E. cuniculi*-infected mice is primarily dependent on the αβ T cell subset of the host.

Next, we directly evaluated the role of γδ T cells on the cytotoxic response against *E. cuniculi*-infected cells. γδ T cells from the mice infected with *E. cuniculi* were isolated by magnetic separation. At day 15 p.i., γδ T cell-enriched and γδ T cell-depleted fractions were cultured in the presence of irradiated spores and feeder cells. After 5 day of incubation, the cytotoxic effect of purified γδ T cells and the remnant population was measured. At an E:T ratio of 20:1, the purified γδ T cell fraction exhibited a background level of cytolysis of infected targets (data not shown). Conversely, at the same E:T ratio, the γδ T cell-depleted population showed a target cell lysis of 38.82 ± 7.56%. These findings rule out the role of γδ T cells in the cytotoxic activity against *E. cuniculi*-infected cells.

**Lack of γδ T cells results in reduced CD8+ T cell response**

As stated above, earlier studies in our laboratory have demonstrated that cytotoxic T cell activity against *E. cuniculi*-infected cells plays a major role in the eradication of infection in the host (6). However, the complete absence of lytic activity by splenocytes from α−/− mice suggests that γδ T cells are not involved in the cytotoxic activity against *E. cuniculi*-infected cells. This was directly confirmed by the studies in which purified γδ T cells were unable to lyse *E. cuniculi*-infected macrophages. Because CD8+ T cells are the major effector cells during *E. cuniculi* infection, we used δ−/− mice to assay the role of γδ T cells in the regulation of CD8+ T cell response during *E. cuniculi* infection. To determine...
the cytotoxic response in the absence of γδ T cells, the pCTL frequency of affinity-purified CD8+ T cells against E. cuniculi infection in δ−/− mice was evaluated. A significantly lower pCTL frequency (p < 0.05) in response to E. cuniculi infection was observed in δ−/− mice (Fig. 8). The pCTL frequency of the CD8+ T cell population in the knockout mice was 1/1.3 × 10^4 compared with 1/1.2 × 10^5 cells in the parental C57BL/6 controls (Fig. 8). Similarly, in a duplicate assay, the pCTL frequency of the CD8+ T cell population in the δ−/− mice was 1/2.2 × 10^4 in comparison to 1/2.8 × 10^3 in the WT mice.

To confirm that down-regulation of the CD8+ CTL response against E. cuniculi infection is due to the generation of fewer Ag-specific T cells, we evaluated the ppf of CD8+ T cells from either WT or δ−/− mice against a lethal dose of 1 × 10^7 spores of E. cuniculi (Fig. 9). The pCTL frequency of the CD8+ T cells from the pooled splenocytes (three mice per group) were isolated and cultured in presence of E. cuniculi spores and irradiated feeder cells. After 1 wk in culture, ppf of CD8+ T cells was determined. Data are representative of one of the two separate experiments. Statistical significance was determined using the Student’s t test (p < 0.05).

Adoptive transfer of CD8+ T cells from δ−/− mice is unable to protect naive CD8−/− mice against E. cuniculi challenge

Previous studies from our laboratory have demonstrated that adoptive transfer of immune CD8+ T cells from parental mice to the CD8−/− animals protects them from lethal E. cuniculi infection (6). We analyzed the ability of the CD8+ T cells from δ−/− mice to protect the CD8−/− host from E. cuniculi challenge. Purified CD8+ T cells (>95% pure) were isolated at day 15 p.i and adoptively transferred to naive CD8−/− mice. At 24 h after transfer, the mice were challenged with a lethal dose (1 × 10^7 spores) of E. cuniculi. Very minimal protection was observed when CD8+ T cells from δ−/− mice were used for the transfer (Fig. 10). In contrast, only one of five mice treated with immune CD8+ T cells from WT mice survived E. cuniculi challenge. Nonimmune CD8+ T cells from either WT or δ−/− mice were unable to confer protection to CD8−/− animals.

Subsequently, we determined whether the protective immunity transferred to the naive CD8−/− mice depends on αβ-bearing CD8+ T cells. For this purpose, CD8+TCRαβ+ T cells were separated from the spleen cells of infected δ−/− and WT mice (>95% pure). Purified CD8+αβ+ T cells (5 × 10^4) were injected in naive CD8−/− mice, which were subsequently challenged 1 day later.
with $1 \times 10^7$ spores of *E. cuniculi*. The animals that received αβ⁺CD8⁺ T cells from δ⁻/⁻ mice were unable to survive *E. cuniculi* challenge (Fig. 11). Conversely, CD8⁻/⁻ mice that were treated with αβ⁺CD8⁺ T cells from parental C57BL/6 animals were able to withstand *E. cuniculi* challenge.

**Discussion**

Our studies demonstrate an early and significant rise of γδ T cells in response to *E. cuniculi* infection. The importance of the γδ T cells was confirmed in vivo with the δ⁻/⁻ mice showing an increased susceptibility to *E. cuniculi* infection. However, in the same experiments, αβ⁺ T cell-deficient mice exhibited less resistance to *E. cuniculi* infection compared with δ⁻/⁻ mice. These observations suggest that, although they may not be potentially as important as αβ⁺ T cells, γδ T cells may play an auxiliary role in the protection against *E. cuniculi* infection. It has been suggested that γδ T cells can act as an important first line of defense against infection with intracellular pathogens such as *L. monocytogenes*, *P. yoelii*, *Mycobacterium tuberculosis*, and *T. gondii* (14, 27–29).

To determine whether γδ T cells are the primary effector cells during early *E. cuniculi* infection, splenocytes from infected α⁺/⁻ and δ⁻/⁻ mice were analyzed for cytotoxic activity against infected targets. The spleen cells from the mice lacking αβ⁺ T cells exhibited background levels of cytotoxic effect on *E. cuniculi*-infected macrophages, suggesting that γδ T cells may not be involved in the lysis of infected cells. This was further confirmed by the observation that the purified γδ T cell population was unable to lyse the *E. cuniculi*-infected targets. These studies undermine the possibility that γδ T cells are the major effector cells during *E. cuniculi* infection.

In addition to their effector function, recent studies have recognized a regulatory role for γδ T cells (16, 17). γδ T cells, due to their ability to produce cytokines, have been shown to establish the primary immune response against *L. monocytogenes* and *N. brasilienis* (18). In a recent study, depletion of γδ T cells in the immune animals resulted in the down-regulation of CD8⁺ T cell response against *L. monocytogenes* (12). However, McKenna et al. recently reported that γδ T cells are a component of the early immunity directed against malarial parasites and are not required for induction of effector αβ⁺ T cell response (30). Thus, the role of γδ T cells in the regulation of αβ⁺ T cell immune response during microbial infection, although described, is not well established. Our current findings strongly suggest that CD8⁺ T cell induction in response to *E. cuniculi* infection is down-regulated in the absence of γδ T cells. Mice lacking γδ T cells show a significantly lowered frequency of Ag-specific CD8⁺ T cells as determined by ppf and pCTL frequency assays. Moreover, adoptive transfer of immune CD8⁺ T cells from δ⁻/⁻ mice failed to protect naive CD8⁻/⁻ mice. Conversely, as observed earlier (6), CD8⁻/⁻ mice that received immune CD8⁺ T cells from WT mice were protected against lethal *E. cuniculi* infection. To rule out the possibility that protective immunity transferred to naive CD8⁻/⁻ mice is not due to CD8⁺ T cells bearing γδ TCR, we isolated a pure population of αβ⁺CD8⁺ T cells. Purified αβ⁺CD8⁺ T cells from WT mice protected the naive CD8⁻/⁻ mice as a result of a lethal *E. cuniculi* infection. Conversely, αβ⁺ CD8⁺ T cells isolated from δ⁻/⁻ animals were unable to protect naive CD8⁻/⁻ mice challenged with an *E. cuniculi* infection. Our observations suggest that, due to their cytokine-producing ability, γδ T cells may be important for inducing CD8⁺ effector T cells during *E. cuniculi* infection. This view is strengthened by the observation that splenocytes from δ⁻/⁻ mice showed an almost 10-fold reduction in IFN-γ message compared with parental WT mice at day 7 p.i. Moreover, significant levels of IFN-γ-positive γδ T cells were observed at days 7 and 15 p.i. Furthermore, exogenous treatment of δ⁻/⁻ mice with IFN-γ enabled them to withstand high challenge doses of *E. cuniculi* infection. The importance of IFN-γ in the induction of class I-restricted CD8⁺ CTL response has been reported in parasitic and viral infections (31). Based on these reports and current observations, we postulate that γδ T cells may be an important component for the induction of an adaptive immune response against *E. cuniculi* infection. Due to their early rise, this T cell subset is an important source of cytokines, which induce the CD8⁺ T cell response against the parasite. A recent study from our laboratory reported that adequate CD8⁺ T cell immune response against *E. cuniculi* infection can be launched in CD4⁺ T cell-deficient mice (32). These findings raised an important question about the mechanism of CD8⁺ T cell priming in the absence of conventional CD4⁺ T cells. Our current observations suggest that γδ T cells are important for the induction of CD8⁺ T cell effector immunity during *E. cuniculi* infection. Mice lacking γδ T cells have a subdued CD8⁺ T cell response and cannot efficiently eradicate the parasites like the normal animals. It will be important to determine whether, in the absence of CD4⁺ T cells, γδ T cells can maintain optimal CD8⁺ T cell immunity against *E. cuniculi* infection. This is an important question, because HIV-infected patients who suffer a
major defect in CD4+ T cell immunity are unable to control infection by *E. cuniculi*. Ongoing studies in our laboratory should be able to provide answers to these important questions.

References


